No. 23-2357

In the

United States Court of Appeals

for the

Federal Circuit

ALNYLAM PHARMACEUTICALS, INC.,

Plaintiff-Appellant,

– v. –

MODERNA, INC., MODERNATX, INC., AND MODERNA US, INC.,

Defendants-Appellees.

On appeal from a final judgment of the
United States District Court for the District of Delaware
Case Nos. 1:22-cv-00335-CFC, 1:22-cv-00925-CFC

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PATENT CLAIM LANGUAGE AT ISSUE

’933 Patent

Claim 18: A cationic lipid comprising a primary group and two biodegradable hydrophobic tails, wherein the primary group comprises (i) a head group that optionally comprises a primary, secondary, or tertiary amine, and (ii) a central moiety to which the head group and the two biodegradable hydrophobic tails are directly bonded; the central moiety is a central carbon or nitrogen atom; each biodegradable hydrophobic tail independently has the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), wherein the biodegradable group is —OC(O)— or —C(O)O—; for at least one biodegradable hydrophobic tail, the terminal hydrophobic chain in the biodegradable hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl, and the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26; in at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to

12 carbon atoms; and the lipid has a pKa in the range of about 4 to about 11 and a logP of at least 10.1.

'979 Patent

Claim 1: A lipid particle comprising: (i) a nucleic acid, (ii) 35-65 mol % of a cationic lipid, (iii) 3-12 mol % distearoylphosphatidylcholine (DSPC), (iv) 15-45 mol % cholesterol, and (v) 0.5-10 mol % of a PEG-modified lipid, wherein the mol % is based on 100% total moles of lipids in the lipid particle; and the cationic lipid comprises a head group, two hydrophobic tails, and a central moiety to which the head group and the two hydrophobic tails are directly bonded, wherein (a) the central moiety is a central carbon or nitrogen atom; (b) each hydrophobic tail independently has the formula -(hydrophobic chain)-(ester group)-(hydrophobic chain), wherein the ester group is —OC(O)— or —C(O)O—; and (c) for at least one hydrophobic tail, (I) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the ester group; (II) the hydrophobic tail has the formula — R^{12}-M^1-R^{13}, wherein R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the ester group, and R^{13} is a branched C_{10}-C_{20} alkyl; (III) the total carbon atom content of the tail — R^{12}-M^1-

R^{13} is 21 to 26; and (IV) the ester group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms.

Claim 18: A method for preparing a lipid particle mixture comprising mixing a first solution comprising an organic solvent, a cationic lipid, distearoylphosphatidylcholine (DSPC), cholesterol, and a PEG-modified lipid, with a second solution comprising a nucleic acid and water to form a mixture containing lipid particles, wherein each lipid particle comprises (i) the nucleic acid, (ii) 35-65 mol % of the cationic lipid, (iii) 3-12 mol % distearoylphosphatidylcholine (DSPC), (iv) 15-45 mol % cholesterol, and (v) 0.5-10 mol % of the PEG-modified lipid, and wherein the mol % is based on 100% total moles of lipids in the lipid particle, and the cationic lipid comprises a head group, two hydrophobic tails and a central moiety to which the head group and the two hydrophobic tails are directly bonded, wherein (a) the central moiety is a central carbon or nitrogen atom; (b) each hydrophobic tail independently has the formula -(hydrophobic chain)-(ester group)-(hydrophobic chain), wherein the ester group is —OC(O)— or —C(O)O—; and (c) for at least one hydrophobic tail, (I) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl, where the branch-

ing occurs at the α-position relative to the ester group; (II) the hydrophobic tail has the formula — R^{12}-M^1-R^{13}, wherein R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the ester group, R^{13} is a branched C_{10}-C_{20} alkyl; (III) the total carbon atom content of the tail — R^{12}-M^1-R^{13} is 21 to 26; and (IV) the ester group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms.

UNITED STATES COURT OF APPEALS
FOR THE FEDERAL CIRCUIT

CERTIFICATE OF INTEREST

Case Number 2023-2357

Short Case Caption Alnylam Pharmaceuticals, Inc. v. Moderna, Inc.

Filing Party/Entity Alnylam Pharmaceuticals, Inc.

Instructions:

1. Complete each section of the form and select none or N/A if appropriate.
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I certify the following information and any attached sheets are accurate and complete to the best of my knowledge.

Date: 12/04/2023

Signature: /s/ William G. Gaede, III

Name: William G. Gaede, III

1. Represented Entities. Fed. Cir. R. 47.4(a)(1).	**2. Real Party in Interest.** Fed. Cir. R. 47.4(a)(2).	**3. Parent Corporations and Stockholders.** Fed. Cir. R. 47.4(a)(3).
Provide the full names of all entities represented by undersigned counsel in this case.	Provide the full names of all real parties in interest for the entities. Do not list the real parties if they are the same as the entities. ☑ None/Not Applicable	Provide the full names of all parent corporations for the entities and all publicly held companies that own 10% or more stock in the entities. ☑ None/Not Applicable
Alnylam Pharmaceuticals, Inc.		

☐ Additional pages attached

4. Legal Representatives. List all law firms, partners, and associates that (a) appeared for the entities in the originating court or agency or (b) are expected to appear in this court for the entities. Do not include those who have already entered an appearance in this court. Fed. Cir. R. 47.4(a)(4).

☐ None/Not Applicable ☐ Additional pages attached

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5. Related Cases. Other than the originating case(s) for this case, are there related or prior cases that meet the criteria under Fed. Cir. R. 47.5(a)?

☑ Yes (file separate notice; see below) ☐ No ☐ N/A (amicus/movant)

If yes, concurrently file a separate Notice of Related Case Information that complies with Fed. Cir. R. 47.5(b). **Please do not duplicate information.** This separate Notice must only be filed with the first Certificate of Interest or, subsequently, if information changes during the pendency of the appeal. Fed. Cir. R. 47.5(b).

6. Organizational Victims and Bankruptcy Cases. Provide any information required under Fed. R. App. P. 26.1(b) (organizational victims in criminal cases) and 26.1(c) (bankruptcy case debtors and trustees). Fed. Cir. R. 47.4(a)(6).

☑ None/Not Applicable ☐ Additional pages attached

N/A		

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STATEMENT OF RELATED CASES

This is an appeal from a final judgment of the United States District Court for the District of Delaware, case numbers 1:22-cv-00335-CFC and 1:22-cv-00925-CFC. The Court's decision in this case may affect a pending case in the United States District Court for the District of Delaware, *Alnylam Pharmaceuticals, Inc. v. Pfizer Inc.*, *et al.*, 22-cv-336-CFC, where Alnylam accuses Pfizer Inc., Pharmacia & Upjohn Co. LLC, BioNTech SE, and BioNTech Manufacturing GmbH (collectively, "Pfizer") of infringing U.S. Patent Nos. 11,246,933 ("the '933 Patent") and 11,382,979 ("the '979 Patent"). The Court's decision may also affect pending cases in the United States District Court for the District of Delaware, *Alnylam Pharmaceuticals, Inc. v. Moderna, Inc.*, *et al.*, 23-cv-580-CFC and *Alnylam Pharmaceuticals, Inc. v. Pfizer Inc.*, *et al.*, 23-cv-578-CFC, where Alnylam accuses Appellees and Pfizer, respectively, of infringing one or more of related U.S. Patent Nos. 11,590,229 ("the '229 Patent"), 11,612,657 ("the '657 Patent"), 11,633,479 ("the '479 Patent"), and 11,633,480 ("the '480 Patent").

JURISDICTIONAL STATEMENT

This is an appeal from a stipulated final judgment entered on August 30, 2023, in a patent case following the district court's August 21, 2023 claim construction order. Appx1-2; Appx3-6; Appx10. The district court had jurisdiction under 28 U.S.C. §§ 1331 and 1338. Alnylam Pharmaceuticals, Inc. timely filed its notice of appeal on August 31, 2023. This Court has jurisdiction under 28 U.S.C. § 1295(a).

INTRODUCTION

This appeal rests on claim construction and poses a single question: Under this Court's exacting standards for lexicography, did the patentees express reasonable clarity, deliberateness, and precision to restrict and narrow the terms "branched alkyl" and "branched C_{10}-C_{20} alkyl" when the claim language, the patent specification, and the prosecution history contradict such narrowing? This Court's precedent says no.

Appellant Alnylam Pharmaceuticals, Inc. ("Alnylam") proposed that these terms carry their full ordinary and customary meanings consistent with their usage in the intrinsic record. The terms relate to the portion of Alnylam's novel lipids, referred to as the tail, which contains "branched alkyls" at the alpha (first) position next to the biodegradable portion (moiety) of the tail. Specifically, and to encompass this plain meaning, Alnylam proposed (1) "branched alkyl" means "a saturated hydrocarbon moiety that is not a straight chain" and (2) "branched C_{10}-C_{20} alkyl" means "a saturated hydrocarbon moiety that has 10 to 20 carbons and is not a straight chain." The district court agreed that alkyls are saturated hydrocarbon moieties, but construed the terms to further require that the branched alpha carbon atom in the alkyl group must be bound to *at least three* other carbon at-

oms, reading out from the plain claim language that the alpha-branched alkyl group may be bound to two carbons atoms.[1]

In so doing, the district court erred in elevating *a portion of a single sentence* in the 540-column long patent specification to a lexicographic fiat. Specifically, the district court relied upon the following sentence: "[u]nless otherwise specified, the terms 'branched alkyl', 'branched alkenyl,' and 'branched alkynyl' refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group." That sentence, however, with its qualifier—"unless otherwise specified"—when read in context of the rest of the intrinsic evidence shows that the patentees did not intend to narrow the terms as the district court did.

Tellingly, the district court's narrow construction excludes disclosed alpha alkyl branched embodiments and formulas identified as part of the "present invention," and alpha branched alkyl structures binding to two

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[1] The district court construed "branched alkyl" as "[a] saturated hydrocarbon moiety group in which one carbon atom in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group." Appx3-4. Similarly, the court construed "branched C_{10}-C_{20} alkyl" as "A saturated hydrocarbon moiety group with 10 to 20 carbon atoms and in which one carbon atom in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group." Appx4. A carbon atom may bind to up to four other atoms. *See* Appx4953.

carbons that the patentees expressly represented during prosecution as falling within the scope of the claims. Accordingly, the district court's construction should be rejected under lexicography's exacting standards and the terms' ordinary and customary meanings as proposed by Alnylam should govern.

To the extent this Court finds lexicography, it should hold that the full definition applies (*i.e.*, including the "unless otherwise specified"), and that consistent with the language and drawings, find that the claims, the written description and prosecution history specifies that a branched alkyl at the alpha position may include structures where the alpha branched alkyl group binds to only two carbon atoms. Here too, the district court erred by applying an overly exacting standard that effectively disregarded the intrinsic record's rich teachings that in the context of an alpha branched carbon next to the biodegradable group, the intrinsic record specifies that two-carbon binding is within the claim scope.

The stipulated judgment of non-infringement, which is based solely on the district court's erroneous claim construction, should be vacated.

ISSUE PRESENTED FOR REVIEW

The principal issue presented is whether the district court erred in limiting the terms "branched alkyl" and "branched C_{10}-C_{20} alkyl" through lexicography to require one carbon atom in the group to be bound to at

least three other carbon atoms, where the claims are not so limited, and the specification and prosecution history specify numerous examples of branching where a carbon atom is bound to two or three other carbon atoms.

STATEMENT OF THE CASE

A. Factual Background

1. Alnylam was the first to obtain FDA approval of an RNA therapeutic.

Alnylam is a recognized pioneer in the development of RNA therapeutics and RNA delivery technology. Beginning more than a decade before the COVID pandemic, Alnylam began developing—and in time—patenting its critical lipid nanoparticle ("LNP") delivery technology for the safe and effective transport of delicate RNA materials to human cells. In 2018, Alnylam utilized LNP technology to deliver the world's first approved RNAi therapeutic, ONPATTRO® (patisiran). ONPATTRO® is currently approved for the treatment of polyneuropathy (a nervous system disorder) caused by an illness called hereditary ATTR (hATTR) amyloidosis. Appx744-745.

Alnylam has also developed an additional delivery modality distinct from its LNP technology, termed GalNAc Delivery, which is utilized in four marketed products, GIVLAARI® (givosiran), approved in 2019, and

OXLUMO® (lumasiran), approved in 2020, and AMVUTTRA® (vutrisiran), approved in 2022, all marketed by Alnylam, and LEQVIO® (inclisiran), approved in 2021, developed initially by Alnylam and licensed to Novartis.

2. The '933 and '979 Patents cover technology underlying RNA therapeutics.

Alnylam's '933 Patent and '979 Patent (collectively, the "Patents-in-Suit") cover groundbreaking technology that allowed for the development of RNA-based vaccines and therapies, including Appellee Moderna's COVID-19 vaccine. The promise of nucleic acids as therapeutics was recognized long ago, but the ability to safely and effectively deliver the fragile nucleic acids to cells in the body remained elusive. The core innovation of the Patents-in-Suit is a class of improved cationic lipids[2] that can effectively deliver nucleic acid payloads and then biodegrade to aid in elimination from the body.

Nucleic acids are the primary way cells store and transmit genetic information. Appx5031. The two main classes of nucleic acids are DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). *Id.* Beginning in the 1980s, scientists sought to develop medicines, including vaccines, using

[2] The district court construed "cationic lipid" to carry its "[p]lain and ordinary meaning, which is 'a lipid that is positively charged or that may be protonated at physiological pH.'" Appx3. That construction is not at issue in this appeal.

nucleic acids. Appx717. But there was a challenge: nucleic acids are fragile, making it difficult to deliver them to the target in the body without degradation. Appx741. So, scientists had to develop a way to protect the nucleic acids—essentially to package them for safe delivery to the target. Appx717.

Scientists knew that certain naturally occurring lipids form cell membranes, which act as protective bubbles around our cells. A key feature of these naturally occurring protective membranes is that their hydrophobic tails and hydrophilic head groups allow the lipids to self-assemble in particular orientations in a process called aggregation. The hydrophobic tails are repelled from the water and arrange themselves on the inside of bilayers, while the head groups, which are less hydrophobic than the tails, preferentially interact with the water.

These naturally occurring lipids are negatively charged, which makes them incompatible for use with nucleic acids, because nucleic acids are also negatively charged and molecules with the same charge repel each other. Therefore, while scientists could appreciate the desirable properties of the naturally occurring lipids, the nucleic acids would repel these lipids. To address this, scientists began to develop lipids that could be positively charged, *i.e.*, cationic lipids, for the delivery of nucleic acids. Appx718. These lipids had the same core features of the naturally occurring nega-

tively charged lipids, including tails and head groups, and in their initial formulation carried a permanent positive charge. These permanently positively charged lipids, however, failed to provide a safe and effective delivery mechanism due to toxicity and other in vivo issues. *Id.*

To address the toxicity and other issues, scientists, like the patentees of the Patents-in-Suit, began to develop lipids where the extent of the positive charge depended on the pH of the solution around the lipids. Appx718. Different parts of cells have different pH ranges, so scientists focused on calibrating lipids to have limited to no charge when it was beneficial, but also be charged at other times, to aid in the delivery of the nucleic acid payload to cells in the body. Appx719, Appx744. Unfortunately, issues with toxicity and other in vivo properties persisted. The inventions in the Patents-in-Suit address these issues through a new class of cationic lipids that are biodegradable and contain specified branching of the tails relative to the biodegradable group. Appx747.

a) The claimed cationic lipids

The claimed cationic lipids are comprised of three parts: the head group, the central moiety, and at least two biodegradable hydrophobic tails. One or more of the tails is branched ("branched alkyl") at the alpha

position[3] next to the biodegradable group in the tail.[4] *See, e.g.*, Appx320 ('933 Patent) at 538:13-38. The branched alkyl allows for a higher number of total carbons in the tail for a given tail length, for achieving optimal properties, while the biodegradable group facilitates elimination of the cationic lipid from the body after delivery of the nucleic acid. Appx52 ('933 Patent) at 1:61-2:6.

These biodegradable cationic lipids, in combination with three other lipids, encapsulate the nucleic acids in LNPs. *See, e.g.*, Appx675 ("a neutral phospholipid, cholesterol, a polyethylene-glycol (PEG)-lipid, and an ionizable cationic lipid"); Appx675; Appx2635-2636 ('979 Patent) at claims 1, 18. These LNPs can be used to safely and effectively deliver nucleic acids, like the mRNA in Moderna's COVID-19 vaccine, through the human body. Appx701, Appx711-712.

Claim 18 of the '933 Patent is illustrative and provides:

> A cationic lipid comprising a primary group and two biodegradable hydrophobic tails, wherein

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[3] "Alpha position" is also written as "α-position."

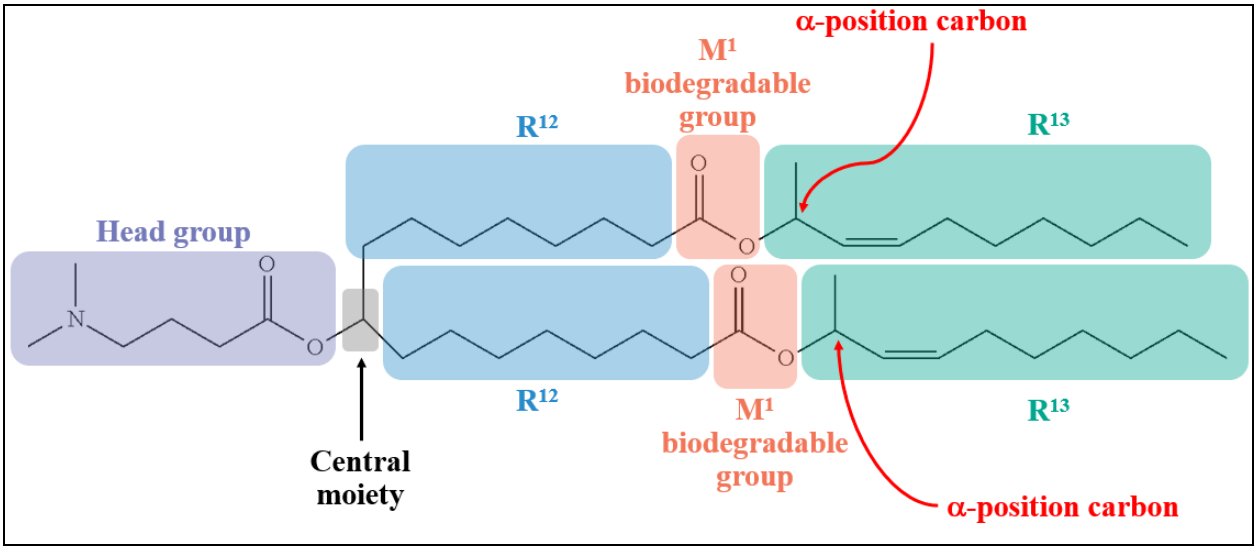
[4] As agreed by the parties, "alpha branching" means "where the branching occurs at a carbon atom next to the [biodegradable/ester] group." Appx5501.

> the primary group comprises (i) a head group that optionally comprises a primary, secondary, or tertiary amine, and (ii) a central moiety to which the head group and the two biodegradable hydrophobic tails are directly bonded;
>
> the central moiety is a central carbon or nitrogen atom;
>
> each biodegradable hydrophobic tail independently has the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), wherein the biodegradable group is —OC(O)— or —C(O)O—;
>
> for at least one biodegradable hydrophobic tail, the terminal hydrophobic chain in the biodegradable hydrophobic tail is a *branched alkyl, where the branching occurs at the α-position relative to the biodegradable group* and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, *M^1 is the biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl*, and the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26;
>
> in at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms; and
>
> the lipid has a pKa in the range of about 4 to about 11 and a logP of at least 10.1.

Appx320 (emphases added); *see also* Appx2635-2636 ('979 Patent) at claims 1, 18.[5]

[5] Claim 1 of the '979 Patent recites a lipid particle including the claimed cationic lipid, while Claim 18 of the '979 Patent recites a method of preparing a lipid particle mixture including the claimed cationic lipid.

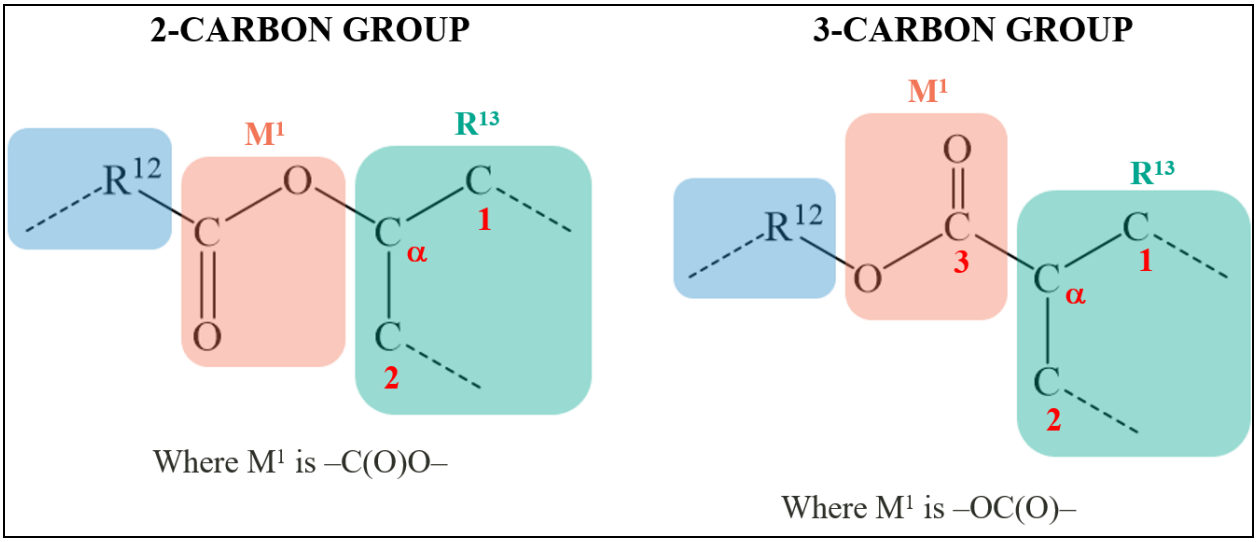
The structure of the at least one biodegradable hydrophobic tail includes a "branched alkyl, where the branching occurs at the α-position relative to the biodegradable group and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}." Appx320 ('933 Patent) at 538:27-29. This "branched alkyl" structure, which is modified by the requirement that the branching occur at the alpha position relative to (next to) the biodegradable group, can be understood with reference to the following exemplary branched alkenyl[6] from the specification annotated below:



Appx68 ('933 Patent) at 34:32-43; Appx69 (36:4, 36:58); Appx81 (60:50-65); Appx5652.

[6] As explained in the Patents-in-Suit, an "alkyl" differs from an "alkenyl" due to the alkenyl having one or more carbon-carbon double bonds, whereas the alkyl has no carbon-carbon double bonds. Appx257 ('933 Patent) at 411:53-54, 411:62-64. For purposes of this example, that difference is immaterial.

As claimed, "M^1 is the biodegradable group" and has the formula —OC(O)— or —C(O)O— (depicted above). Appx320 ('933 Patent) at 538:23-24. R^{13} is a branched C_{10}-C_{20} alkyl "where the branching occurs at the α-position relative to the biodegradable group [M^1]." Appx320 ('933 Patent) at 538:27-29. Thus, depending on the formula of M^1, —OC(O)— or —C(O)O—, the carbon in R^{13} at the alpha position may be bound to two (as depicted above) or three other carbon atoms (also referred to herein as a "2-carbon group" or "3-carbon group," respectively).[7] This is illustrated below:



Appx5647 (annotated).

[7] It is also possible for the alpha-branched carbon to bind to four carbons if the biodegradable group contains the formula —OC(O)— and branches into three carbon chains consistent with the claim language.

The Patents-in-Suit include dependent claims directed specifically to —C(O)O— as the M^1 group and accordingly expressly contemplate a 2-carbon group. For example, Claim 20 of the '933 Patent recites:

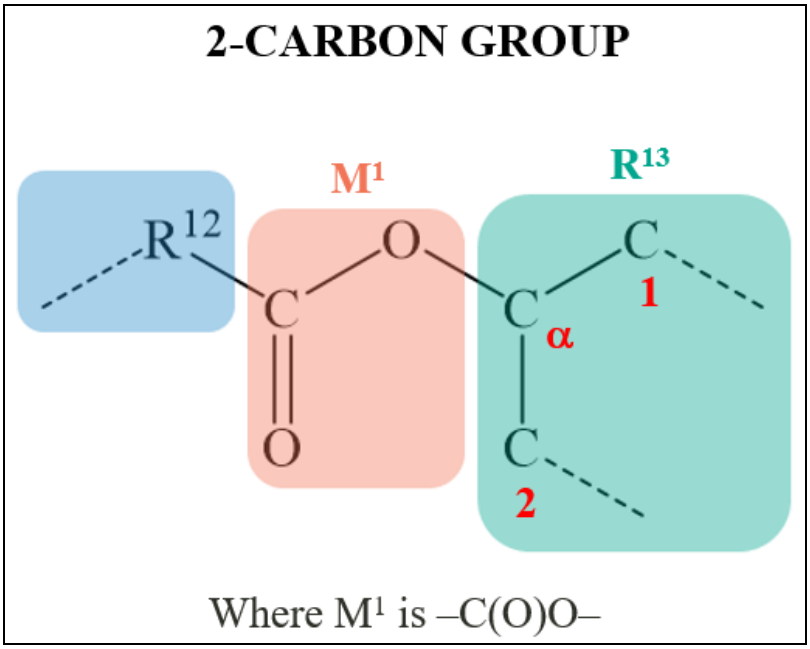
> The cationic lipid of claim 18, wherein the *biodegradable group is — C(O)O—*.

Appx320 ('933 Patent) at 538:41-42 (emphasis added).

Claim 5 of the '979 Patent recites:

> The lipid particle of claim 1, wherein *the ester group is — C(O)O—*.

Appx2635 ('979 Patent) at 494:52-53 (emphasis added). This structure is the structure depicted above on the left, *viz*.:



Thus, Claim 20 of the '933 Patent and Claim 5 of the '979 Patent expressly specify a branched alkyl group where the alpha carbon is bound to only two other carbons.

Similarly, the Patents-in-Suit include dependent claims directed specifically to —O(C)O— as the M^1 group and accordingly expressly contemplate a 3-carbon group. For example, Claim 19 of the '933 Patent recites:

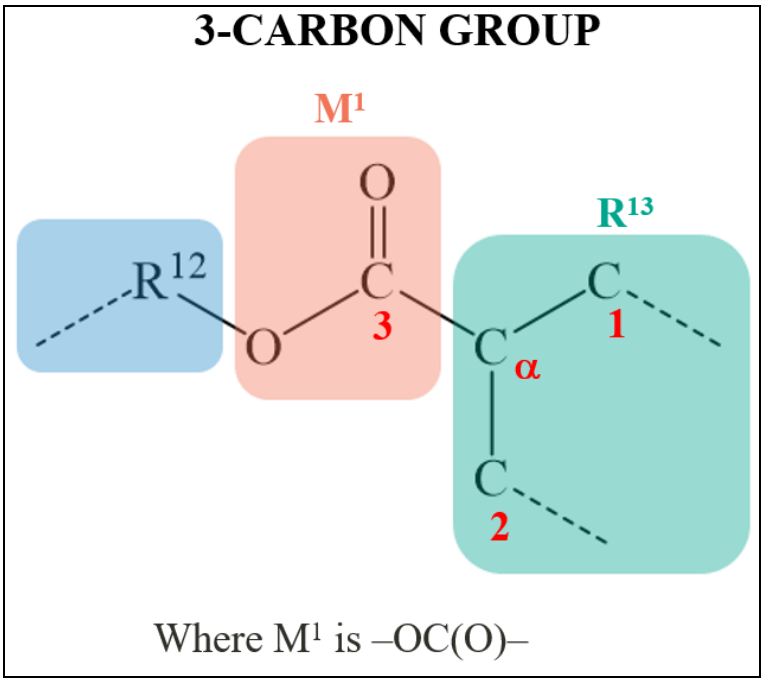
> The cationic lipid of claim 18, wherein the *biodegradable group is —OC(O)—*.

Appx320 ('933 Patent) at 538:39-40 (emphasis added).

Claim 4 of the '979 Patent recites:

> The lipid particle of claim 1, wherein *the ester group is —OC(O)—*.

Appx2635 ('979 Patent) at 494:49-50 (emphasis added). This structure is depicted above on the right, *viz*.:



In another instance, the patentees claimed the specific number of carbons at the point of branching. For example, dependent Claim 14 (not asserted in the case) claims a 3-carbon group:

> The cationic lipid of claim 1, wherein the branched alkyl group has only one carbon atom which is bound to three other carbon atoms.

Appx320 ('933 Patent, claim 14). No such limitation is found in the independent claims.

The independent claims of the Patents-in-Suit thus allow for both a 2-carbon group and a 3-carbon group, depending on the structure of M^1.

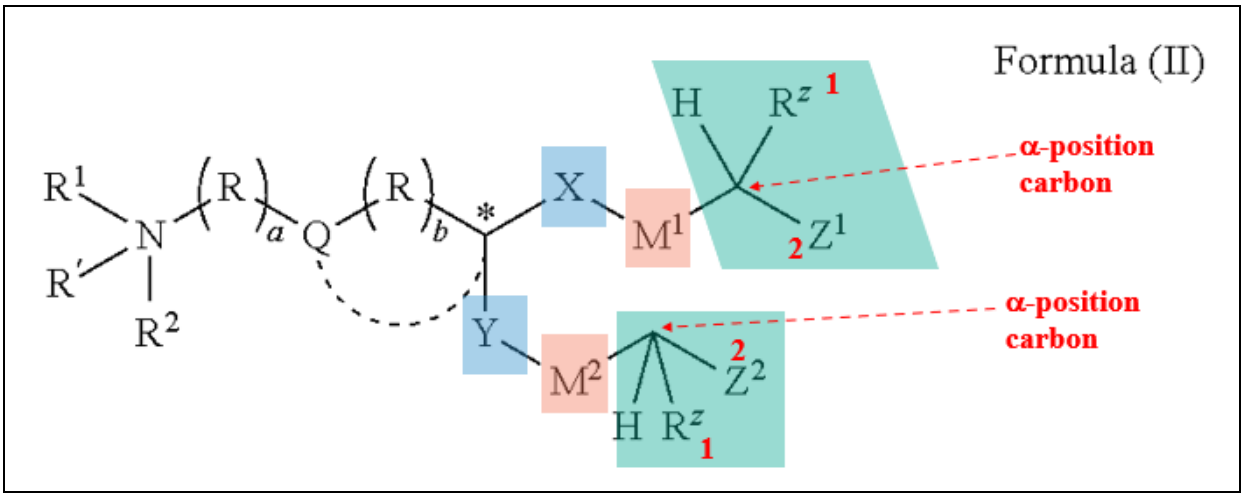
b) The written description.

The written description begins by explaining that the "present invention relates to a cationic lipid and PEG lipid suitable for forming nucleic

acid-lipid particles." Appx52 ('933 Patent) at 1:61-2:2. These lipid particles may be used for delivering an active agent such as a nucleic acid. The written description thereafter describes cationic lipids of Formulas (I) through (VIII). Formulas (I), (II), and (VIII) depict branching at the alpha position.[8]

The written description describes what an alpha "branched alkyl" is in the context of the Formulas, which depict alpha branched 2-carbon groups. For example, Formula (II) discloses "branched alkyl at the alpha position adjacent to the biodegradable group" (Appx53 ('933 Patent) at 3:63-67). The formula discloses two carbons binding to the branched alpha-position carbon if M1 is —C(O)O— or three carbons binding to the branched alpha position carbon if M1 is —O(C)O—. The branched alkyl groups are annotated in green below:

[8] The other formulas do not expressly depict branching at the alpha position. Specifically, in Formulas (III), (IIIA), and (IV), the branching is not at the alpha position and Formulas (V), (VIA), (VIB), and (VII) do not have branched alkyls. Formula (VIII) has asymmetrical hydrophobic groups but is a generic depiction that may cover formula (I)-(VII) and accordingly would be the same as Formulas (I) and (II) for "branched alkyl."



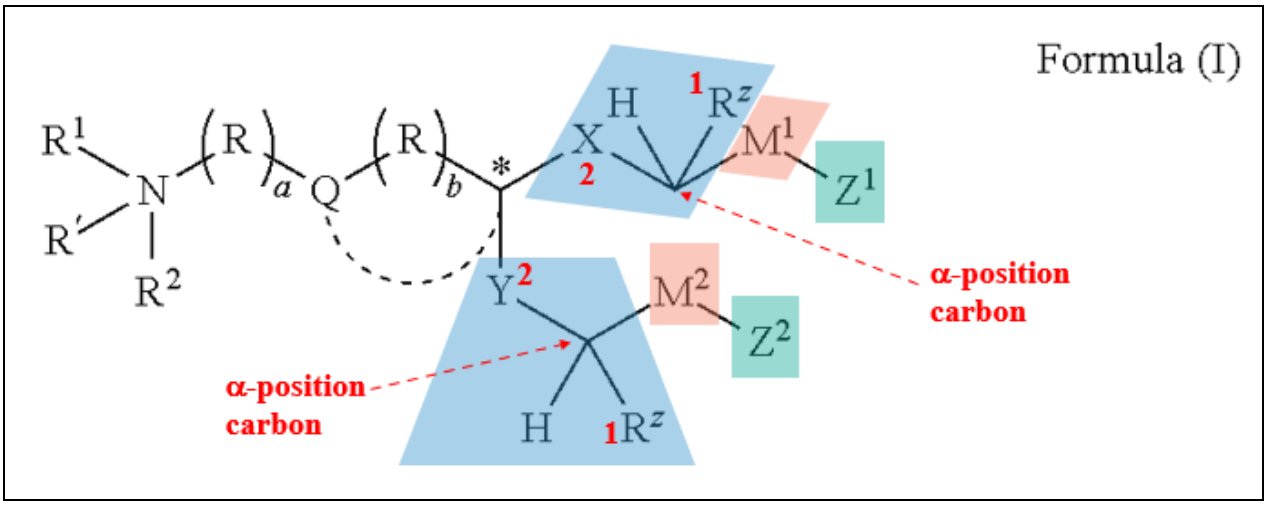
Appx53 ('933 Patent) at 4:1-10 (annotated); Appx4515 (Certificate of Correction); Appx5096.[9]

Like the patents' claims, in Formula (II), M^1 is "a biodegradable group (e.g., (—OC(O)— or —C(O)O—, …)." Appx54 ('933 Patent) at 5:1-18. Where M^1 is —C(O)O—, the carbon at the alpha position to M^1 is attached to two other carbons, Z^1 and R^z, as well as a hydrogen (H) atom, as the Formula depicts above. Appx54 ('933 Patent) at 5:18 ("each occurrence of

[9] While Formulas (I) and (II) fall outside the asserted claims, they are representative of structures consistent with the language used in the claims and describe the two-carbon binding structures as "branched alkyl at the alpha position adjacent to the biodegradable group." Like the claimed structures, Formula (II) includes R^{12}-M^1-R^{13} with branching in R^{13} at the alpha position adjacent to M^1. *See* Appx53 ('933 Patent) at 4:1-10; Appx4515 (Certificate of Correction). Formula (I) includes branching at the alpha position adjacent to M^1, however that branching is in R^{12}—not R^{13}. Appx52 ('933 Patent) at 2:11-20; Appx4514 (Certificate of Correction).

R^z is, independently, C_1-C_8 alkyl"), 5:22 ("Z^1 and Z^2 are each independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl").

Similarly, with reference to Formula (I), the specification expressly states that it "has a branched alkyl at the alpha position adjacent to the biodegradable group" (Appx52 ('933 Patent) at 2:7-11), and sets forth the formula, provided below with the branched alkyl annotated in blue.

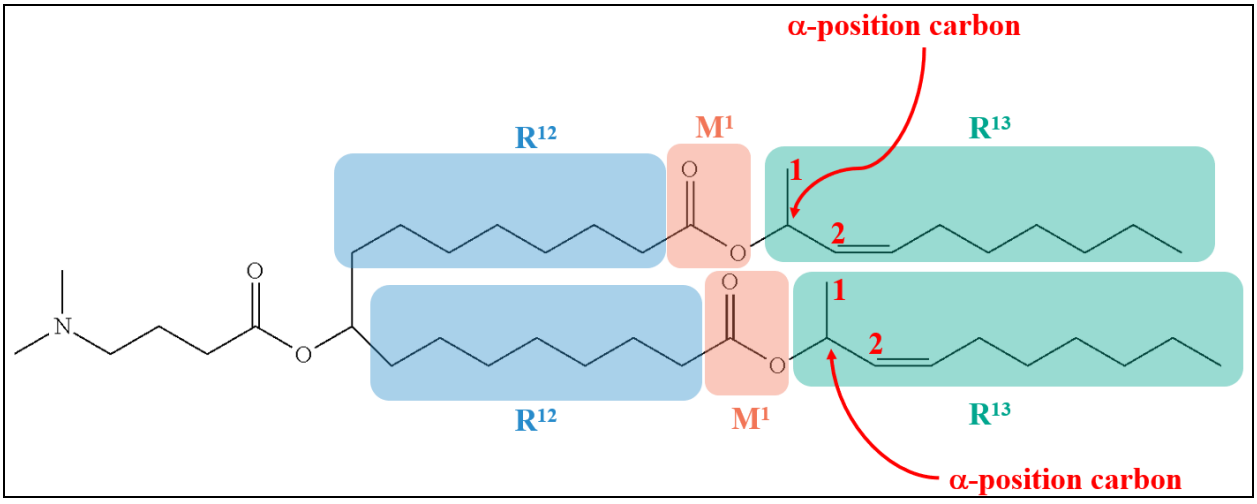


Appx52 ('933 Patent) at 2:11-20 (annotated); Appx4514 (Certificate of Correction); Appx5096.

Again, this formula depicts branching where one carbon is bound to two other carbons and a hydrogen (H) atom (as reflected in the blue above), when M^1 or M^2 is —OC(O)—. Appx53 ('933 Patent) at 3:3-4 ("M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—. . .")). The first atom in each of "X" and "Y" is a carbon, respectively, as is the first atom in "R^z." Appx53 ('933 Patent) at 3:1-2 ("X and Y are

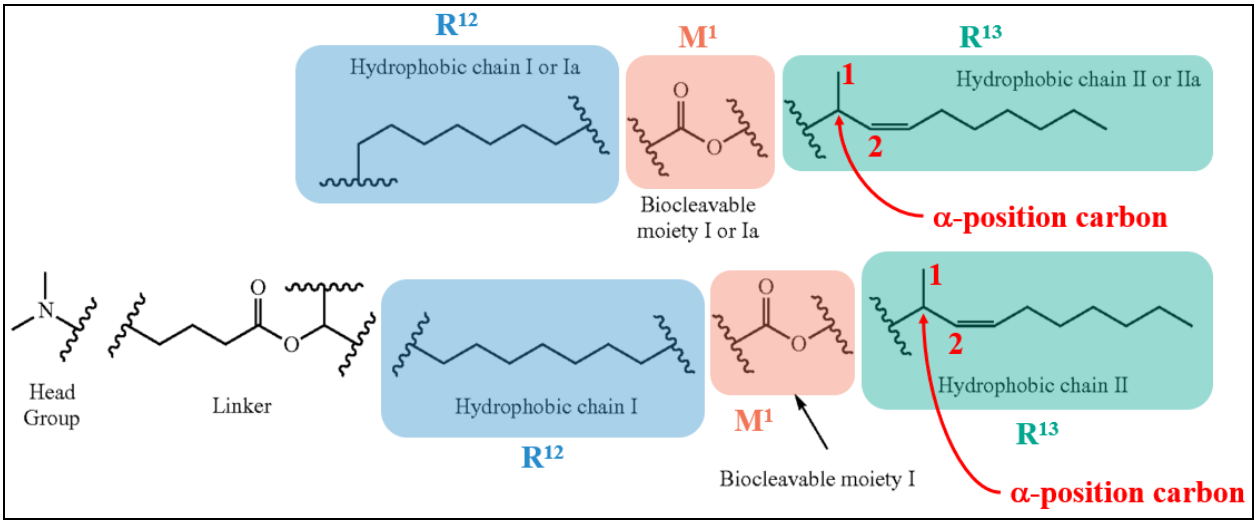
each, independently, alkylene . . .); 3:21-22 ("each occurrence of R^z is, independently, C_1-C_8 alkyl (e.g., methyl, ethyl, isopropyl . . ."). Thus, when M^1 or M^2 is —OC(O)—, the portion of the tail in Formula (I) annotated above in blue is a 2-carbon group that binds to the first oxygen in the —OC(O)— group, as shown above.

After describing the Formulas disclosing two or three carbon "branched alkyl" groups at the alpha position, the specification provides a "detailed description" of the invention. Appx66 ('933 Patent) at 29:62-64. Under the section "The Cationic Lipid" (*id*. at 30:7), the specification discloses various embodiments of the cationic lipid containing a 2-carbon group. For example, "Compound 1" (annotated below) depicts branching at the alpha position adjacent to the biodegradable group where a carbon is attached to two other carbons:



Appx68 ('933 Patent at 34:32-43) (annotated). Again, M^1 is —C(O)O— and at the alpha position next to the M^1 biodegradable group is a carbon branched to two other carbons, as well as a hydrogen atom (not shown). This 2-carbon group structure is shown again in columns 35 and 36, a third time in columns 37 and 38 of the patent specification, and a fourth time at columns 59 and 60.

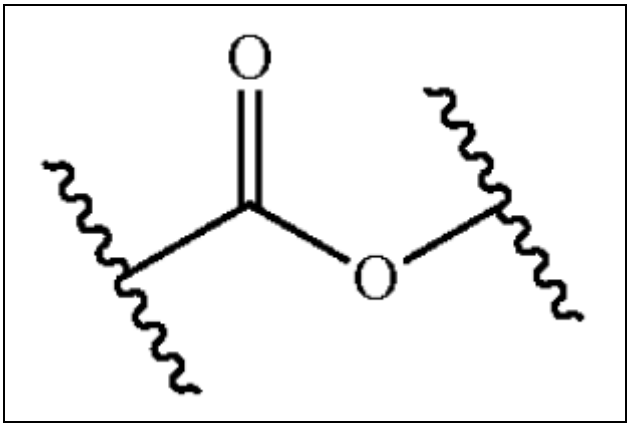
Using this compound as an exemplary structure, the specification explains that this compound "can be thought of as a combination of a headgroup, a central moiety, and two hydrophobic groups as follows:"



Appx82 ('933 Patent) at cols. 61-62, lines 1-20 (annotated). As depicted above and disclosed in the written description various combinations of "Head Group," "Linker," "Hydrophobic Chain," and "Biocleavable moiety" are contemplated. Following this exemplary structure, the written description states "[t]he *present invention* includes compounds composed of *any*

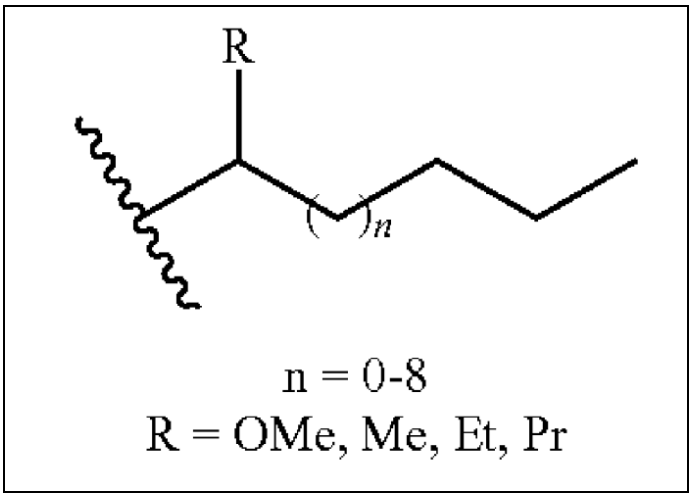
combination of the head, linker, hydrophobic chain I, and hydrophobic chain II groups listed below." *Id.* at 61:22-26 (emphases added).

Further elaborating on the "present invention," the specification then depicts representative chains and moieties that may be combined to comprise the tails. Table 2D discloses "[r]epresentative biodegradable moieties I and/or Ia and combinations thereof" (M^1) of the "present invention," including the —C(O)O— structure (reproduced below).

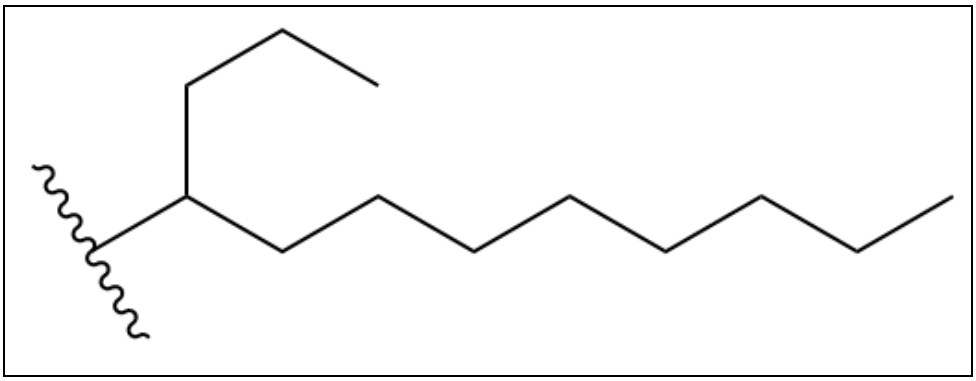


Appx87 ('933 Patent) at 72:1-10.

These biodegradable moieties may be combined with the terminal "[r]epresentative hydrophobic chain II and/or II hydrophobic chains" (R^{13}) of the "present invention" that are depicted in Table 2E, which include:

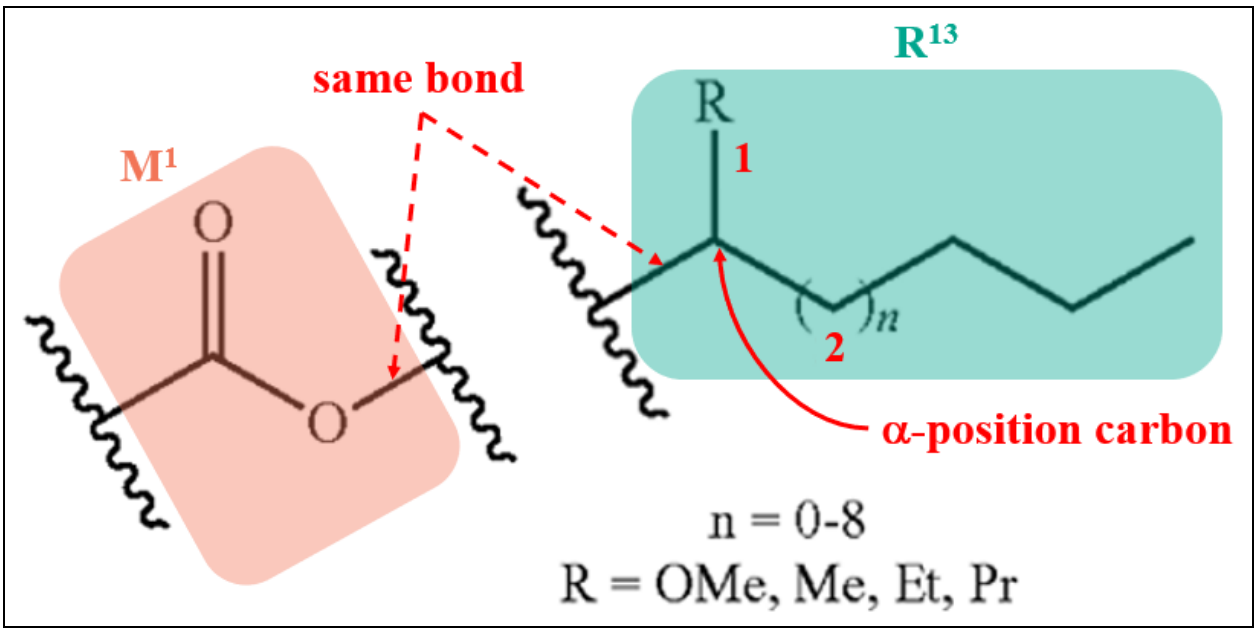


Appx88 ('933 Patent) at 74:52-57 (where R could be a chain of one, two, or three carbons without additional branching); Appx4952 (showing structures for Me, Et and Pr). This formula depicts an alpha-branched chain of up to 15 carbons, where (like the others discussed above) the branching point is a carbon at the alpha position connected to two other carbons. An example (appx4499) where R is Pr (a C_3 alkyl chain) and n is 5 is:



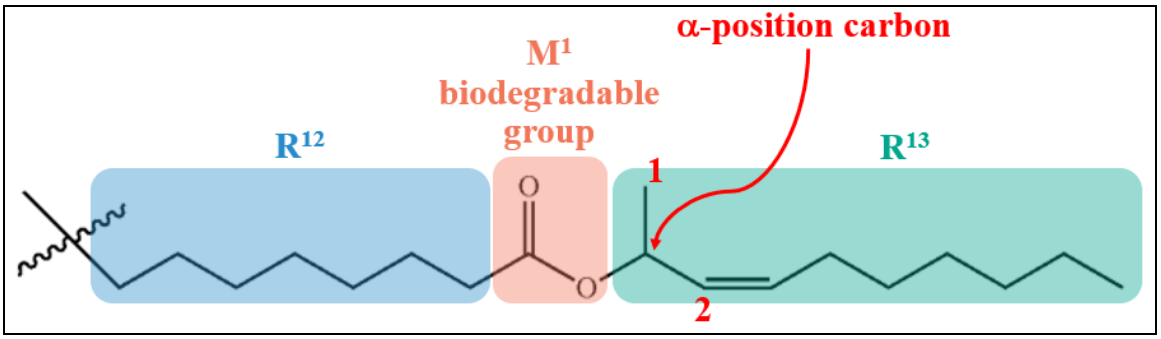
Thus, taking these representative structures together the written description "of the present invention" expressly discloses a "combination" of a

biodegradable moiety (M^1) and hydrophobic chain (R^{13}) that specifies an alpha branched carbon bound to two other carbons:



Appx87 ('933 Patent) at 72:1-10; Appx88 ('933 Patent) at 74:52-57 (annotated above with numbers at two carbons in red and to show how these moieties combine).

Other hydrophobic groups of the "*present invention*" (Appx70 ('933 Patent) at col 37:21-26) are included in Table 1C of the Patents-in-Suit, which again includes an alpha branched alkyl 2-carbon hydrophobic chain (R^{13}) following a —C(O)O— structure in M^1. Appx73, Appx76 ('933 Patent) at cols. 43-44, 49-50(annotated).

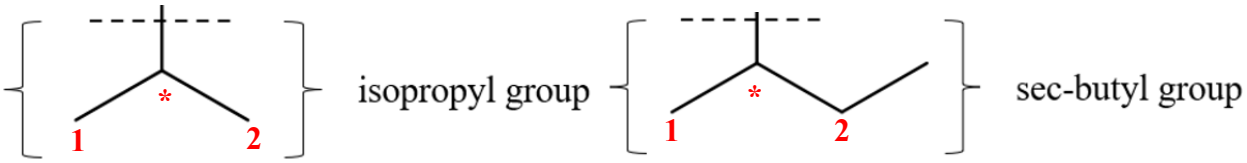


The text of the written description includes examples of branched alkyls commonly understood to have a point of branching where a carbon is connected to two other carbons. For example, the written description in the Definitions section broadly specifies various representative branched alkyl groups:

> The terms "alkyl" and "alkylene" refer to *a straight or branched chain saturated hydrocarbon moiety*. In one embodiment, the alkyl group is a straight chain saturated hydrocarbon. Unless otherwise specified, the "alkyl" or "alkylene" group contains from 1 to 24 carbon atoms. Representative saturated straight chain alkyl groups include methyl, ethyl, n-propyl, n-butyl, n-pentyl, and n-hexyl. *Representative saturated branched alkyl groups include isopropyl, sec-butyl, isobutyl, tert-butyl, and isopentyl*.

Appx257 ('933 Patent) at 411:53-61 (emphases added). As shown from the quoted text above, the written description lists "isopropyl" and "sec-butyl" as examples of a representative branched alkyl group. Appx257 ('933 Patent) at 411:60-61. In each of these branched alkyl groups, a carbon at the point of branching is bound to only two other carbons, as shown below. *See*

Appx4952-4953 (illustrating the chemical structure for isopropyl and sec-butyl with the branch point indicated by "*").



Thus, the written description repeatedly discloses cationic lipid structures with alpha branched alkyl 2-carbon groups.

The written description in the Definitions section further states:

> Unless otherwise specified, the terms "branched alkyl", "branched alkenyl," and "branched alkynyl" refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group.

Appx257 ('933 Patent) at 412:13-18.

c) The prosecution history.

The prosecution history specifies that the claimed cationic lipids include an alpha-branched 2-carbon group. During the prosecution of the '933 Patent, the examiner rejected the pending claims as anticipated by WO '493. *See* Appx4940.

In response, the applicants amended the pending claims to add the formula $-R^{12}-M^{1}-R^{13}$ and total carbon content of at least one of the biodegradable hydrophobic tails. This included pending Claim 20 that issued ul-

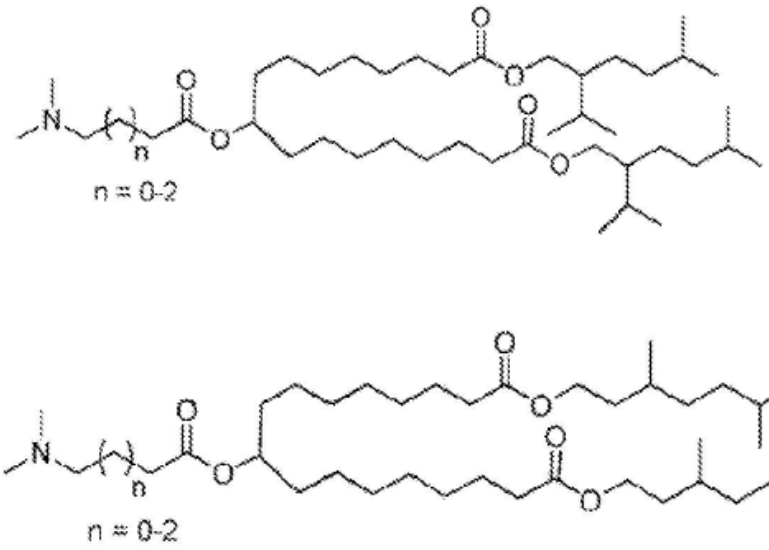
timately as Claim 18 of the '933 Patent, which is the asserted independent claim in this case:

> 20. (Currently Amended) A cationic lipid comprising a primary group and two biodegradable hydrophobic tails, wherein
>
> the primary group comprises (i) a head group that optionally comprises a primary, secondary, or tertiary amine, and (ii) a central moiety to which the head group and the two biodegradable hydrophobic tails are directly bonded;
>
> the central moiety is a central carbon or nitrogen atom;
>
> each biodegradable hydrophobic tail independently has the formula –(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), wherein the biodegradable group is -OC(O)- or -C(O)O-;
>
> for at least one biodegradable hydrophobic tail, the terminal hydrophobic chain in the biodegradable hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group and the biodegradable hydrophobic tail has the formula $-R^{12}-M^1-R^{13}$, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is a biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl, and the total carbon atom content of the tail $-R^{12}-M^1-R^{13}$ is 21 to 26;
>
> in at least one hydrophobic tail, the biodegradable group is separated from the terminus of the hydrophobic tail by from 6 to 12 carbon atoms; and
>
> the lipid has a pKa in the range of about 4 to about 11 and a logP of at least 10.1.

Appx4911 ('311 Application File History, Oct. 14, 2021 Response to Non-Final Office Action). As shown above, the pending claims already included the language "the terminal hydrophobic chain in the biodegradable hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group." *Id. See also* Appx4940.

The applicants then identified 2-carbon groups as covered by the pending claims. Specifically, the applicants distinguished the cited prior art compounds (reproduced below). Appx4940-4941 ('311 Application File History, Oct. 14, 2021 Response to Non-Final Office Action).

Page 51 of WO '493

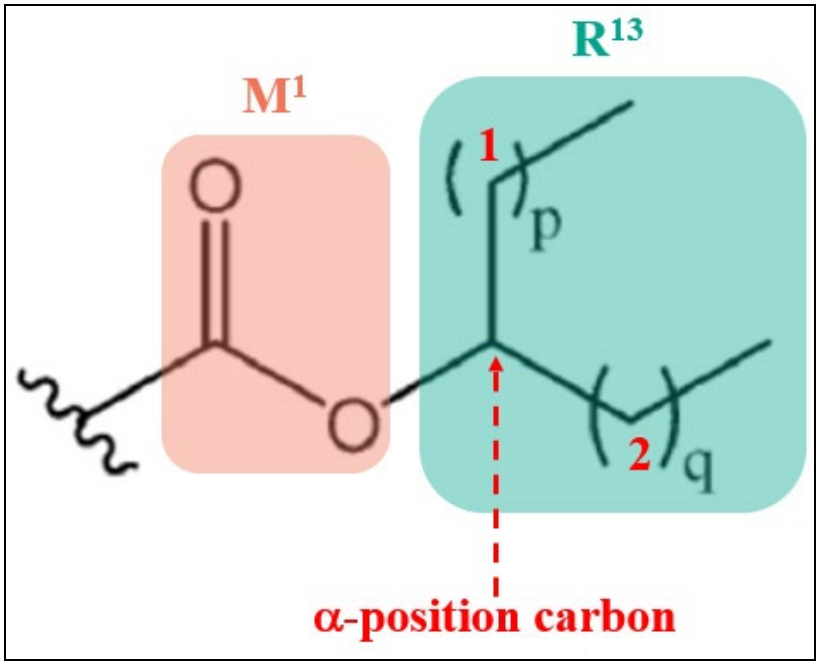


Unlike the pending claims, the prior art compounds have branching at the β- and γ-positions relative to the oxygen in the biodegradable group.[10]

The applicants explained that the prior art compounds "do not have branching in the terminal hydrophobic chain [(R^{13})] at the α-position relative to the biodegradable group [(M^1)] as recited in the pending claims. *Such compounds with branching at the α-position would have a moiety as shown below* (assuming the biodegradable group is an ester and the varia-

[10] The β- and γ-positions, refer to the second and third carbons from the biodegradable group, respectively.

bles p and q are integers).” Appx4941 (’311 Application File History, Oct. 14, 2021 Response to Non-Final Office Action) (emphases added). Applicants then set forth this “moiety”:



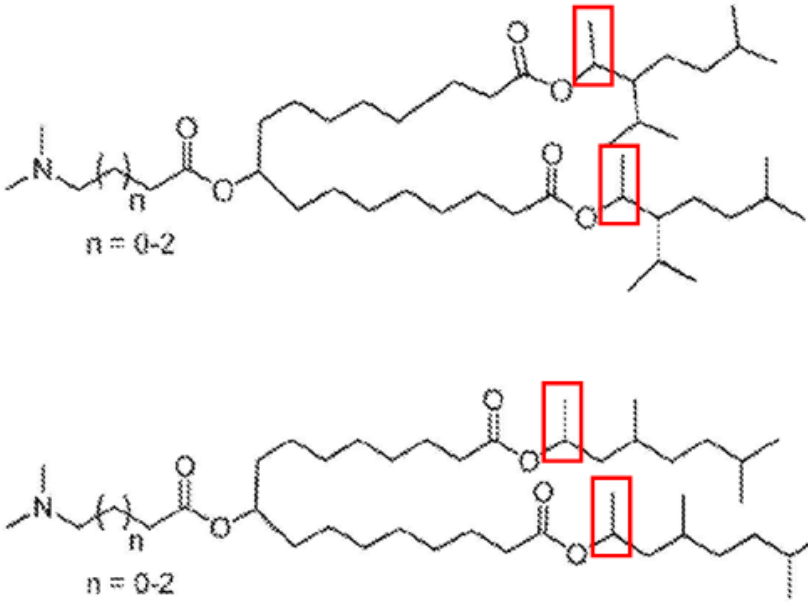
Id. (annotated to show M^1, R^{13}, and the carbon counting). This example moiety unequivocally shows an alpha-branched 2-carbon group binding to the C(O)O biodegradable group.

The applicants distinguished other prior art compounds that showed 2-carbon branched alkyl groups at the alpha position. The applicants did so based on *total carbon atom content* in the tails and not on the ground that the pending claims did not encompass two-carbon alpha branching. The compounds on page 58 of WO ’493 were distinguished on this basis.

> Claims 1 and 20 recite that the total carbon atom content of the tail -R^{12}-M^1-R^{13} is 21 to 26. Similarly, claim 14 recites that the total number of carbon atoms in the hydrophobic tail is from 21 to 26. In contrast, the total carbon contents of the tails in the compounds on page 58 of WO '493 are only 18 and 19.

Appx4941 ('311 Application File History, Oct. 14, 2021 Response to Non-Final Office Action) (highlighting added).

Page 58 of WO '493

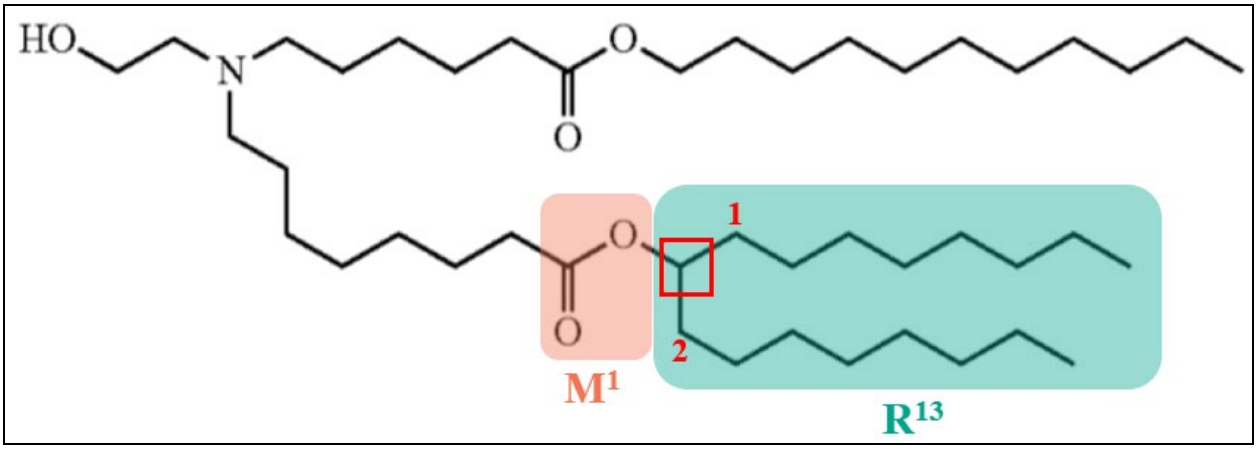


Id. (annotated). As shown above, these prior art compounds included a 2-carbon group branched at the alpha position relative to the biodegradable group. By distinguishing these compounds based on total tail carbon atom content, and not by alpha branching or the number of carbons at the point of branching, the applicants acknowledged that the pending claims covered

a 2-carbon group at the alpha position in R^{13} relative to the biodegradable moiety (M^1).

3. The accused product contains a 2-carbon group

It is undisputed that Moderna's COVID-19 vaccine contains a cationic lipid, referred to as SM-102, which has the following structure:



Appx43 (annotated with a red box at the branching at the alpha position and red numerals at the carbons adjacent to the branching point); Appx679. As can be seen above, SM-102 has branching at the alpha position relative to the biodegradable group and has only two carbons bound to the carbon at the point of branching.

B. Procedural background

Alnylam initiated this action against Moderna on March 17, 2022 when it filed a complaint alleging infringement of the '933 Patent. Appx33. Alnylam subsequently filed a second complaint on July 12, 2022 alleging infringement of the '979 Patent. The district court consolidated both ac-

tions for all purposes. Alnylam also brought similar actions against Pfizer that BioNTech later voluntarily joined.

After briefing on claim construction, the district court conducted a *Markman* hearing on August 9, 2023 where Alnylam, Moderna, and Pfizer/BioNTech presented arguments.

At the hearing, Chief Judge Connolly heard arguments on the "branched alkyl" terms and adopted Moderna's proposed constructions. Appx10, Appx5559 (Tr. at 145:20-25). In so doing, Chief Judge Connolly explained:

> So the reason, just to be clear, why I'm interpreting all of these terms consistent with defendants' position is because there is clear and unequivocal lexigraphy. And it comes at 412, Lines 13 through 18. And as I mentioned at Column 411, Line 53 and 54.

Appx10, Appx5559 (Tr. at 145:20-146:16). The passages from the specification cited by the court provide as follows:

> The terms "alkyl" and "alkylene" refer to a straight or branched chain saturated hydrocarbon moiety.

Appx257 ('933 Patent) at 411:53-54.

> Unless otherwise specified, the terms "branched alkyl", "branched alkenyl," and "branched alkynyl" refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group.

Appx257 ('933 Patent) at 412:13-18. Chief Judge Connolly concluded his remarks by further explaining "I think this is an easy call. It's not complicated at all." Appx10, Appx5559 (Tr. at 146:17-19).

Later that day, after the *Markman* hearing, Chief Judge Connolly issued an Order reiterating his opinion on lexicography with reference to Column 412 of the '933 Patent. Appx5519-5522.[11]

Chief Judge Connolly ordered the parties to jointly prepare a claim construction order consistent with his constructions on the record at the hearing. Appx5563. The parties submitted a proposed order, which the district court entered on August 21, 2023. Appx3-6.

Pursuant to a stipulation, the parties submitted a joint motion for entry of final judgment of non-infringement in light of the court's claim

[11] This Order addresses the term "R^{13} is a branched C_{10}-C_{20} alkyl" and is specific to an issue raised by the Pfizer/BioNTech defendants about whether "R^{13} and M^1 can be distinct but still share a carbon atom." Appx5521-5522. Because the issues addressed by the court there were not raised by Moderna and are not germane to infringement or invalidity claims raised with respect to Moderna, Alnylam does not address this Order in detail. *See GPNE Corp. v. Apple Inc.*, 830 F.3d 1365, 1372–73 (Fed. Cir. 2016) (explaining that where a "court has resolved the questions about claim scope that were raised by the parties, it is under no obligation to address other potential ambiguities that have no bearing on the operative scope of the claim"); *Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999).

construction order, which the court entered on August 30, 2023. Appx1-2; Appx5665-5671.

SUMMARY OF THE ARGUMENT

The terms "branched alkyl" and "branched C_{10}-C_{20} alkyl"[12] should be given their ordinary and customary meaning: "a saturated hydrocarbon moiety that is not a straight chain" and "a saturated hydrocarbon moiety that has 10 to 20 carbons and is not a straight chain," respectively. The correct construction of these terms includes an alpha-branched carbon atom bound to two other carbon atoms ("2-carbon group"). Such a construction comports with the claim language, written description, and prosecution history.

The district court committed legal error when it narrowed the terms to exclude 2-carbon groups, incorrectly holding that the specification evinces a clear intent to limit the plain claim scope through lexicography.

A. The intrinsic record demonstrates a clear intent to claim 2-carbon groups, not to artificially exclude them from the plain meaning of the term "branched alkyl." By drafting independent and dependent claims that encompass 2-carbon groups, and by repeatedly disclosing 2-carbon groups and characterizing them as "the present invention," the patentees unam-

[12] The only difference between these terms is that "branched C_{10}-C_{20} alkyl" specifies the number of carbons in the chain.

biguously demonstrated their intent that their claims would cover those embodiments. As this Court has repeatedly explained, "[w]e normally do not interpret claim terms in a way that excludes embodiments disclosed in the specification." *E.g.*, *Oatey Co. v. IPS Corp.*, 514 F.3d 1271, 1276 (Fed. Cir. 2008).

This fact is further evidenced by express statements in the prosecution history unequivocally depicting as within the claim scope an alpha-branched carbon binding to two other carbons in R^{13} and an oxygen in the biodegradable group (M^1).

B. Particularly in light of this clear evidence from the intrinsic record—all of which the district court essentially disregarded—the district court erred in finding lexicography. With respect to the "branched alkyl," the written description itself provides numerous indicators that the patentees did not intend to narrow the terms as the district court held. And by using the qualifier "unless otherwise specified," the patentees signaled that they never intended for the statement relied on by the district court to be so limiting.

The facts here therefore align with the many cases in which this Court has rejected lexicography. *See, e.g.*, *Apple Inc. v. Corephotonics, Ltd.*, 81 F.4th 1353, 1359 (Fed. Cir. 2023); *Baxalta Inc. v. Genentech, Inc.*, 972 F.3d 1341, 1349 (Fed. Cir. 2020); *Ecolab, Inc. v. FMC Corp.*, 569 F.3d

1335, 1345 (Fed. Cir. 2009); *Merck & Co., Inc. v. Teva Pharms. USA, Inc.*, 395 F.3d 1364, 1370 (Fed. Cir. 2005). In each of those cases, the absence of clear intent to redefine claim terms in a manner at odds with their plain and ordinary meaning resulted in no lexicography. Just so here.

C. Finally, even if (contrary to our principal submission) lexicography were to apply, the *full* definition necessarily applies—and that full definition includes the "unless otherwise specified" clause. Consistent with the patent language and drawings, the Court should find that the claims, the written description, and prosecution history *do* "otherwise specify" that a branched alkyl at the alpha position may include structures where the alpha-branched alkyl group binds to only two carbon atoms.

For all of these reasons, the Court should reverse the judgment of non-infringement, and remand for further proceedings.

STANDARD OF REVIEW

"Claim construction is ultimately a question of law, decided de novo on review, as are the intrinsic-evidence aspects of a claim-construction analysis." *Intel Corp. v. Qualcomm Inc.*, 21 F.4th 801, 808 (Fed. Cir. 2021). The Court applies "clear error review when reviewing subsidiary factfinding in patent claim construction." *Teva Pharms. USA, Inc. v. Sandoz, Inc.*, 574 U.S. 318, 331 (2015).

ARGUMENT

I. THE DISTRICT COURT ERRED IN HOLDING THAT THE PATENTEES ACTED AS THEIR OWN LEXICOGRAPHER.

"It is a 'bedrock principle' of patent law that 'the claims of a patent define the invention to which the patentee is entitled the right to exclude.'" *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312 (Fed. Cir. 2005) (en banc) (quoting *Innova/Pure Water, Inc. v. Safari Water Filtration Sys., Inc.*, 381 F.3d 1111, 1115 (Fed. Cir. 2004)). There is a "heavy presumption" that claim terms carry their "full ordinary and customary meaning, unless [the accused infringer] can show the patentee expressly relinquished claim scope." *Epistar Corp. v. Int'l Trade Com'n,* 566 F.3d 1321, 1334 (Fed. Cir. 2009).

"There are only two exceptions" to the maxim that claim terms should be afforded their full ordinary and customary meaning, "1) when a patentee sets out a definition and acts as his own lexicographer, or 2) when the patentee disavows the full scope of a claim term either in the specification or during prosecution." *Thorner v. Sony Computer Entm't Am. LLC*, 669 F.3d 1362, 1365 (Fed. Cir. 2012). Here, the district court determined there is lexicography.

But a statement is not lexicography merely because it is set out in the form of a definition. *See, e.g.*, *Abbott Labs. v. Andrx Pharms., Inc.*,

473 F.3d 1196, 1210 (Fed. Cir. 2007) (finding the specification statement "a pharmaceutically acceptable polymer *is*" did not "unambiguously signify that the description provided is definitional.").

More is needed. A "patentee must clearly express [the] *intent* in the written description" to "assign to a term a unique definition that is different from its ordinary and customary meaning." *Helmsderfer v Bobrick Washroom Equip., Inc.*, 527 F.3d 1381 (Fed. Cir. 2008) (emphasis added). "When a patentee acts as his own lexicographer in redefining the meaning of particular claim terms away from their ordinary meaning, he must clearly express that intent in the written description." *Merck & Co.*, 395 F.3d at 1370; *see also GE Lighting Sols., LLC v. AgiLight, Inc.*, 750 F.3d 1304, 1309 (Fed. Cir. 2014); *Abbott Labs. v. Syntron Bioresearch, Inc.*, 334 F.3d 1343, 1354 (Fed. Cir. 2003); *Union Carbide Chems. & Plastics Tech. Corp. v. Shell Oil Co.,* 308 F.3d 1167, 1177-78 (Fed. Cir. 2002); *In re Paulsen*, 30 F.3d 1475, 1480 (Fed. Cir. 1994); *Renishaw PLC v. Marposs Societa' per Azioni*, 158 F.3d 1243, 1249 (Fed. Cir. 1998).

The standard for lexicography is "exacting." *GE Lighting Sols.,* 750 F.3d at 1309. This is because "the statement in the specification must have sufficient clarity to put one reasonably skilled in the art on notice that the inventor intended to redefine the claim term." *Merck & Co.,* 395 F.3d at 1370. "The patentee's lexicography must … appear with rea-

sonable clarity, deliberateness, and precision before it can affect the claim." *Syntron Bioresearch*, 334 F.3d at 1354.

Where there is lack of reasonable clarity, deliberateness, and precision in the definition, courts have refused to find lexicographic definition. *See, e.g.*, *id.* at 1355 (declining to find lexicographic redefinition despite express definition "[a]s used herein, 'analyte' refers . . ."); *Merck & Co.,* 395 F.3d at 1370-71 (finding the specification statement "ambiguous" in redefining "about"); *Ecolab*, 569 F.3d at 1345 (declining to accept the patentee's definition of the term "sanitize," finding the definition ambiguous); *Andrx Pharms.,* 473 F.3d at 1210.

Here, the full breadth of the claims themselves, the numerous examples of 2-carbon groups in the written description that are part of the "present invention," the non-limiting definitional statement, and the clear statements specifying the plain claim scope in the prosecution history all indicate an intent *not* to artificially exclude 2-carbon groups from the ordinary meaning of the terms "branched alkyl" and "branched C_{10}-C_{20} alkyl." They certainly do not demonstrate that the patentees unambiguously and intentionally limited the terms through a lexicographical definition.

A. The intrinsic record demonstrates the patentees' intent not to limit the "branched alkyl" terms.

The patentees' plain intent not to exclude 2-carbon groups from the scope of the claims is signaled throughout the intrinsic record.

Claim construction—including the question whether a patentee has acted as its own lexicographer—must be determined with consideration of the patent as a whole and not based on any single isolated portion. *Baxalta*, 972 F.3d at 1347 (in the context of a potentially definitional statement, "claim construction requires that we 'consider the specification as a whole, and [ ] read all portions of the written description, if possible, in a manner that renders the patent internally consistent.'") (quoting *Budde v. Harley-Davidson, Inc.*, 250 F.3d 1369, 1379-80 (Fed. Cir. 2001)); *Pfizer, Inc. v. Teva Pharms., USA, Inc.*, 429 F.3d 1364, 1373 (Fed. Cir. 2005) ("It is necessary to consider the specification as a whole, and to read all portions of the written description, if possible, in a manner that renders the patent internally consistent."); *SanDisk Corp. v. Memorex Prods., Inc.*, 415 F.3d 1278, 1285 (Fed. Cir. 2005) ("The court must always read the claims in view of the *full* specification.").

Here, the intrinsic record objectively shows that the patentees did not intend to limit the claimed "branched alkyl" to groups just having an alpha-branched carbon bound to at least three other carbons. Indeed,

(i) the claim language itself, (ii) the written description replete with embodiments and formulas disclosing 2-carbon alpha-branched alkyl groups, including identifying them as part of "the present invention," and (iii) the prosecution emphatically stating the clear intent to cover such structures all underscore that there is no act of lexicography. *See supra* Section Factual Background A.2.

As explained in detail below, the claims require "a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where … M^1 is the biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl." The claims further recite that the biodegradable ester group M^1 includes —C(O)O—, meaning the alpha branched alkyl is immediately next to the "O" of the —C(O)O— group. This claimed structure expressly covers a 2-carbon group and this conclusion is informed by the written description and prosecution history.

By disregarding this rich intrinsic record and effectively finding that the patentees had disclaimed such embodiments and described scope, the district court committed legal error. *See Sequoia Tech. LLC v Dell, Inc.*, 66 F.4th 1317, 1328 (Fed. Cir. 2023) ("While not dispositive, we find it unlikely that an inventor would define an invention such that an element of a preferred embodiment is superfluous."); *GE Lighting Sols.*, 750 F.3d at

1311 ("[W]here claims can reasonably [be] interpreted to include a specific embodiment, it is incorrect to construe the claims to exclude that embodiment, absent probative evidence on the contrary.") (quoting *Oatey Co.*, 514 F.3d at 1277); *Oatey Co.*, 514 F.3d at 1276 ("We normally do not interpret claim terms in a way that excludes embodiments disclosed in the specification.").

1. The claims cover 2-carbon alpha-branched alkyl groups.

Starting with the claims of the Patents-in-Suit, each would encompass a 2-carbon group. For example, exemplary Claim 18 of the '933 Patent recites, in part, that:

> each biodegradable hydrophobic tail independently has the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), wherein the *biodegradable group is —OC(O)— or —C(O)O—*;
>
> for at least one biodegradable hydrophobic tail, the terminal hydrophobic chain in the biodegradable hydrophobic tail is *a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group* and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, *M^1 is the biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl*, and the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26;

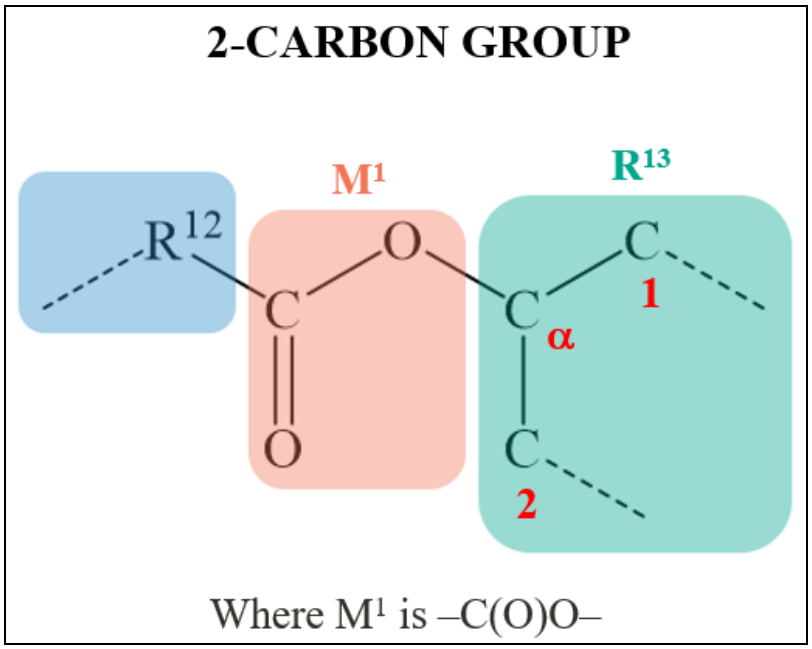
Appx320 (emphases added); *see also* Appx2635 ('979 Patent claim 1).

Claim 18 defines the general formula of the hydrophobic tail as -R^{12}-M^1-R^{13}, where M^1 is the biodegradable group, which can be either —

OC(O)— or —C(O)O—, and where R^{13} is a branched alkyl that is branched at the alpha position relative to the biodegradable group. The claim thus expressly allows either orientation of the biodegradable group (—OC(O)— or —C(O)O—), which means that the branched-alkyl carbon at the alpha position in R^{13} can bind to either two or three other carbons.

That is, by reciting that the biodegradable ester group M^1 includes —C(O)O—, meaning the branched alkyl is immediately next to the "O" of the —C(O)O— group ("where the branching occurs at the α-position relative to the biodegradable group")[13], the claim language expressly contemplates the 2-carbon group illustrated below:

[13] As discussed in the Factual Background, the parties agreed that this phrase modifying "branched alkyl" means "where the branching occurs at a carbon next to the [biodegradable/ester] group." Appx4438; Appx5501-5502 (Amended Joint Claim Construction Chart).



Thus, the alpha-branched alkyl that binds to 2-carbons and to the oxygen of the biodegradable group is expressly contemplated by the claim language itself.[14]

Likewise, dependent Claim 20 of the '933 Patent and Claim 5 of the '979 Patent expressly call out "—C(O)O—" as the biodegradable/ester group M^1 that would encompass such a two-carbon alpha-branched alkyl

[14] Before the district court, Moderna presented a structure containing a —C(O)O— oriented ester and a 3-carbon group. Moderna argued that, because its structure could satisfy the claim language, the claims do not "otherwise specify" the meaning of "branched alkyl" to also include 2-carbon groups. Appx4485-4487. But this alpha-branched structure does not expressly appear in any of the patents' drawings, unlike the specific two-carbon bound alpha branched drawings, and Moderna's identification of a structure within the claim scope is no basis whatsoever to impose an artificial limitation on that claim scope.

group. Appx320 ('933 Patent) at 538:39-40; Appx2635 ('979 Patent) at 494:49-50. These claims thus specify (and therefore "otherwise specify," if that test is relevant, *see* pages 67-70, *infra*) a two-carbon bound alpha-branched alkyl group and clearly show the intent to cover such alpha-branched alkyl structures that bind to two carbons and to the oxygen of the biodegradeable group.

Language of dependent Claim 14 of the '933 Patent also demonstrates that the asserted claims allow alpha branched 2-carbon group or 3-carbon group. Claim 14, dependent on claim 1 which includes similar branched alkyl language as claim 18, recites only a 3-carbon group:

> The cationic lipid of claim 1, wherein the branched alkyl group has only one carbon atom which is *bound to three other carbon atoms*.

Appx320 ('933 Patent claim 14) (emphasis added). "An independent claim impliedly embraces more subject matter than its narrower dependent claim." *Intamin, Ltd. v. Magnetar Techs., Corp.*, 483 F.3d 1328, 1335 (Fed. Cir. 2007); *see also AK Steel Corp. v. Sollac & Ugine*, 344 F.3d 1234, 1242 (Fed. Cir. 2003) ("Under the doctrine of claim differentiation, dependent claims are presumed to be of narrower scope than the independent claims from which they depend."). Claim 14 thus "raises a presumption that the limitation in question is not found in the independent claim." *Liebel-*

Flarsheim Co. v. Medrad, Inc., 358 F.3d 898, 910 (Fed. Cir. 2004).[15] Specifically, from Claim 14 it is presumed that the independent claims allow an alpha-branched 2-carbon group or 3-carbon group when the M^1 group is —C(O)O—.

Collectively, the claim language evinces the patentees' intent that their claims are not restricted to alpha-branched alkyls that must bind to at least three other carbons.

2. The written description evinces an intent not to limit the claims to only 3-carbon groups.

Beyond the clear claim language, the written description confirms that the district court erred in applying a limiting construction. The written description contains a number of formulas, figures, and embodiments that the district court's limiting construction effectively reads out and would be effectively disclaimed.

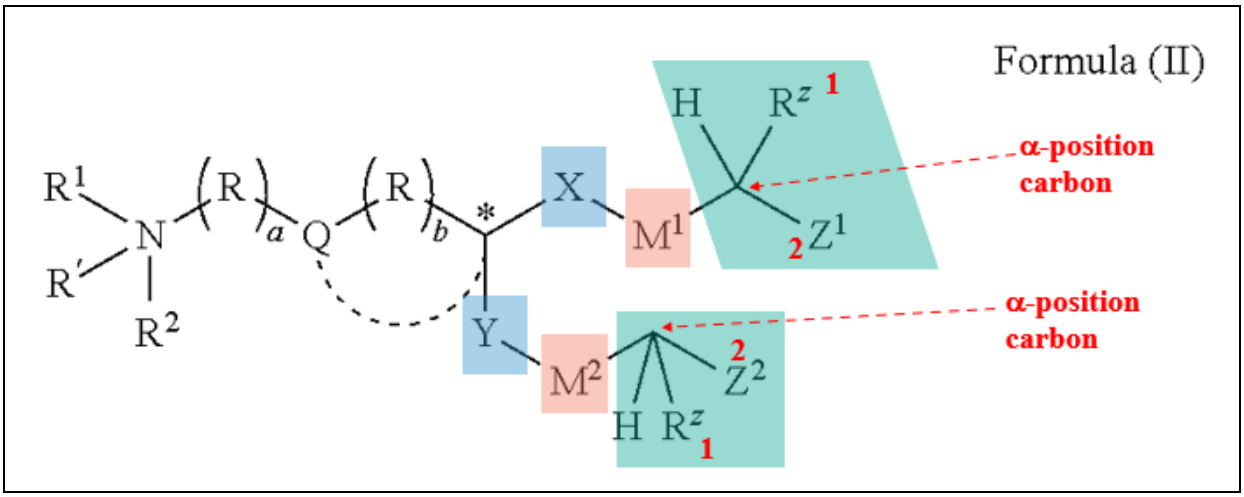
To start with, the patent's figures and formulas are highly germane to the objective manifestations of the patentee's intent to encompass al-

[15] Although claims 1 and 14 are unasserted, they are highly relevant. Indeed, "[o]ther claims of the patent in question, both asserted and *unasserted*, can also be valuable sources of enlightenment as to the meaning of a claim term." *Phillips*, 415 F.3d at 1314-15 (citing *Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1582 (Fed. Cir. 1996)) (emphasis added). This is "[b]ecause claim terms are normally used consistently throughout the patent, the usage of a term in one claim can often illuminate the meaning of the same term in other claims." *Id*.

pha-branched alkyls that are bound to only two carbons. *CVI/Beta Ventures, Inc. v. Tura LP*, 112 F.3d 1146, 1153 (Fed. Cir. 1997) ("Patent drawings are highly relevant in construing the … limitations of the claims."); *Ariad Pharms., Inc. v. Eli Lilly & Co.*, 598 F.3d 1336, 1350, 1352 (Fed. Cir. 2010) (en banc) ("the description requirement does not demand any particular form of disclosure" but can be demonstrated by "structure, formula, chemical name, physical properties or other properties"); *Advanced Steel Recovery, LLC v. X-Body Equip., Inc.*, 808 F.3d 1313 (Fed. Cir. 2015) (finding a patent's drawings supported a district court construction of a patent's claims requiring a certain structure).

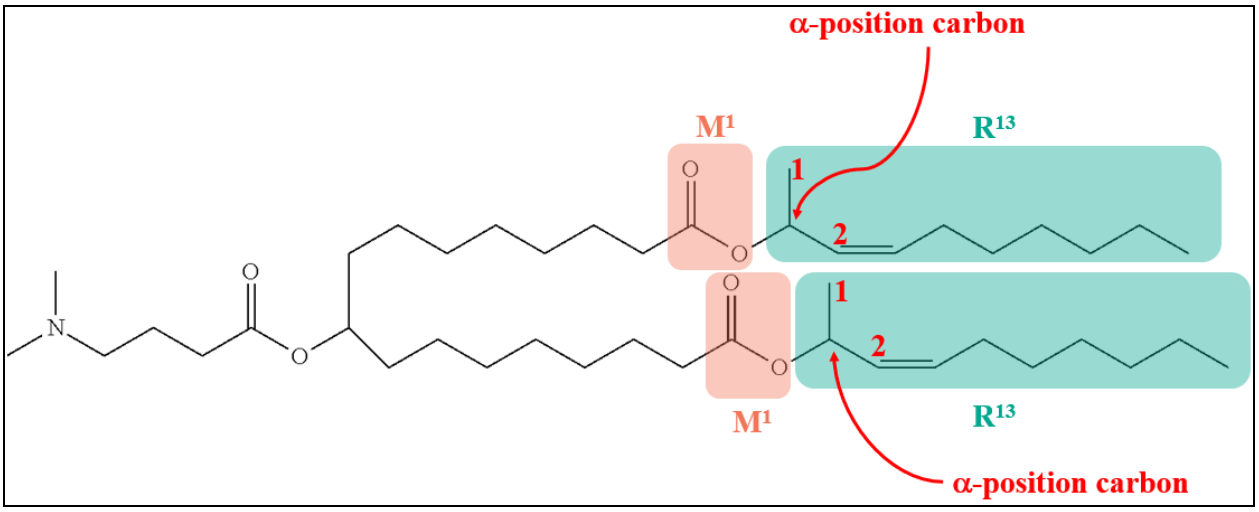
Each of the patents' Formulas with alpha branching (Formulas I, II, and VIII) depict an alpha-branched alkyl carbon bound to two other carbons plus a hydrogen atom and thus binding to an oxygen in the biodegradable group. And the patent specification expressly identifies these structures as "branched alkyl." Appx52 ('933 Patent) at 2:7-9 ("the cationic lipid is a compound of Formula (I), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added); Appx53 ('933 Patent) at 3:63-65 ("the cationic lipid is a compound of Formula (II), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added).

Specifically, the alpha-branched carbon is bound to a hydrogen (H) atom and two carbon atoms in R^z and X, R^z and Y, R^z and Z^1, or R^z and Z^2 in Formulas (I) and (II). Appx52 ('933 Patent) at 2:11-20; Appx4514 (Certificate of Correction); Appx53 ('933 Patent) at 4:1-10; Appx4515 (Certificate of Correction); *supra* Factual Background A.2. For example, Formula (II) depicts:



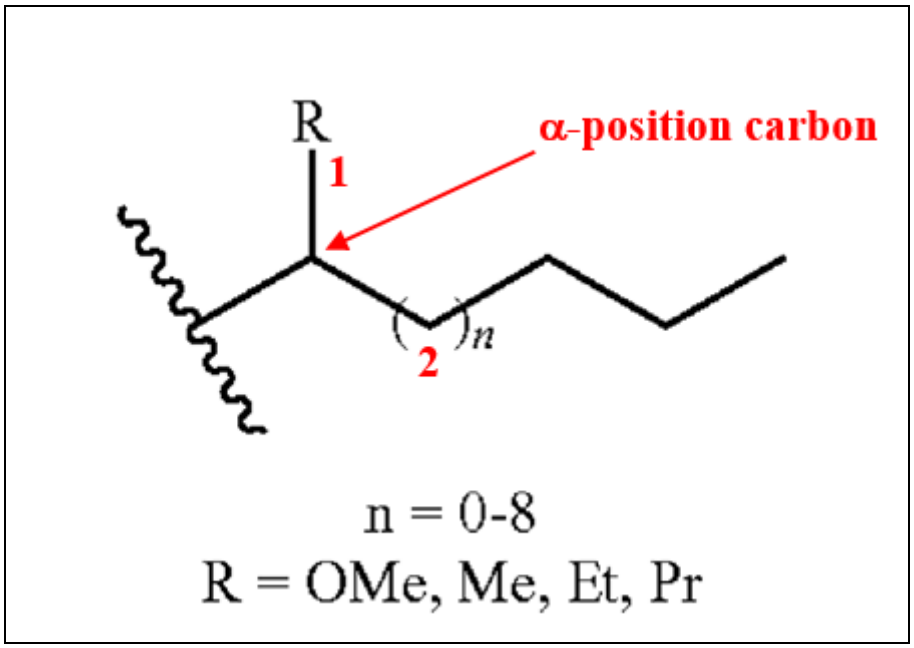
These formulas objectively specify that two-carbon binding is contemplated when the "branched alkyl" is at the alpha position.

The exemplary Compound 1 (below) repeatedly disclosed in the specification likewise has two carbons bound to the alpha branched carbon in R^{13}. Appx68 ('933 Patent at 34:32-43); Appx69 ('933 Patent at 35-36); Appx70 ('933 Patent at 37-38); Appx81 (60:50-65); *supra* Factual Background A.2.

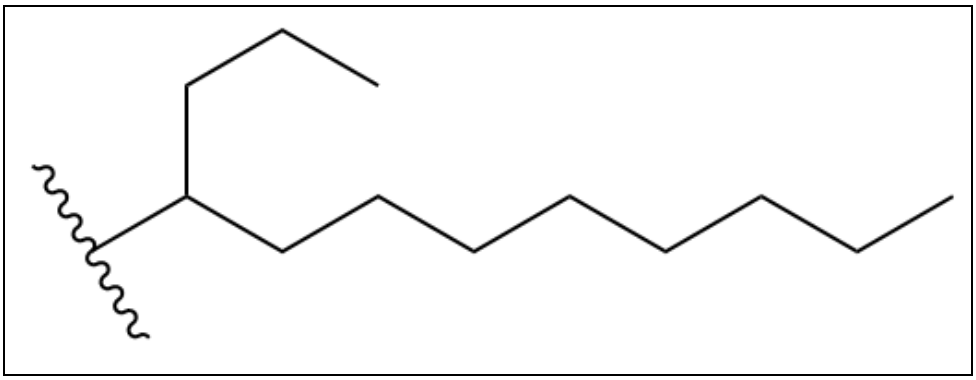


See, e.g., Appx68 ('933 Patent at 34:32-43) (annotated).

In addition, the written description expressly calls out as the "*present invention*" structures that would encompass a branched alkyl binding to two carbons. Appx82 ('933 Patent at 61:23-26). As discussed in greater detail in the Factual Background, such two-carbon bound, alpha branched alkyls are plainly specified. For example, the "representative hydrophobic chain II of the present invention" (*i.e.*, tail element R^{13}) disclosed in the specification includes 2-carbon group as set forth below:

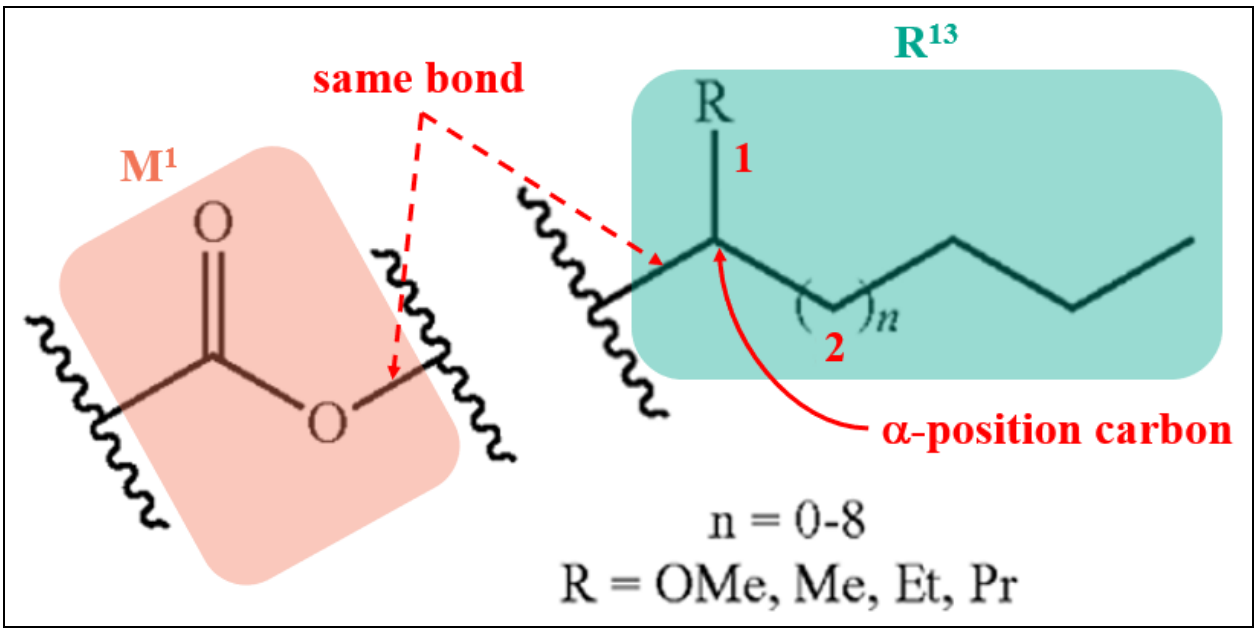


Appx88 ('933 Patent) at 74:52-57 (annotated above with numbers at two carbons in red); Appx4952 (showing structures for Me, Et and Pr). This formula depicts an alpha-branched chain of up to 15 carbons, where (like the others discussed above) the branching point is a carbon at the alpha position connected to two other carbons and a hydrogen (H) atom. An example is:



Appx4499. Thus, taking the disclosed R^{13} structure and combining it with a biodegradable moiety (M^1) of the "present invention," results in a

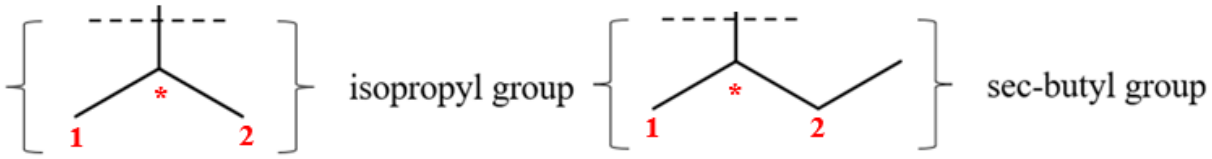
branched alkyl 2-carbon group, contrary to the limiting lexicography the district court imposed:



Appx87 ('933 Patent) at 72:1-10; Appx88 ('933 Patent) at 74:52-57 (annotated above with numbers at two carbons in red and to show how these moieties combine).[16]

[16] Moderna argued before the district court that the specification's "discussion" of the various figures depicting an alpha-branched carbon binding to two carbons in R^{13} and to the oxygen in M^1 nowhere used the term "branched alkyl." Appx4506-4507. Should Moderna resurrect it here, that argument has no merit. The specification expressly describes and shows a "branched alkyl" at the alpha position as binding to two carbons with the third bond to a hydrogen. Appx52 ('933 Patent) at 2:7-9 ("the cationic lipid is a compound of Formula (I), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added); Appx53 ('933 Patent) at 3:63-65 ("the cationic lipid is a compound of Formula (II), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added). Further, the drawings show it. A drawing is no less in-

More, in the Definitions section, the written description defines the term "alkyl" as "a straight or branched chain saturated hydrocarbon moiety." Appx257 ('933 Patent) at 411:53-61. The definition of "alkyl" goes on to state: "*Representative saturated branched alkyl groups include isopropyl, sec-butyl, isobutyl, tert-butyl, and isopentyl*." *Id.* at 411:60-61 (emphases added). "Isopropyl" and "sec-butyl" each contains a carbon branched to two other carbons as set forth below:



Appx4952-4953; Appx5011-5012; Appx5663. These "representative" branched alkyl groups plainly confirm that there was no intent to limit "branched alkyl" to three- or more-carbon groups only. Indeed, the express inclusion of "isopropyl" and "sec-butyl" as being "representative" branched

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formative than words. *CVI/Beta Ventures,* 112 F.3d at 1146. The specification sets forth the terms alkyl, alkenyl, and alkynyl and describe them as either a straight chain or a branched chain. Appx257 ('933 Patent at 411:53-54, 62-63 and 412:6-7). The record contained the commonsense textbook quote that a straight carbon chain is distinguished from a branched carbon chain because the "difference between the two is that you can draw a line connecting all the carbons of a straight-chain alkane without retracing your step or lifting your pencil from the paper." Appx5009. The specification figures themselves using the language of chemistry show that the "branched alkyl" at the alpha position relative to the biodegradable group M^1 may bind two carbons.

alkyl groups demonstrates that 2-carbon groups *must* be within the scope of the "branched alkyl" term.

Rather than read the entire specification as a whole to determine whether the requisite and exacting reasonable clarity, deliberateness, and precision for lexicography existed, the district court disregarded the formulas and examples that contradicted its construction. This was legal error. *Knowles Elecs. LLC v. Iancu*, 886 F.3d 1369, 1375 (Fed. Cir. 2018) ("claim construction that does not encompass a disclosed embodiment is . . . rarely, if ever, correct.").

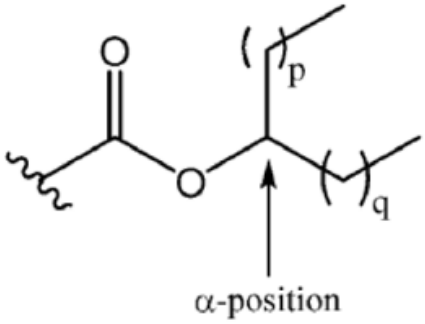
3. During prosecution, the applicants expressly identified 2-carbon groups as covered by the claims and distinguishing the prior art.

If the claims and written description were not enough (they are), the prosecution history of the '933 Patent specifies that the claim term "branched alkyl" includes 2-carbon groups. "[T]he prosecution history can often inform the meaning of the claim language by demonstrating how the inventor understood the invention and whether the inventor limited the invention in the course of prosecution, making the claim scope narrower than it would otherwise be." *Phillips*, 415 F.3d at 1317.

During prosecution, the applicants informed the Patent Office examiner that a 2-carbon structure they depicted is covered by the issued patent claims (pending at that time). Appx4941 ('311 Application File History,

Oct. 14, 2021 Response to Non-Final Office Action) at 10. Specifically, the prosecution history's figure (at Appx4941 and reproduced with yellow highlighting below) unequivocally shows a 2-carbon group branched at the alpha position relative to the biodegradable group —C(O)O—.

The compounds on page 51 of WO '493 do not have branching in the terminal hydrophobic chain at the α-position relative to the biodegradable group as recited ***in the pending claims***. *Such compounds* with ***branching at the α-position*** would have a moiety as shown below (assuming the biodegradable group is an ester and the variables p and q are integers):



[17]

This prosecution history specifies exactly what the applicants understood as an example of a branched alkyl at the alpha position binding to two carbons. Moreover, the applicants' reliance on this structure to distinguish the prior art shows their intent and understanding that such struc-

[17] Contrary to Moderna's claims below, the chemical structure drawn here is not a depiction of the prior art. Rather, it is a structure illustrating branching at the alpha position as within the claim scope.

ture falls within the scope of the claims. Any suggestion that this Court should not consider this portion of the intrinsic record should be rejected.[18]

That the applicants considered 2-carbon groups to be covered by the asserted claims is further evidenced by their response to prior art compounds disclosed in page 58 of WO '493. The prior art compounds included 2-carbons bound to the branching carbon at the alpha position relative to the biodegradable group. Appx4941 ('311 Application File History, Oct. 14, 2021 Response to Non-Final Office Action) at 10. The applicants, however, distinguished those compounds based on *total carbon atom content of the tail and not that two-carbon binding alpha-branched alkyls were not covered by the claims.*

The patentees' prosecution history provides objective notice that the claims are not limited to at least three carbon binding, alpha branched al-

[18] Before the district court, Moderna argued that Alnylam's reliance on the prosecution history is misplaced because the office action response cited here purportedly does not expressly discuss the "branched alkyl" or "branched C_{10}-C_{20} alkyl" terms. But this ignores that the statements to the Examiner were provided in response to the applicants amending the pending claims to add "branched C_{10}-C_{20} alkyl." Appx4911. It ignores the plain recitation to the pending claims. And it ignores the plain claim language that the term "branched alkyl" is modified by the phrase "where the branching is at the alpha position," and that the term "branched alkyl" must be read in the claim's context. *Phillips*, 415 F.3d at 1314 ("To begin with, the context in which a term is used in the asserted claim can be highly instructive.").

kyls. *See Phillips*, 415 F.3d at 1317 ("An invention is construed not only in the light of the claims, but also with reference to the file wrapper or prosecution history in the Patent Office.") (quoting *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 33 (1966)).

* * *

In summary, the asserted claims allow for an alpha branched 2-carbon group. First, the independent claims themselves recite that the biodegradable ester group M^1 includes —C(O)O—, meaning the branched alkyl is immediately next to the "O" of the —C(O)O— group and the carbon at the alpha position to the "O" can be branchd to two other carbons. The breadth of the independent claims is confirmed by the dependent claims that expressly call out the —C(O)O—group or expressly limiting themselves to 3-carbon group structures. Second, the written description calls out as "branched alkyl" alpha-branched two-carbon group structures and additionally specifies numerous examples of the "present invention" that require this alpha-branched structure. And finally, the patentees during prosecution identified alpha branched 2-carbon structures as covered by the now issued claimed and distinguished prior art with reference to this type of branched structure. Accordingly, the intrinsic record as a whole compels the conclusion that the asserted claims allow for an alpha

branched 2-carbon group. But the district court's oral decision did not specifically address *any* of this critical material. *See* Appx5558-5563.

B. The district court erred in holding that the patentee engaged in lexicography and thus excluded disclosed embodiments.

Without squarely addressing any of this evidence revealing the patentees' intent—that is, a plain intent to claim, rather than exclude, 2-carbon groups—the district court relied on a single, qualified sentence in the written description's Definitions section. But that single sentence does not establish, under this Court's exacting standards for acts of lexicography contrary to ordinary meaning, an intent to exclude from the claim scope two-carbon alpha-branched alkyls.

1. The relevant language from the specification's Definitions section provides:

> Unless otherwise specified, the terms "branched alkyl", "branched alkenyl," and "branched alkynyl" refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group.

Appx257 ('933 Patent) at 412:13-18.

This statement cannot bear the weight placed upon it by the district court, for a whole host of reasons.

First, this definition of "branched alkyl" starts with the qualifier "unless otherwise specified." The addition of the qualifier was purposeful. If the patentees had intended to limit every "branched alkyl" to require that one carbon atom in the group is bound to at least three other carbon atoms, they would not have started the definition with a qualifier*, i.e.*, "unless otherwise specified." The qualifier signals to the reader to consider the context in which the term "branched alkyl" appears. The very language shows the patentees' explicit intent *not* to limit the scope of "branched alkyl" to only the three-carbon structure stated thereafter. *Cf., e.g.*, *Hill v. Schilling*, 578 Fed. App'x 456, 460 (5th Cir. 2014) (applying the commonsense proposition that statutory phrase "unless otherwise specified" renders what follows not an inexorable command); *United States v. Kluger*, 722 F.3d 549, 558 (3d Cir. 2013) ("[B]y including the phrase 'unless otherwise specified,' the relevant conduct provision" in a sentencing guideline "admits of exceptions."); *Abbadessa v. Tegu*, 154 A.2d 483, 486-487 (Vt. 1959) ("[B]y the addition of the words 'unless otherwise provided,'" statutory provision "is rendered subservient to all other provisions in the law which may run counter to it. … Since the legislature has otherwise provided, effect must be given accordingly.").

In fact, as explained above (*see supra* pages 9-16, 42-46), the claims (independent and certain dependent claims) themselves *do* "otherwise

specify" a branched alkyl group where the carbon is bound to only two other carbons due to the claimed biodegradable/ester group. *See* Appx320 ('933 Patent) at 538:41-42; Appx2635 ('979 Patent) at 494:52-53. Specifically, the independent claims explicitly provide that M^1, the biodegradable ester group, can have the structure —C(O)O—. By reciting that the biodegradable ester group M^1 includes —C(O)O—, meaning the branched alkyl is immediately next to the "O" (oxygen) of the —C(O)O— group, the claim language expressly includes the 2-carbon group.

Second, the remainder of the Definitions section demonstrates that the patentees knew exactly how to define a term without a qualifier when they intended to. Indeed, the patentees defined several terms without a qualifier and others with a qualifier—and, in some instances, arranged the terms with and without a qualifier following one after the other. *Compare* Appx257 ('933 Patent) at 411:53-54 ("The terms 'alkyl' and 'alkylene' refer to a straight or branched chain saturated hydrocarbon moiety."); 411:27-30 ("The term 'biodegradable cationic lipid' refers to . . ."); 411:35-37 ("As used herein, the term 'biodegradable group' refers to . . ."); 411:50-52 ("As used herein, an 'aliphatic' group is a . . ."); 411:53-54 ("The terms 'alkyl' and 'alkylene' refer to . . ."); 411:62-64 ("The term 'alkeynyl' refers to . . ."); 412:6-8 ("The term 'aklynyl' refers to . . ."); 412:36-39 ("The term 'aryl' refers to . . .") *with id.* at 412:13-20 ("*Unless otherwise specified*, the terms

'branched alkyl', 'branched alkenyl', and 'branched alkynyl' refer to an . . ."); 412:21-24 ("*Unless otherwise specified*, the term 'acyl' refers to a . . .") (emphasis added).

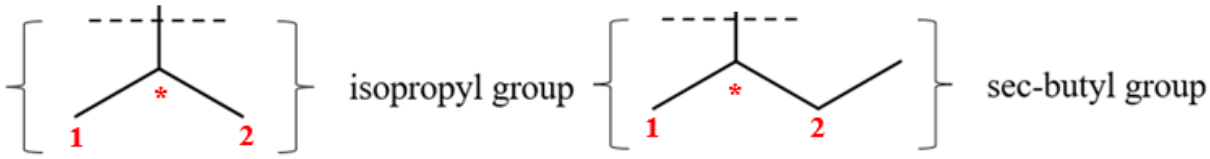
By deliberately choosing to add a qualifier to the language at issue here (when they did not do so elsewhere), the patentees conveyed their intent *not* to provide the closed-ended lexicography for "branched alkyl" that the district court imposed to exclude two-carbon bound, alpha-branched alkyls. *Abbott Laboratories v. Andrx Pharmaceuticals, Inc.* is instructive. 473 F.3d 1196. There, the specification provided that "a pharmaceutically acceptable polymer *is*" a particular subset of polymers. *Id*. at 1210 (emphasis in original). The Court explained that while the "word 'is' may signify that a patentee is serving as its own lexicographer," it was in contrast to other unambiguous definitions such as "'Pharmaceutically acceptable' as used herein, *means* . . . .", and "does not as unambiguously signify that the description provided is definitional." *Id*. (emphasis in original).

Here, the patentees' use of the qualifier "unless otherwise stated … refer to," renders their lack of unambiguous definitional intent even more stark than in *Abbott*. The qualifier indicates that structures beyond those identified in the rest of the statement are included and the term is open-ended. In that sense, the phrase is akin to "for example" and "may also include"—phrases this Court has found to indicate a lack of intent to limit a

term's natural scope through lexicography. *Baxalta*, 972 F.3d at 1349 ("the written description's use of 'may also include,' '*e.g.*,' 'such as,' and '*etc*.' makes clear the patentee did not intend this excerpt" to be definitional); *Novro Corp. v. Boston Sci. Corp.*, 955 F.3d 35, 44 (Fed. Cir. 2020) (reversing the lower court's definition of "therapy signal" based on the phrase "therapy signals (e.g., electrical impulses)" because it "does not meet the exacting standard for redefining 'therapy signal.'"); *Acme Scale Co., Inc. v. LTS Scale Co., LLC*, 615 Fed. App'x 673, 679 (Fed. Cir. 2015) ("Rather, because the definition employs 'broad, inclusive, and non-limiting expressions like 'broadly', 'include', 'such as', 'for example', and 'but not limited to,' the inclusion of devices such as forklifts, flatbed trucks and pallet trucks do not suggest that the '946 patent was intended to be limited strictly to these devices.").

Third, the same Definitions section containing the language seized upon by the district court also defines the term "alkyl" as "a straight or branched chain saturated hydrocarbon moiety." Appx257 ('933 Patent) at 411:53-61. The definition of "alkyl" goes on to state: "*Representative saturated <u>branched alkyl</u> groups include <u>isopropyl</u>, <u>sec-butyl</u>, isobutyl, tert-*

butyl, and isopentyl." *Id.* at 411:60-61 (emphases added).[19] "Isopropyl" and "sec-butyl" each contains a carbon branched to two other carbons as set forth below:



Appx4952-4953; Appx5011-5012; Appx5663. Identifying such two-carbon groups as "representative" branched alkyls evinces further an intent not to limit "branched alkyl"—the very same term these two-carbon groups are "representative" of—to three- or more-carbon groups only. The district court's decision to the contrary violates this Court's repeated admonition to "read all portions of the written description, if possible, in a manner that renders the patent internally consistent." *Baxalta*, 972 F.3d at 1347.

Fourth, the fact that this statement appears under the heading Definitions is not dispositive. As the Court has repeatedly explained, "claim construction requires that we 'consider the specification as a whole, and read all portions of the written description, if possible, in a manner that

[19] This disclosure demonstrates that the written description lays out a duality where an alkyl is either straight or branched. *See* Appx4952-4953; Appx5009. Alnylam's proposed construction of the "branched alkyl" terms is thus consistent with the disclosure that indicates that these terms connote a chain saturated hydrocarbon moiety that is not straight.

renders the patent internally consistent.'" *Baxalta*, 972 F.3d at 1347; *see also, e.g.*, *Merck & Co.,* 395 F.3d at 1370.

In sum, the patentees did not "clearly set out [their] own definition" of the disputed term "branched alkyl" with "'reasonable clarity, deliberateness, and precision,' and thus failed to act as [their] own lexicographer" by disclaiming the full scope of that term's ordinary meaning. *Merck & Co.,* 395 F.3d at 1371. Rather, by starting the "branched alkyl" definition with the qualifier "unless otherwise specified," the patentees signaled their intent that the term "branched alkyl" is not a closed-ended act of lexicography that excludes two-carbon bound, alpha branched alkyls. [20]

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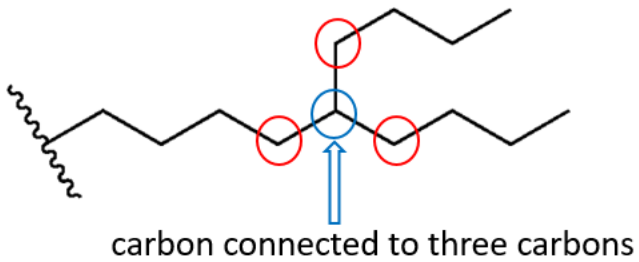
[20] Before the district court, Moderna incorrectly argued that the alleged definition of three carbon binding applied "to every single specific example of an R^{13} branched alkyl group in the written description." It further set forth such an "example":



Appx4505, Appx4484 citing, *inter alia*, Appx79 ('933 Patent at 55:45). Notably, the preceding figure and the others Moderna cited do not depict branching *at the alpha positi*on (first) "relative to the biodegradable group," as the claim language plainly requires. The above figure shows branching at the *fifth* carbon position relative to the biodegradable moiety

2. The district court thus excluded embodiments repeatedly specified in the patents and disclosed as part of "the present invention." *See supra* Argument Section I.A.2. Under similar facts, this Court has repeatedly rejected lexicography.

For example, in *Baxalta*, the district court identified two sentences in the written description that it determined defined the term "antibody." *Baxalta*, 972 F.3d at 1347. Based on those sentences, the district court limited the term "antibody" to, *inter alia*, a molecule "consisting of two identical heavy chains (H chains) and two identical light chains (L chains)." *Id.* at 1344. This Court rejected the district court's construction in favor of the broader "comprising two heavy chains (H chains) and two light chains (L chains)." *Id.* at 1349. In doing so, this Court found that the written description included specific disclosures of various types of antibodies "all of which do not comport with the district court's construction." *Id.* at 1347. One such example was "bispecific antibodies," which the parties agreed "do

---

M^1 and not at the first alpha position. For the claimed "branched alkyl, where the branching occurs at the alpha position relative to the biodegradable group," as discussed, the written description and prosecution history is replete with examples of an alpha branched carbon binding to two other carbons. *See* Section Factual Background A.2, *supra*. Moderna's erroneous district court argument turning a blind eye to the claim language and the intrinsic record cannot support limiting the claims under lexicography's exacting and unambiguous standards.

not consist of two identical H chains and two identical L chains and thus fall outside the district court's construction." *Id.*

Ultimately, the Court held "that the district court erred in selecting the narrower construction, which is inconsistent with the written description and the plain language of the claim." *Id.* at 1349. The same is true here. The district court adopted a claim construction excluding disclosed examples where one carbon atom in the branched group is bound to only two other carbon atoms. The district court's construction thus is inconsistent with the use of the terms in the patent and must be rejected.

Similarly, this Court recently vacated a Patent Trial and Appeal Board's claim construction where a portion of the written description was "suggestive of an effort by the patentee to be its own lexicographer" but "there is no indication in the claims, specification, or otherwise that the patentee meant to claim their invention more narrowly." *Apple Inc.*, 81 F.4th 1353 at 1358-1359. The Court first observed that the claim itself did not specifically limit the term in the manner the Board determined. *Id.* at 1358. It then looked to the rest of the specification and found that it disclosed various embodiments that would have been excluded under the Board's construction. *Id.* Accordingly, the Court rejected the Board's construction in favor of one "more in line with the intrinsic evidence." *Id.* at 1359.

In *Abbott*, despite an express definition of the term "analyte," the Court declined to find lexicography, finding that that the "specification provides two alterative definitions" for "analyte" and thus did not provide the requisite clarity, deliberateness, and precision. 334 F.3d at 1355. Accordingly, the Court construed "analyte" to have its ordinary meaning. *Cf. supra* pages 52, 61-62 (explaining that the Definitions section here lists multiple two-carbon alkyls as "representative" "branched alkyls").

In *Merck & Co.*, the Court found the specification statement "ambiguous" in redefining "about," explaining that the statement was "amenable to a second (and more reasonable interpretation)" and based on disclosure in the specification construed "about" to have its ordinary meaning of "approximately." 395 F.3d at 1370–71.

Similarly, in *Ecolab,* the Court declined to accept the patentee's definition of the term "sanitize," finding the definition ambiguous because it did not indicate when the consumption of the meat product would occur—immediately after the application of the antimicrobial compound PAA or after the meat is cooked. 569 F.3d at 1345. The Court concluded that "the district court did not err when it construed the term 'sanitize' to mean that the treated meat has become safe for human handling and post-cooking consumption." *Id*.

Likewise, here, the Court should reject the district court's imposition of lexicography contrary to the intrinsic record, just as it has done time and again. *See Apple*, 81 F.4th at 1359 ("Our caselaw counsels against interpreting the claims in a way that would omit a disclosed embodiment absent clear evidence to the contrary.") (citing *Sequoia Tech.,.*, 66 F.4th at 1327); *see also Andrx Pharms.,* 473 F.3d at 1211 (rejecting district court's lexicography determination where district court's definition of "pharmaceutically acceptable polymer" to be "a water-soluble hydrophilic polymer" "would not cover some of the very polymers listed because they are not water-soluble"); *Azure Networks, LLC v. CSR PLC*, 771 F.3d 1336, 1349 (Fed. Cir. 2014) *vacated on other grounds*, 575 U.S. 959, 959 (2015) ("This one indicium therefore is simply not a strong enough suggestion that the inventor intended to displace a well-established term of art.").

C. Even if lexicography applies, the district court failed to adopt the patentees' chosen definition, which would include a 2-carbon alpha-branched alkyl.

Even if this Court were to enforce the definition of branched alkyl as lexicography, the district court erred. It failed to give weight to the definition's "unless otherwise specified" language and the intrinsic record's clear "specify[ing]" "otherwise" through text, formulas, and figures. As discussed above, this record shows that in the context of alpha branching the claims

encompass an alpha-branched carbon that binds to two carbons in R^{13} and to the oxygen in the biodegradable group (M^1).

Instead, the district court imposed a new standard requiring an unduly heightened showing for something to be specified otherwise. *See* Appx8, Appx5555 (Tr. at 129:15-18). For example, the district court—through its questioning—indicated it sought some exacting statements in the written description to show the patentees specified otherwise: "So when you say, 'exacting standards,' doesn't the 'otherwise specified' have to meet the exacting standard? It's part of the lexicography." *Id.* The district court further expressed the belief that "it would have to be done specifically; *i.e.*, in an instance, in every instance in which you want to depart from the lexicography" and "has to be done clearly and unequivocally." Appx10, Appx5559 (Tr. at 146:3-9).

These statements by the district court reveal a misunderstanding of this Court's lexicography precedents: The Court requires an exacting standard before it will conclude that a patentee has departed from the ordinary meaning of a term; it does not follow that *exceptions or provisos* from a lexicographic definition—which essentially allow a return to the ordinary meaning—must also be strictly construed. "[T]he description requirement does not demand any particular form of disclosure" but can be demonstrated by "structure, formula, chemical name, physical properties

or other properties." *Ariad Pharms.*, 598 F.3d at 1350, 1352; *CVI/Beta*, 112 F.3d at 1153. Thus, the fact that the intrinsic records' embodiments and formulas are disclosed as drawings and the patent does not expressly label them "otherwise specified" is of no particular significance.

The district court did not identify any authority for its holding, which is contrary to this Court's well-established precedent that "claim construction requires that we 'consider the specification as a whole, and read all portions of the written description, if possible, in a manner that renders the patent internally consistent.'" *Baxalta*, 972 F.3d at 1347. Importantly, the express scope of the independent claims allows for a 2-carbon group that the district court's construction excluded. That fact is further evidenced by dependent Claim 20 of the '933 Patent and dependent Claim 5 of the '979 Patent, which both expressly otherwise specify a branched alkyl group where the carbon is branched to two other carbons due to the claimed "—C(O)O—" biodegradable/ester group. *See* Appx320 ('933 Patent) at 538:41-42; Appx2635 ('979 Patent) at 494:52-53. Likewise, dependent Claim 14's three carbon limitation "raises a presumption that the limitation in question is not found in the independent claim." *Liebel-Flarsheim*, 358 F.3d at 910.

The patent written description expressly specifies these alpha-branched two-carbon bound structures as a "branched alkyl." Appx52

('933 Patent) at 2:7-9 ("the cationic lipid is a compound of Formula (I), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added); Appx53 ('933 Patent) at 3:63-65 ("the cationic lipid is a compound of Formula (II), which has a *branched alkyl* at the alpha position adjacent to the biodegradable group") (emphasis added). And the multiple depictions of alpha-branched alkyls bonded to two carbons in the specification and prosecution history specify otherwise from the alleged three-carbon lexicography when applied to the Patents' claimed "branched alkyls, where the branching occurs at the alpha position relative to the biodegradable group." *See* Appx320 ('933 Patent) at 538:27-29; *see Baxalta*, 972 F.3d at 1347.

Consequently, if this Court finds an unambiguous act of lexicography, it should further enforce the entire definition and hold that the intrinsic record repeatedly "otherwise specified" that in the context of alpha branching, the alpha position carbon may bind to two or more carbons.

CONCLUSION

For the foregoing reasons, Alnylam respectfully requests that the Court construe "branched alkyl" and "branched C_{10}-C_{20} alkyl" to have their ordinary and customary meanings, reverse the district court's judgment of non-infringement, and remand for further proceedings.

Respectfully submitted,

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Dated: December 4, 2023

ADDENDUM

TABLE TO THE ADDENDUM

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ALNYLAM PHARMACEUTICALS, INC., Plaintiff, v. MODERNA, INC., MODERNATX, INC., and MODERNA US, INC., Defendants.	)	C.A. No. 22-335-CFC (CONSOLIDATED)

~~[PROPOSED]~~ **FINAL JUDGMENT**

Pursuant to and for the reasons set forth in Plaintiff Alnylam Pharmaceuticals, Inc. ("Alnylam") and Defendants Moderna, Inc., ModernaTX, Inc., and Moderna US, Inc.'s (collectively, "Moderna") August 25, 2023 Stipulation And Joint Motion For Entry Of Final Judgment Of Non-Infringement ("Stipulation"), the Court ENTERS FINAL JUDGMENT of:

1. non-infringement of all asserted claims of U.S. Patent No. 11,246,933 ("the '933 Patent") and U.S. Patent No. 11,382,979 ("the '979 Patent") (collectively, the "Patents-in-Suit") in view of the Court's Claim Construction Order (D.I. 125).

The Court also DISMISSES WITHOUT PREJUDICE Moderna's affirmative defenses and counterclaims.

Each party is to bear its own costs and attorneys' fees incurred through the date of the Stipulation.

This is a final, appealable judgment.

IT IS SO ORDERED.

SO ORDERED, this 30th day of August, 2023.

United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ALNYLAM PHARMACEUTICALS, INC., Plaintiff, v. MODERNA, INC., MODERNATX, INC., and MODERNA US, INC., Defendants.	C.A. No. 22-cv-335-CFC (CONSOLIDATED)

~~[PROPOSED]~~ CLAIM CONSTRUCTION ORDER

The Court, having considered the parties' briefing on claim construction (D.I. 95), and in accordance with the reasoning set forth during the claim construction hearing on August 9, 2023 (D.I. 115), and the Court's subsequent August 9, 2023 Order (D.I. 114), IT IS HEREBY ORDERED that the terms of U.S. Patent Nos. 11,246,933 (the "'933 Patent") and 11,382,979 (the "'979 Patent") set forth below are construed as follows:

Claim Term	Claim Number(s)	Construction
"cationic lipid"	All asserted claims of the '933 Patent All asserted claims of the '979 Patent	Plain and ordinary meaning, which is "a lipid that is positively charged or that may be protonated at physiological pH"
"branched alkyl"	All asserted claims of the '933 Patent	"A saturated hydrocarbon moiety group in which one carbon atom

Claim Term	Claim Number(s)	Construction
	All asserted claims of the ’979 Patent	in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group.”
“branched C_{10}-C_{20} alkyl”	All asserted claims of the ’933 Patent All asserted claims of the ’979 Patent	“A saturated hydrocarbon moiety group with 10 to 20 carbon atoms and in which one carbon atom in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group”
“R^{13} is a branched C_{10}-C_{20} alkyl”		“R^{13} is a saturated hydrocarbon moiety group with 10 to 20 carbon atoms and in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group.”

Further, the parties have agreed to the following constructions, as set forth in the Joint Claim Construction Brief (D.I. 95):

Term	Agreed Construction
“directly bonded”	“Covalently bonded without any intervening atoms”
“primary group”	“the head group and central moiety”
“optionally comprises a primary, secondary, or tertiary amine”	“may or may not contain an amine, where the amine may be primary, secondary, or tertiary”
“where the branching occurs at the α-position relative to the [biodegradable/ester] group”	“where the branching occurs at a carbon atom next to the [biodegradable/ester] group”
“nucleic acid”	“a molecule composed of nucleotides, including modified nucleotides”
“an RNA”	“comprising ribonucleic acid”

The construction of the term "head group" is held in abeyance until such time as this Court conducts an additional hearing as discussed during the August 9, 2023 hearing, if necessary.

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Dated: August 16, 2023

IT IS SO ORDERED this 21st day of August, 2023.

Chief Judge Colm F. Connolly

IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

ALNYLAM PHARMACEUTICALS, INC.,)
Plaintiff,)
v.) C.A. No. 22-cv-335-CFC
MODERNA, INC., MODERNATX, INC., and MODERNA US, INC.,)
Defendants.)

Wednesday, August 9, 2023
9:05 a.m.
Markman Hearing

844 King Street
Wilmington, Delaware

BEFORE: THE HONORABLE COLM F. CONNOLLY
United States District Court Judge

APPEARANCES:

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For the BioNTech Defendant

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P R O C E E D I N G S

(Proceedings commenced in the courtroom beginning at 9:05 a.m.)

THE COURT: Good morning. Please be seated.

All right. Mr. Townsend.

MR. TOWNSEND: Good morning, Judge Connolly. Ethan Townsend from McDermott Will & Emery on behalf of Plaintiff Alnylam.

With me at counsel table is William Gaede, Sarah Fischer, Sarah Columbia, and Ian Brooks. Also here are -- is Indrani Franchini, general counsel, Alnylam, and Steve Bossone, chief intellectual property officer.

THE COURT: Mr. Farnan, how are you?

MR. FARNAN: Good morning, Your Honor. Michael Farnan for Moderna.

With me today at counsel table from Cooley is Chad Shear and Geoff Biegler. And also from Cooley in the gallery is Betsy Flanagan and Brianna Chamberlin. And from Moderna, we have Kelli Powell.

THE COURT: All right. Thank you. Who do we have next?

Mr. Silverstein.

MR. SILVERSTEIN: Good morning, Your Honor. Alan Silverstein of Connolly Gallagher for defendants Pfizer and Pharmacia and UpJohn.

With me today are Michael Johnson, Sara Horton and Dan Constantinescu from the Willkie Farr firm. Also with me today are Karen Shen and Jeff Myers from Pfizer.

THE COURT: Okay. Mr. Tigan.

MR. TIGAN: Good morning, Your Honor. Jeremy Tigan with Morris, Nichols on behalf of BioNTech. I'm joined by three of my colleagues from Winston & Strawn today, Charles Klein, Claire Fundakowski, and Jovial Wong. Our client representatives are here as well, Raymond Parker, Jessica Mackay and Vinny Lee.

THE COURT: Okay. Thank you.

Good morning, everybody. So I guess the first term is the cationic term, right?

MS. COLUMBIA: Good morning, Your Honor. May I approach and hand the slides up to the bench?

THE COURT: Sure. Go ahead, please.

MS. COLUMBIA: Good morning, Your Honor. Sarah Columbia on behalf of the plaintiff, Alnylam.

I will be speaking to the cationic lipid term and the head group term, which I think all the parties agree sort of rise and fall together.

And my colleague, Bill Gaede, will be speaking

exact same argument.

So you do have express written description about alpha branched alkyls. You have also express written description using the term "branched alkyl groups" showing binding to only two carbons. You have express examples, drawings in the specification, particularly at the four areas identified for that figure, plus -- that ends in Column 60, plus you also have all the different diagrams that are inherent within the drawing at Column 74 that we looked at. So given the standards -- exacting standards for lexicography, does not exist here, Your Honor, in the context of an alpha branched alkyl. The specification as a whole teaches clearly to the contrary.

THE COURT: So when you say, "exacting standards," doesn't the "otherwise specified" have to meet the exacting standard? It's part of the lexicography.

MR. GAEDE: It doesn't mean that you have to use the specific words, Your Honor.

THE COURT: I didn't ask that question. Just answer the question.

Doesn't the "otherwise specified" or the otherwise specification have to meet the exacting standard of lexicography? It's part of the lexicography,

right?

MR. GAEDE: I'm not going to quarrel with you on that, because I think we have. In other words, we have through -- in the prosecution history, all the points and specifications, the plain claim language itself, nowhere does it say that there must be three carbon bonds in the context of R13. That language is wholly absent from --

THE COURT: You didn't ask me to construe R13. I'm construing "branched alkyl" and "branched C10-C20 alkyl," right? And then I'm construing "R13 is a branched C10-C20 alkyl", right?

MR. GAEDE: Right.

THE COURT: In other words, every disputed term I'm asked to construe is includes "branched alkyl."

MR. GAEDE: Yes, correct. I would just make the important point, even though it is agreed upon construction, that "branched alkyl" is modified by the language wherein it occurs at the alpha position. So you can't read that out of the definition of "branched alkyl" is my only point.

And every example in the specification, then, of that fully meets our constructions.

THE COURT: All right. Anything else?

MR. GAEDE: Nothing, Your Honor. Thank you.

MR. BIEGLER: Good morning, Your Honor, Jeff Biegler from Cooley on behalf of Moderna. I'm going to start off and then Mr. Johnson might have some additional words to say for Pfizer.

Just to start, can we go to Slide 33, please.

There was a lot of discussion of the structure that appears at Columns 59 to 60 and carrying over to 61. Sorry, that's not it. 33.

THE COURT: You've got me at, what?

MR. BIEGLER: Column 59 to 60 carrying over to 61.

THE COURT: Okay.

MR. BIEGLER: This is the structure that there was a lot of discussion about. It starts at end of the page that has Columns 59 and 60. And I think I heard counsel --

THE COURT: Now, it's also at the bottom of Column 35 and 36, right, just so we are on the same page?

MR. BIEGLER: Exactly. Correct.

I think I heard counsel for Alnylam say that one of the defendants had cited this as an example of a branched alkyl. I want to just clarify first that that's incorrect.

The cite that Pfizer had in their brief ended at Line 35 of that page. This is at the bottom of the

page. This starts another discussion that's going on to discuss different parts of a representative lipid, which is what this is. So there was no concession on behalf of Pfizer that this showed a branched alkyl. That's just incorrect, first of all.

And there's a lot of discussion about this potentially being somehow read out of the claims by Moderna and Pfizer's construction, but there's no discussion in the specification that suggests this is a branched alkyl. It doesn't say that. There's nothing that says this is alpha branching. That's all detail that counsel added.

THE COURT: Right.

MR. BIEGLER: So the specification doesn't even use this as an example of a branched alkyl.

THE COURT: Because, I've got to tell you. Well, you can tell from my question I was taken by that, and I was going to actually press you, well, if this is a branched alkyl, does it meet the definition of 412? But you're saying you've never, nor has Pfizer, ever conceded that this is a branched alkyl?

MR. BIEGLER: No. We --

THE COURT: All right. Now let me just -- can I see in the briefs? What do they point to in the briefs that they said you said it, and then let me --

have to take my word for it; you can take the inventor's word for it.

If we can go to Slide 29. Sorry, 28. The numbering is off. There we go.

There is another claim in the patent, Your Honor, Claim 14, which depends from Claim 1. These aren't asserted, but Claim 1 has almost identical language as far as the alpha branching and branched alkyl groups go. You'll see in the second clause Claim 1 requires a branched alkyl "wherein the branching occurs at the alpha position relative to the biodegradable group," just like the claims we're talking about.

The inventors added a dependent claim, Claim 14, that specifically says, "Wherein the branched alkyl group has only one carbon atom which is bound to three other carbon atoms." So they were specifying, because there could be potentially more than one. And they're saying, here, it's only one.

And if we can go to Slide 34. Next one, please. Keep going. There we go. Next one. One more.

So counsel was discussing the file history, and showed how the inventors differentiate some prior art. And there was sort of two different groups of lipids that were differentiated. And he showed, I think, this same blow-out of the prior art, which is the box

that has Page 58 of WO493. And I think he said the inventors didn't differentiate this based on a carbon being bound to three other carbons.

That's just false. The inventors actually added that Claim 14 that we just looked at. It was Claim 32. At the time, they added this claim to overcome this prior art. And so the inventors said, new Claim 32 recites that the branched alkyl group has only one carbon atom which is bound to three other carbon atoms. The compounds in the prior art each have three carbon atoms, which are bound to three carbon atoms.

So this is explicitly showing that they are using the inventor's definition and specifying that, for this dependent claim, there's only one of these such groups, not more than one. And that's how they differentiated the prior art.

And I would submit, Your Honor, you asked what does it mean to "otherwise specify"? They could have done that very simply, either by using language like this, they could have just said for the claim, "There are none of these types of groups."

They could have also just drawn up the chemical structure, which is the most common way of claiming things in this area. We saw Formula II come up. If they had wanted to claim Formula II, they could have

claimed Formula II. That's not what they did. They decided to use the language that they specifically defined in the specification.

And not only that, if there is some clarification that the definition doesn't apply, that would need to come in the claims because the definition itself anticipates that there might be some scenarios in which it doesn't apply. So if there's an example in the specification that, you know, refers to a branched alkyl, where that's not the case, that's otherwise specified.

THE COURT: It's got to be specific. I mean, that's the whole point, right.

MR. BIEGLER: Exactly.

THE COURT: It's got to be a specific instance of it being used where there is this otherwise specification.

MR. BIEGLER: And so how do you do that? You draw a chemical structure.

THE COURT: It has to be, also, clear and unequivocal, right? Or clear and -- not clear and convincing. I guess it's clear and unequivocal.

MR. BIEGLER: Deliberate.

THE COURT: All right.

MR. BIEGLER: Because I think, Your Honor, Formula II is probably the closest. You asked me for the

closest example they have in the specification that otherwise specifies. The closest is probably Formula II. But at best, that otherwise specifies, the claims don't do that. The inventors clarified through the dependent claims that this definition could apply to these claims with Moderna and Pfizer's definition of branched alkyl.

THE COURT: All right. Hold on one second.

So let me ask you, defendants -- I mean, I think the defendants have the better arguments here by far. I mean, I don't think there's -- there's nothing like clear and deliberate otherwise specifying to escape from what is absolutely clear and unequivocal lexicography.

I mean, it's in the definition section. And it says, at Column 412, Lines 13 through 18, quote, "Unless otherwise specified, the terms 'branched alkyl, branched alkenyl, and branched alkynyl' refer to an alkyl, alkenyl or alkynyl group in which one carbon atom in the group is (1) bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group."

Now, Column 411 at Line 52, there's a definition for alkyl and alkylene. And it says that those terms refer to "a straight or branched chain saturated hydrocarbon moiety."

It strikes me, if we're really going to be

precise, we probably should incorporate that, at least for alkyl and alkylene. And, actually, I'm only defining alkyl, so that would clearly apply.

So how about this, the first term you've asked me to interpret is "branched alkyl." Right? That term, it seems to me, should be taken expressly from these two places.

I think both defendants have said I should interpret that to mean an alkyl in one carbon atom in the group(1)is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group, unqote.

I was inclined to construe "branched alkyl" to mean, "A saturated hydrocarbon moiety group in which one carbon atom in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group."

What's defendants' position on that?

MR. BIEGLER: That's fine with Moderna, Your Honor.

THE COURT: Okay. So the reason, just to be clear, why I'm interpreting all of these terms consistent with the defendants' position is because there is clear and unequivocal lexicography. And it comes at 412, Lines 13 through 18. And as I mentioned at Column 411, Line 53 and 54.

There has to be some specification otherwise to depart from that lexicography. By definition, specification is specific. So it would have to be done specifically; i.e., in an instance, in every instance in which you want to depart from the lexicography.

Furthermore, I think to be consistent with Federal case law, providing that specification otherwise, has to be done clearly and unequivocally. And the plaintiffs have come nowhere close to demonstrating that.

They asked me to engage in this kind of, you know, follow-the-dots logic. They don't point to anything specific or express in the claims, which is the most important thing, nor do they point to anything even in the written description that would support that there was a departure from the lexicography provided in Column 412.

And, frankly, it's pretty easy to come up, I mean, I think this is an easy call. It's not complicated at all. And, you know, just for the record, I'll refer to the *Thorner against Sony Computer* case, 669 F.3d 1362, Page 1365, where this black letter law set out that if you want to depart from the plain and ordinary meaning of a term, there's only two exceptions. And the first is when the patentee sets out a definition and acts as her own lexicographer, and the second is when the patentee

disavows the full scope of the claim, either in the written description or during prosecution. They must be, in both instances, clear and unmistakable, and we just don't have that here.

What we do have, as I've already mentioned, is clear and unmistakable lexicography. All right. Now, so that applies to all the disputed terms, that general finding and dictates the outcome.

I've already construed the branched alkyl term.

The next term in dispute is the branched C10-C20 alkyl. I'm inclined, but I'm happy to hear from the defendants, to construe that term as "A saturated hydrocarbon moiety group with 10 to 20 carbon atoms and in which one carbon atom in the group (1) is bound to at least three other carbon atoms, and (2) is not a ring atom of a cyclic group," unquote.

MR. JOHNSON: I think the only thing, Your Honor, is it -- for the term for Pfizer that makes clear that that is talking specifically about R13 is that --

THE COURT: I haven't gotten to R13 yet.

MR. JOHNSON: Okay. I'm sorry.

THE COURT: I mean, well, no, I don't know. Look, this is the way you present it to me. I don't know that I would have presented it this way, but you all did.

You did it. Whenever parties agree, I'm inclined to do what they agree to.

So this term you've asked me to construe is, quote, "branched C10-C20 alkyl."

The third term you asked me to construe is really a sentence, "R13 is a branched C10-C20 alkyl." I haven't gotten to that yet.

MR. JOHNSON: Sorry.

THE COURT: So do you have any objection to what I've just said about construing, quote, "branched C10-C20 alkyl" unquote?

MR. JOHNSON: I do to the extent that -- well, I guess it depends a little bit on what you're going to say about R13.

But to me, the term is "R13 is this." So we need that to reflect that those things are all part of R13 and not part of M1, for example.

THE COURT: Well, I was going to construe -- again, you didn't ask me to construe R13. You asked me to construe "R13 is a branched C10-C20 alkyl." Right?

And I was going to construe it to mean, quote, "R13 is a saturated hydrocarbon moiety group with 10 to 20 carbon atoms and in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group," unquote.

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(12) **United States Patent**
Maier et al.

(10) **Patent No.: US 11,246,933 B1**
(45) **Date of Patent: *Feb. 15, 2022**

(54) **BIODEGRADABLE LIPIDS FOR THE DELIVERY OF ACTIVE AGENTS**

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(57) **ABSTRACT**

The present invention relates to a cationic lipid having one or more biodegradable groups located in a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid. These cationic lipids may be incorporated into a lipid particle for delivering an active agent, such as a nucleic acid. The invention also relates to lipid particles comprising a neutral lipid, a lipid capable of reducing aggregation, a cationic lipid of the present invention, and optionally, a sterol. The lipid particle may further include a therapeutic agent such as a nucleic acid.

28 Claims, No Drawings

Specification includes a Sequence Listing.

Related U.S. Application Data

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BIODEGRADABLE LIPIDS FOR THE DELIVERY OF ACTIVE AGENTS

This application is a continuation of U.S. patent application Ser. No. 16/520,183, filed Jul. 23, 2019, which is a continuation of U.S. patent application Ser. No. 14/677,801, filed Apr. 2, 2015, now U.S. Pat. No. 10,369,226, which is a continuation of U.S. patent application Ser. No. 13/708,383, filed Dec. 7, 2012, now U.S. Pat. No. 9,061,063, which claims the benefit of U.S. Provisional Application No. 61/568,133, filed Dec. 7, 2011, and U.S. Provisional Application No. 61/623,274, filed Apr. 12, 2012, each of which is hereby incorporated by reference.

TECHNICAL FIELD

The present invention relates to biodegradable lipids and to their use for the delivery of active agents such as nucleic acids.

BACKGROUND

Therapeutic nucleic acids include, e.g., small interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, immune stimulating nucleic acids, antisense, antagomir, antimir, microRNA mimic, supermir, U1 adaptor, and aptamer. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both in vitro and in vivo models. In addition, siRNA constructs are currently being evaluated in clinical studies.

However, two problems currently faced by siRNA or miRNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind the protein RISC when administered systemically as the free siRNA or miRNA. Lipid nanoparticles formed from cationic lipids with other lipid components, such as cholesterol and PEG lipids, and oligonucleotides (such as siRNA and miRNA) have been used to facilitate the cellular uptake of the oligonucleotides.

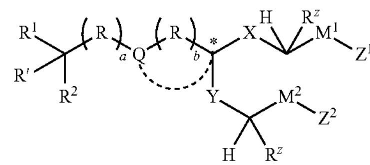
There remains a need for improved cationic lipids and lipid nanoparticles for the delivery of oligonucleotides. Preferably, these lipid nanoparticles would provide high drug:lipid ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to the patient.

SUMMARY

The present invention relates to a cationic lipid and PEG lipid suitable for forming nucleic acid-lipid particles. Each of the cationic and PEG lipids of the present invention includes one or more biodegradable groups. The biodegradable groups are located in a lipidic moiety (e.g., a hydrophobic chain) of the cationic or PEG lipid. These cationic and PEG lipids may be incorporated into a lipid particle for delivering an active agent, such as a nucleic acid (e.g., an siRNA). The incorporation of the biodegradable group(s) into the lipid results in faster metabolism and removal of the lipid from the body following delivery of the active agent to a target area. As a result, these lipids have lower toxicity than similar lipids without the biodegradable groups.

In one embodiment, the cationic lipid is a compound of formula (I), which has a branched alkyl at the alpha position adjacent to the biodegradable group (between the biodegradable group and the teriary carbon):

Formula (I)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl);

with respect to R^1 and R^2,

(i) R^1 and R^2 are each, independently, optionally substituted alkyl, alkenyl, alkynyl, cycloalkylalkyl, heterocycle, or R^{10};

(ii) R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocyclic ring; or

(iii) one of R^1 and R^2 is optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, or heterocycle, and the other forms a 4-10 member heterocyclic ring or heteroaryl (e.g., a 6-member ring) with (a) the adjacent nitrogen atom and (b) the $(R)_a$ group adjacent to the nitrogen atom;

each occurrence of R is, independently, —(CR^3R^4)—;

each occurrence of R^3 and R^4 are, independently H, halogen, OH, alkyl, alkoxy, —NH_2, R^{10}, alkylamino, or dialkylamino (in one preferred embodiment, each occurrence of R^3 and R^4 are, independently H or C_1-C_4 alkyl);

each occurrence of R^{10} is independently selected from PEG and polymers based on poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein (i) the PEG or polymer is linear or branched, (ii) the PEG or polymer is polymerized by n subunits, (iii) n is a number-averaged degree of polymerization between 10 and 200 units, and (iv) wherein the compound of formula has at most two R^{10} groups (preferably at most one R^{10} group);

the dashed line to Q is absent or a bond;

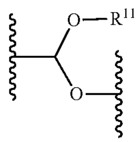
when the dashed line to Q is absent then Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —C(O)N(R^4)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —O—N═C(R^5)—, —C(R^5)═N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)═N—O—C(O)—; or

when the dashed line to Q is a bond then (i) b is 0 and (ii) Q and the tertiary carbon adjacent to it (C*) form a substituted or unsubstituted, mono- or bi-cyclic heterocyclic group having from 5 to 10 ring atoms (e.g., the heteroatoms in the heterocyclic group are selected from O and S, preferably O);

each occurrence of R^5 is, independently, H or alkyl (e.g. C_1-C_4 alkyl);

X and Y are each, independently, alkylene or alkenylene (e.g., C_4 to C_{20} alkylene or C_4 to C_{20} alkenylene);

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



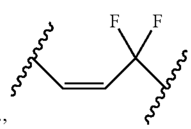
(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

each occurrence of R^z is, independently, C_1-C_8 alkyl (e.g., methyl, ethyl, isopropyl, n-butyl, n-pentyl, or n-hexyl);

a is 1, 2, 3, 4, 5 or 6;

b is 0, 1, 2, or 3; and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g., ).

The $R'R^1R^2$N—(R)$_a$-Q-(R)$_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2$N—(R)$_a$-Q-(R)$_b$— is $(CH_3)_2$N—$(CH_2)_3$—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2$N—$(CH_2)_3$—C(CH_3)═N—O—.

In one embodiment, R^1 and R^2 are both alkyl (e.g., methyl).

In a further embodiment, a is 3. In another embodiment, b is 0.

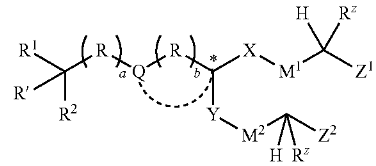
In a further embodiment, a is 3, b is 0 and R is —CH_2—. In yet a further embodiment, a is 3, b is 0, R is —CH_2— and Q is —C(O)O—. In another embodiment, R^1 and R^2 are methyl, a is 3, b is 0, R is —CH_2— and Q is —C(O)O—.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

In further embodiments, M^1 and M^2 are each, independently, —OC(O)— or —C(O)O—. For example, in one embodiment, M^1 and M^2 are each —C(O)O—.

In another embodiment, the cationic lipid is a compound of formula (II), which has a branched alkyl at the alpha position adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 o Z^2):

Formula (II)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl);

with respect to R^1 and R^2,

(i) R^1 and R^2 are each, independently, optionally substituted alkyl, alkenyl, alkynyl, cycloalkylalkyl, heterocycle, or R^{10};

(ii) R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocylic ring; or

(iii) one of R^1 and R^2 is optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, or heterocycle, and the other forms a 4-10 member heterocyclic ring or heteroaryl (e.g., a 6-member ring) with (a) the adjacent nitrogen atom and (b) the (R)$_a$ group adjacent to the nitrogen atom;

each occurrence of R is, independently, —(CR^3R^4)—;

each occurrence of R^3 and R^4 are, independently H, halogen, OH, alkyl, alkoxy, —NH_2, R^{10}, alkylamino, or dialkylamino (in one preferred embodiment, each occurrence of R^3 and R^4 are, independently H or C_1-C_4 alkyl);

each occurrence of R^{10} is independently selected from PEG and polymers based on poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein (i) the PEG or polymer is linear or branched, (ii) the PEG or polymer is polymerized by n subunits, (iii) n is a number-averaged degree of polymerization between 10 and 200 units, and (iv) wherein the compound of formula has at most two R^{10} groups (preferably at most one R^{10} group);

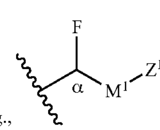
the dashed line to Q is absent or a bond;

when the dashed line to Q is absent then Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —C(O)N(R^4)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —O—N═C(R^5)—, —C(R^5)═N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)═N—O—C(O)—; or

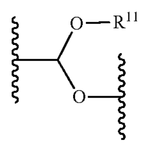
when the dashed line to Q is a bond then (i) b is 0 and (ii) Q and the tertiary carbon adjacent to it (C*) form a substituted or unsubstituted, mono- or bi-cyclic heterocyclic group having from 5 to 10 ring atoms (e.g., the heteroatoms in the heterocyclic group are selected from O and S, preferably O);

each occurrence of R^5 is, independently, H or alkyl;

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group

(e.g., );

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



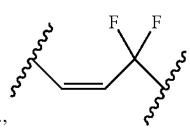
(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

each occurrence of R^z is, independently, C_1-C_8 alkyl (e.g., methyl, ethyl, isopropyl);

a is 1, 2, 3, 4, 5 or 6;

b is 0, 1, 2, or 3; and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein (i) the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g., )

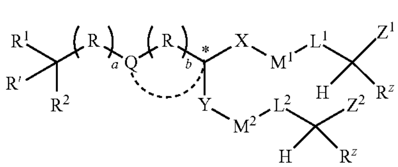
and (ii) the terminus of at least one of Z^1 and Z^2 is separated from the group M^1 or M^2 by at least 8 carbon atoms.

In another embodiment, X and Y are each, independently —(CH_2)$_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —(CH_2)$_6$—. In another embodiment, X and Y are —(CH_2)$_7$—. In yet another embodiment, X and Y are —(CH_2)$_9$—. In yet another embodiment, X and Y are —(CH_2)$_8$—.

The R'R^1R^2N—(R)$_a$-Q-(R)$_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—(R)$_a$-Q-(R)$_b$— is (CH_3)$_2$N—(CH_2)$_3$—C(O)O—, (CH_3)$_2$N—(CH_2)$_2$—NH—C(O)O—, (CH_3)$_2$N—(CH_2)$_2$—OC(O)—NH—, or (CH_3)$_2$N—(CH_2)$_3$—C(CH_3)═N—O—.

In another embodiment, the cationic lipid is a compound of formula (III), which has a branching point at a position that is 2-6 carbon atoms (i.e., at the beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position(ζ)) adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (III)



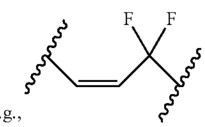
or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, R^z, a, and b are defined as in formula (I);

L^1 and L^2 are each, independently, C_1-C_5 alkylene or C_2-C_5 alkenylene;

X and Y are each, independently, alkylene (e.g., C_4 to C_{20} alkylene or C_6-C_8 alkylene) or alkenylene (e.g., C_4 to C_{20} alkenylene); and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g., ),

and with the proviso that the terminus of at least one of Z^1 and Z^2 is separated from the group M^1 or M^2 by at least 8 carbon atoms.

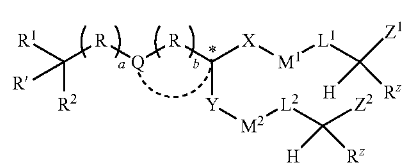
In one embodiment, L^1 and L^2 are each —CH_2—. In another embodiment, L^1 and L^2 are each —(CH_2)$_2$—. In one embodiment, L^1 and L^2 are each —(CH_2)$_3$—. In yet another embodiment, L^1 and L^2 are each —(CH_2)$_4$—. In yet another embodiment, L^1 and L^2 are each —(CH_2)$_5$—. In yet another embodiment, L^1 and L^2 are each —CH_2—CH═CH—. In a preferred embodiment, L^1 and L^2 are each —CH_2— or —(CH_2)$_2$.

In one embodiment, X and Y are each, independently —(CH_2)$_n$, wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —(CH_2)$_7$—. In another exemplary embodiment, X and Y are —(CH_2)$_8$—. In yet another exemplary embodiment, X and Y are —(CH_2)$_9$—.

The R'R^1R^2N—(R)$_a$-Q-(R)$_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—(R)$_a$-Q-(R)$_b$— is (CH_3)$_2$N—(CH_2)$_3$—C(O)O—, (CH_3)$_2$N—(CH_2)$_2$—NH—C(O)O—, (CH_3)$_2$N—(CH_2)$_2$—OC(O)—NH—, or (CH_3)$_2$N—(CH_2)$_3$—C(CH_3)═N—O—.

In another embodiment, the cationic lipid is a compound of formula (IIIA), which has a branching point at a position that is 2-6 carbon atoms (i.e., at the beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position(ζ)) from the biodegradable groups M^1 and M^2 (i.e., between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (IIIA)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

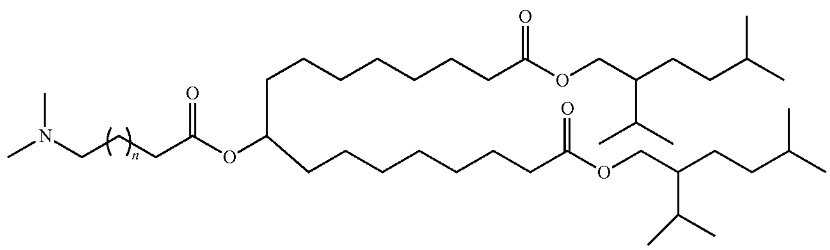
each R^z is, independently, C_1-C_8 alkyl (e.g., C_3-C_6 alkyl or C_2-C_3 alkyl);

L^1 and L^2 are each, independently, C_1-C_5 alkylene (e.g., C_2-C_3 alkylene) or C_2-C_5 alkenylene;

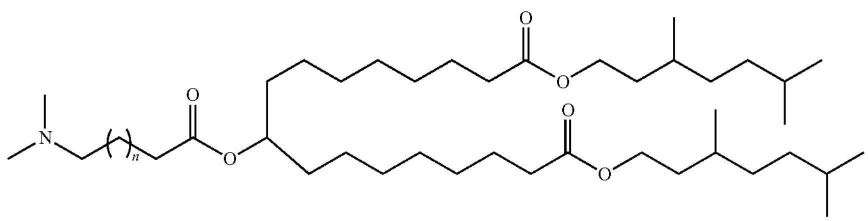
X and Y are each, independently, alkylene (e.g., C_4 to C_{20} alkylene or C_7-C_9 alkylene) or alkenylene (e.g., C_4 to C_{20} alkenylene or C_7-C_9 alkenylene); and

Z^1 and Z^2 are each, independently, C_1-C_8 alkyl (e.g., C_1-C_6 alkyl, such as C_1, C_3 or C_5 alkyl) or C_2-C_8 alkenyl (such as C_2-C_6 alkenyl);

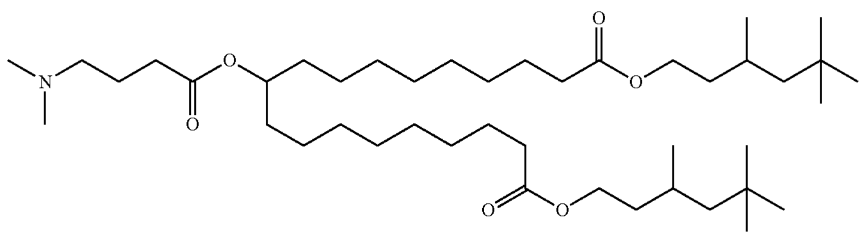
wherein said cationic lipid is not one selected from:

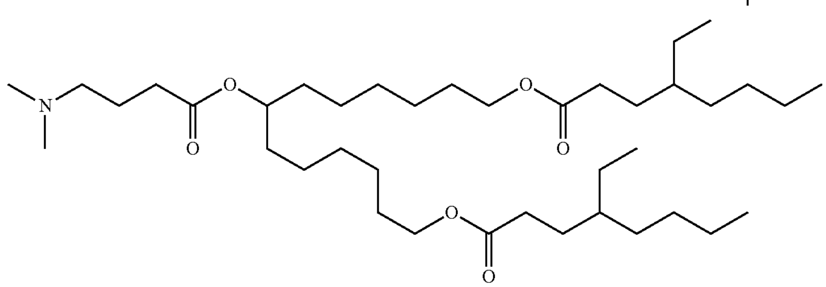


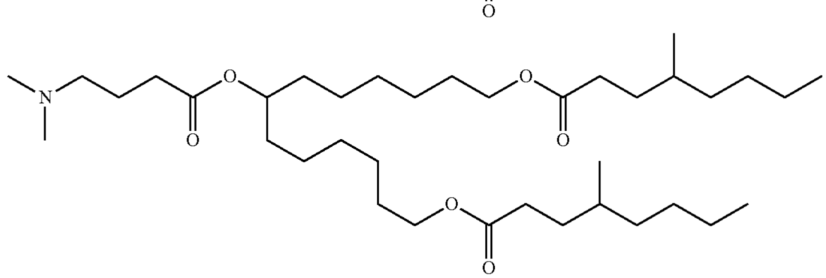
n = 0-2

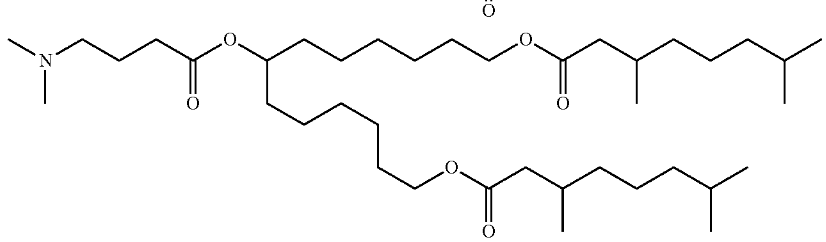


n = 0-2









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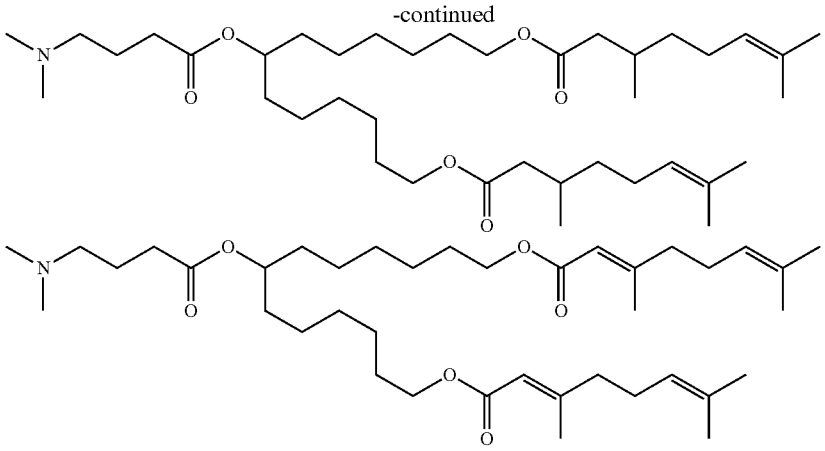


In one embodiment, L^1 and L^2 are each —$(CH_2)_2$—. In another embodiment, L^1 and L^2 are each —$(CH_2)_3$—.

In one embodiment, X and Y are each, independently —$(CH_2)_n$ wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, 4 to 12 or 7-9. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_7$—. In yet another exemplary embodiment, X and Y are —$(CH_2)_9$.

In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and L^2).

The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—$C(CH_3)$=N—O—.

In one preferred embodiment, Z^1 and Z^2 are branched alkyl or branched alkenyl groups.

In one embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 alkyl (such as a C_3-C_6 alkyl). In another embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 branched alkyl (such as a C_3-C_6 branched alkyl). In yet another embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 straight alkyl (such as a C_3-C_6 straight alkyl).

In one embodiment of formula (IIIA), the branching point is at the second position (the β-position) from the biodegradable groups M^1 and M^2 in each tail. Z^1, Z^2, and each R^z can be C_3-C_8 alkyl (e.g., a C_3-C_6 alkyl), such as a C_3-C_8 branched alkyl (e.g., a C_3-C_6 branched alkyl) or a C_3-C_8 straight alkyl (e.g., a C_3-C_6 straight alkyl). In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and/or L^2).

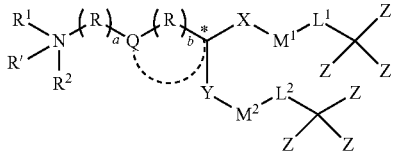
In one embodiment of formula (IIIA), the branching point is at the third position (the γ-position) from the biodegradable groups M^1 and M^2 in each tail. Z^1, Z^2, and each R^z can be C_3-C_8 alkyl (e.g., a C_3-C_6 alkyl), such as a C_3-C_8 branched alkyl (e.g., a C_3-C_6 branched alkyl) or a C_3-C_8 straight alkyl (e.g., a C_3-C_6 straight alkyl). In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and/or L^2).

In one embodiment of formula (IIIA), the branching point is at the third position (the γ-position) from the biodegradable groups M^1 and M^2 in each tail.

In another embodiment of formula (IIIA), M^1 and/or M^2 are not —O(C(O)— (where the oxygen atom in M^1 and/or M^2 is bound to the variable X, and the carbonyl in M^1 and/or M^2 is bound to the variable L^1 and/or L^2). In yet another embodiment of formula (IIIA), Z^1, Z^2, and R^z are not C_3-C_{10} cycloalkyl(C_1-C_6 alkyl).

In another embodiment, the cationic lipid is a compound of formula (IV), which has a branching point at a position that is 2-6 carbon atoms (i.e., at beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position(ζ)) adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (IV)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

L^1 and L^2 are each, independently, C_1-C_5 alkylene or C_2-C_5 alkenylene;

X and Y are each, independently, alkylene or alkenylene (e.g., C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene); and

each occurrence of Z is independently C_1-C_4 alkyl (preferably, methyl).

For example, in one embodiment, -L^1-$C(Z)_3$ is —$CH_2C(CH_3)_3$. In another embodiment, -L^1-$C(Z)_3$ is —$CH_2CH_2C(CH_3)_3$.

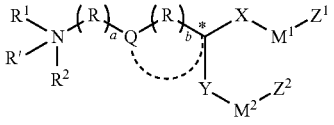
In one embodiment, the total carbon atom content of each tail (e.g., —X-M^1-L^1-$C(Z)_3$ or —Y-M^2-L^2-$C(Z)_3$) is from about 17 to about 26. For example, the total carbon atom content can be from about 19 to about 26 or from about 21 to about 26.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In

another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

In one embodiment, the cationic lipid is a compound of formula (V), which has an alkoxy or thioalkoxy (i.e., —S-alkyl) group substitution on at least one tail:

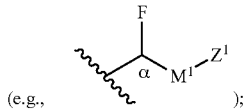
Formula (V)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

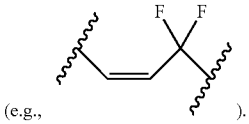
R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



(e.g.,);

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein (i) the C_8-C_{14} alkyl or C_8-C_{14} alkenyl of at least one of Z^1 and Z^2 is substituted by one or more alkoxy (e.g., a C_1-C_4 alkoxy such as —OCH_3) or thioalkoxy (e.g., a C_1-C_4 thioalkoxy such as —SCH_3) groups, and (ii) the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2



(e.g.,).

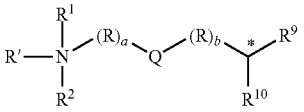
In one embodiment, the alkoxy substitution on Z^1 and/or Z^2 is at the beta position from the M^1 and/or M^2 group.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

The R'R^1R^2N—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2$N—$(CH_2)_3$—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2$N—$(CH_2)_3$—C(CH_3)=N—O—.

In one embodiment, the cationic lipid is a compound of formula (VIA), which has one or more fluoro substituents on at least one tail at a position that is either alpha to a double bond or alpha to a biodegradable group:

Formula (VIA)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R^1, R^2, R, a, and b are as defined with respect to formula (I);

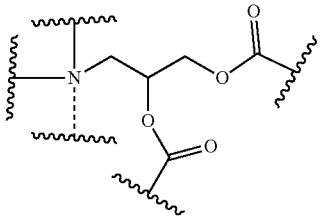
Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —C(O)N(R^4)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —O—N=C(R^5)—, —C(R^5)=N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)=N—O—C(O)—;

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl); and

each of R^9 and R^{10} are independently C_{12}-C_{24} alkyl (e.g., C_{12}-C_{20} alkyl), C_{12}-C_{24} alkenyl (e.g., C_{12}-C_{20} alkenyl), or C_{12}-C_{24} alkoxy (e.g., C_{12}-C_{20} alkoxy) (a) having one or more biodegradable groups and (b) optionally substituted with one or more fluorine atoms at a position which is (i) alpha to a biodegradable group and between the biodegradable group and the tertiary carbon atom marked with an asterisk (*), or (ii) alpha to a carbon-carbon double bond and between the double bond and the terminus of the R^9 or R^{10} group; each biodegradable group independently interrupts the C_{12}-C_{24} alkyl, alkenyl, or alkoxy group or is substituted at the terminus of the C_{12}-C_{24} alkyl, alkenyl, or alkoxy group, wherein

(i) at least one of R^9 and R^{10} contains a fluoro group;

(ii) the compound does not contain the following moiety:



wherein ---- is an optional bond; and

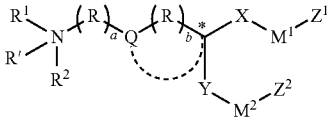
(iii) the terminus of R^9 and R^{10} is separated from the tertiary carbon atom marked with an asterisk (*) by a chain of 8 or more atoms (e.g., 12 or 14 or more atoms).

In one preferred embodiment, the terminus of R^9 and R^{10} is separated from the tertiary carbon atom marked with an asterisk (*) by a chain of 18-22 carbon atoms (e.g., 18-20 carbon atoms).

In another embodiment, the terminus of the R^9 and/or R^{10} has the formula —C(O)O—CF_3.

In another embodiment, the cationic lipid is a compound of formula (VIB), which has one or more fluoro substituents on at least one tail at a position that is either alpha to a double bond or alpha to a biodegradable group:

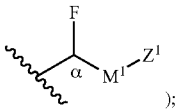
Formula (VIB)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

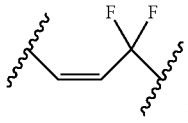
R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group and



(e.g.,);

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein said C_8-C_{14} alkenyl is optionally substituted by one or more fluorine atoms at a position that is alpha to a double bond

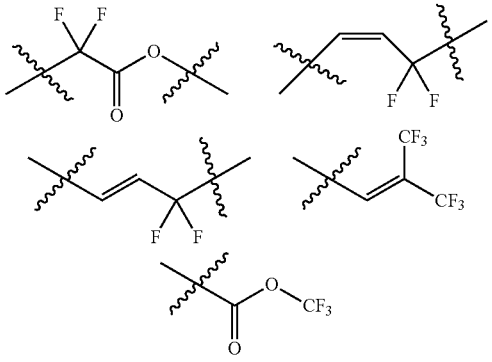


(e.g.,).

wherein at least one of X, Y, Z^1, and Z^2 contains a fluorine atom.

In one embodiment, at least one of Z^1 and Z^2 is substituted by two fluoro groups at a position that is either alpha to a double bond or alpha to a biodegradable group. In one embodiment, at least one of Z^1 and Z^2 has a terminal —CF_3 group at a position that is alpha to a biodegradable group (i.e., at least one of Z^1 and Z^2 terminates with an —C(O)OCF_3 group).

For example, at least one of Z^1 and Z^2 may include one or more of the following moieties:

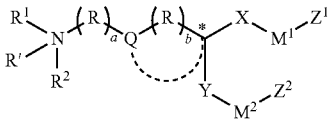


In one embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_7$—. In another exemplary embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—$C(CH_3)$=N—O—.

In one embodiment, the cationic lipid is a compound of formula (VII), which has an acetal group as a biodegradable group in at least one tail:

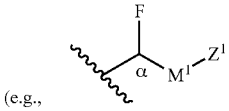
Formula (VII)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

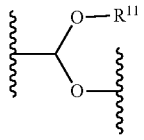
R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



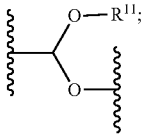
(e.g.,);

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —$C(R^5)$=N—, —N=$C(R^5)$—, —$C(R^5)$=N—O—, —O—N=$C(R^5)$—, —C(O)(NR^5)—, —$N(R^5)$C(O)—, —C(S)(NR^5)—, —$N(R^5)$C(O)—, —$N(R^5)$C(O)$N(R^5)$—, —OC(O)O—, —$OSi(R^5)_2O$—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



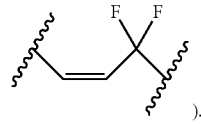
(wherein R^{11} is a C_4-C_{10} alkyl or C_4-C_{10} alkenyl));

with the proviso that at least one of M^1 and M^2 is



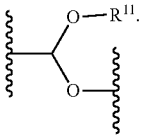
and

Z^1 and Z^2 are each, independently, C_4-C_{14} alkyl or C_4-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2



(e.g.,).

In one embodiment, each of M^1 and M^2 is



In another embodiment, X and Y are each, independently $—(CH_2)_n—$ wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are $—(CH_2)_6—$. In another embodiment, X and Y are $—(CH_2)_7—$. In yet another embodiment, X and Y are $—(CH_2)_9—$. In yet another embodiment, X and Y are $—(CH_2)_8—$.

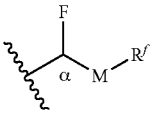
The $R'R^1R^2N—(R)_a\text{-}Q\text{-}(R)_b—$ group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N—(R)_a\text{-}Q\text{-}(R)_b—$ is $(CH_3)_2N—(CH_2)_3—C(O)O—$, $(CH_3)_2N—(CH_2)_2—NH—C(O)O—$, $(CH_3)_2N—(CH_2)_2—OC(O)—NH—$, or $(CH_3)_2N—(CH_2)_3—C(CH_3)=N—O—$.

In another embodiment, the present invention relates to a cationic lipid or a salt thereof having:

(i) a central carbon atom,

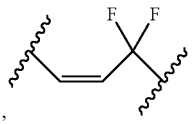
(ii) a nitrogen containing head group directly bound to the central carbon atom, and

(iii) two hydrophobic tails directly bound to the central carbon atom, wherein each hydrophobic tail is of the formula $—R^e\text{-}M\text{-}R^f$ where R^e is a C_4-C_{14} alkyl or alkenyl, M is a biodegradable group, and R^f is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of $—R^e\text{-}M\text{-}R^f$ is at most 20 atoms (i.e. the total length of the tail from the first carbon atom after the central carbon atom to a terminus of the tail is at most 20), and (ii) the group $—R^e\text{-}M\text{-}R^f$ has at least 20 carbon atoms (e.g., at least 21 atoms). Optionally, the alkyl or alkenyl group in R^e may be substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



(e.g.,).

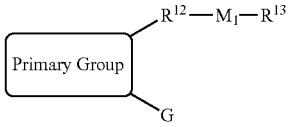
Also, optionally, the alkenyl group in R^f may be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of R^f



(e.g.,).

In one embodiment, the cationic lipid of the present invention (such as of formulas I-VII) has assymetrical hydrophobic groups (i.e., the two hydrophobic groups have different chemical formulas). For example, the cationic lipid can have the formula:

Formula (VIII)

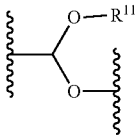


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

G is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl (e.g., a n-C_8 alkyl n-C_9 alkyl, or n-C_{10} alkyl);

R^{12} is a branched or unbranched alkylene or alkenylene (e.g., C_6-C_{20} alkylene or C_6-C_{20} alkenylene such as C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene);

M_1 is a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S, $—C(R^5)=N—$, $—N=C(R^5)—$, $—C(R^5)=N—O—$, $—O—N=C(R^5)—$, $—C(O)(NR^5)—$, $—N(R^5)C(O)—$, $—C(S)(NR^5)—$, $—N(R^5)C(O)—$, $—N(R^5)C(O)N(R^5)—$, —OC(O)O—, $—OSi(R^5)_2O—$, $—C(O)(CR^3R^4)C(O)O—$, $—OC(O)(CR^3R^4)C(O)—$, or

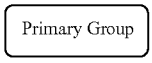


(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

R^3 and R^4 are defined as in formula (I);

each occurrence of R^5 is, independently, H or alkyl (e.g., C_1-C_4 alkyl);

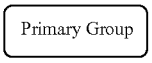
R^{13} is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl;



comprises a protonatable group having a pK_a of from about 4 to about 13, more preferably from about 5 to about 8 (e.g. from about 5 to about 7, or from about 5 to about 6.5, or from about 5.5 to about 6.5, or from about 6 to about 6.5).

In one embodiment, the primary group includes (i) a head group, and (ii) a central moiety (e.g., a central carbon atom) to which both the hydrophobic tails are directly bonded. Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group.

Representative



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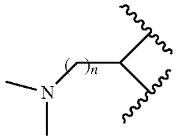
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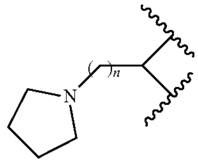
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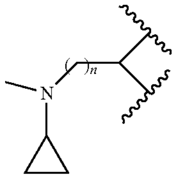
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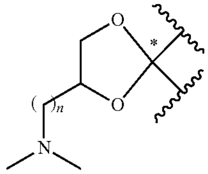
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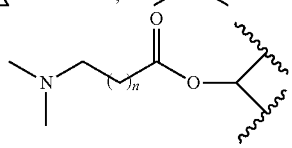
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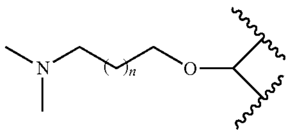
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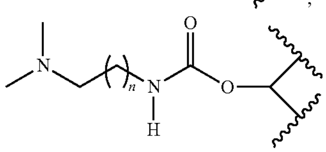
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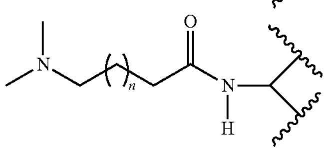
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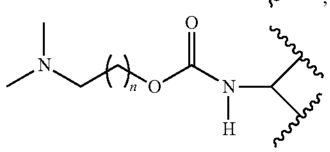

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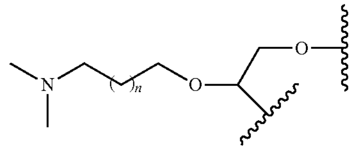
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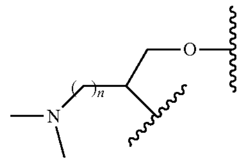
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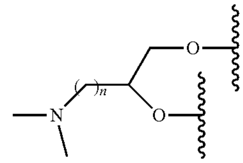
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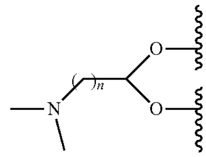
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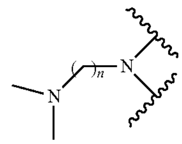
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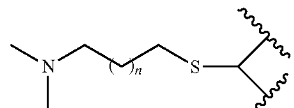
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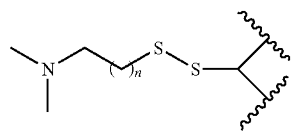
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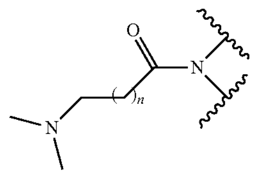
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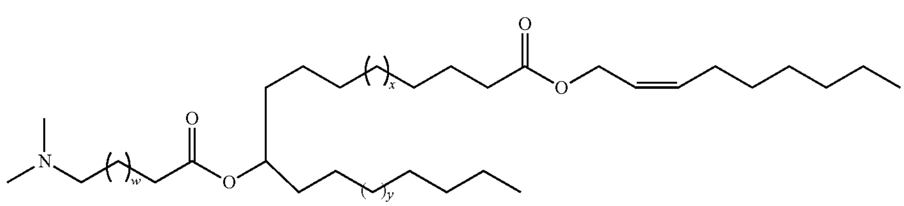
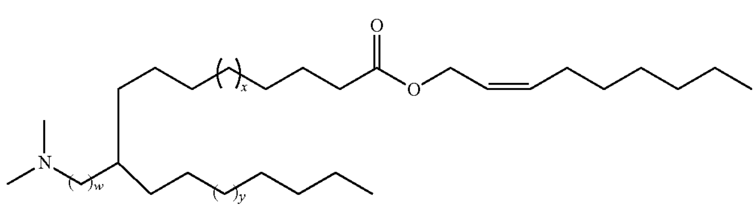
where n is 0-6.

Representative asymmetrical cationic lipids include:





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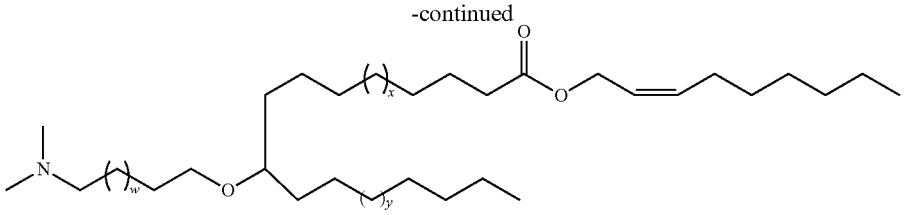
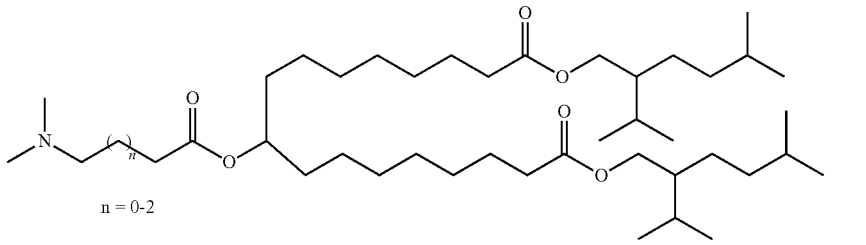


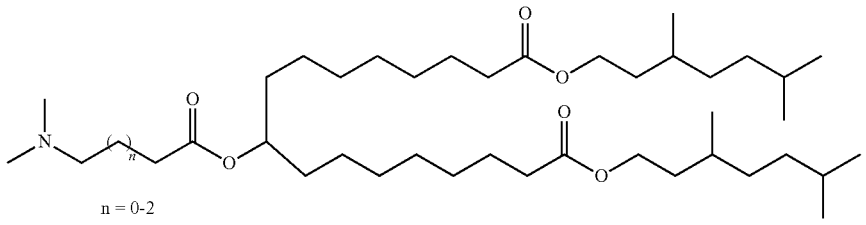
wherein w is 0, 1, 2, or 3; and x and y are each independently 1, 2, 3, 4, 5, 6, or 7.

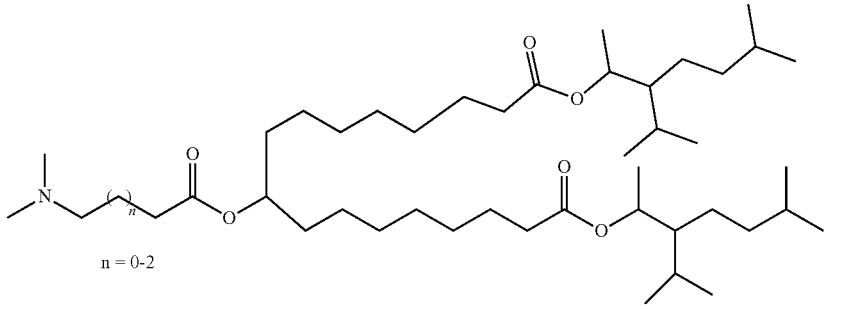
In a preferred embodiment of the aforementioned biodegradable cationic lipids, the biodegradable cationic lipid has a log P value of at least 10.1 (as calculated by the software available at http://www.molinspiration.com/services/logp.html from Molinspiration Cheminformatics of Slovensky Grob, Slovak Republic). More preferably, the log P value is at least 10.2 or 10.3.

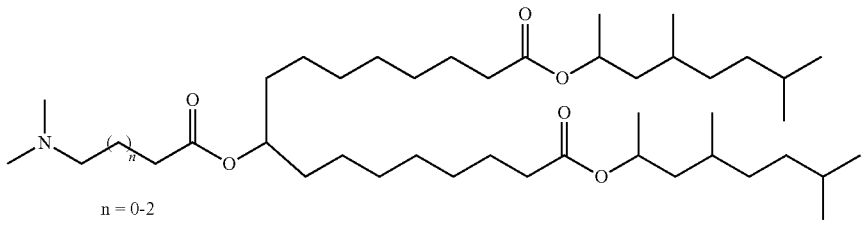
In another preferred embodiment of the aforementioned biodegradable cationic lipids, the biodegradable cationic lipid in the lipid nanoparticle has a HPLC retention time (relative to the retention time of cholesterol in the lipid nanoparticle), hereafter referred to as t_{lipid}–t_{chol}, of at least 1.4. (The HPLC parameters are provided in the examples below. Unless otherwise specified, the formulation of the lipid nanoparticle used is that described in Example 31). More preferably, the t_{lipid}–t_{chol} value is at least 1.75, 2.0, or 2.25.

In another embodiment, the biodegradable cationic lipid of the present invention is not one selected from:









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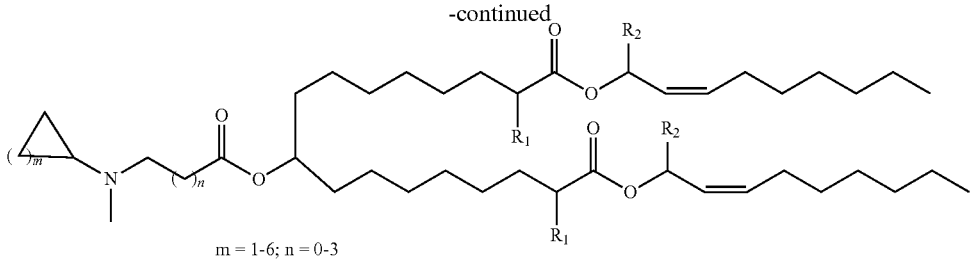
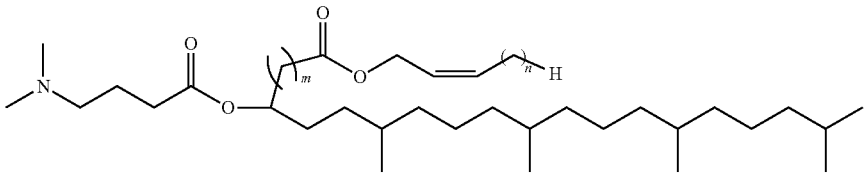
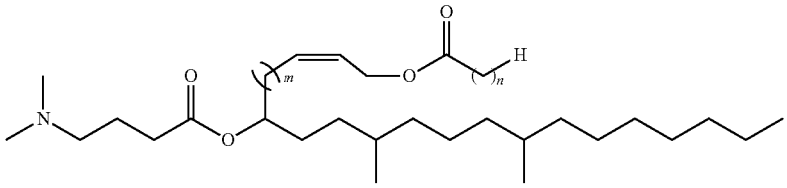


m = 1-6; n = 0-3

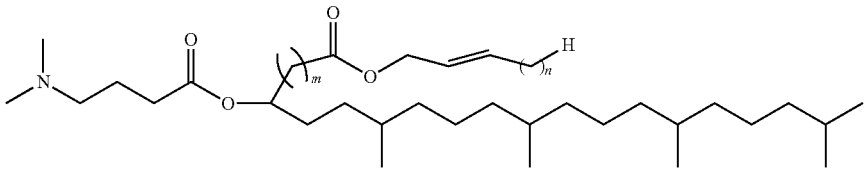
$R_1 = R_2$ = Me, Et, iPr etc.



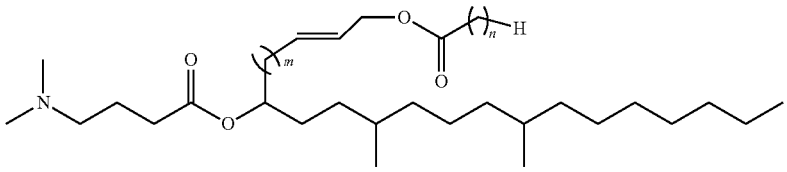
where m and n are integers, and m+n=13



where m and n are integers, and m+n=13



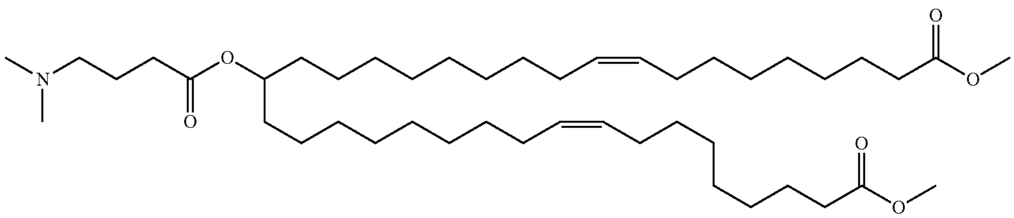
where m and n are integers, and m+n=13

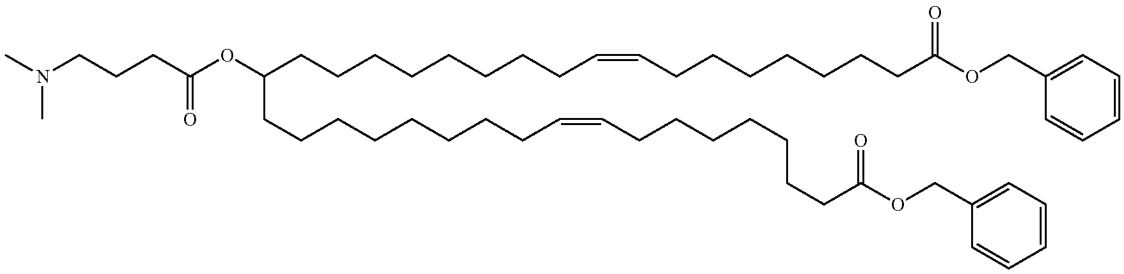


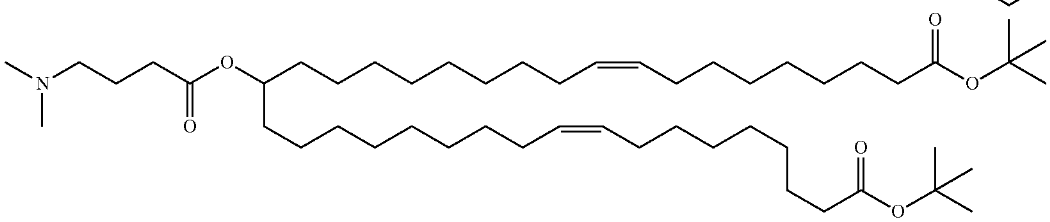
where m and n are integers, and m+n=13

In yet another embodiment, the biodegradable cationic lipid is not one selected from those disclosed in International Publication No. WO 2011/153493 and U.S. Patent Publication No. 2012/0027803, both of which are hereby incorporated by reference.

Yet another embodiment is a biodegradable cationic lipid having (i) a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and (2) one or more biodegradable groups (such as an ester group) located in the mid- or distal section of a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid, with the proviso that the compound is not selected from







In another embodiment, the biodegradable cationic lipid is not one selected from those disclosed in International Publication No. WO 2011/153493 and U.S. Patent Publication No. 2012/0027803, both of which are hereby incorporated by reference. The incorporation of the biodegradable group (s) into the cationic lipid results in faster metabolism and removal of the cationic lipid from the body following delivery of the active pharmaceutical ingredient to a target area. In a preferred embodiment, the cationic lipid includes a branched alkyl or branched alkenyl group in its biodegradable group(s). In another preferred embodiment, the cationic lipid has a log P of at least 10.2 or 10.3. In yet another preferred embodiment, the cationic lipid has a $t_{lipid}-t_{chol}$, of at least 1.75, 2.0, or 2.25. The cationic lipid preferably has a pKa of from about 4 to about 7 (such as 6.0 to 6.5).

In one embodiment, the cationic lipid having a log P value of at least 10.1 and/or a $t_{lipid}-t_{chol}$, of at least 1.4 comprises (a) a head group (preferably a nitrogen containing head group, such as the head groups described herein), (b) at least two hydrophobic tails, each of the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), and (c) a linker group (for instance, a single central carbon atom) which is bound to the head group and the hydrophobic tails. The cationic lipid preferably has one, two, three, four or more of the properties listed below:

(i) a pKa of from about 4 to about 7 (such as 6.0 to 6.5);

(ii) in at least one hydrophobic tail (and preferably all hydrophobic tails), the biodegradable group is separated from the terminus of the hydrophobic tail by from about 6 to about 12 carbon atoms (for instance, 6 to 8 carbon atoms or 8 to 12 carbon atoms),

(iii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the chain length from the linker group to the terminus of the hydrophobic tail is at most 21 (e.g., at most 20, or from about 17 to about 21, from about 18 to about 20, or from about 16 to about 18) (The atom(s) in the linker group are not counted when calculating the chain length.);

(iv) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms in the hydrophobic tail is from about 17 to about 26 (such as from about 19 to about 26, or from about 21 to about 26);

(v) for at least one hydrophobic tail (and preferably all hydrophobic tails), the number of carbon atoms between the linker group and the biodegradable group ranges from about 5 to about 10 (for example, 6 to 10, or 7 to 9);

(vi) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms between the linker group and the terminus of the hydrophobic tail is from about 15 to about 20 (such as from 16 to 20, 16 to 18, or 18 to 20);

(vii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms between the biodegradable group and the terminus of the hydrophobic tail is from about 12 to about 18 (such as from 13 to 25);

(viii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl or alkenyl group, for example, where the branching occurs at the α, β, γ, or δ position on the hydrophobic chain relative to the biodegradable group;

(ix) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid has an in vivo half life ($t_{1/2}$) in the liver of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours;

(x) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from the liver in mice with a greater than 10-fold reduction in lipid levels relative to C_{max} within the first 24 hours post-dose;

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(xi) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from the spleen in mice with an equal or greater than 10-fold reduction in lipid levels relative to C_{max} within the first 168 hours post-dose; and

(xii) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from plasma with a terminal plasma half-life ($t_{1/2\beta}$) in rodents and non-human primates of 48 hours or shorter.

The present invention embodies compounds having any combination of some or all of the aforementioned properties. These properties provide a cationic lipid which remains intact until delivery of an active agent, such as a nucleic acid, after which cleavage of the hydrophobic tail occurs in vivo. For instance, the compounds can have all of properties (i) to (viii) (in addition to the log P or t_{lipid}–t_{chol} value). In another embodiment, the compounds have properties (i), (ii), (iii), and (viii). In yet another embodiment, the compounds have properties (i), (ii), (iii), (v), (vi), and (viii).

Another embodiment is a method of preparing a cationic lipid comprising:

(a) designing a cationic lipid having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii)); and

(b) synthesizing the cationic lipid of step (a). The cationic lipid in step (a) may comprises (a) a head group (preferably a nitrogen containing head group, such as the head groups described herein), (b) at least two hydrophobic tails, each of the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), and (c) a linker group (for instance, a single central carbon atom) which is bound to the head group and the hydrophobic tails. Step (a) may comprise:

(a)(i) preparing one or more cationic lipids having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii);

(a)(ii) screening the cationic lipids to determine their efficacy and/or toxicity in lipid nanoparticles; and

(a)(iii) selecting a cationic lipid for synthesis.

Yet another embodiment is a method of designing a cationic lipid comprising:

(a) selecting a cationic lipid having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii)); and

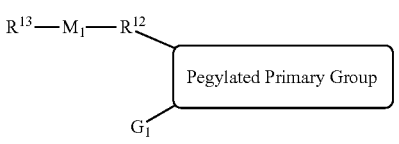
(b) optionally,

(i) preparing one or more cationic lipids having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii);

(ii) screening the cationic lipids to determine their efficacy and/or toxicity in lipid nanoparticles; and

(iii) optionally, selecting a cationic lipid for further development or use.

In one embodiment, the PEG lipid has the formula:



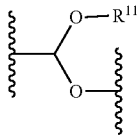
Formula (IX)

wherein

G_1 is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl (e.g., a n-C_8 alkyl n-C_9 alkyl, or n-C_{10} alkyl); or G_1 is —R^{12}-M_1-R^{13};

R^{12} is a branched or unbranched alkylene or alkenylene (e.g., C_6-C_{20} alkylene or C_6-C_{20} alkenylene such as C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene);

M_1 is a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or

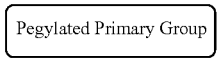


(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

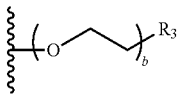
R^3 and R^4 are defined as in formula (I);

each occurrence of R^5 is, independently, H or alkyl (e.g., C_1-C_4 alkyl);

R^{13} is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl;



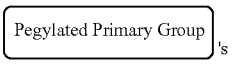
comprises a PEG moiety, such as



moiety wherein b is an integer from 10 to 1,000 (e.g., 5-100, 10-60, 15-50, or 20-45); R^3 is —H, —R^c, or —OR^c; and R^c is —H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, or heterocyclyl.

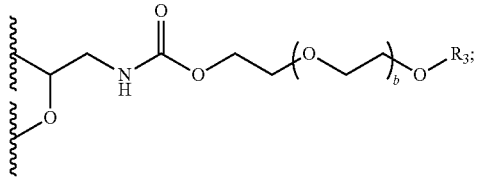
In one embodiment, the pegylated primary group includes (i) a head group having a PEG moiety, and (ii) a central moiety (e.g., a central carbon atom) to which both the hydrophobic tails are directly bonded. Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group.

Representative



's

include, but are not limited to,



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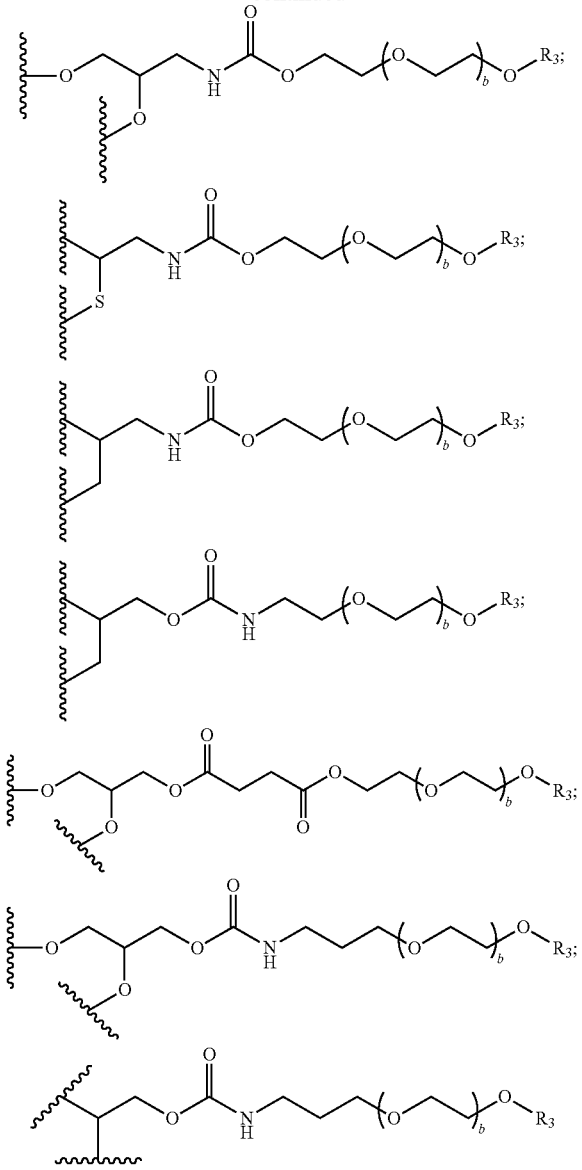
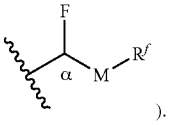


where b is 10-100 (e.g., 20-50 or 40-50)

Another embodiment of the present invention is a PEG lipid (or a salt thereof) having:

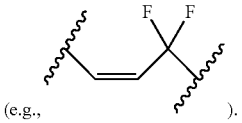
(i) a pegylated primary group including a head group which includes a PEG moiety (e.g., having from 10 to 1000 repeating units such as ethoxy units)), and

(iii) one or more hydrophobic tails (preferably, two hydrophobic tails) directly bound to the pegylated primary group, wherein at least one hydrophobic tail is of the formula —R^e-M-R^f where R^e is a C_4-C_{14} alkyl or alkenyl, M is a biodegradable group, and R^f is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of —R^e-M-R^f is at most 20 atoms (i.e. the total length of the tail from the first carbon atom after the central carbon atom to a terminus of the tail is at most 20), and (ii) the group —R^e-M-R^f has at least 20 carbon atoms (e.g., at least 21 atoms). Optionally, the alkyl or alkenyl group in R^e may be substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



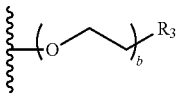
(e.g.,).

Also, optionally, the alkenyl group in R^f may be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of R^f



(e.g.,).

In one embodiment, the pegylated primary group includes (i) a head group having a PEG moiety, and (ii) a central moiety (e.g., a central carbon atom) to which the hydrophobic tails are directly bound. The PEG moiety may have 5-100, 10-60, 15-50, or 20-45 repeating units. For example, the PEG moiety may have the formula



moiety wherein b is an integer from 10 to 1,000 (e.g., 5-100, 10-60, 15-50, or 20-45); R^3 is —H, —R^c, or —OR^c; and R^c is —H, alkyl (e.g., C_1-C_4 alkyl), acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, or heterocyclyl.

Yet another embodiment is a lipid particle that includes a cationic lipid and/or PEG lipid of the present invention. In one embodiment, the lipid particle includes a cationic lipid of the present invention (e.g., of one of formulas (I)-(VIII)). In another embodiment, the lipid particle includes a PEG lipid of the present invention (e.g., of formula (IX)). In yet another embodiment, the lipid particle includes a cationic lipid of the present invention and a PEG lipid of the present invention.

In a preferred embodiment, the lipid particle includes a neutral lipid, a lipid capable of reducing aggregation, a cationic lipid, and optionally, a sterol (e.g., cholesterol). Suitable neutral lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), POPC, DOPE, and SM. Suitable lipids capable of reducing aggregation include, but are not limited to, a PEG lipid, such as PEG-DMA, PEG-DMG, and those of the present invention (e.g., of formula (IX)) or a combination thereof.

The lipid particle may further include an active agent (e.g., a therapeutic agent). The active agent can be a nucleic acid such as a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, or a ribozyme. In a preferred embodiment, the nucleic acid is a siRNA. In another preferred embodiment, the nucleic acid is a miRNA.

In another embodiment, the lipid particle includes a cationic lipid of the present invention, a neutral lipid and a sterol. The lipid particle may further include an active agent, such as a nucleic acid (e.g., an siRNA or miRNA).

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In yet another embodiment, the lipid particle includes a PEG lipid of the present invention, a cationic lipid, a neutral lipid, and a sterol. The lipid particle may further include an active agent, such as a nucleic acid (e.g., an siRNA or miRNA).

The lipid particles described herein may be lipid nanoparticles.

Yet another embodiment of the invention is a pharmaceutical composition which includes a lipid particle of the present invention and a pharmaceutically acceptable carrier.

In one embodiment, the cationic lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

In another embodiment, the PEG lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

In another embodiment, the present invention relates to a method of delivering a nucleic acid molecule comprising administering a nucleic lipid particle comprising (i) the nucleic acid molecule and (ii) a cationic lipid and/or a PEG lipid of the present invention. In one embodiment, the cationic lipid and/or a PEG lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

Yet another aspect is a method of modulating the expression of a target gene in a cell by providing to the cell a lipid particle of the present invention. The active agent can be a nucleic acid selected from a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme. In a preferred embodiment, the nucleic acid is a siRNA or miRNA.

Yet another aspect is a method of treating a disease or disorder characterized by the overexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a nucleic acid selected from an siRNA, a microRNA, and an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense oligonucleotide includes a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof. In a preferred embodiment, the nucleic acid is a siRNA or miRNA.

Yet another aspect is a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

Yet another aspect is a method of inducing an immune response in a subject by providing to the subject a pharmaceutical composition wherein the active agent is an immunostimulatory oligonucleotide.

Yet another aspect is a transfection agent that includes the composition or lipid particles described above, where the composition or lipid particles include a nucleic acid. The agent, when contacted with cells, can efficiently deliver nucleic acids to the cells. Yet another aspect is a method of delivering a nucleic acid to the interior of a cell, by obtaining or forming a composition or lipid particles described above, and contacting the composition or lipid particles with a cell.

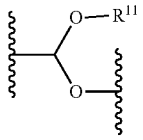
DETAILED DESCRIPTION

In one aspect, the present invention relates to a lipid particle that includes a neutral lipid, a lipid capable of reducing aggregation (e.g., a PEG lipid), a cationic lipid, and optionally a sterol. In certain embodiments, the lipid particle further includes an active agent (e.g., a therapeutic agent). Various exemplary embodiments of these lipids, lipid particles and compositions comprising the same, and their use to deliver therapeutic agents and modulate gene and protein expression are described in further detail below.

The Cationic Lipid

In one embodiment, the cationic lipid is a compound of any one of Formulas I-VIII. The following disclosure represents various embodiments of the compounds described above, including the compounds of Formulas I-VIII.

In one embodiment, M^1 and M^2 are each, independently: —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



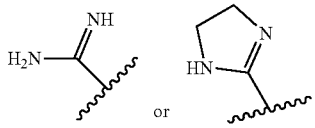
(wherein R^{11} is a C_2-C_8 alkyl or alkenyl).

In another embodiment, M^1 and M^2 are each, independently: —OC(O)—, —C(O)—O—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —O—C(O)O—, —C(O)N(R^5)—, —N(R^5)C(O)—, —C(O)S—, —SC(O)—, —C(S)O—, —OC(S)—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

In yet another embodiment, M^1 and M^2 are each, independently: —C(O)—O—, —OC(O)—, —C(R^5)═N—, —C(R^5)═N—O—, —O—C(O)O—, —C(O)N(R^5)—, —C(O)S—, —C(S)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

In another embodiment, M^1 and M^2 are each —C(O)O—.

In one embodiment, R^1 and R^2 are each, individually, optionally substituted alkyl, cycloalkyl, cycloalkylalkyl, or heterocycle. In one embodiment, R^1 is alkyl and R^2 is alkyl, cycloalkyl or cycloalkylalkyl. In one embodiment, R^1 and R^2 are each, individually, alkyl (e.g., C_1-C_4 alkyl, such as methyl, ethyl, or isopropyl). In one embodiment, R^1 and R^2 are both methyl. In another embodiment, R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocylic ring (e.g., N-methylpiperazinyl). In another embodiment, one of R^1 and R^2 is



(e.g., R^1 is one of the two aforementioned groups and R^2 is hydrogen).

In one embodiment, R' is hydrogen or alkyl. In another embodiment, R' is hydrogen or methyl. In one embodiment, R' is absent. In one embodiment, R' is absent or methyl.

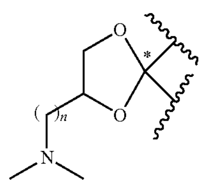
For cationic lipid compounds which contain an atom (e.g., a nitrogen atom) that carries a positive charge, the compound also contains a negatively charged counter ion. The counterion can be any anion, such as an organic or inorganic anion. Suitable examples of anions include, but are not limited to, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-keto-glutarate, α-glycerophosphate, halide (e.g., chloride), sulfate, nitrate, bicarbonate, and carbonate. In one embodiment, the counterion is a halide (e.g., Cl).

In one embodiment each R is, independently, —(CR^3R^4)—, wherein R^3 and R^4 are each, independently, H or alkyl (e.g., C_1-C_4 alkyl). For example, in one embodiment each R is, independently, —(CHR^4)—, wherein each R^4 is, independently H or alkyl (e.g., C_1-C_4 alkyl). In another embodiment, each R is, independently, —CH_2—, —$C(CH_3)_2$— or —CH(iPr)- (where iPr is isopropyl). In another embodiment, each R is —CH_2—.

In another embodiment R^5 is, in each case, hydrogen or methyl. For example, R^5 can be, in each case, hydrogen.

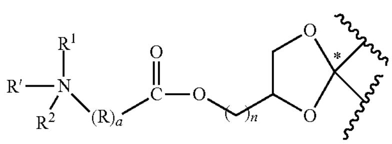
In one embodiment, Q is absent, —C(O)O—, —OC(O)—, —C(O)N(R^5)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —C(R^5)═N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)═N—O—C(O)—. In one embodiment, Q is —C(O)O—.

In one embodiment, the dashed line to Q is absent, b is 0 and $R'R^1R^2N$—$(R)_a$-Q- and the tertiary carbon adjacent to it (C*) form the following group:



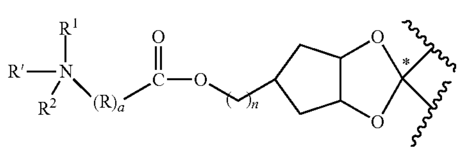
where n is 1 to 4 (e.g., n is 2).

In one embodiment, the dashed line to Q is absent, b is 0 and $R'R^1R^2N$—$(R)_a$-Q- and the tertiary carbon adjacent to it form the following group:

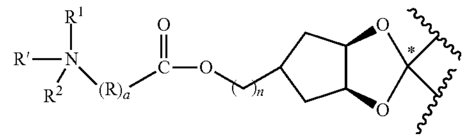


where n is 1 to 4 (e.g., n is 2), and R^1, R^2, R, a, and b are as defined with respect to formula (I). In one embodiment, a is 3.

In one embodiment, the dashed line to Q is absent, b is 0 and $R'R^1R^2N$—$(R)_a$-Q- and the tertiary carbon adjacent to it form the following group:



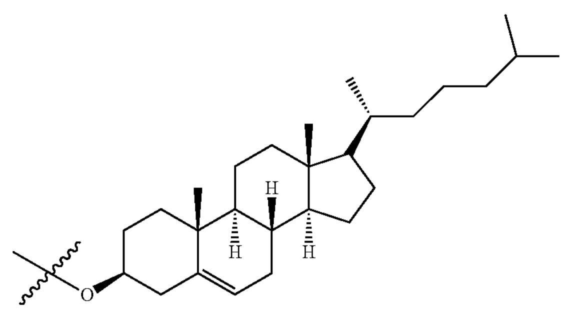
where n is 1 to 4 (e.g., n is 2), and R^1, R^2, R, a, and b are as defined with respect to formula (I). In one embodiment, a is 0. For example, the group can be:



In one embodiment, b is 0. In another embodiment, a is 2, 3, or 4 and b is 0. For example, in one embodiment, a is 3 and b is 0. In another embodiment, a is 3, b is 0, and Q is —C(O)O—.

In certain embodiments, the biodegradable group present in the cationic lipid is selected from an ester (e.g., —C(O)O— or —OC(O)—), disulfide (—S—S—), oxime (e.g., —C(H)═N—O— or —O—N═C(H)—), —C(O)—O—, —OC(O)—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —O—C(O)O—, —C(O)N(R^5), —N(R^5)C(O)—, —C(S)(NR^5)—, (NR^5)C(S)—, —N(R^5)C(O)N(R^5)—, —C(O)S—, —SC(O)—, —C(S)O—, —OC(S)—, —OSi$(R^5)_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

A suitable cholesterol moiety for the cationic lipids of the present invention (including compounds of formulas I-VI) has the formula:



Additional embodiments include a cationic lipid having a head group, one or more hydrophobic tails, and a central moiety between the head group and the one or more tails. The head group can include an amine; for example an amine having a desired pK_a. The pK_a can be influenced by the structure of the lipid, particularly the nature of head group; e.g., the presence, absence, and location of functional groups such as anionic functional groups, hydrogen bond donor functional groups, hydrogen bond acceptor groups, hydrophobic groups (e.g., aliphatic groups), hydrophilic groups (e.g., hydroxyl or methoxy), or aryl groups. The head group amine can be a cationic amine; a primary, secondary, or tertiary amine; the head group can include one amine group (monoamine), two amine groups (diamine), three amine groups (triamine), or a larger number of amine groups, as in an oligoamine or polyamine. The head group can include a functional group that is less strongly basic than an amine, such as, for example, an imidazole, a pyridine, or a guanidinium group. The head group can be zwitterionic. Other head groups are suitable as well.

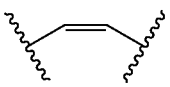
Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central

hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group. Additionally, the central moiety can include, for example, a glyceride linker, an acyclic glyceride analog linker, or a cyclic linker (including a spiro linker, a bicyclic linker, and a polycyclic linker). The central moiety can include functional groups such as an ether, an ester, a phosphate, a phosphonate, a phosphorothioate, a sulfonate, a disulfide, an acetal, a ketal, an imine, a hydrazone, or an oxime. Other central moieties and functional groups are suitable as well.

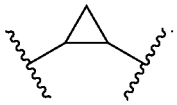
In one embodiment, the cationic lipid is a racemic mixture. In another embodiment, the cationic lipid is enriched in one diastereomer, e.g. the cationic lipid has at least 95%, at least 90%, at least 80% or at least 70% diastereomeric excess. In yet another embodiment, the cationic lipid is enriched in one enantiomer, e.g. the lipid has at least 95%, at least 90%, at least 80% or at least 70% enantiomer excess. In yet another embodiment, the cationic lipid is chirally pure, e.g. is a single optical isomer. In yet another embodiment, the cationic lipid is enriched for one optical isomer.

Where a double bond is present (e.g., a carbon-carbon double bond or carbon-nitrogen double bond), there can be isomerism in the configuration about the double bond (i.e. cis/trans or E/Z isomerism). Where the configuration of a double bond is illustrated in a chemical structure, it is understood that the corresponding isomer can also be present. The amount of isomer present can vary, depending on the relative stabilities of the isomers and the energy required to convert between the isomers. Accordingly, some double bonds are, for practical purposes, present in only a single configuration, whereas others (e.g., where the relative stabilities are similar and the energy of conversion low) may be present as inseparable equilibrium mixture of configurations.

In some cases, a double-bonded unsaturation is replaced by a cyclic unsaturation. The cyclic unsaturation can be a cycloaliphatic unsaturation, e.g., a cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl group.



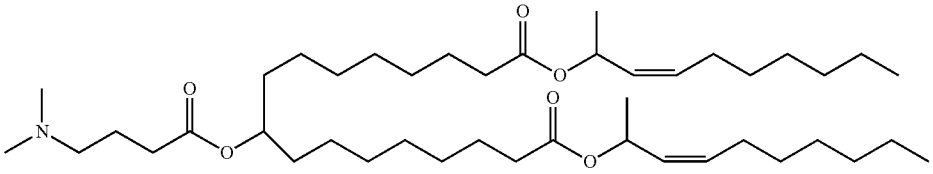
can be replaced by



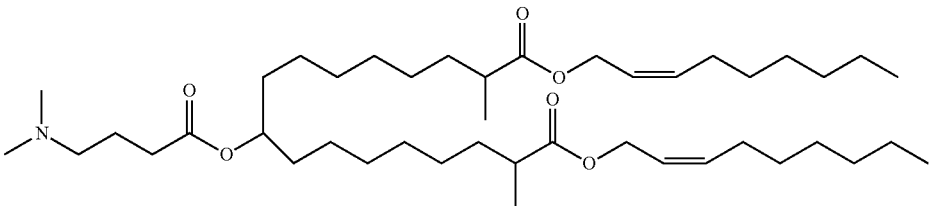
The cationic lipid includes one or more biodegradable groups. The biodegradable group(s) include one or more bonds that may undergo bond breaking reactions in a biological environment, e.g., in an organism, organ, tissue, cell, or organelle. Functional groups that contain a biodegradable bond include, for example, esters, dithiols, and oximes. Biodegradation can be a factor that influences the clearance of the compound from the body when administered to a subject. Biodegredation can be measured in a cell based assay, where a formulation including a cationic lipid is exposed to cells, and samples are taken at various time points. The lipid fractions can be extracted from the cells and separated and analyzed by LC-MS. From the LC-MS data, rates of biodegradation (e.g., as $t_{1/2}$ values) can be measured.

For example, the compound

(Compound 1)



includes an ester linkage in each aliphatic chain, which can undergo hydrolysis in a biological environment, for example, when exposed to, e.g., a lipase or an esterase. The structure of the compound, of course, influences the rate at which the compound undergoes biodegradation. Thus, a compound where the methyl substituent is on the other side of the biodegradable group such as

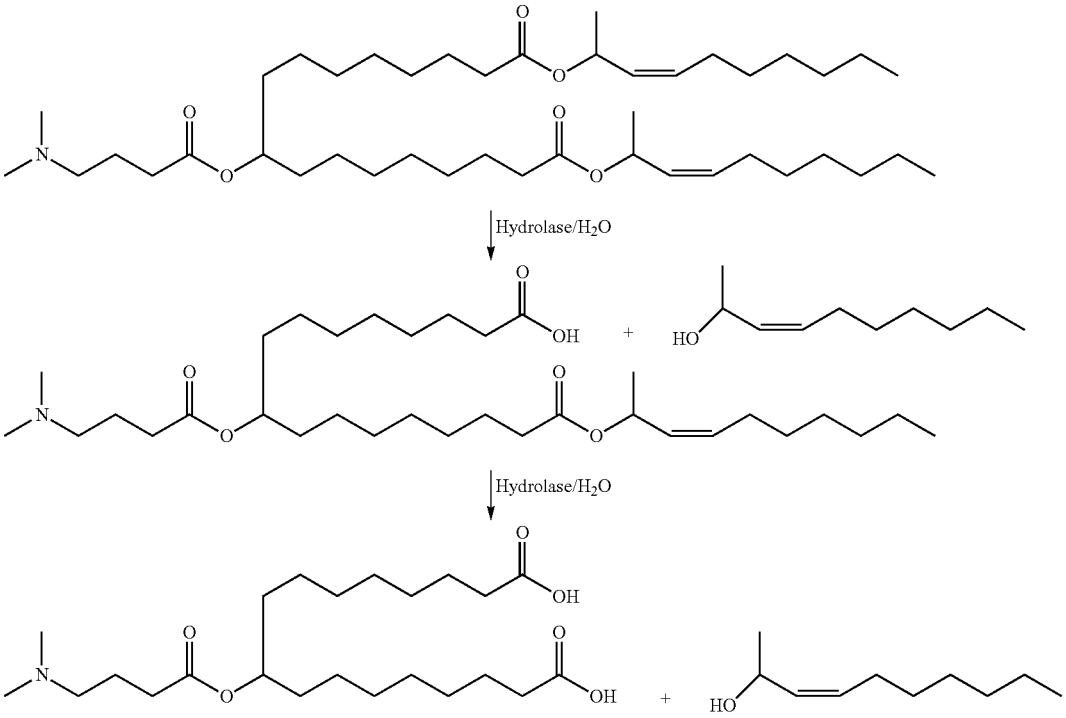


would be expected to exhibit a different rate of biodegradation. Greater effects on that rate would be expected from changes in the structure of the compound at the site of hydrolysis. One modification that can influence the rate of hydrolysis, and thereby influence the rate of biodegradation

In some cases, the cyclic group can be a polycyclic group, e.g., a bicyclic group or tricyclic group. A bicyclic group can be bridged, fused, or have a spiro structure. In some cases, a double bond moiety can be replaced by a cyclopropyl moiety, e.g.,

and clearance from a subject's body, is to make the leaving group of the hydrolysis reaction have a secondary, rather than primary, alcohol.

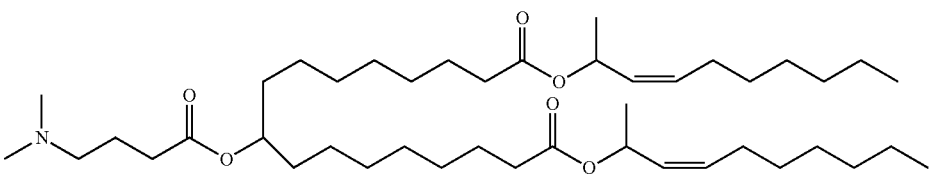
For example, without wishing to be bound by theory, Compound 1 shown above may be metabolized as shown in the scheme below:



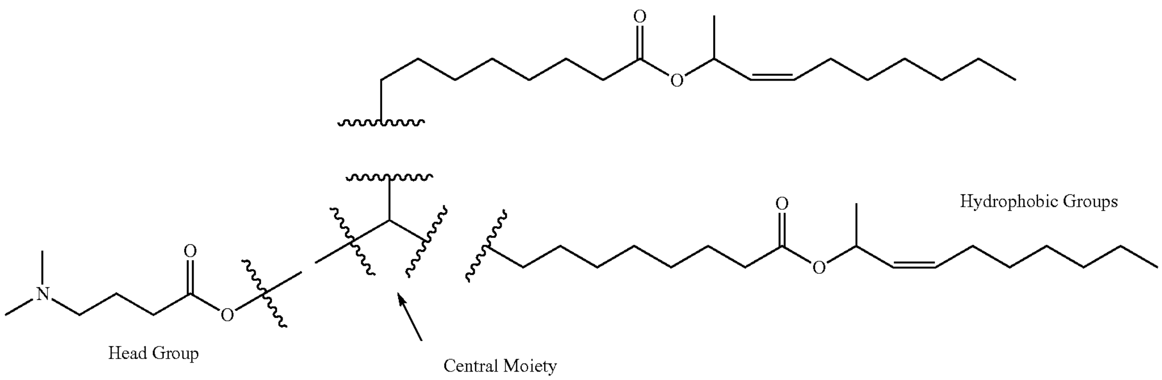
In one embodiment, a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours. The cationic lipid preferably remains intact, or has a half-life sufficient to form a stable lipid nanoparticle which effectively delivers the desired active pharmaceutical ingredient (e.g., a nucleic acid) to its target but thereafter rapidly degrades to minimize any side effects to the subject. For instance, in mice, the cationic lipid preferably has a $t_{1/2}$ in the spleen of from about 1 to about 7 hours.

In another embodiment, a cationic lipid of any of the embodiments described herein containing a biodegradable group or groups has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 10% (e.g., less than about 7.5%, less than about 5%, less than about 2.5%) of that for the same cationic lipid without the biodegrable group or groups.

Some cationic lipids can be conveniently represented as a hydrophobic group combined via a central moiety (such as a carbon atom) with a headgroup. By way of example, the compound:



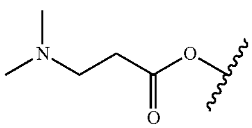
can be thought of as a combination of a headgroup, a central moiety, and two hydrophobic groups as follows:

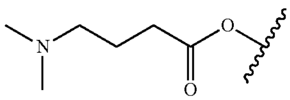


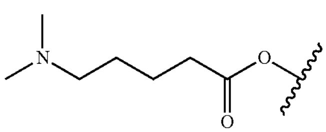
The present invention includes compounds composed of any combination of the head and hydrophobic groups listed below (in combination with a central moiety (such as a central carbon atom).

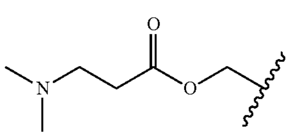
Some suitable head groups include those depicted in Table 1A:

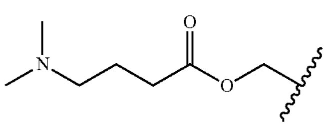
TABLE 1A

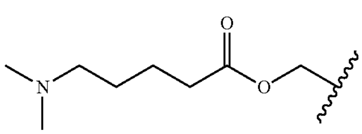












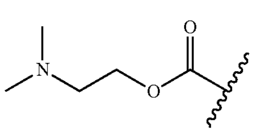
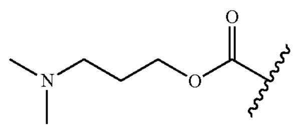
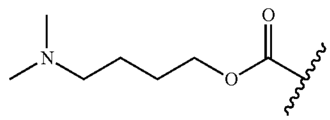
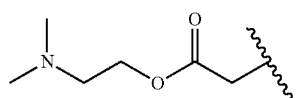
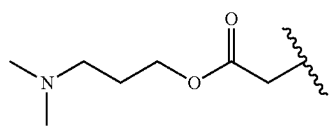


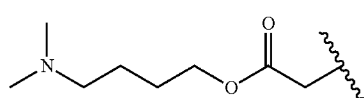
TABLE 1A-continued

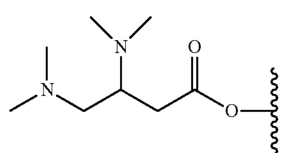


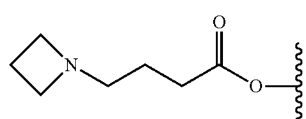












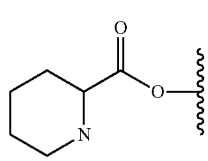
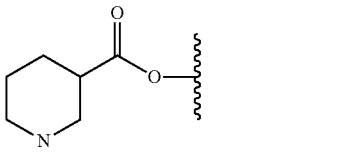
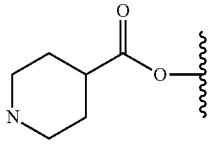
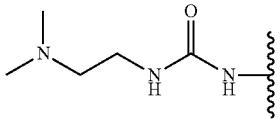
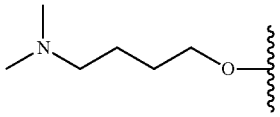


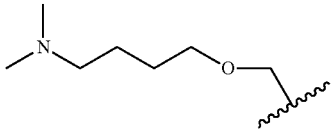
TABLE 1A-continued

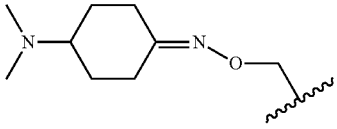


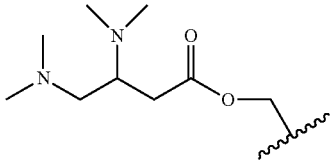


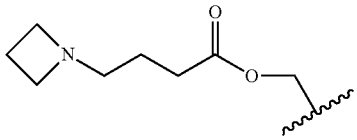


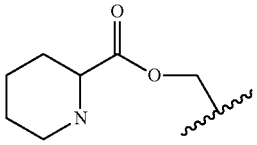












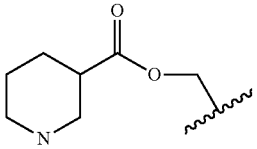
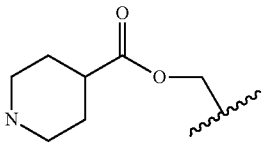
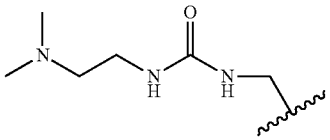
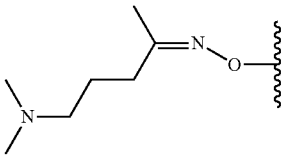
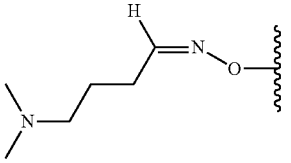


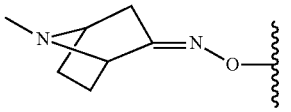
TABLE 1A-continued

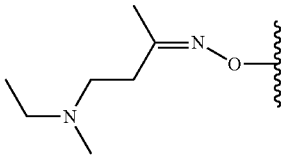


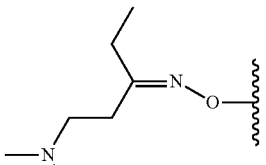


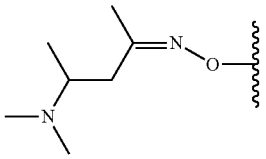












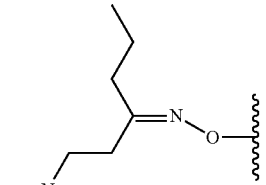


TABLE 1A-continued

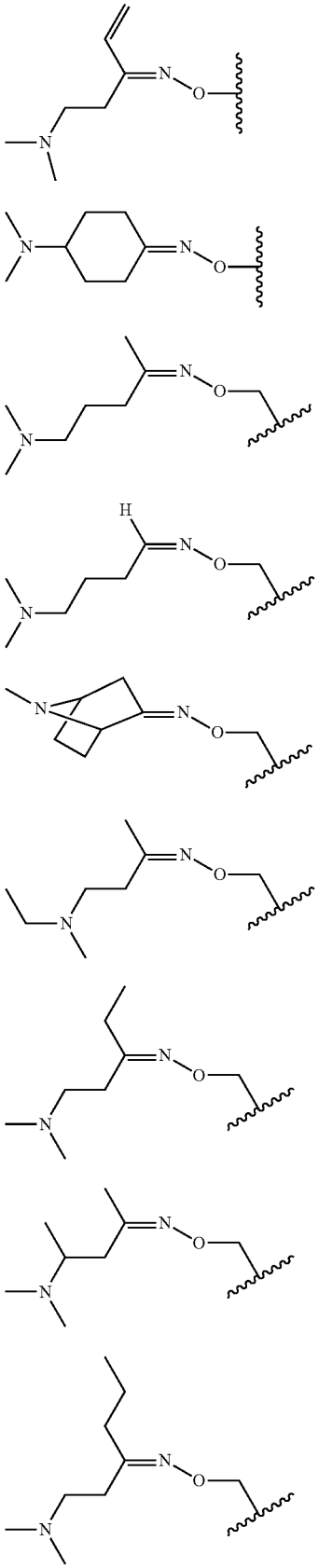
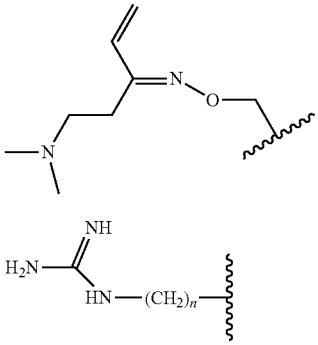
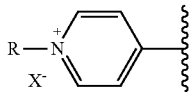


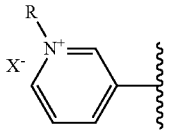
TABLE 1A-continued



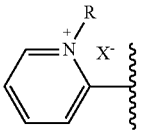
(where n is 0-5)



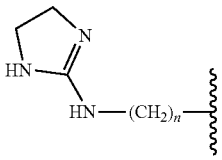
R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)



R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)



R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)



(where n is 0-5)

Suitable primary groups include, but are not limited to, those that are a combination of a head group from table 1A with a central carbon atom. Other suitable primary groups include those in table 1B below:

TABLE 1B

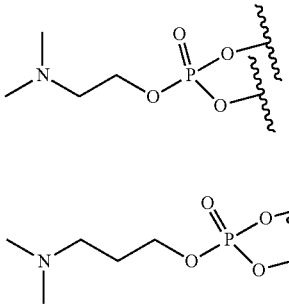


TABLE 1B-continued

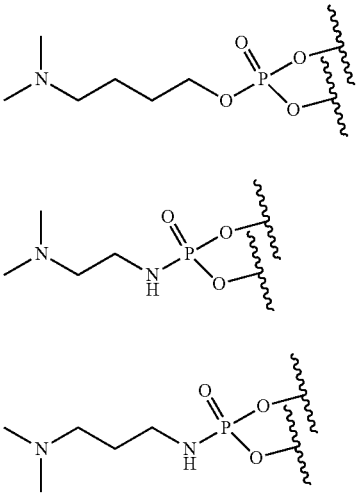
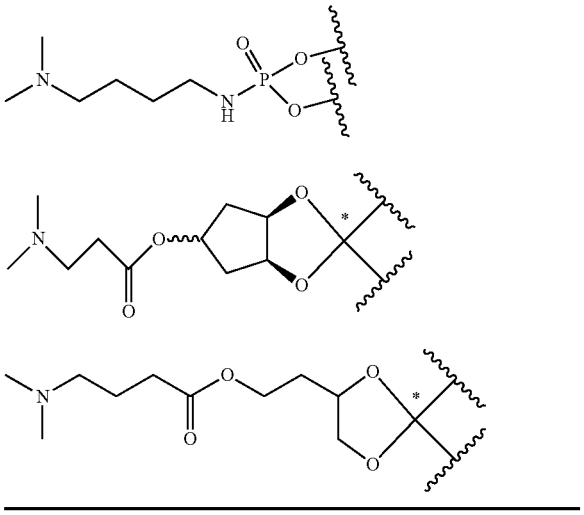


TABLE 1B-continued



Some suitable hydrophobic tail groups include those depicted in Table 1C:

TABLE 1C

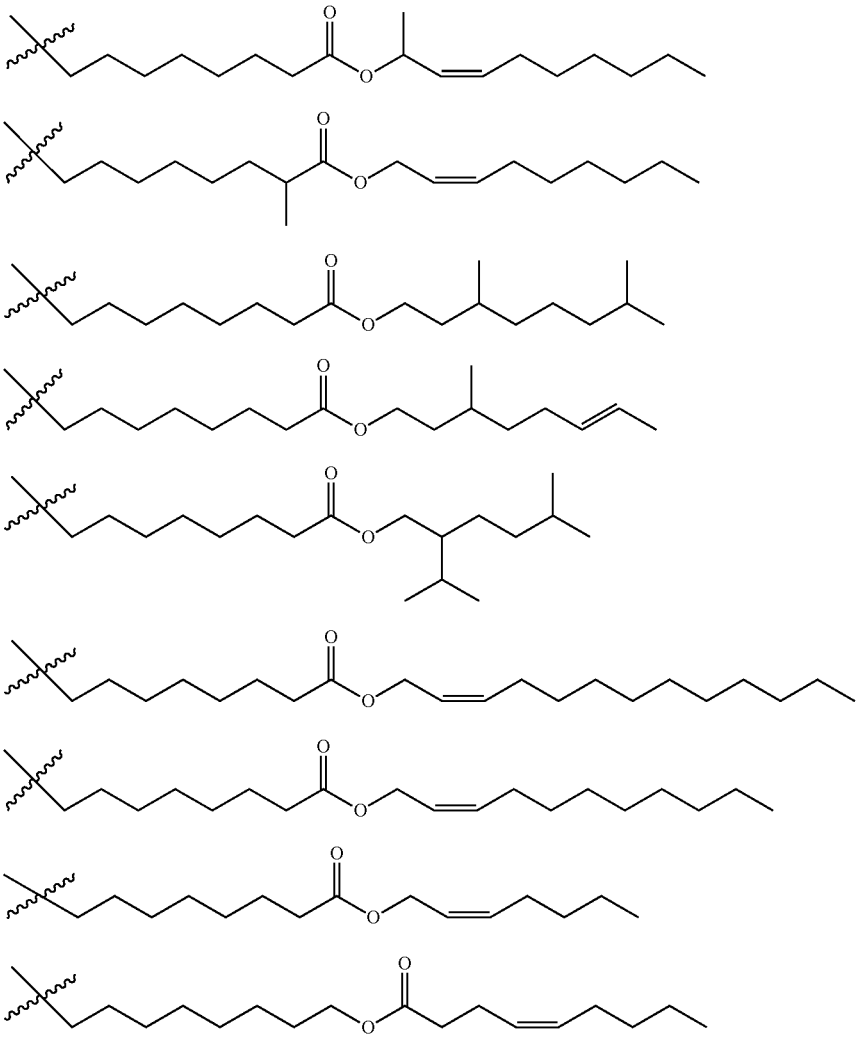
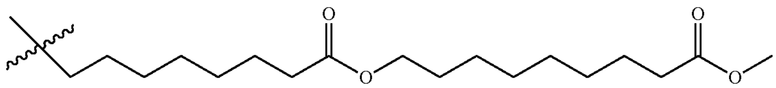
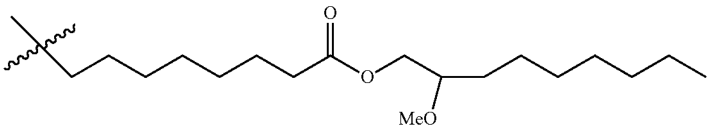
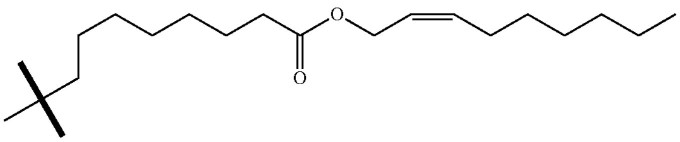
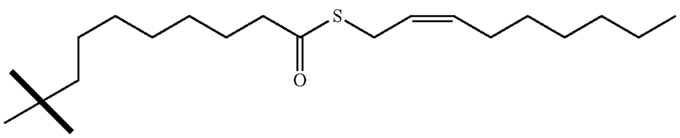


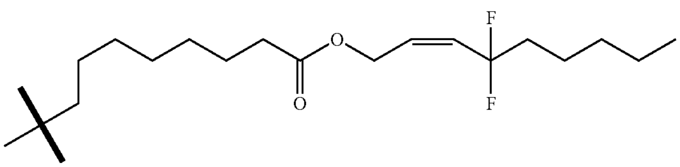
TABLE 1C-continued

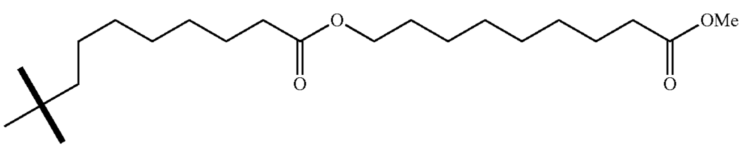


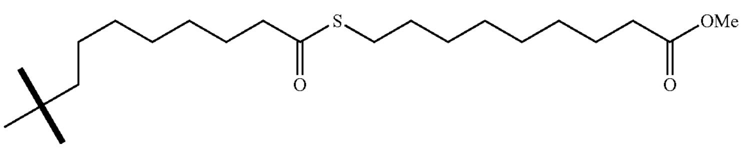


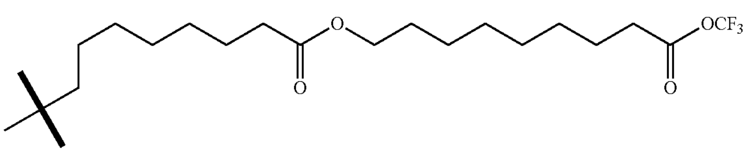


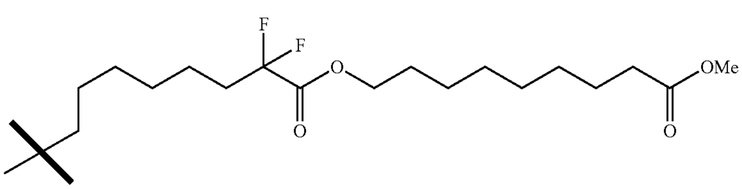


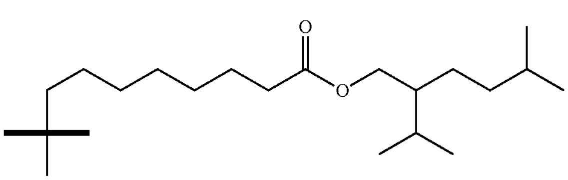












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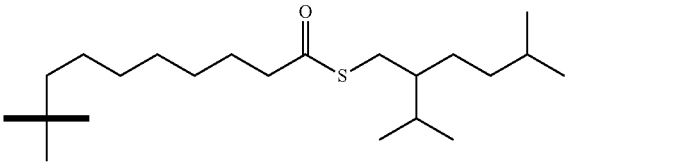
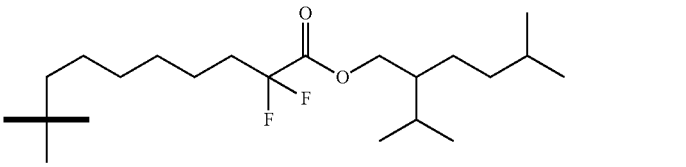
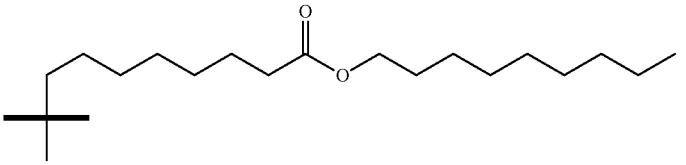
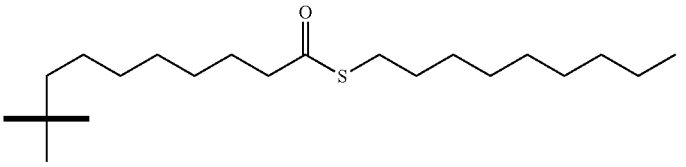
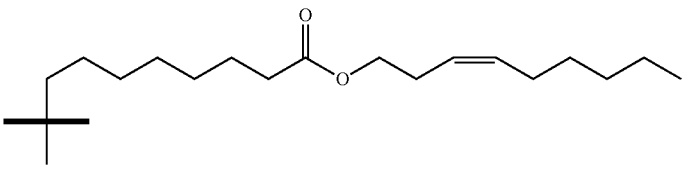
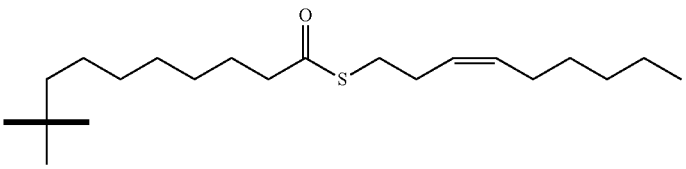


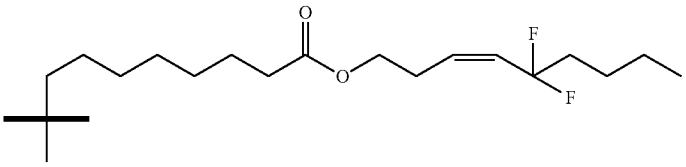


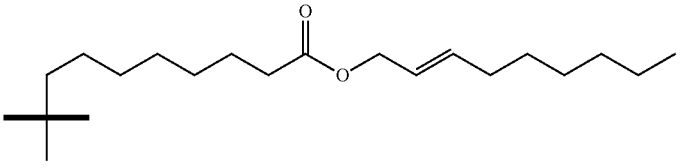












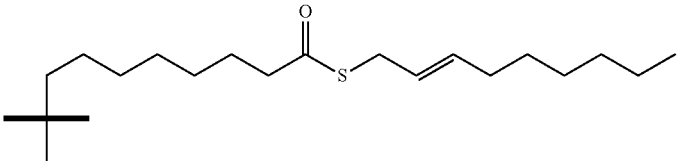
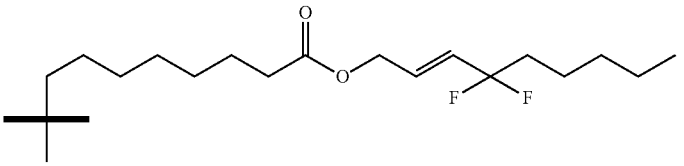
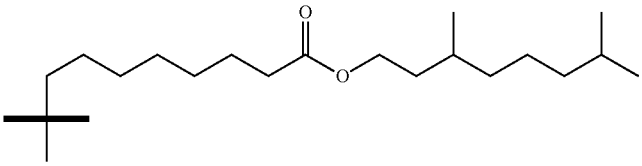
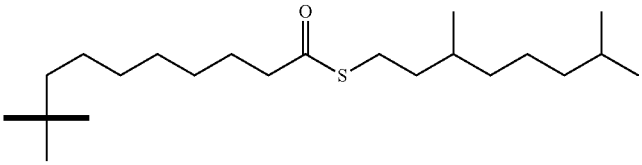
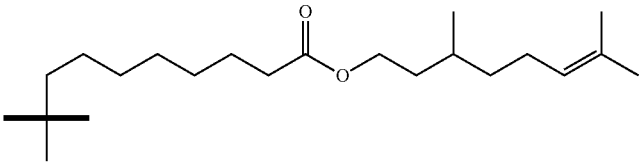


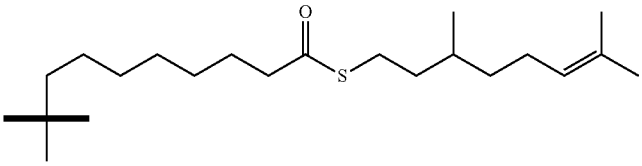
TABLE 1C-continued

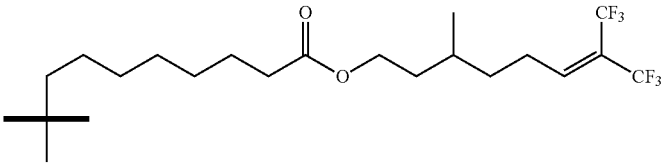


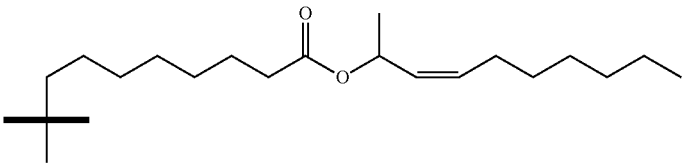


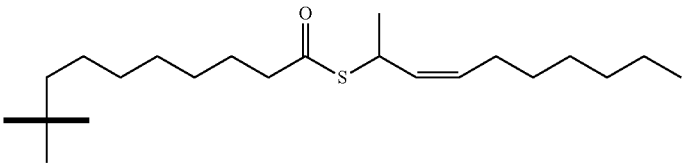












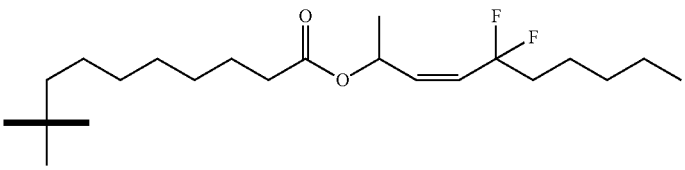


TABLE 1C-continued

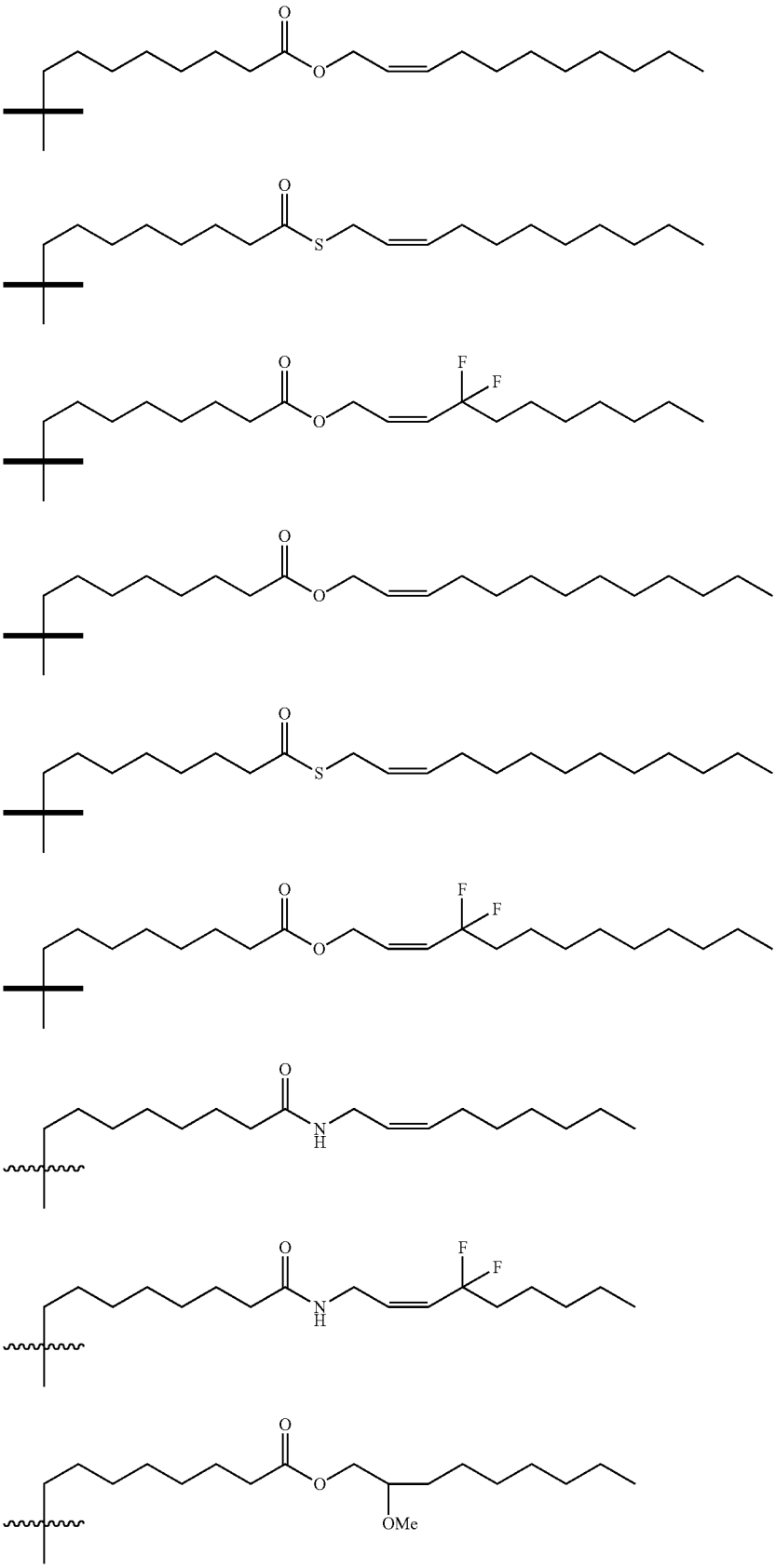
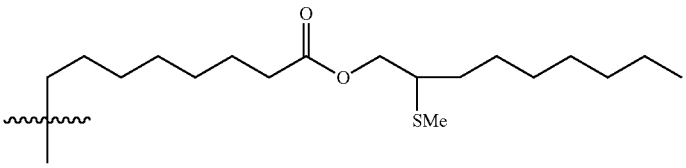
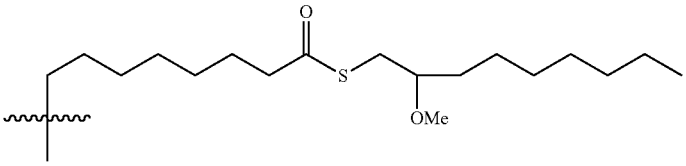
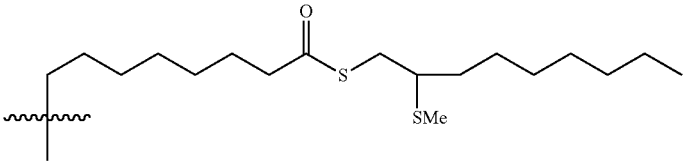
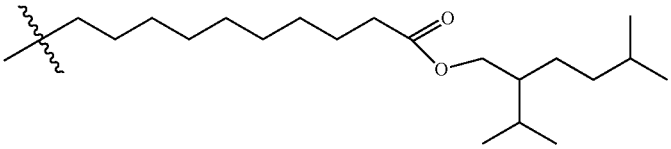


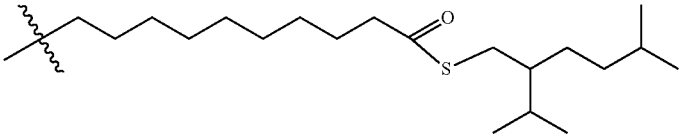
TABLE 1C-continued

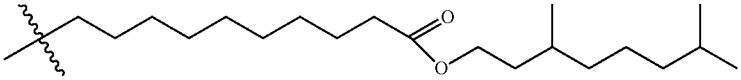


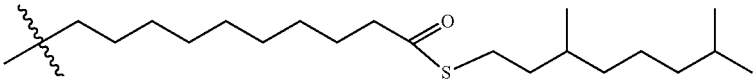


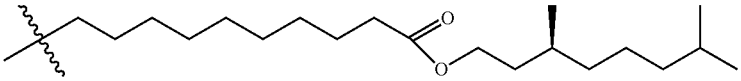


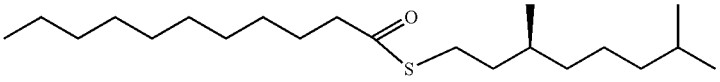


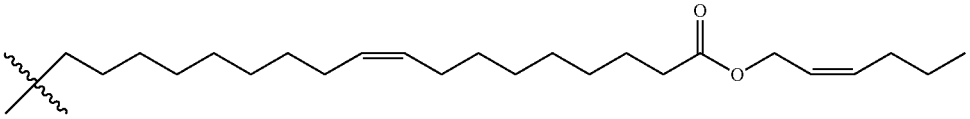












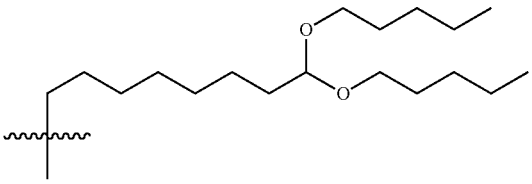


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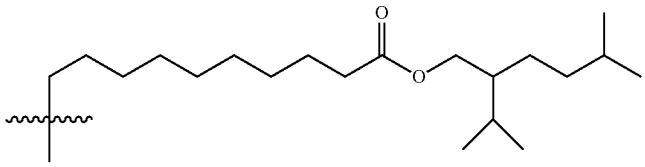
TABLE 1C-continued

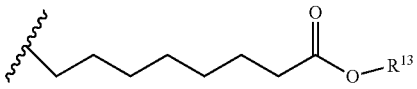


Other suitable tail groups includes those of the formula —R^{12}-M^1-R^{13} where R^{12} is a C_4-C_{14} alkyl or C_4-C_{14} alkenyl, M^1 is a biodegradable group as defined above, and R^{13} is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms (i.e., the total length of the tail from the first carbon after the tertiary carbon (marked with an asterisk) to a terminus of the tail is at most 21), and (ii) the group —R^{12}-M^1-R^{13} has at least 20 carbon atoms (e.g., at least 21 or 22 carbon atoms).

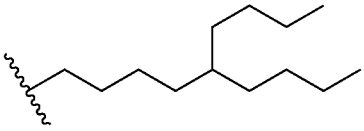
In one preferred embodiment, the chain length of —R^{12}-M^1-R^{13} is at most 21 (e.g., at most 20). For example, the chain length can be from about 17 to about 24 or from about 18 to about 20.

In one embodiment, the total carbon atom content of each tail (—R^{12}-M^1-R^{13}) is from about 17 to about 26. For example, the total carbon atom content can be from about 19 to about 26 or from about 21 to about 26.

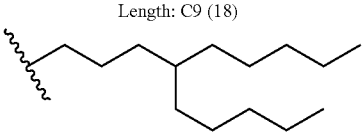
In one embodiment, the tail has the formula:



where R^{13} is an alkyl or alkenyl group having from about 13 to about 17 carbon atoms, and the total carbon length of the tail from the first carbon (the leftmost carbon atom above) to a terminus of the tail is at most 20. Preferably, the tail has from about 22 to about 26 carbon atoms. In one embodiment, the maximum length of R^{13} from its attachment point to the ester group of the compound is 12 carbon atoms (e.g., the maximum length can be 11 carbon atoms). In one preferred embodiment, the branch in the alkyl or alkenyl group is at the δ-position or later from the point of attachment of R^{13} to the ester group. Suitable R^{13} groups include, but are not limited to



C13 (C21)
Length: C9 (18)



C14 (C22)
Length: C9 (18)

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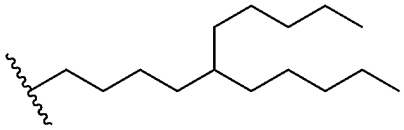
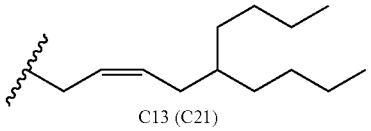
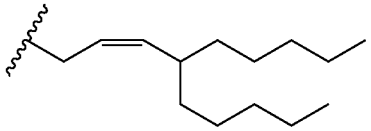


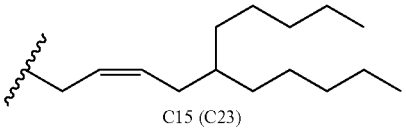
C15 (C23)
Length: C10 (19)



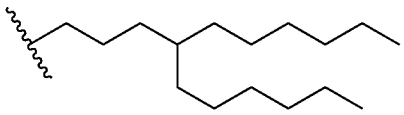
C13 (C21)
Length: C9 (18)



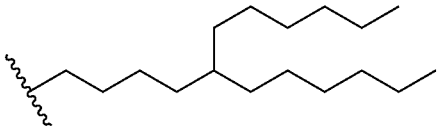
C14 (C22)
Length: C9 (18)



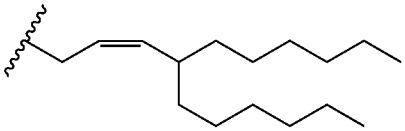
C15 (C23)
Length: C10 (19)



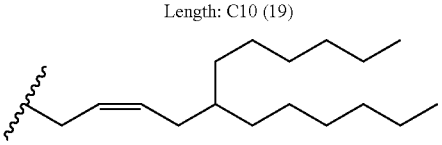
C16 (C24)
Length: C10 (19)



C17 (C25)
Length: C11 (20)

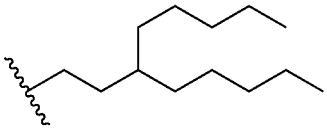


C16 (C24)
Length: C10 (19)

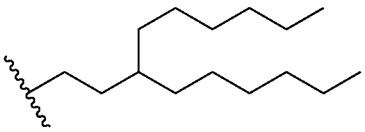


C17 (C25)
Length: C11 (20)

-continued

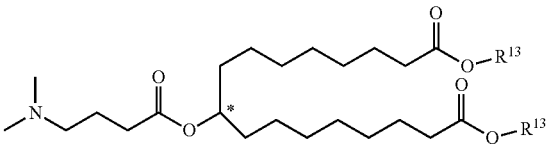


C13 (C21)
Length: C8 (17)



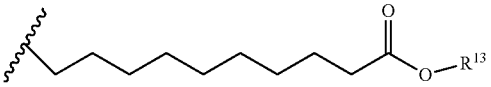
C15 (C23)
Length: C9 (18)

For example, the cationic lipid can be

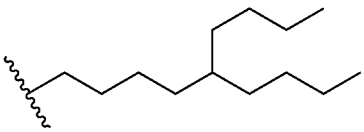


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), where R^{13} is selected from the groups mentioned above.

Another example is a tail of the formula

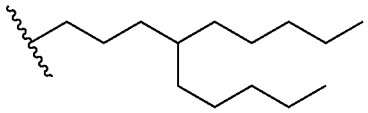


where R^{13} is an alkyl or alkenyl group having from about 13 to about 15 carbon atoms, and the total carbon length of the tail from the first carbon (i.e., the leftmost carbon atom, which is attached to a tertiary carbon) to a terminus of the tail is at most 20. Preferably, the tail has from about 24 to about 26 carbon atoms. In one embodiment, the maximum length of R^{13} from its attachment point to the ester group of the compound is 10 carbon atoms (e.g., the maximum length can be 9 carbon atoms). In one preferred embodiment, the branch in the alkyl or alkenyl group is at the δ-position or later from the point of attachment of R^{13} to the ester group. Suitable R^{13} groups include, but are not limited to

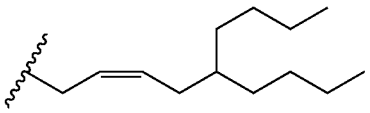


C13 (C23)
Length: C9 (20)

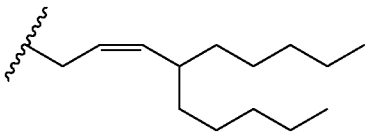
-continued



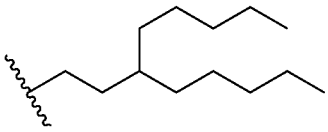
C14 (C24)
Length: C9 (20)



C13 (C23)
Length: C9 (20)

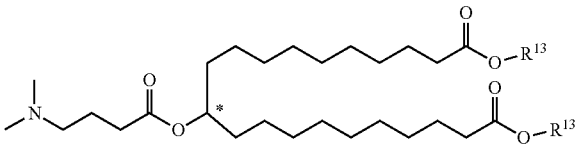


C14 (C24)
Length: C9 (20)



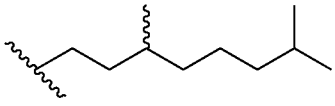
C13 (C24)
Length: C8 (19)

For example, the cationic lipid can be

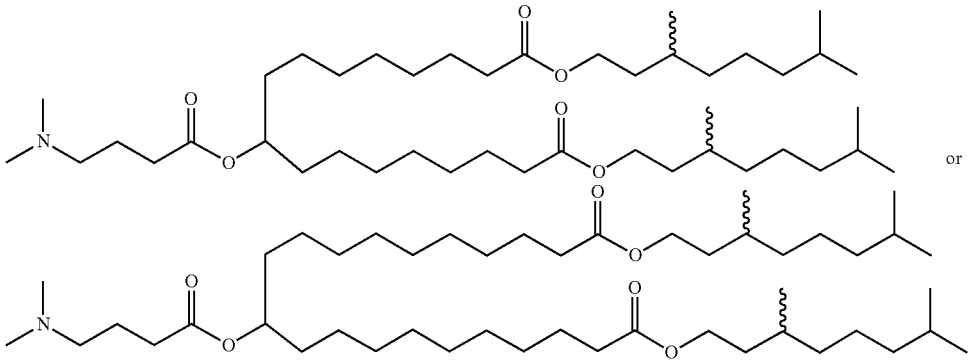


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), where R^{13} is selected from the groups above.

The R^{13} group may be derived from a natural product, such as dihydrocitgronellol, lavandulol, phytol, or dihydrophytol. In one embodiment, the R^{13} group in the tails above is a dihydrocitronellol group (either as a racemic group or a chirally pure group):



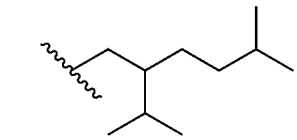
For example, the cationic lipid having a dihydroitronellol group can be



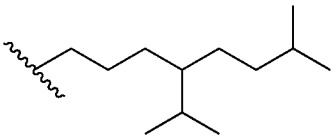
or

or a salt thereof.

In another embodiment, the R^{13} group in the tails above is a lavandulol group or a homolog of it as shown below:

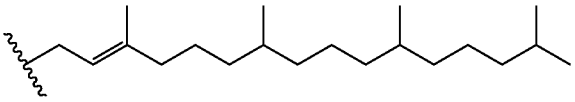


Lavandulol

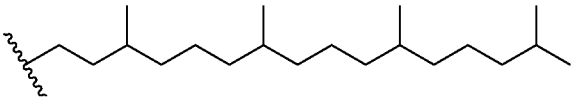


homolog

In another embodiment, the R^{13} group in the tails above is a phytol or dihydro phytol group:

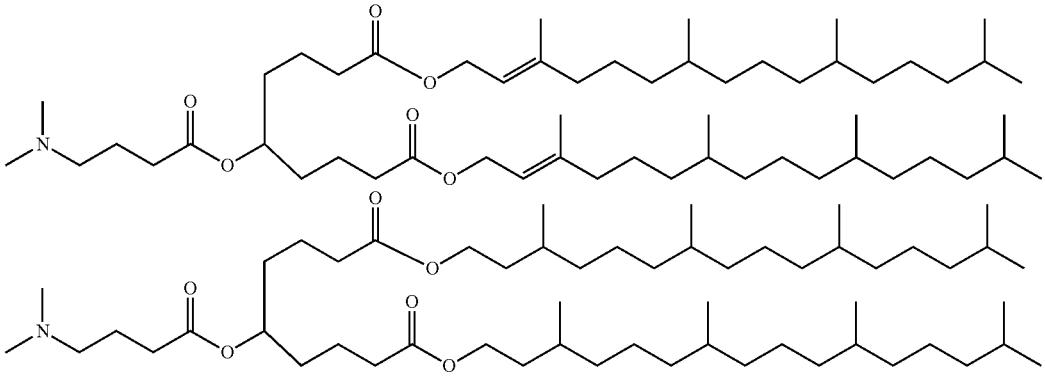


Phytol

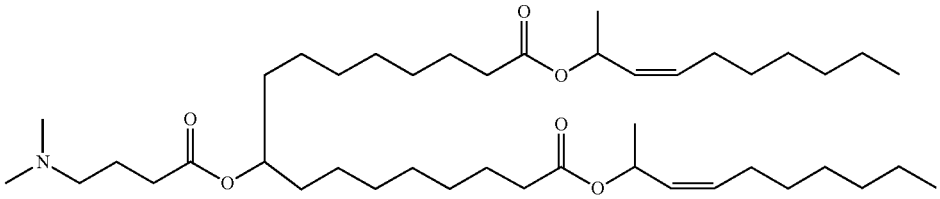


Dihydrophytol

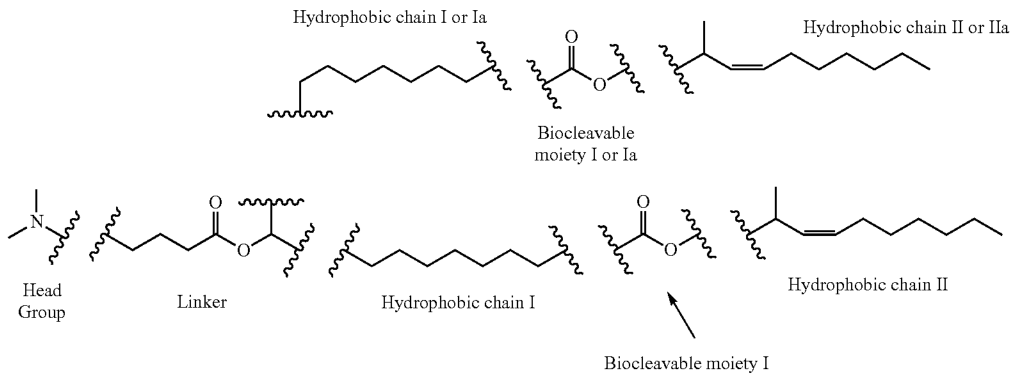
For instance, the cationic lipid can be:



A cationic lipid of the formula:



can also be thought of as a combination of a headgroup, a linker moiety, and two parts of the hydrophobic chains as follows:



Various headgrops, linker moieties, and hydrophobic chains I and II are listed below. The present invention includes compounds composed of any combination of the head, linker, hydrophobic chain I, and hydrophobic chain II groups listed below.

TABLE 2A

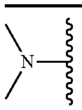
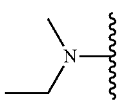
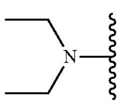
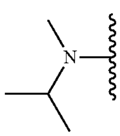
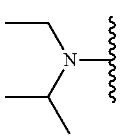
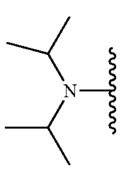
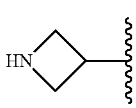
Representative headgroups








TABLE 2A-continued

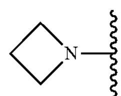
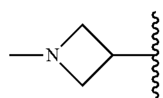
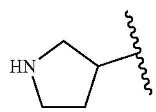
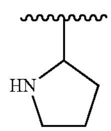
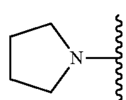
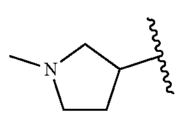
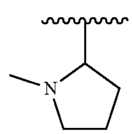
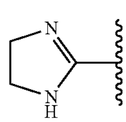
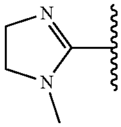
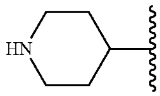
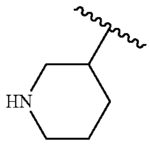
Representative headgroups









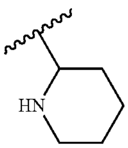
TABLE 2A-continued

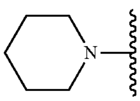
Representative headgroups

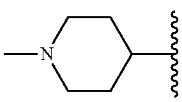


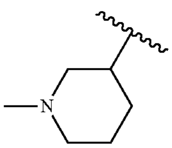


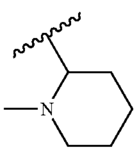


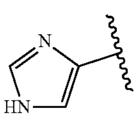


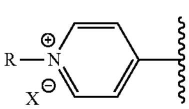




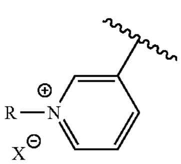








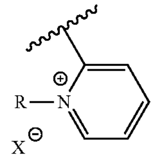
R = H, alkyl; X = halogen



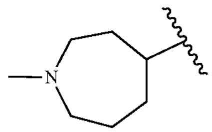
R = H, alkyl; X = halogen

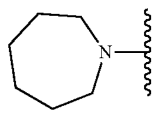
TABLE 2A-continued

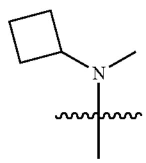
Representative headgroups

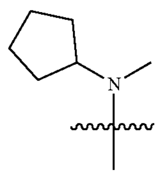


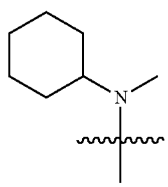
R = H, alkyl; X= halogen

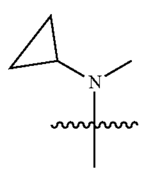


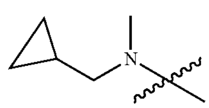


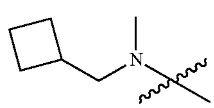












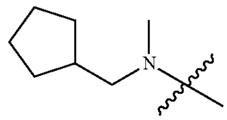


TABLE 2A-continued

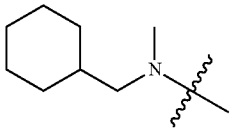
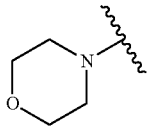
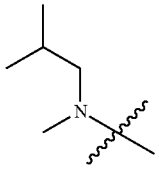
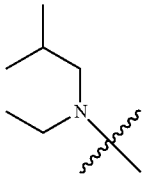
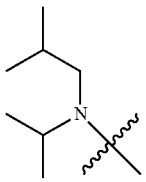
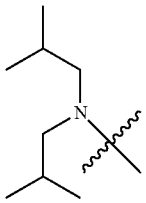
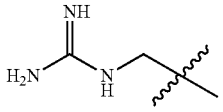
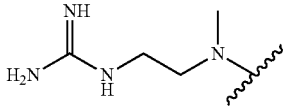
Representative headgroups






(where n is 0-5)



TABLE 2A-continued

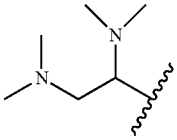
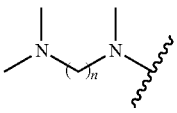
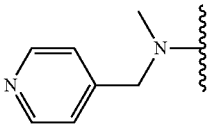
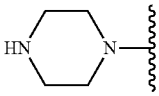
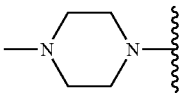
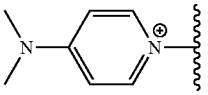
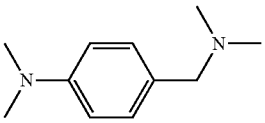
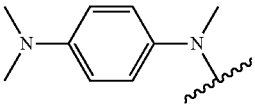
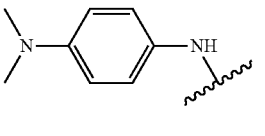
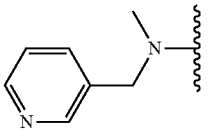
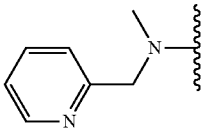
Representative headgroups


n = 0-6










TABLE 2A-continued

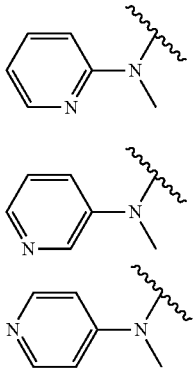
Representative headgroups


TABLE 2B

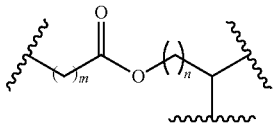
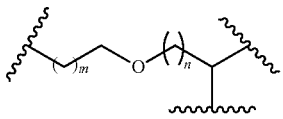
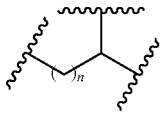
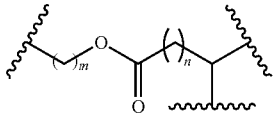
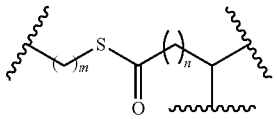
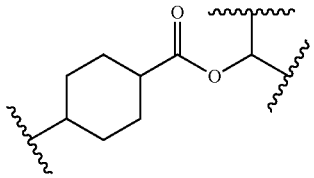
Representative linker groups
 m = 1-5; n = 0-3
 m = 0-5, n = 0-3
 n = 0-5
 m = 0-5; n = 0-3
 m = 0-5; n = 0-3


TABLE 2B-continued

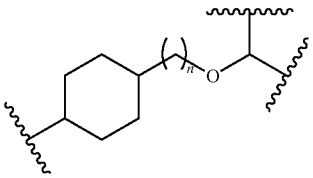
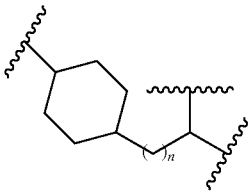
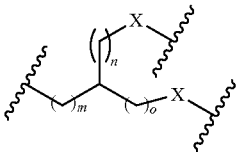
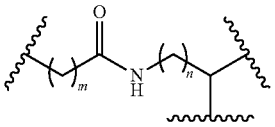
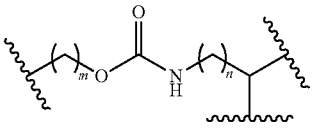
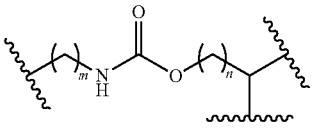
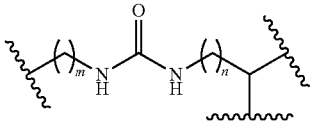
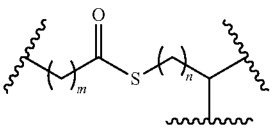
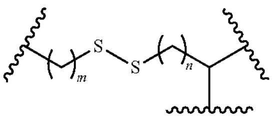
Representative linker groups
 n = 0-3
 n = 0-3
 m = 1-4; n/o = 0-3 x = O or S
 m = 0-5; n = 0-3
 m = 0-5; n = 0-3
 m = 0-5; n = 0-3
 m = 0-5; n = 0-3

TABLE 2B-continued

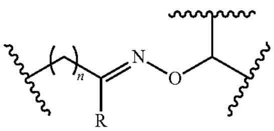
Representative linker groups



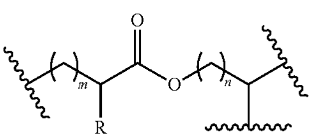
m = 0-5; n = 0-3



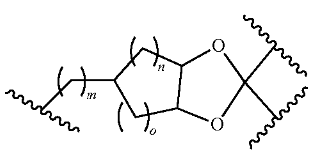
m = 0-5; n = 0-3



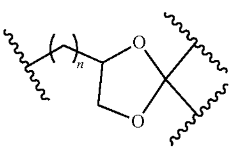
n = 0-5



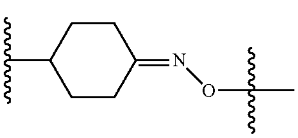
m = 1-4; n = 0-3
R = COOH, COOME, COOEt,
CN, CONH2, CONHMe

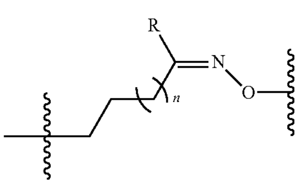


m = 1-4; n/o = 1-3



n = 1-5

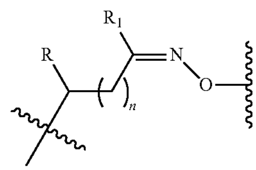




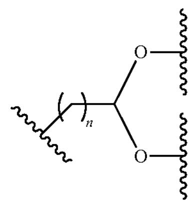
R = H, Me, Et, Pr, allyl

TABLE 2B-continued

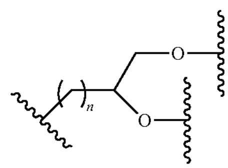
Representative linker groups



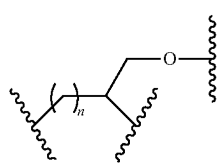
R = Me, Et, Pr, allyl
R1 = Me, Et, Pr, allyl



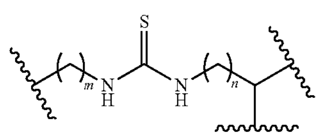
n = 0-6



n = 0-6



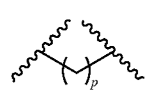
n = 0-6



m = 0-5; n = 0-3

TABLE 2C

Representative hydrophobic chain I and/or Ia, and combination thereof



p = 0-15

TABLE 2C-continued

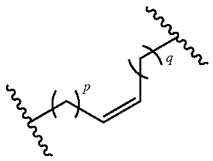
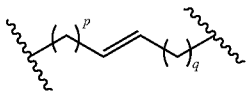
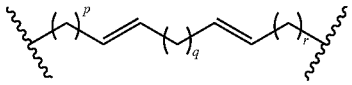
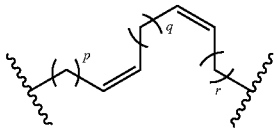
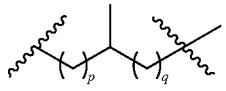
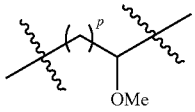
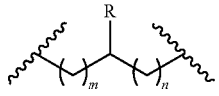
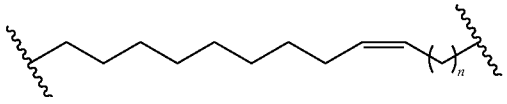
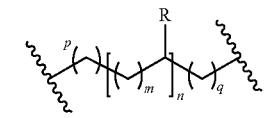
Representative hydrophobic chain I and/or Ia, and combination thereof
 p = 0-15, q = 0-15
 p = 0-15, q = 0-15
 p = 0-15, q = 1-4, r = 0-15
 p = 0-15, q = 1-4, r = 0-15
 p = 0-15, q = 0-6
 p = 0-15
 m = 0-4; n = 0-4; R = Me, Et, Pr, iPr, Bu, iBu
 n = 1-7
 m = 1-4, n = 1-10, p = 0-15, q = 0-15 R = Me, Et, OMe

TABLE 2D

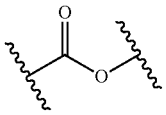
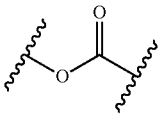
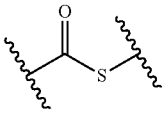
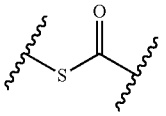
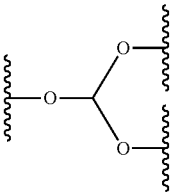
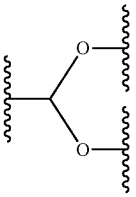
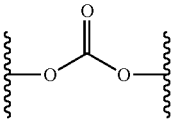
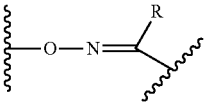
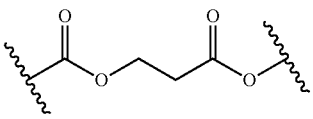
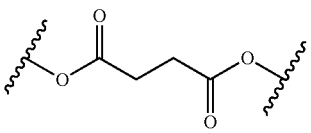
Representative biodegradable moieties I and/or Ia and combinations thereof











TABLE 2D-continued

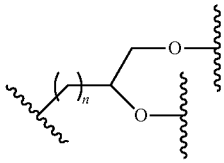
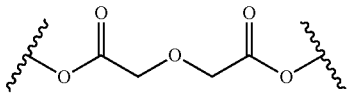
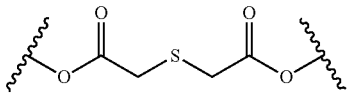
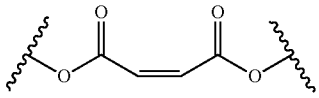
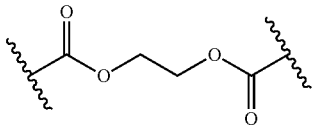
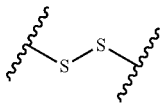
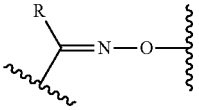
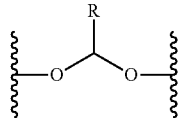
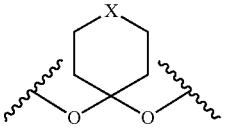
Representative biodegradable moieties I and/or Ia and combinations thereof
 n = 0-6






 R = H, Me, Et, cyclic alkyl, alicylic, aromatic
 X = CH_2, O, S

TABLE 2E

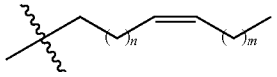
Representative hydrophobic chain II and/or IIa and combinations thereof
 n = 0-6; m = 0-16

TABLE 2E-continued

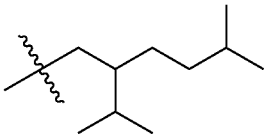
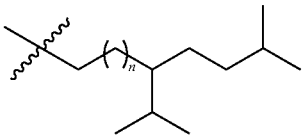
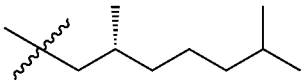
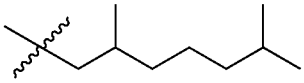
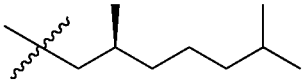
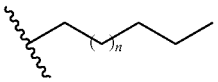
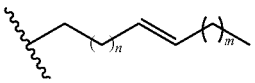
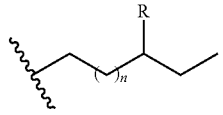
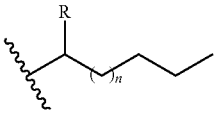
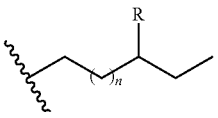
Representative hydrophobic chain II and/or IIa and combinations thereof

 n = 0-6



 n = 0-8
 n = 0-8; m = 0-6
 n = 0-8 R = OMe, Me, Et, n-Pr, n-Bu
 n = 0-8 R = OMe, Me, Et, Pr
 n = 0-8 R = OMe, Me, Et, Pr

TABLE 2E-continued

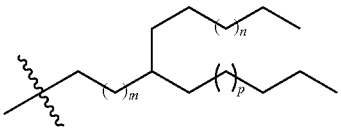
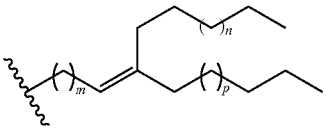
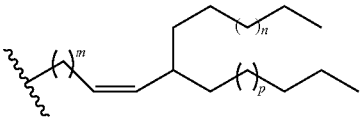
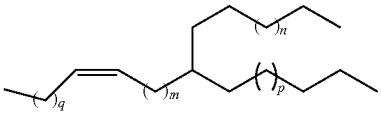
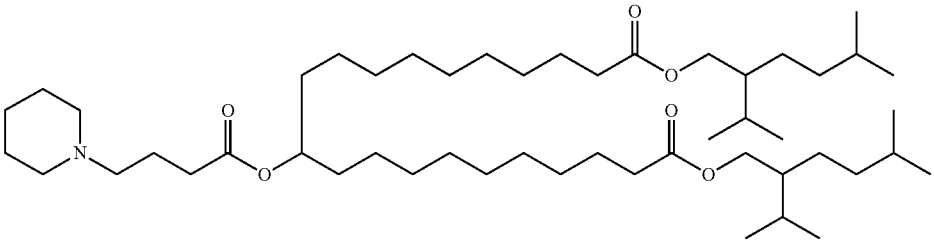
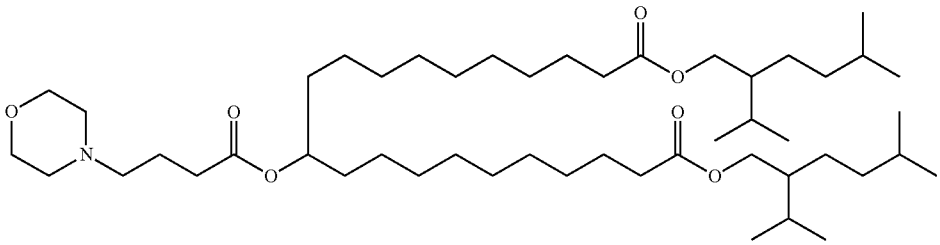
Representative hydrophobic chain II and/or IIa and combinations thereof
 m = 0-6; n = 0-6; p = 0-6
 m = 0-6; n = 0-6; p = 0-6
 m = 0-6; n = 0-6; p = 0-6

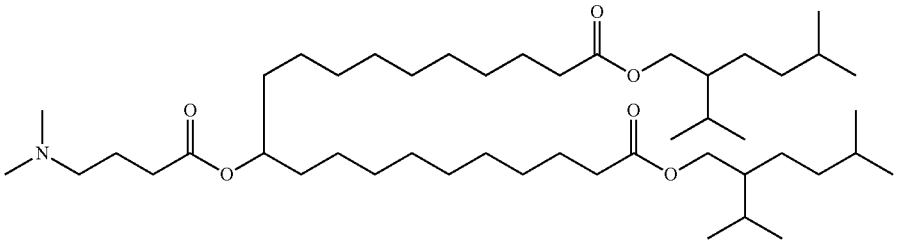
TABLE 2E-continued

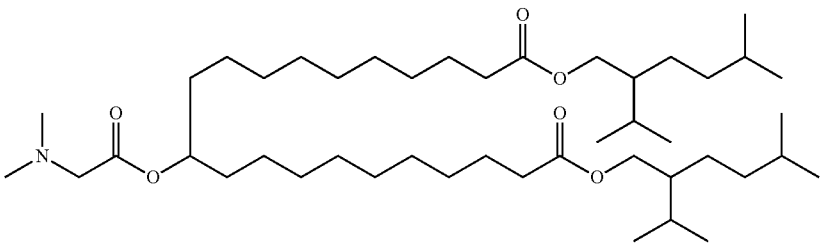
Representative hydrophobic chain II and/or IIa and combinations thereof
 m = 0-6; n = 0-6; p = 0-6; q = 0-6

Other cationic lipids of the present invention include those in Table 3 below. Each asymmetric carbon atom in the compounds below can be either chirally pure (R or S) or racemic. These cationic lipids as well as those in the working examples (such as Examples 36 and 37) are suitable for forming nucleic acid-lipid particles.









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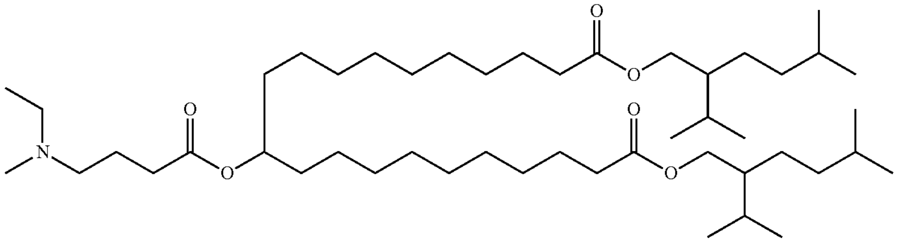

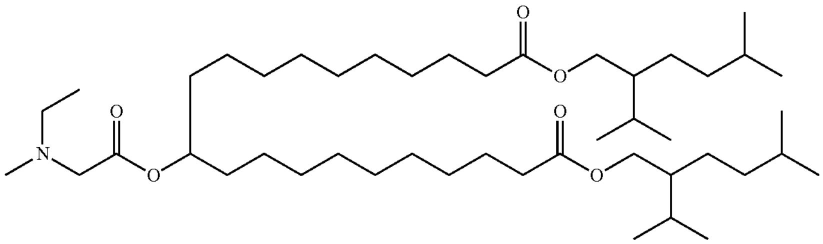

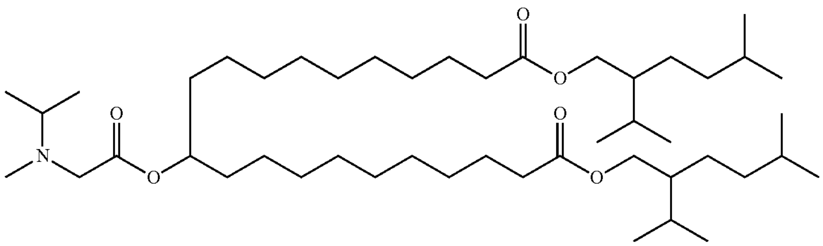

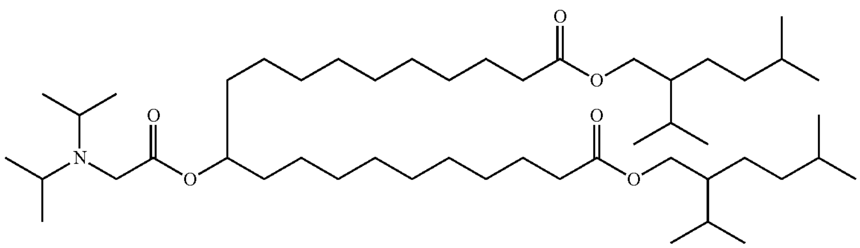

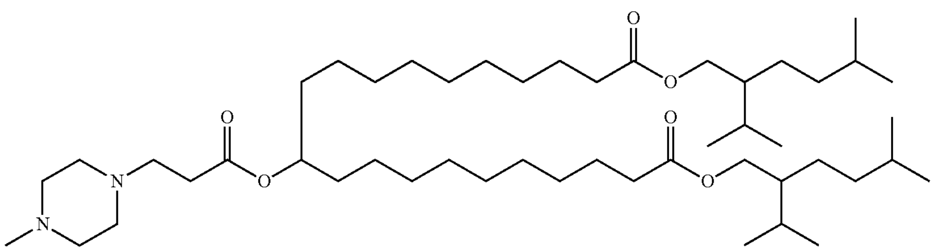

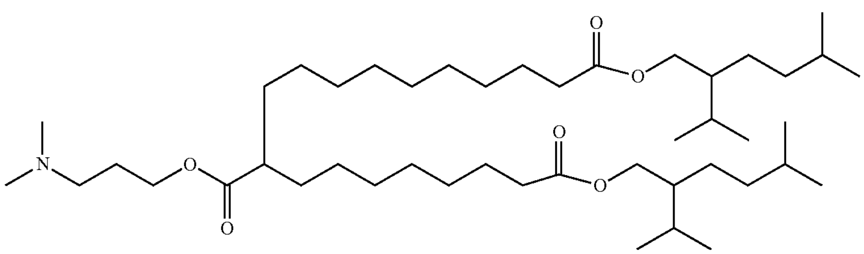

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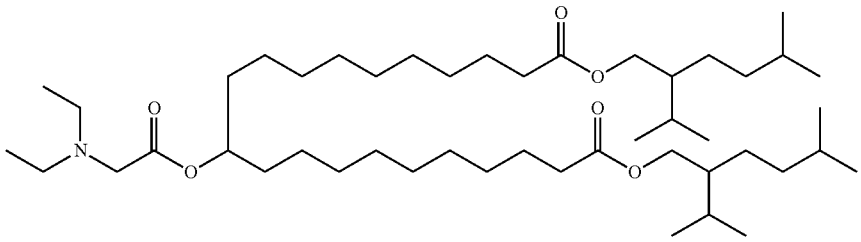

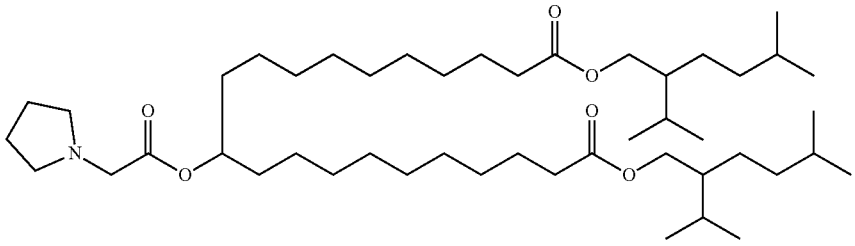

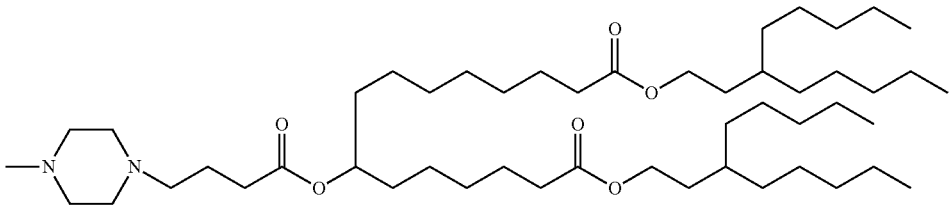

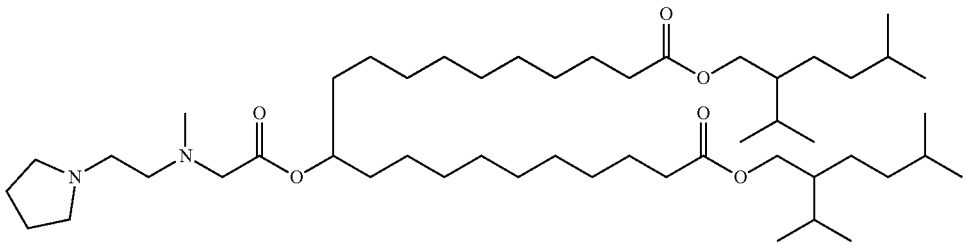

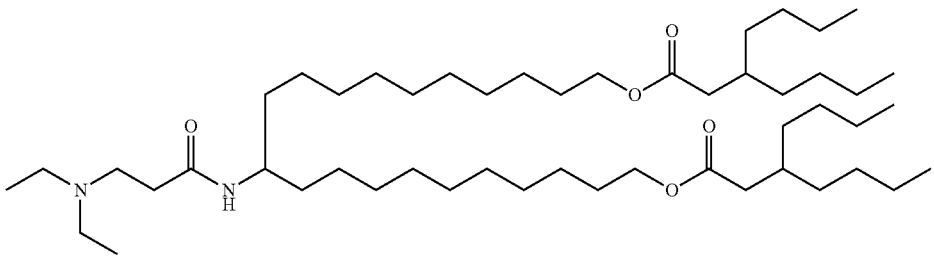

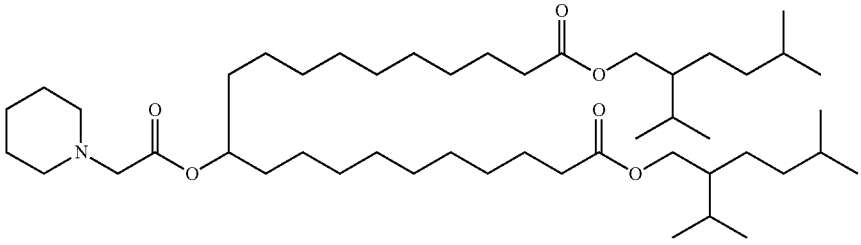

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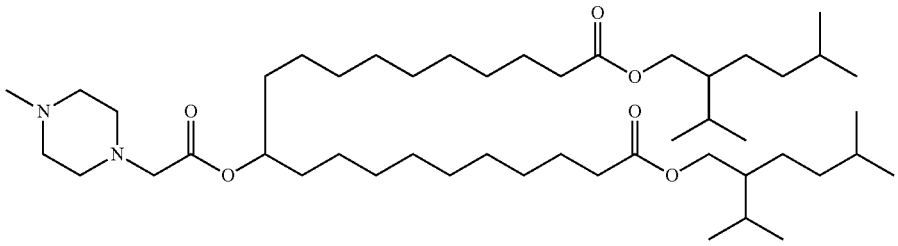

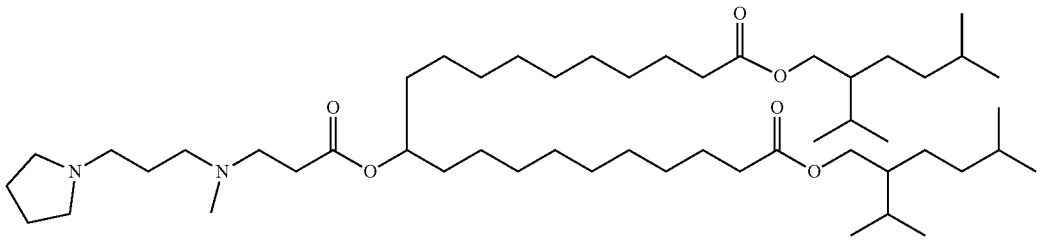

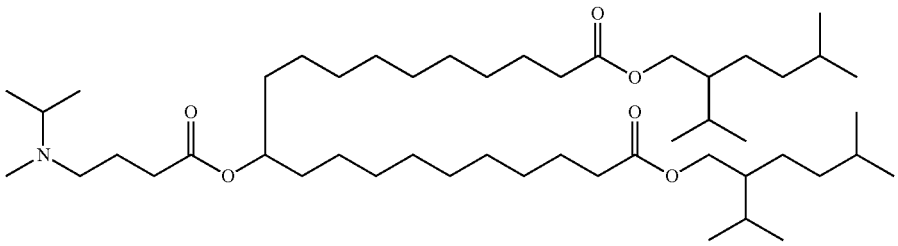

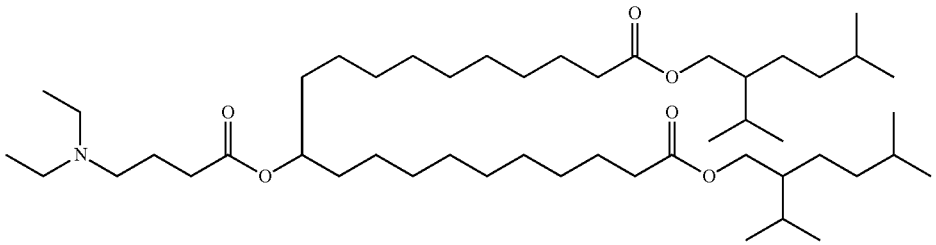

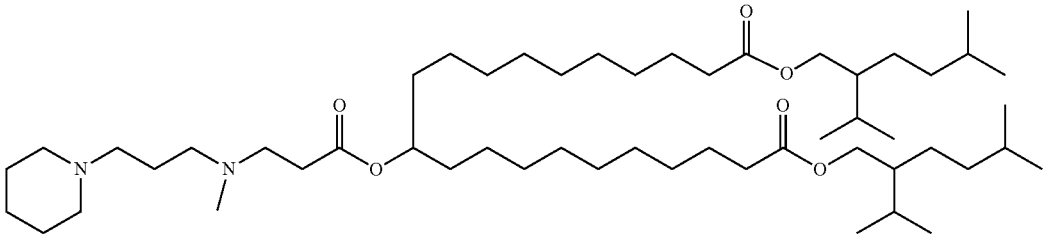

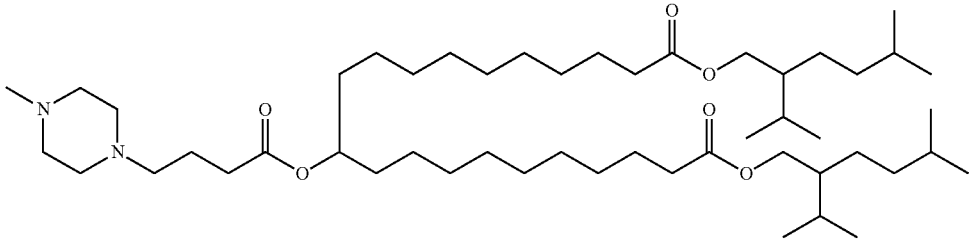

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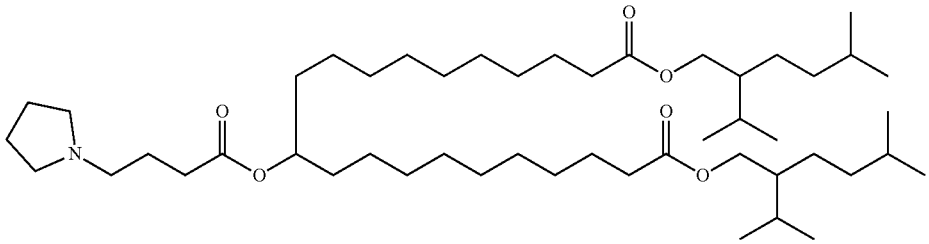

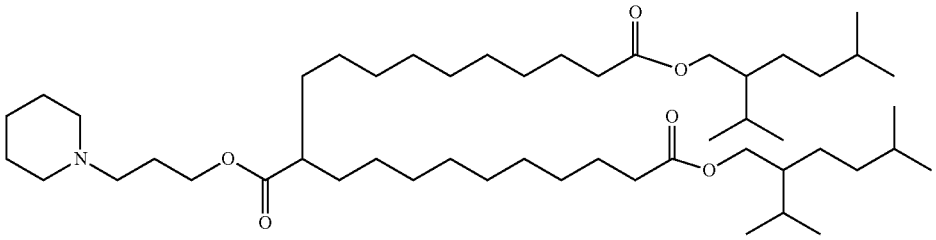

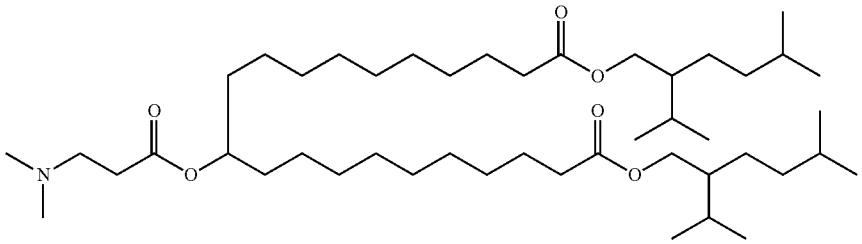

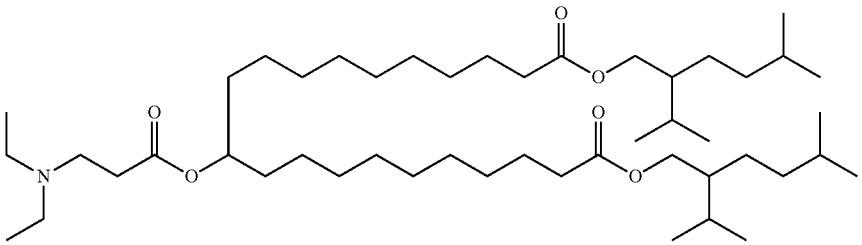

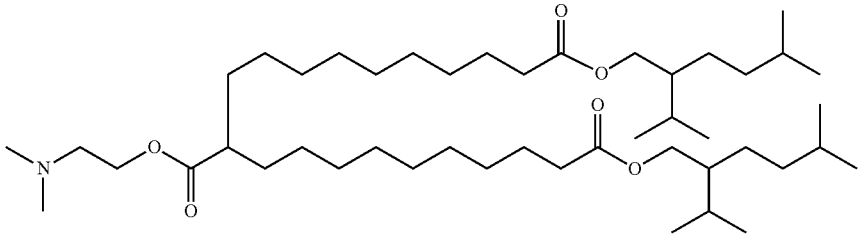

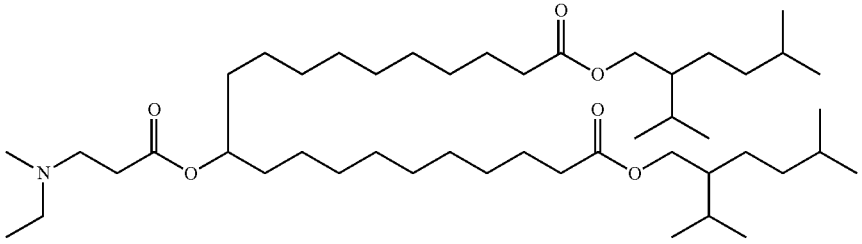

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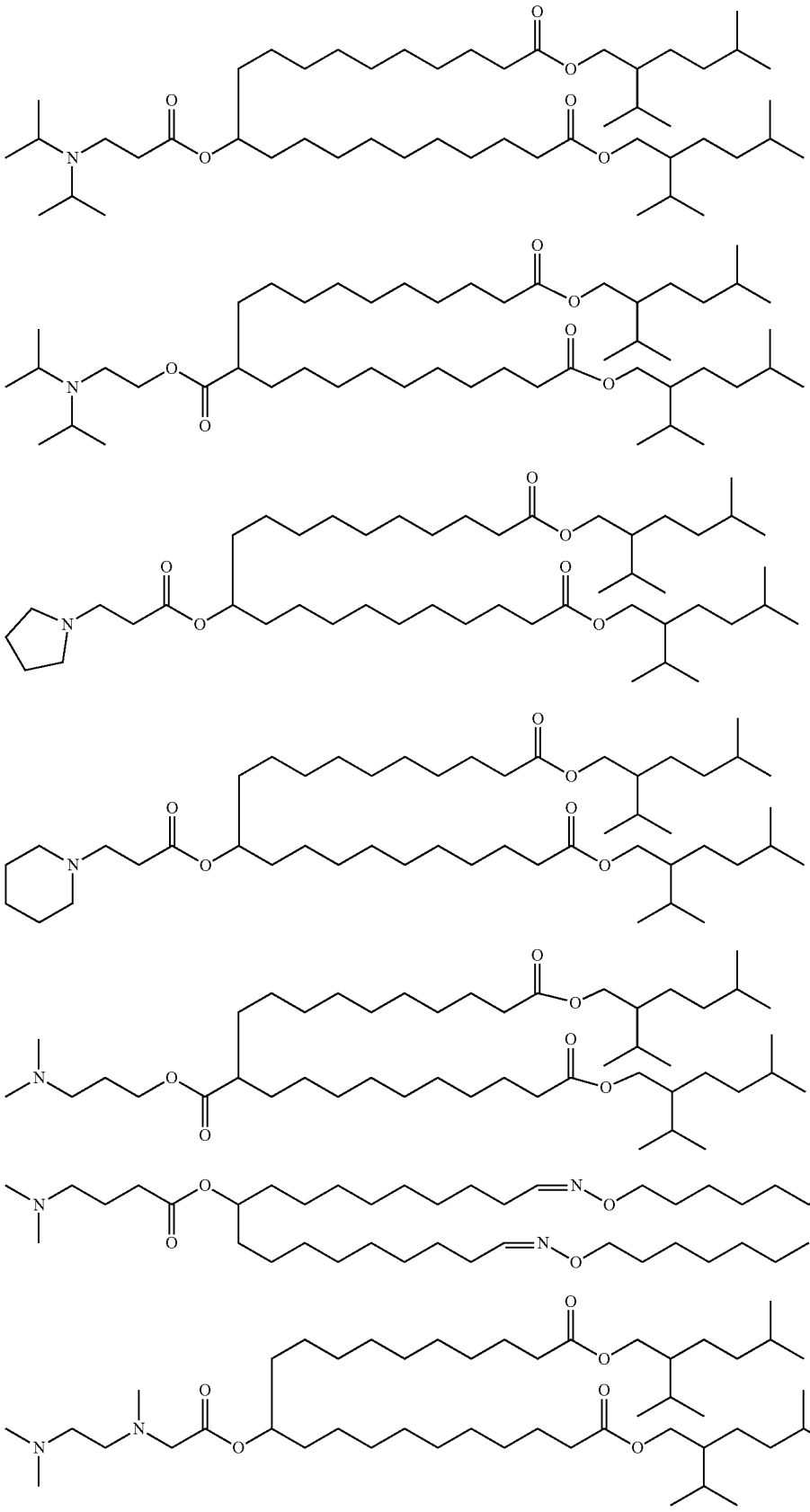

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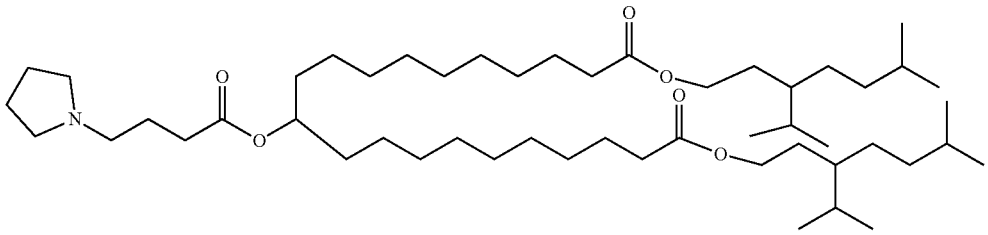

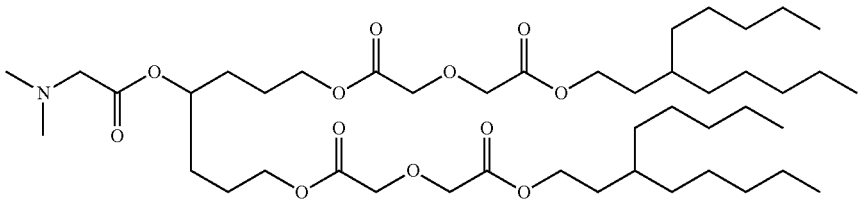

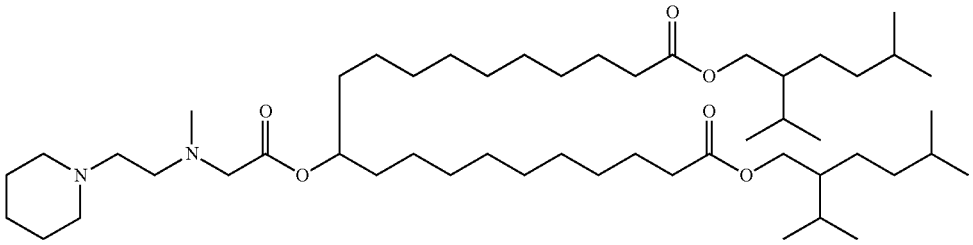

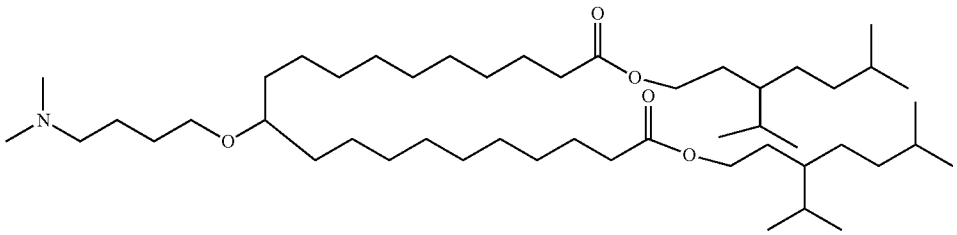

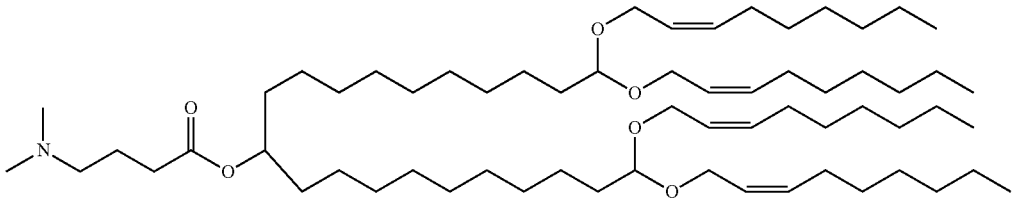

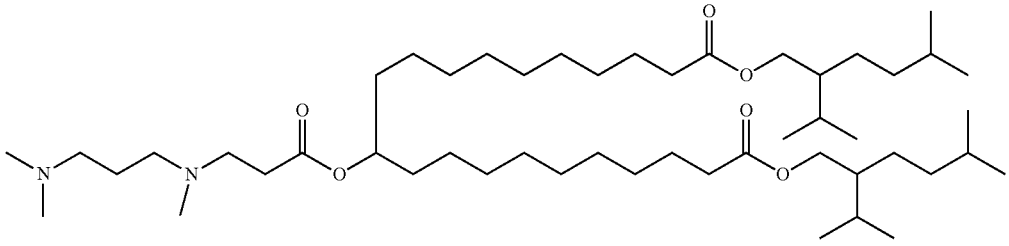

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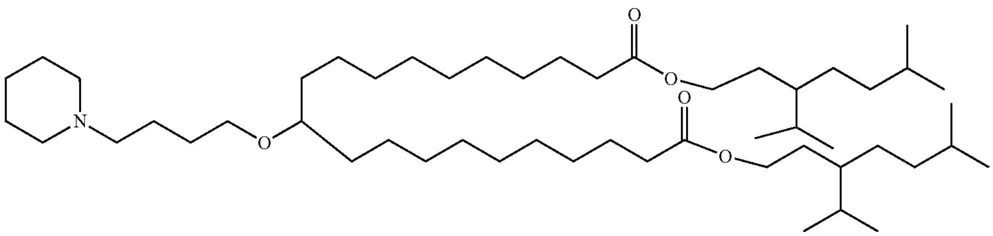

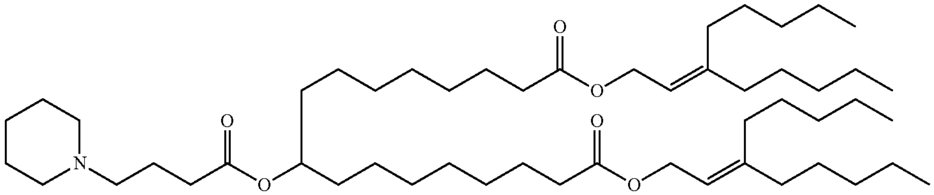

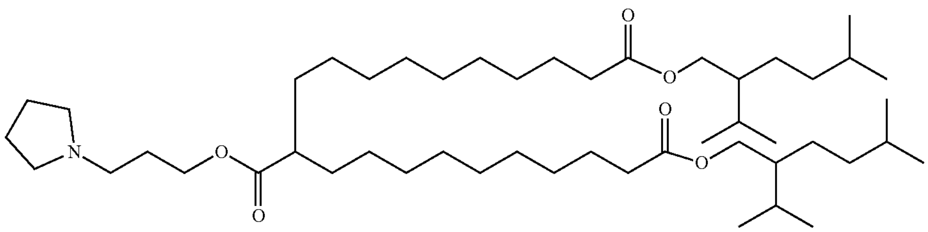

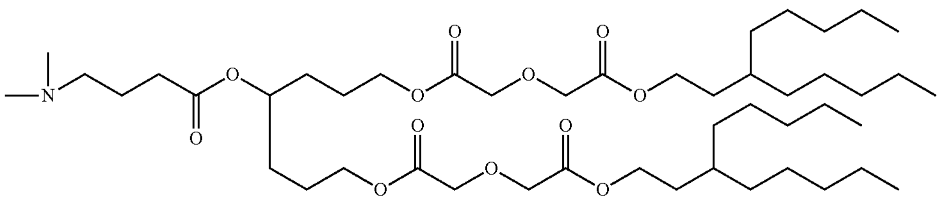

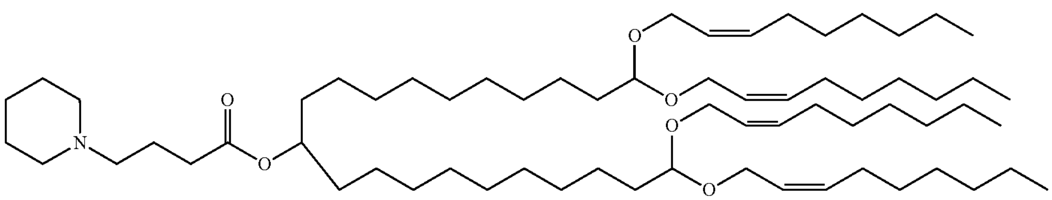

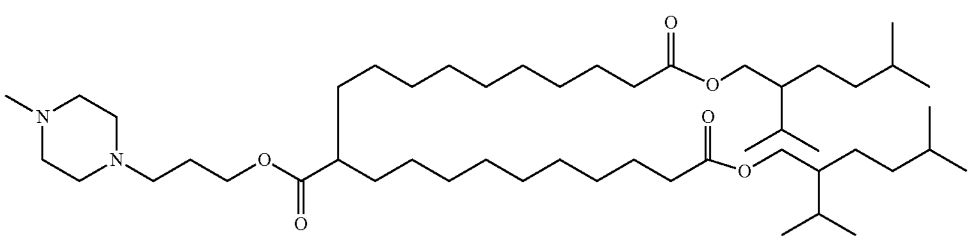

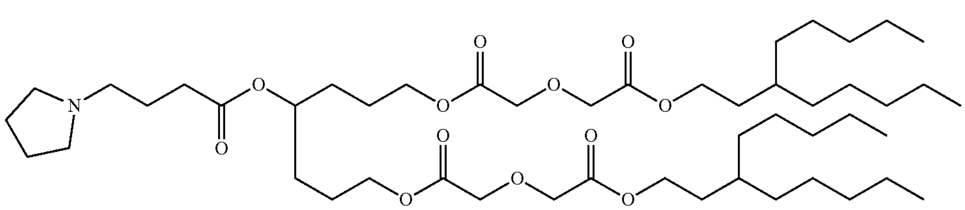

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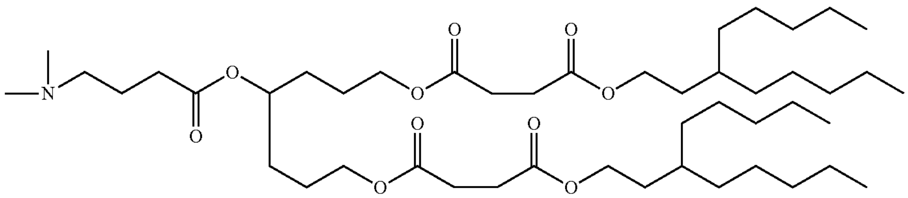

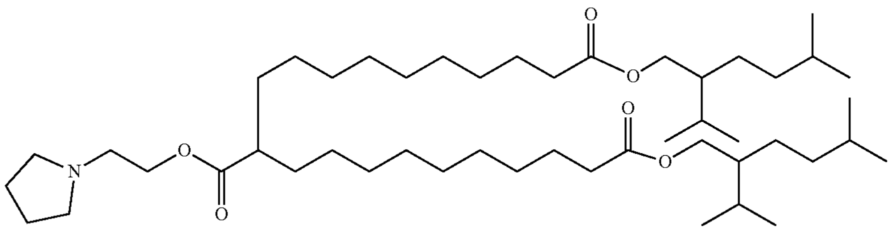

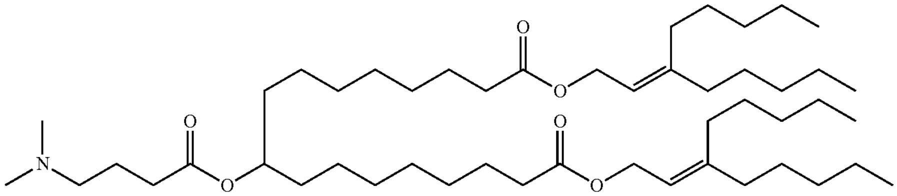

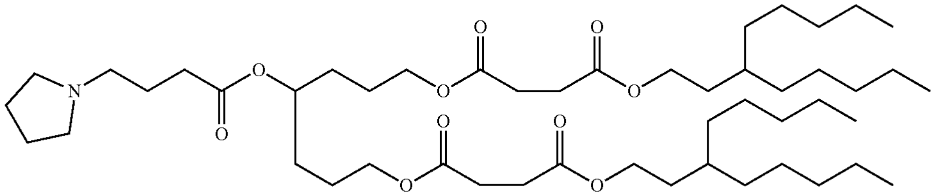

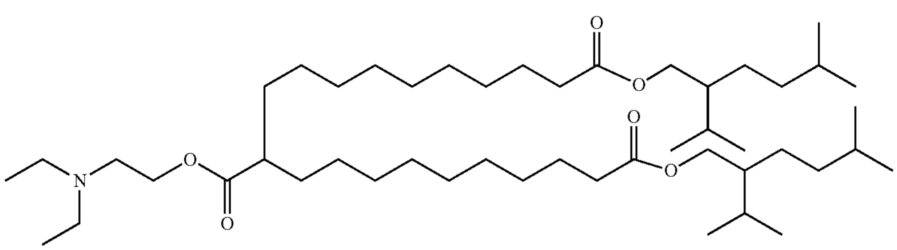

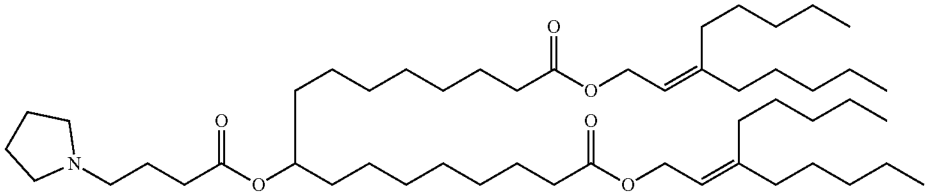

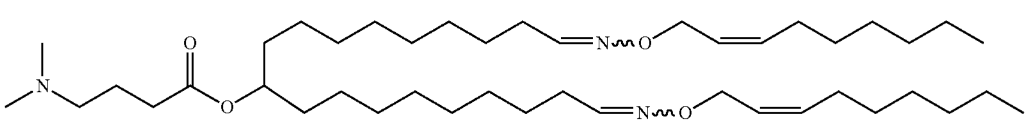

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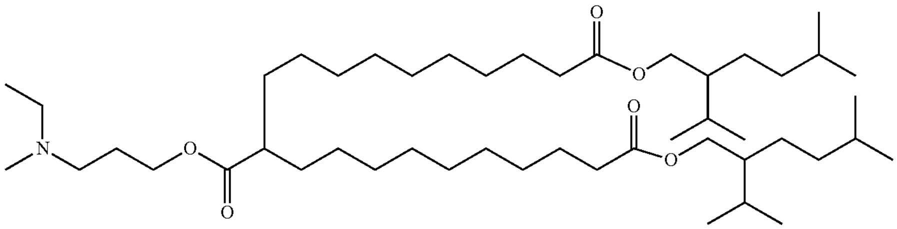

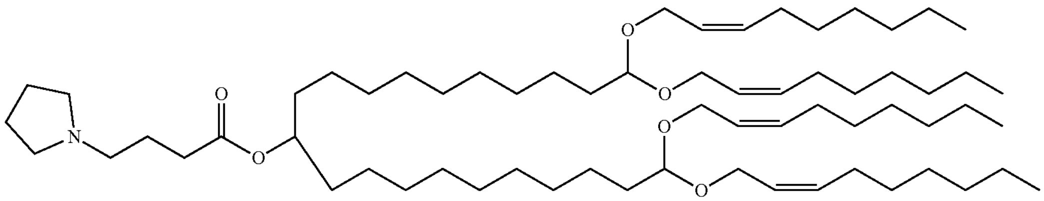

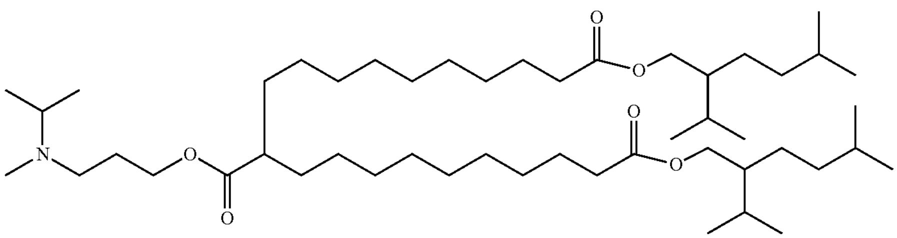

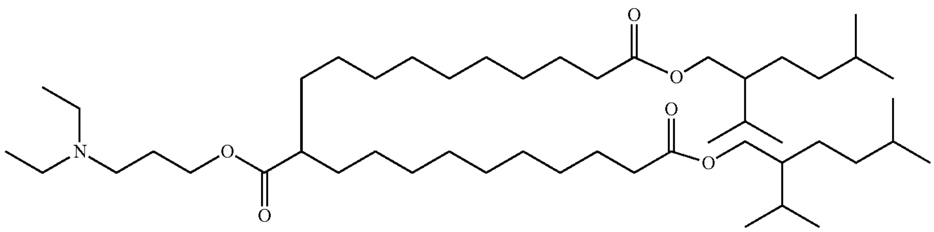

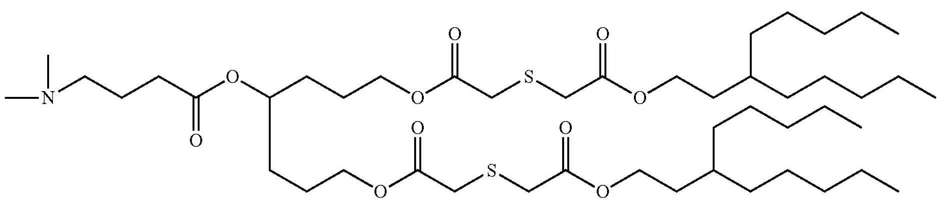

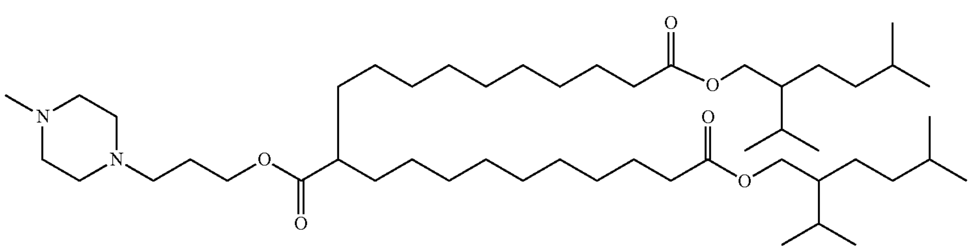

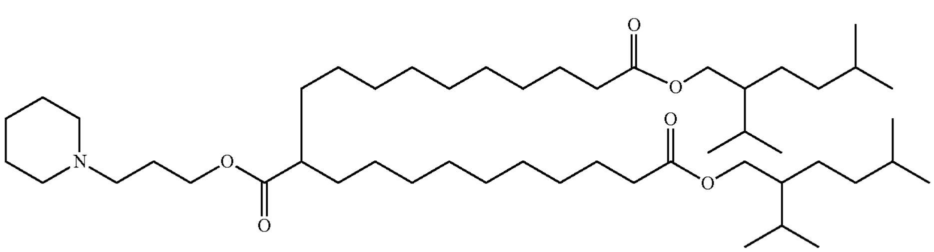

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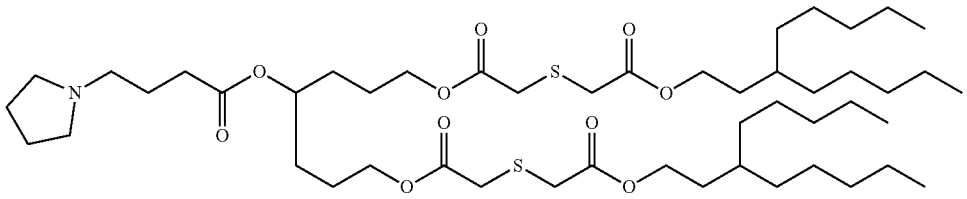

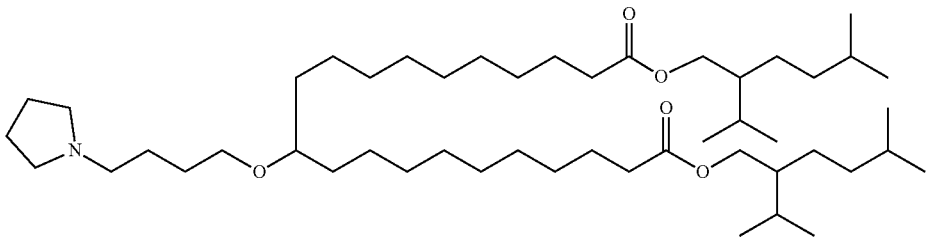

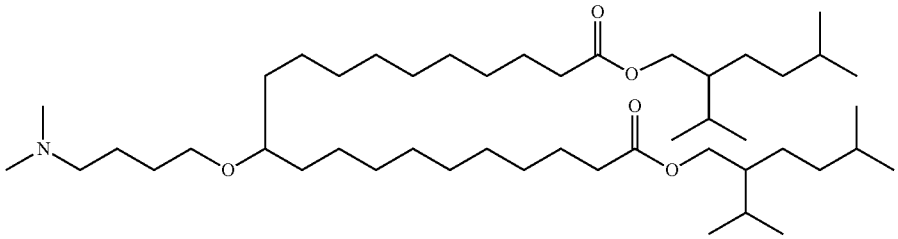

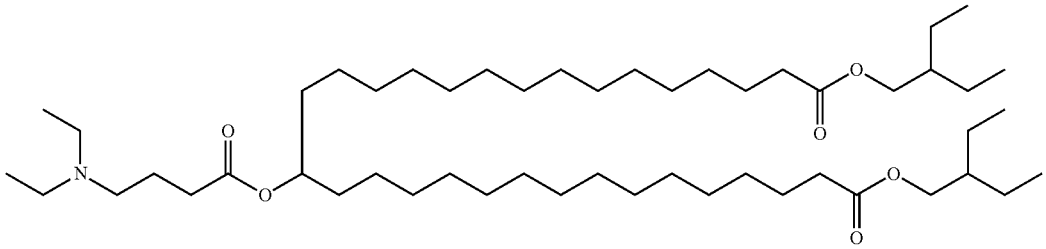

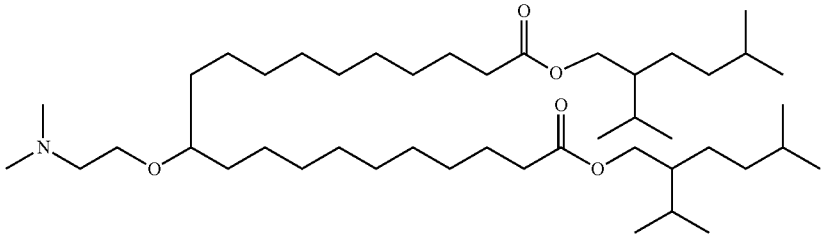

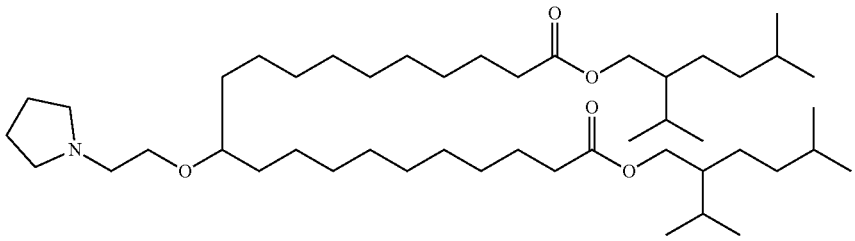

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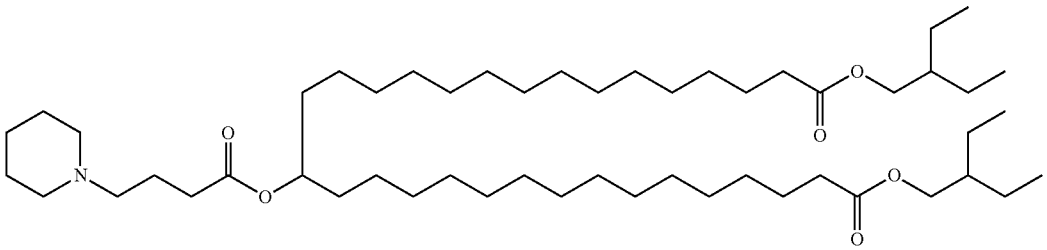

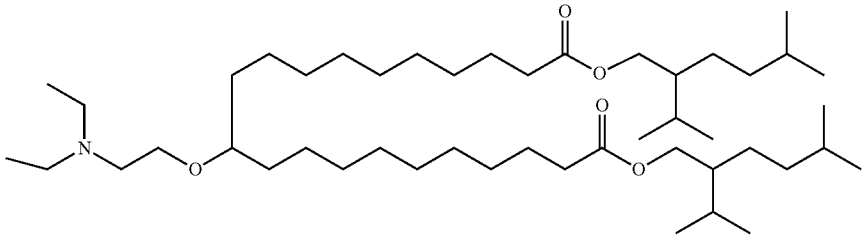

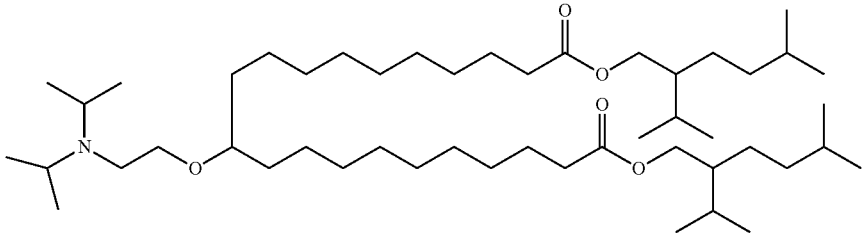

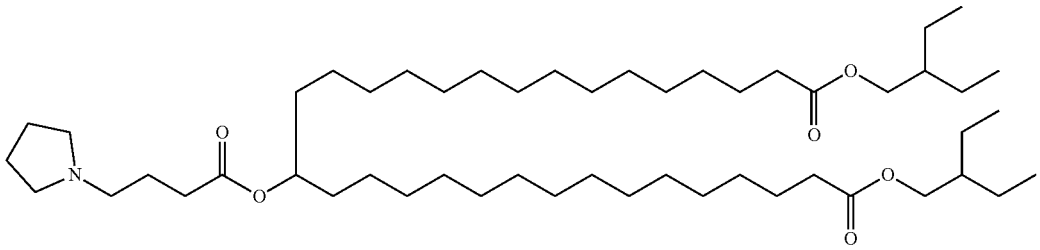

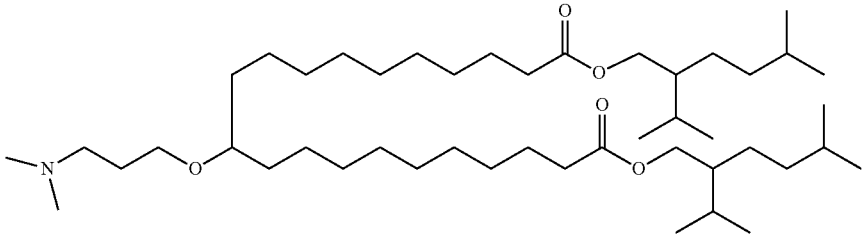

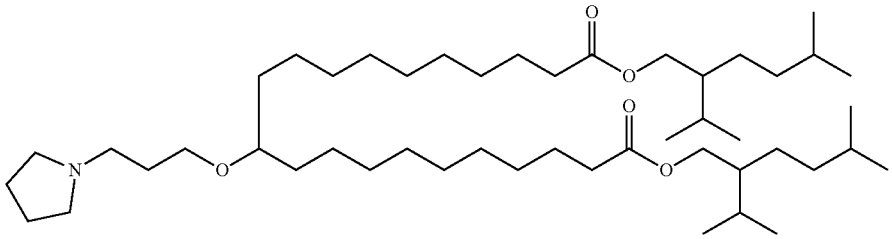

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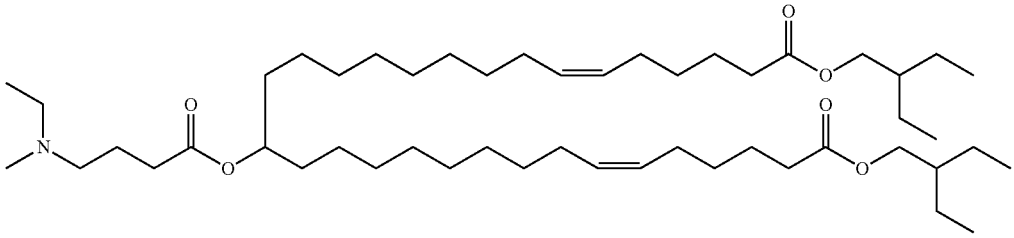

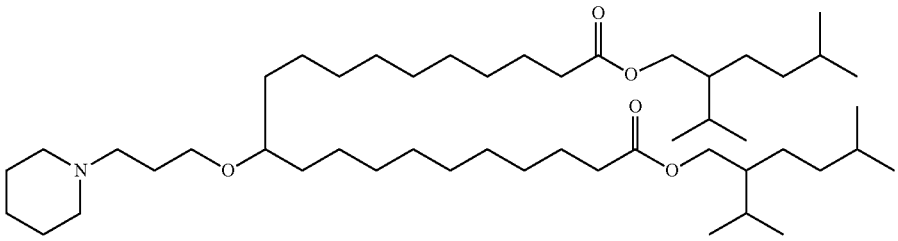

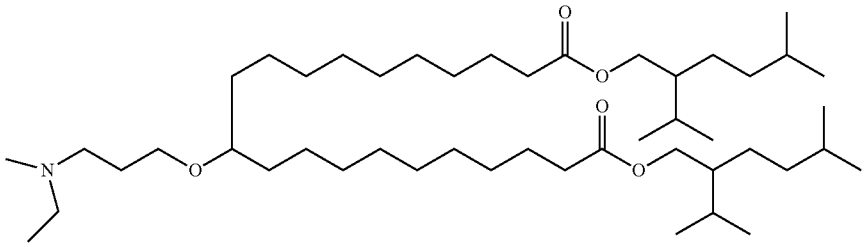

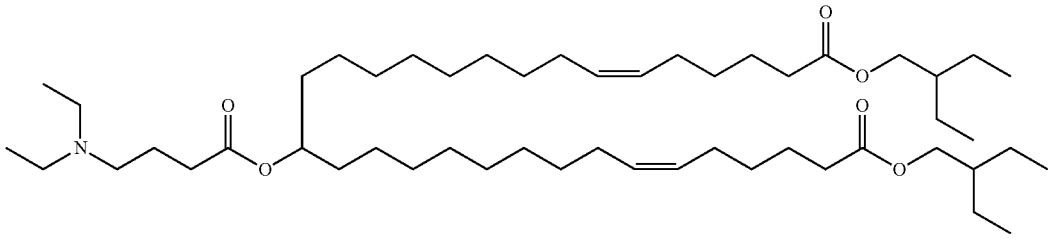

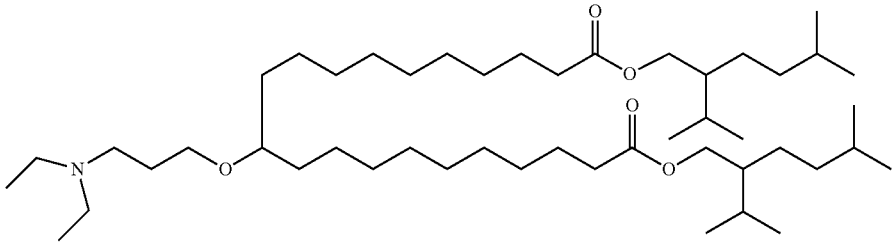

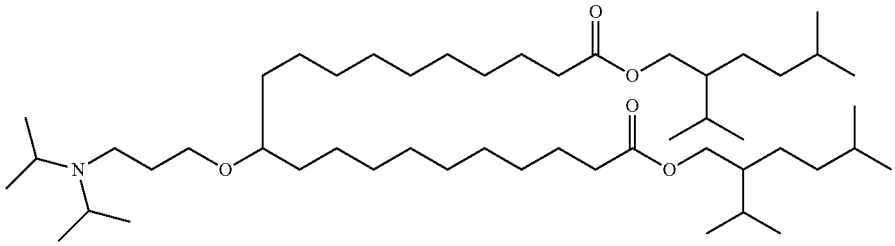

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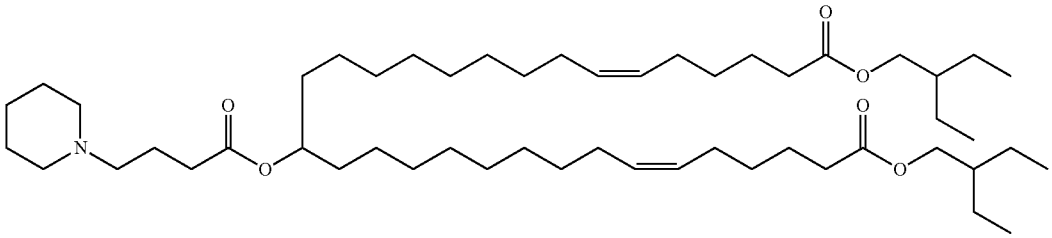

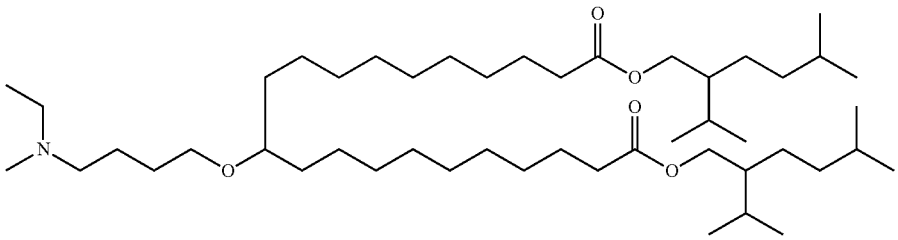

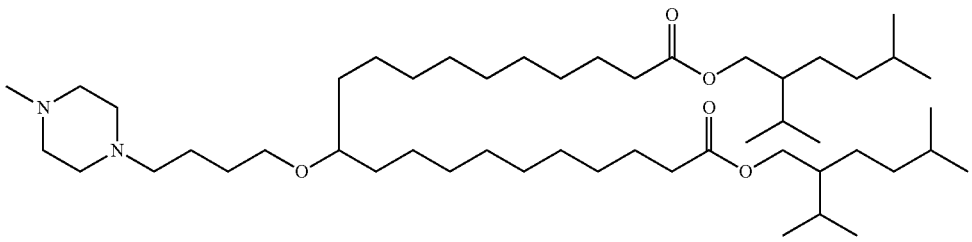

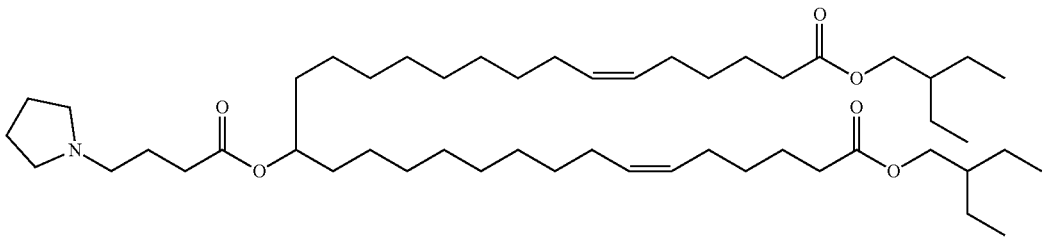

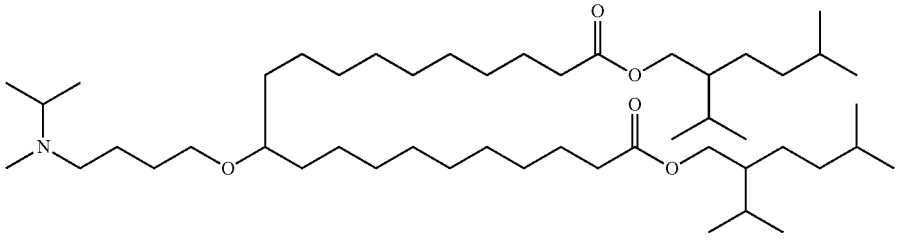

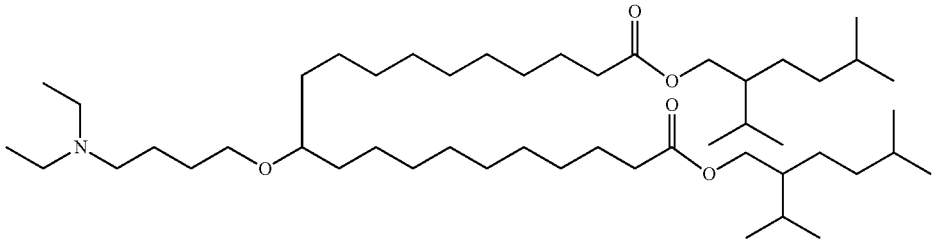

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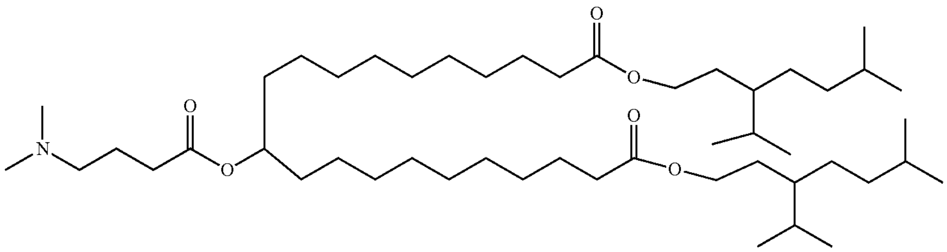

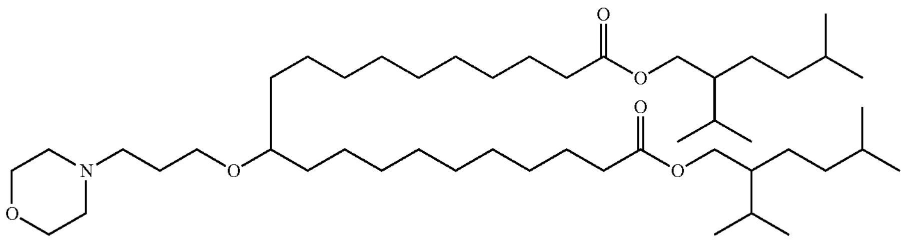

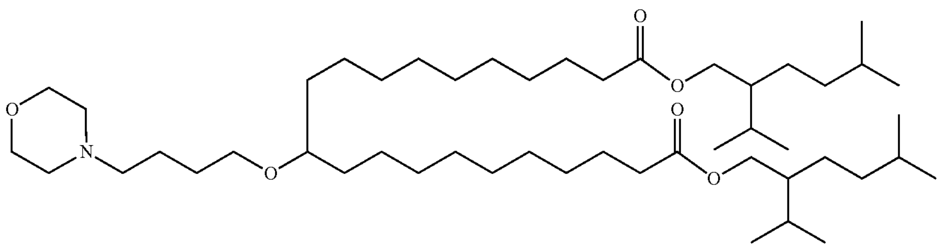

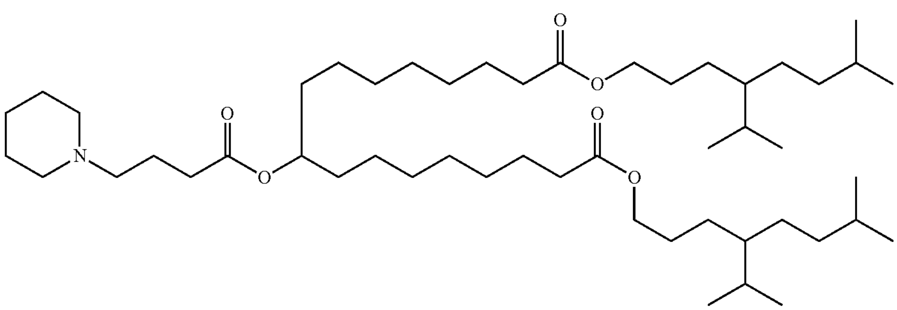

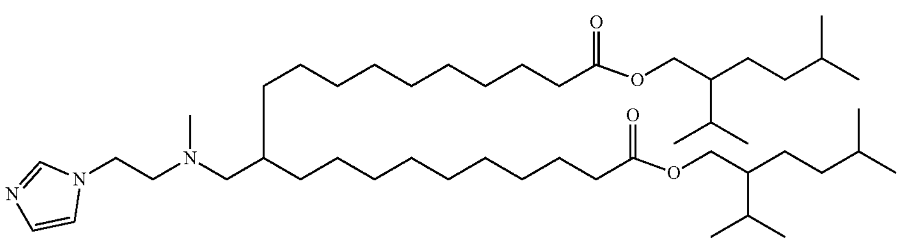

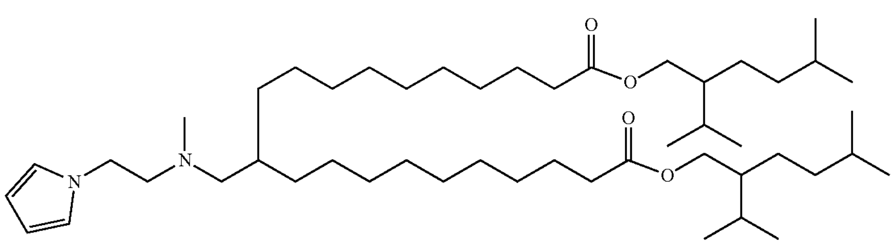

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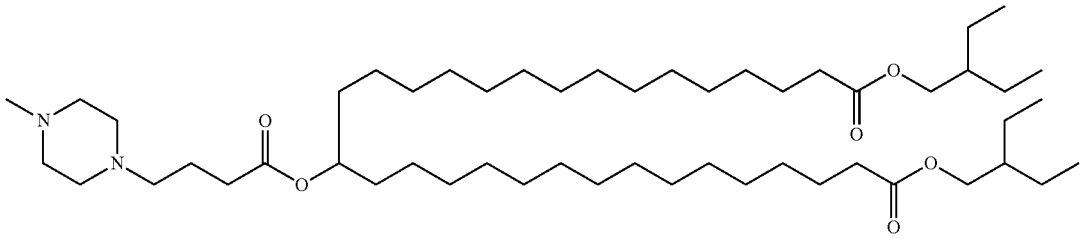

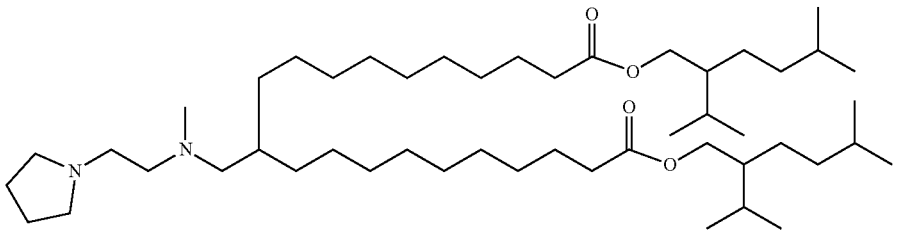

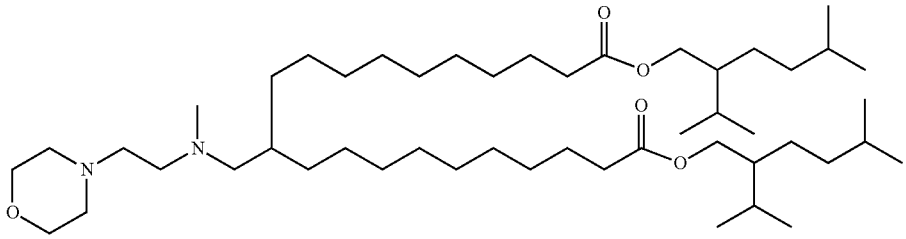

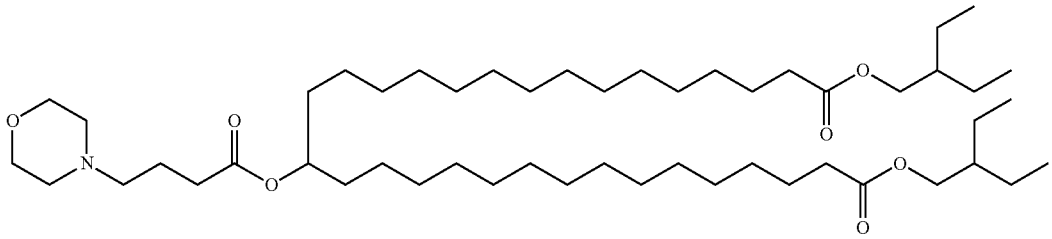

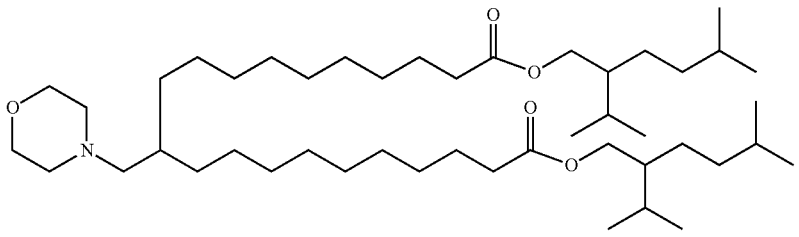

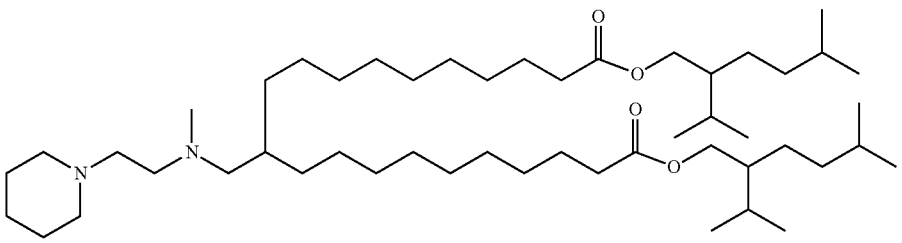

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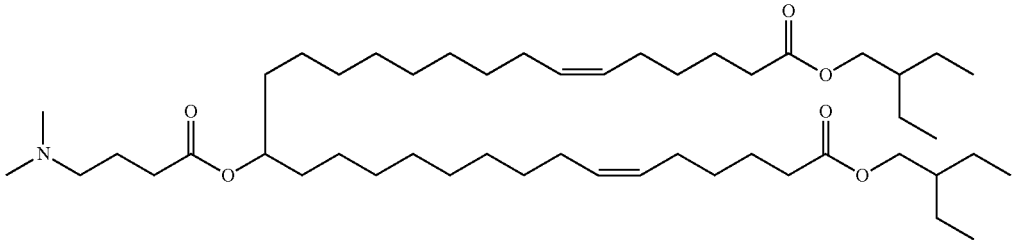

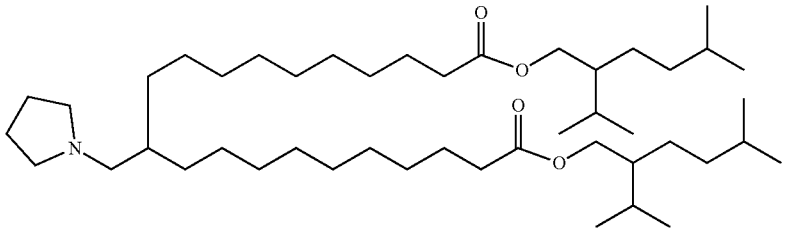

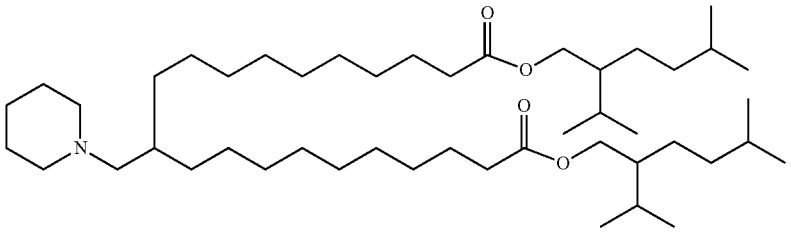

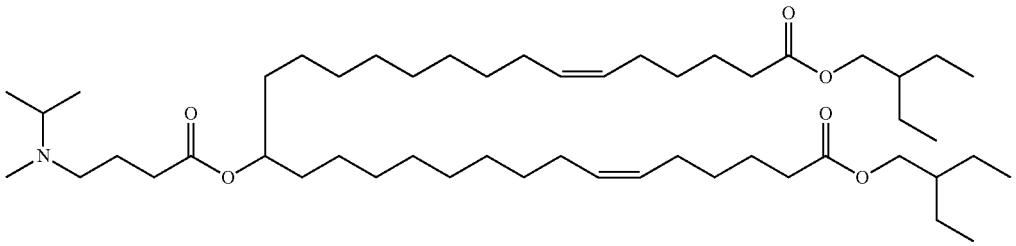

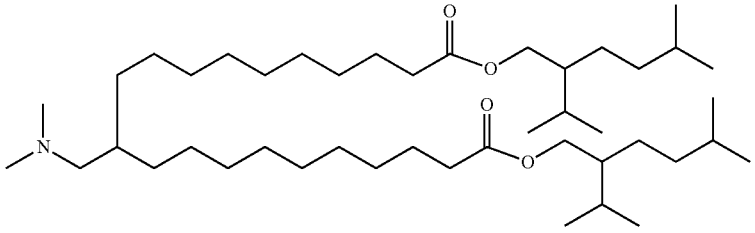

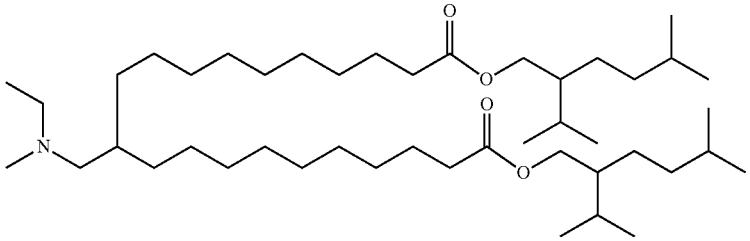

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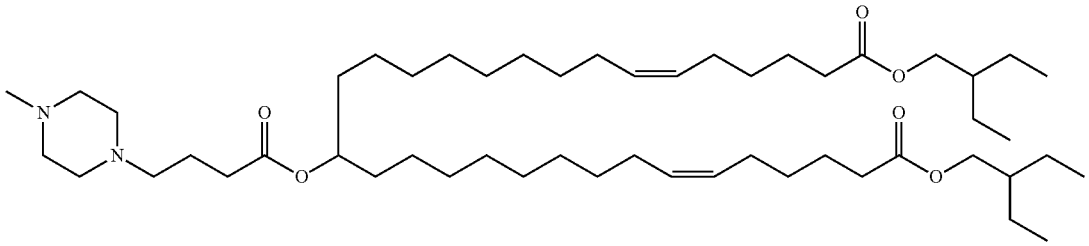

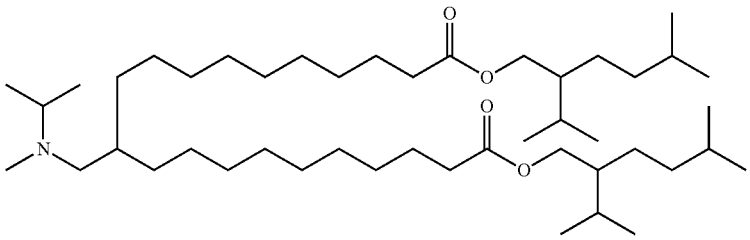

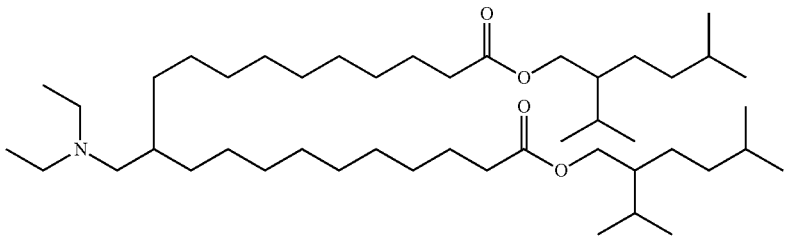

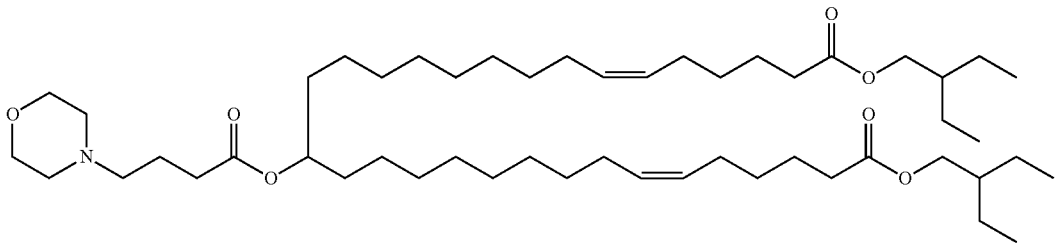

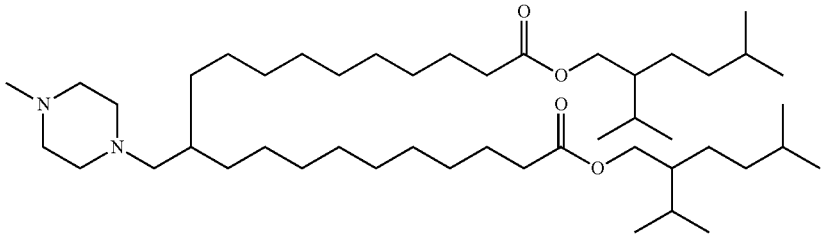

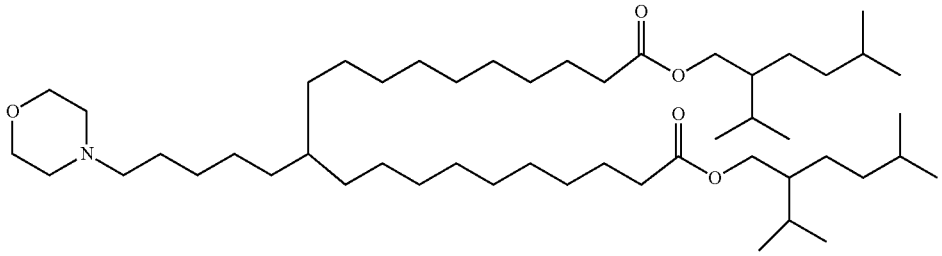

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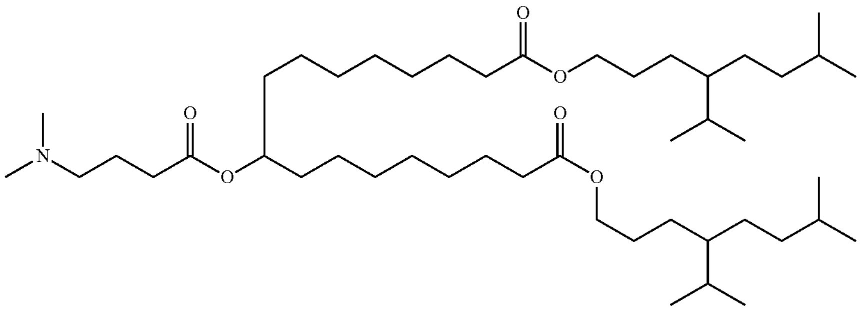

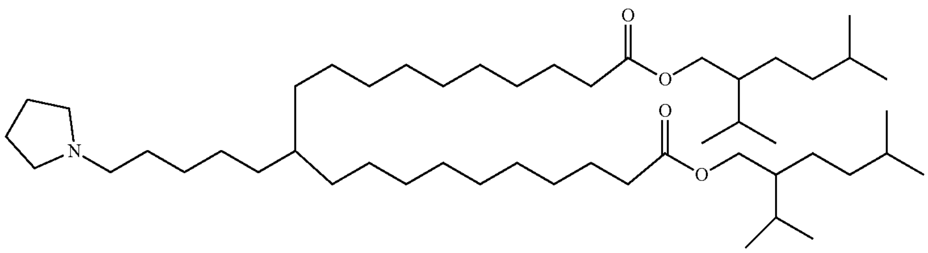

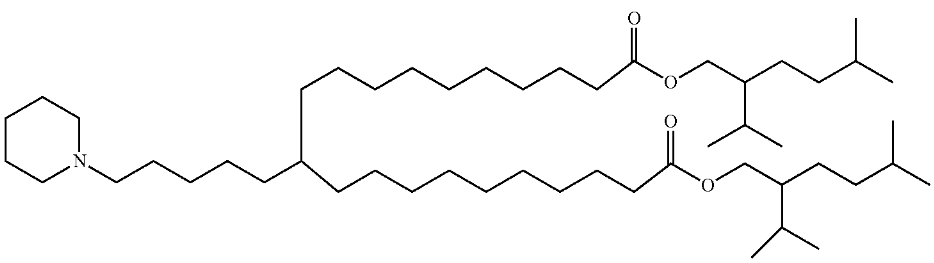

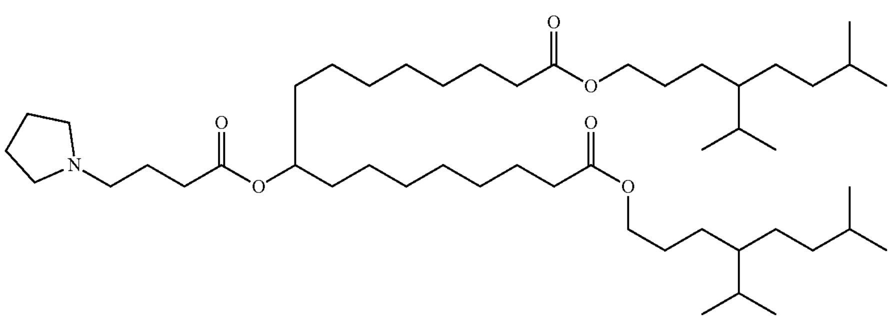

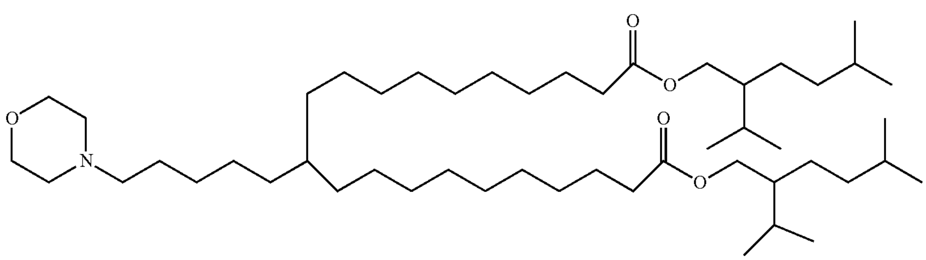

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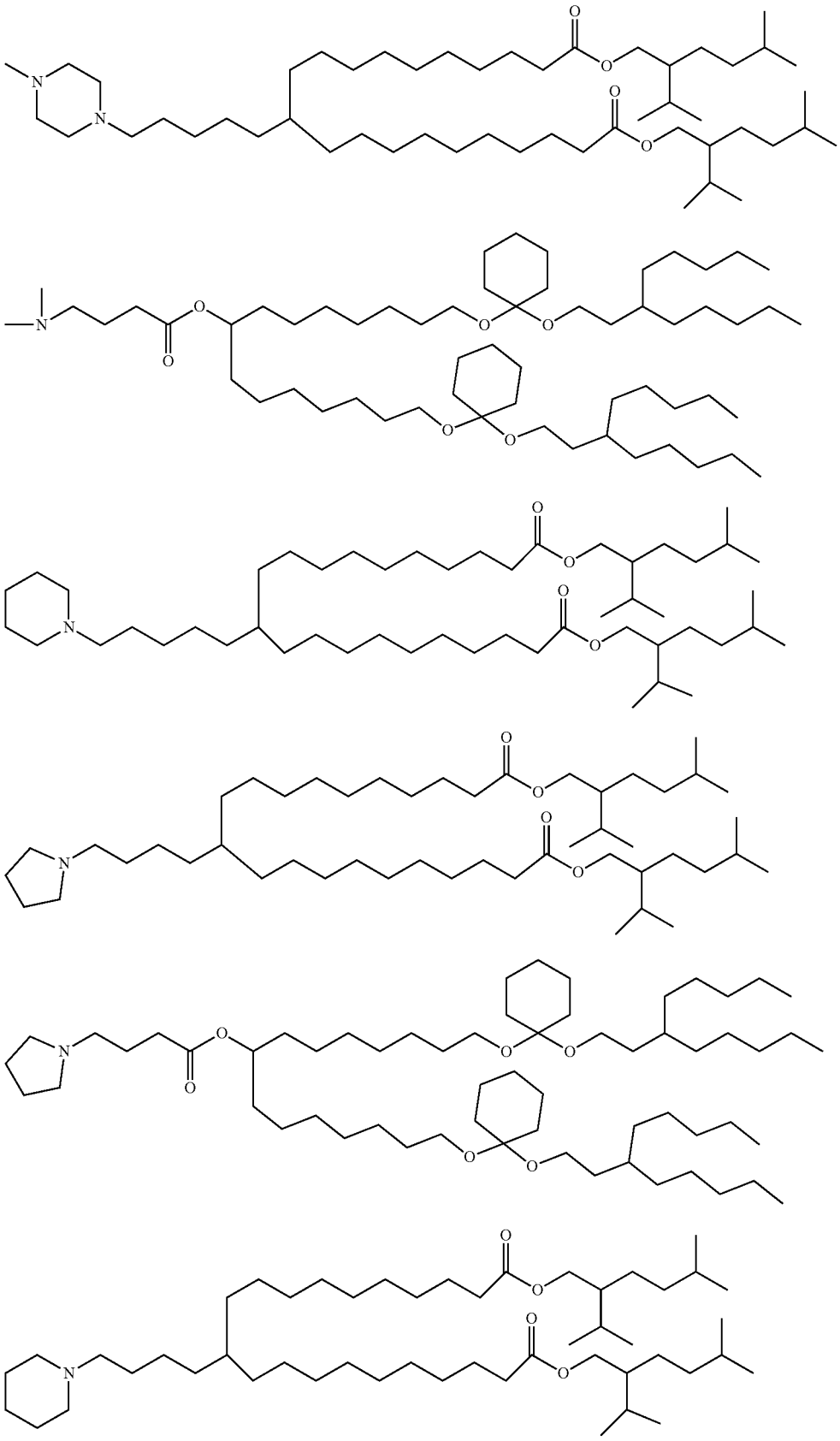

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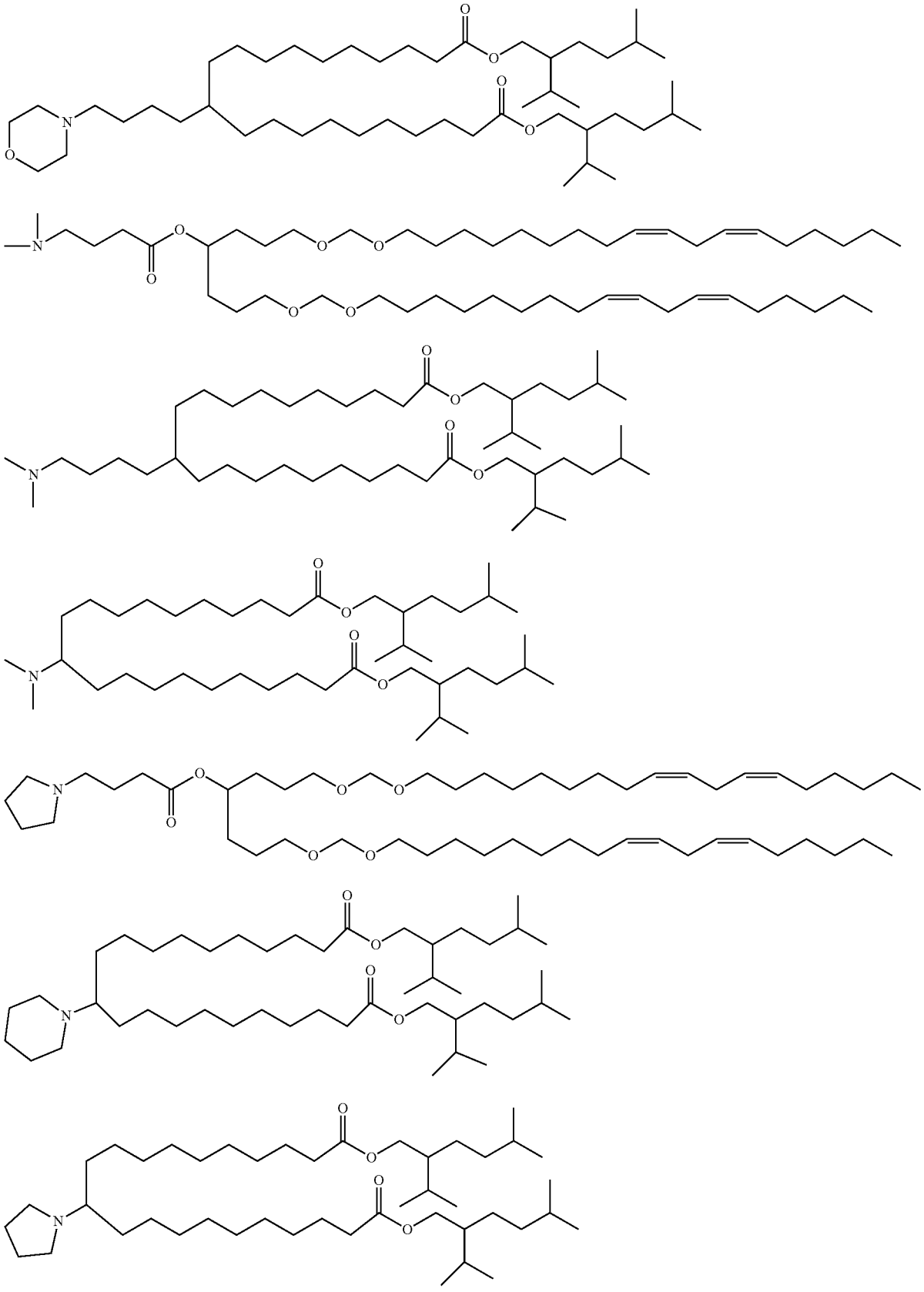

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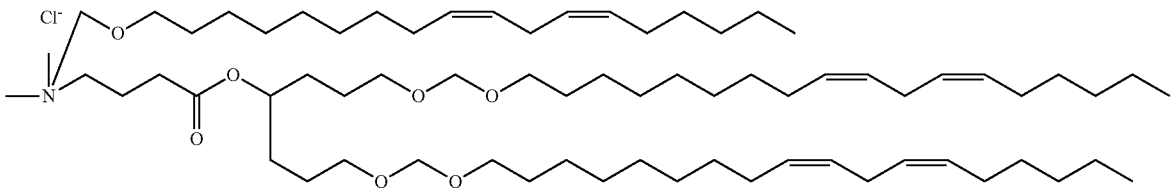

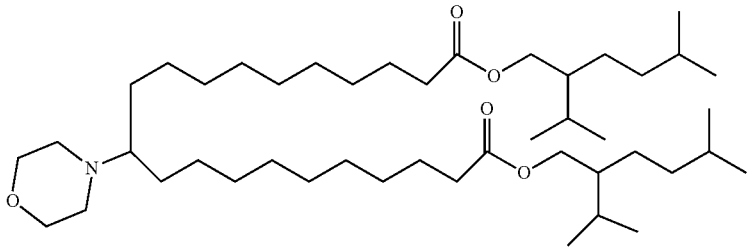

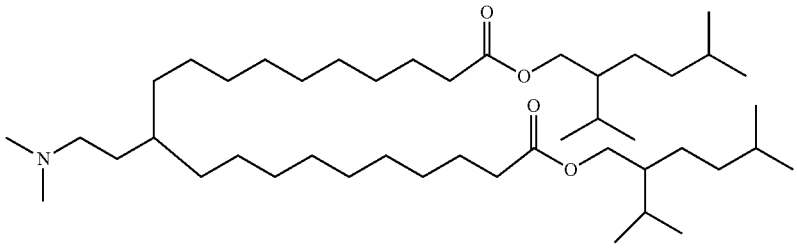

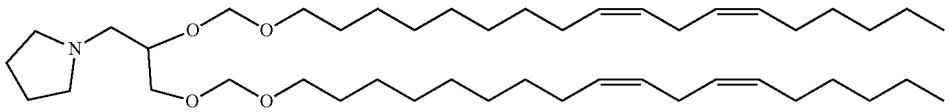

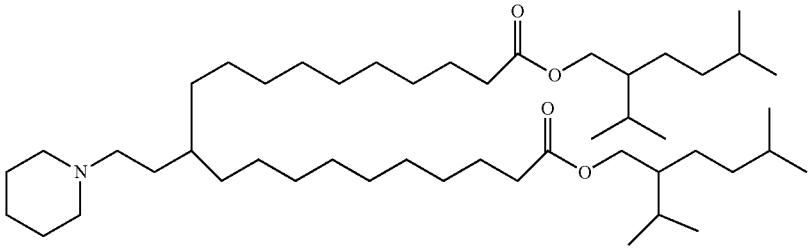

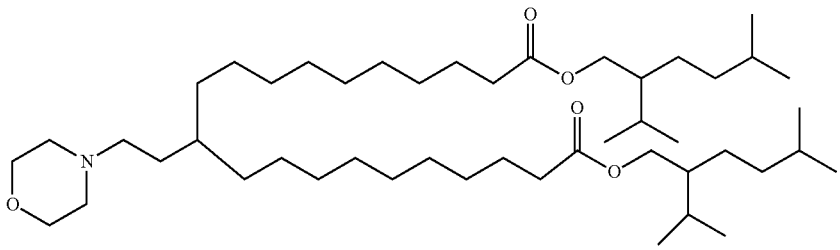

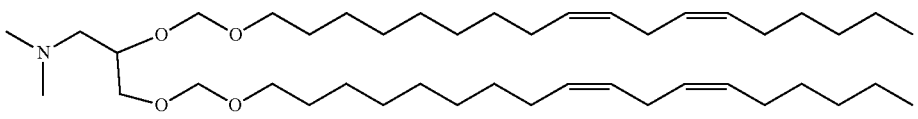

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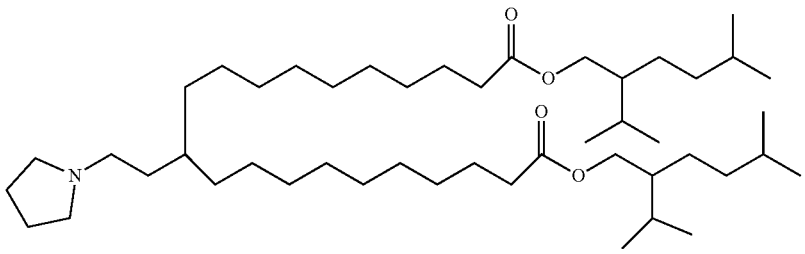

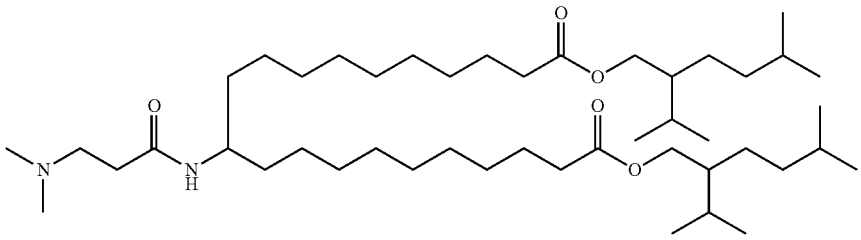

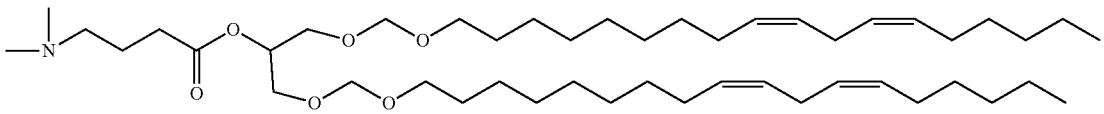

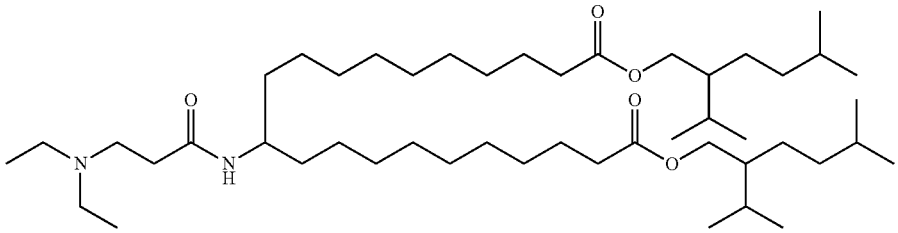

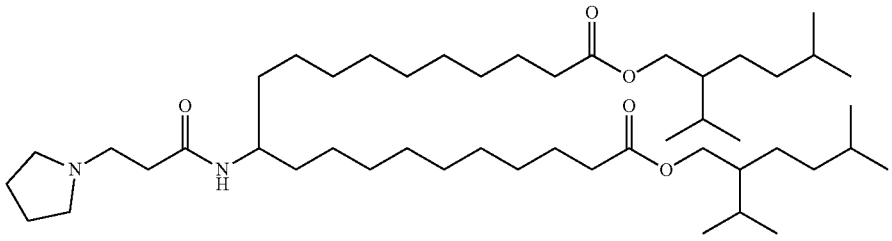

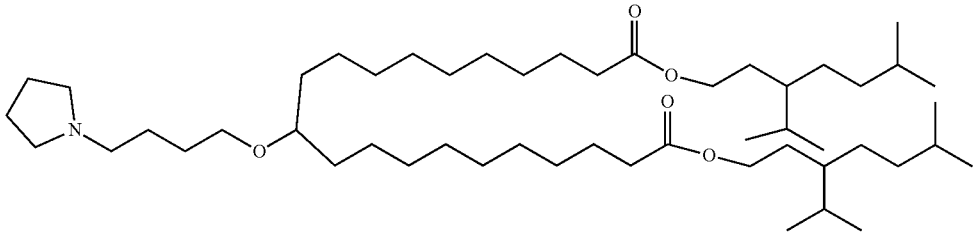

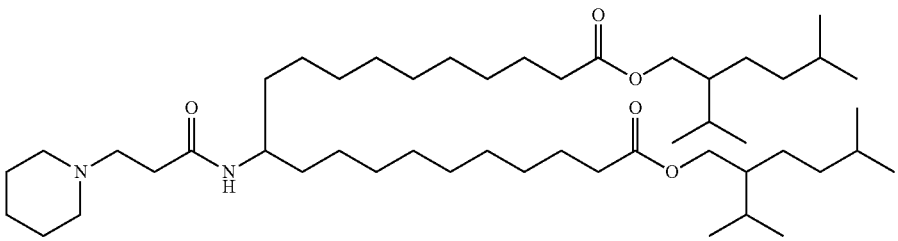

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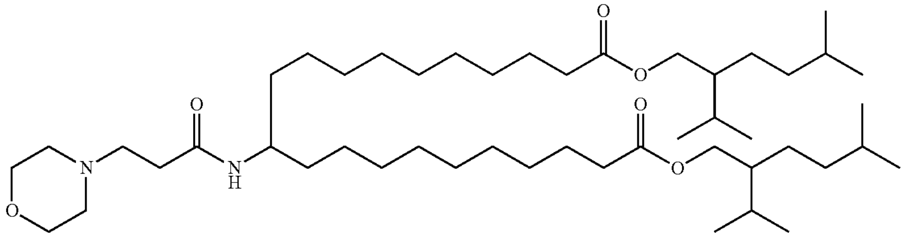

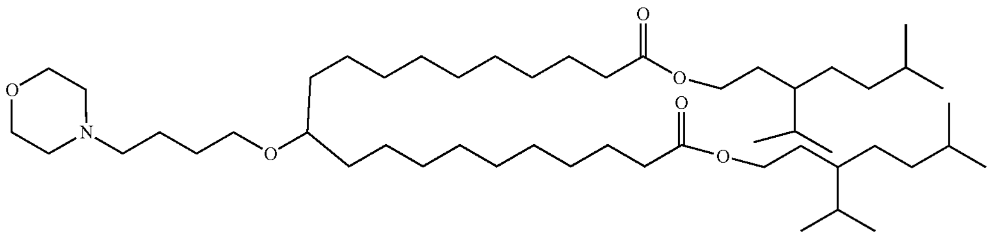

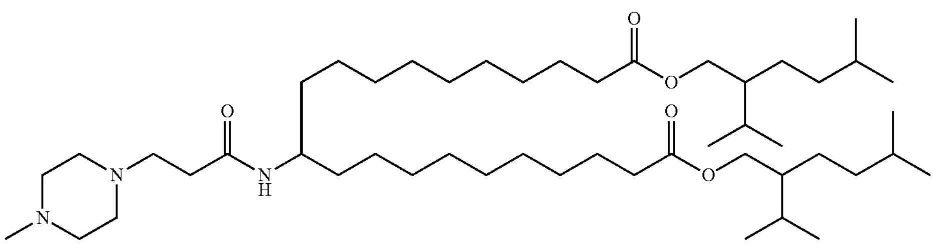

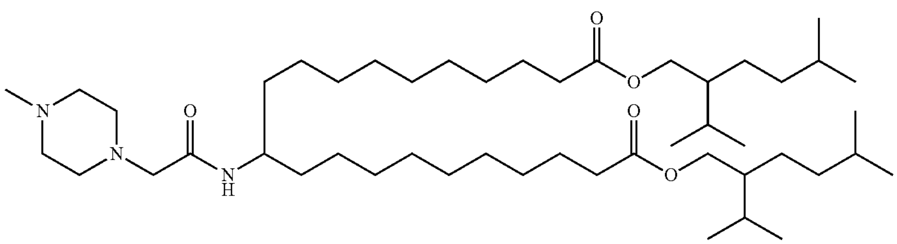

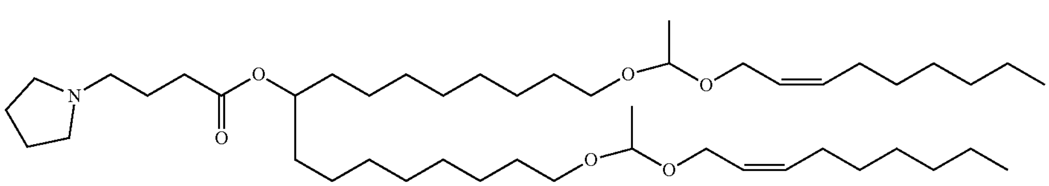

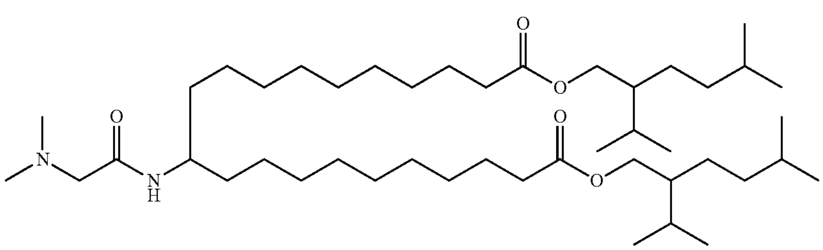

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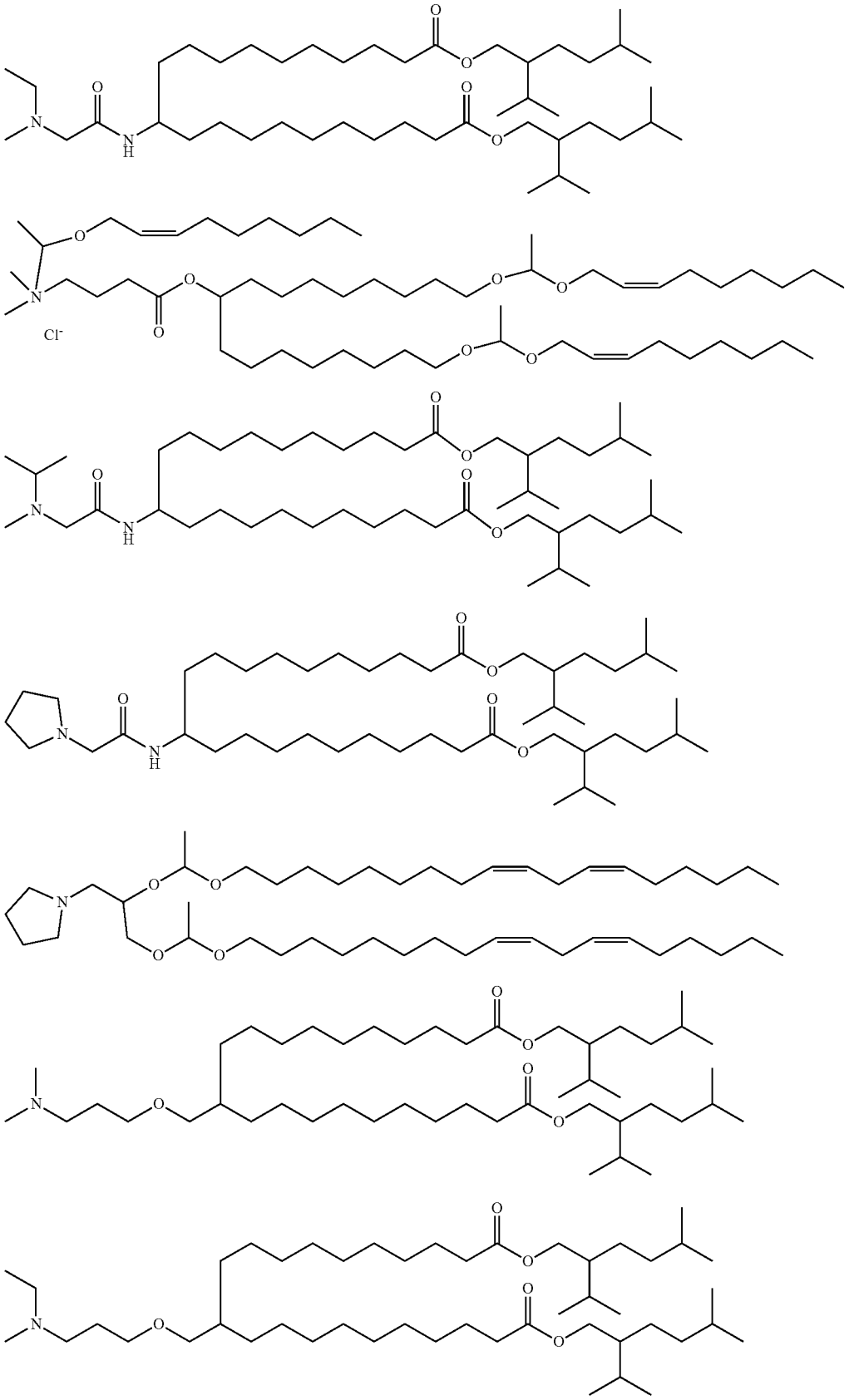

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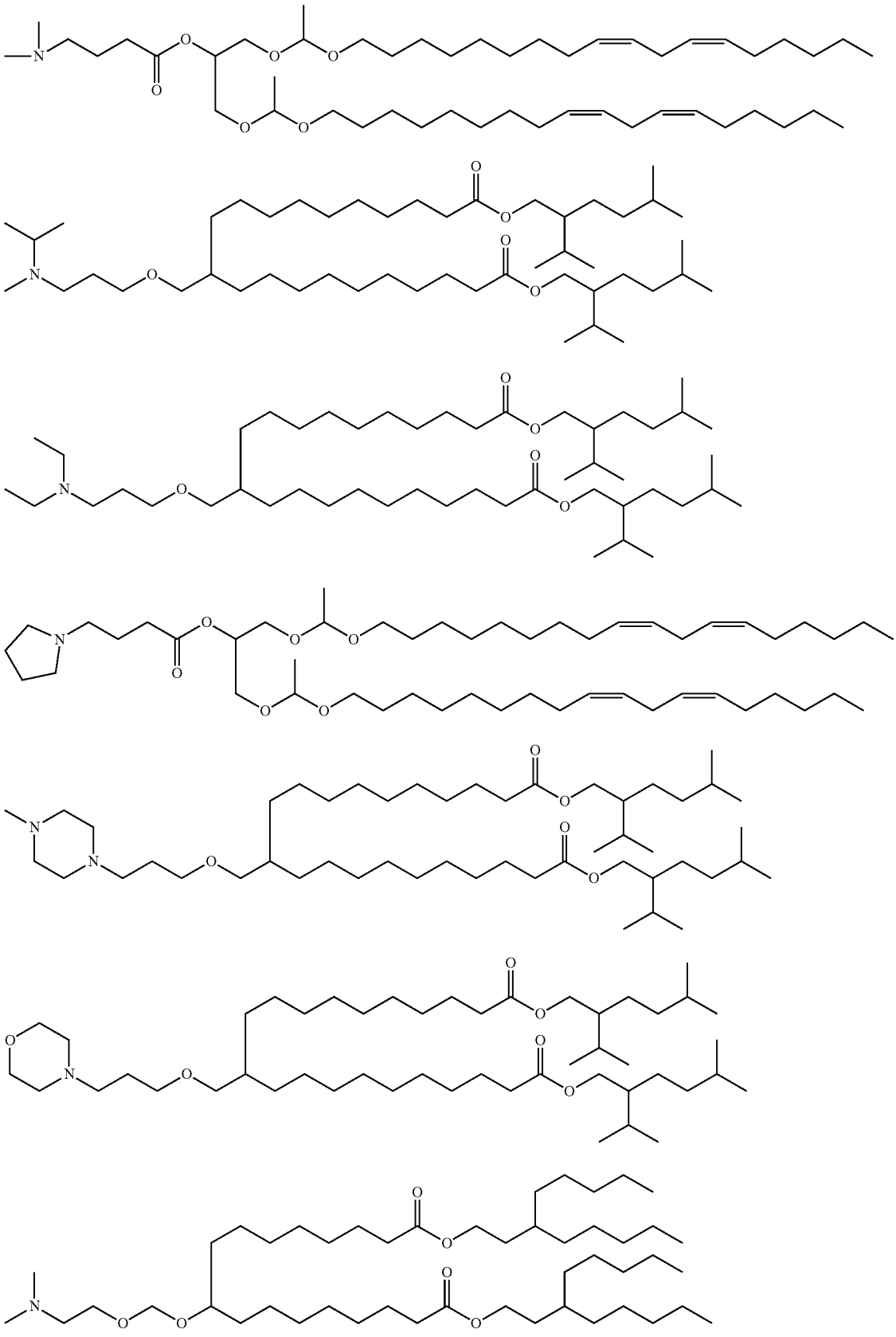

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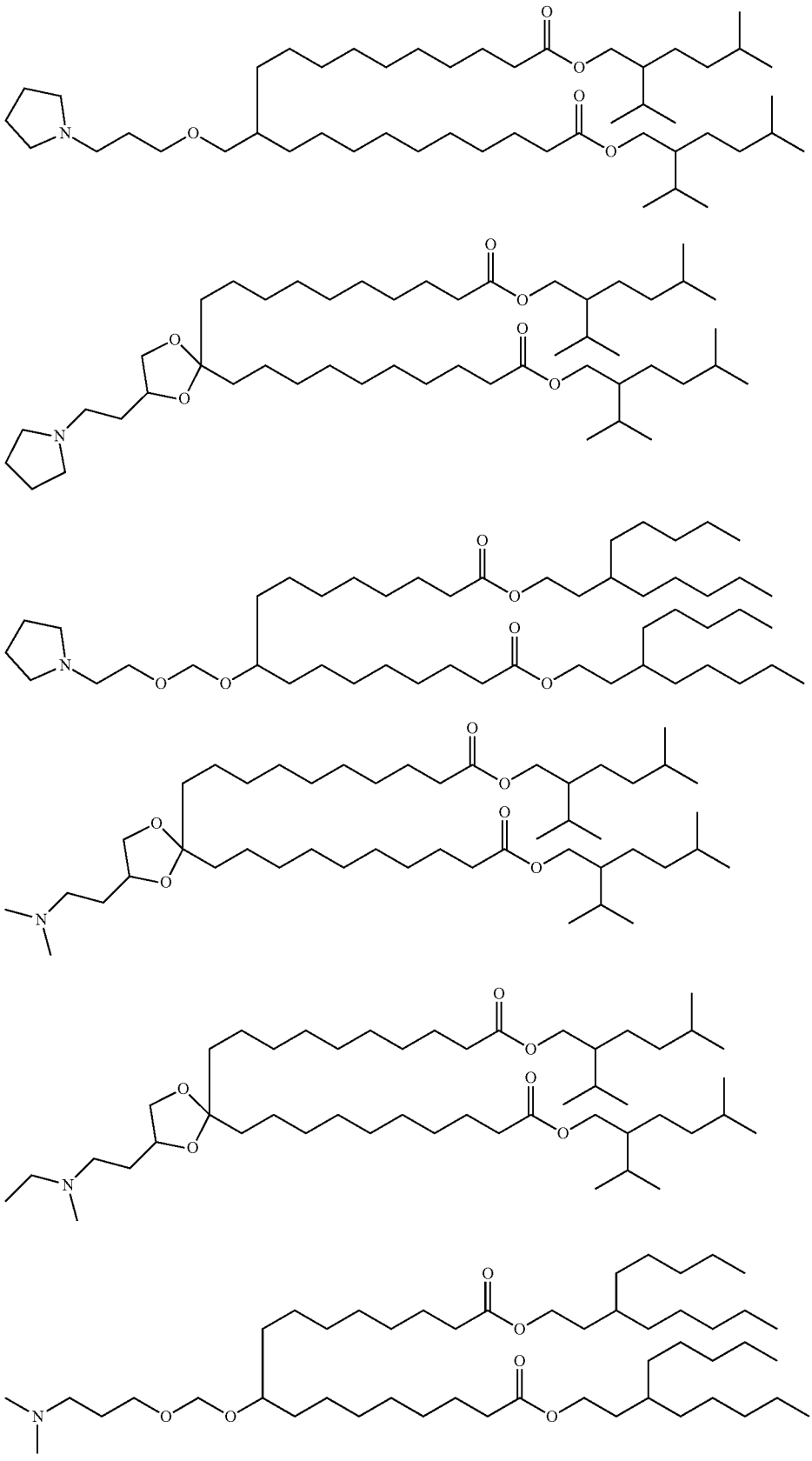

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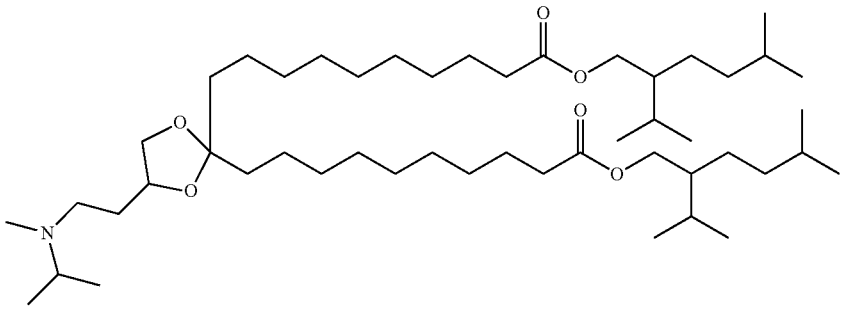

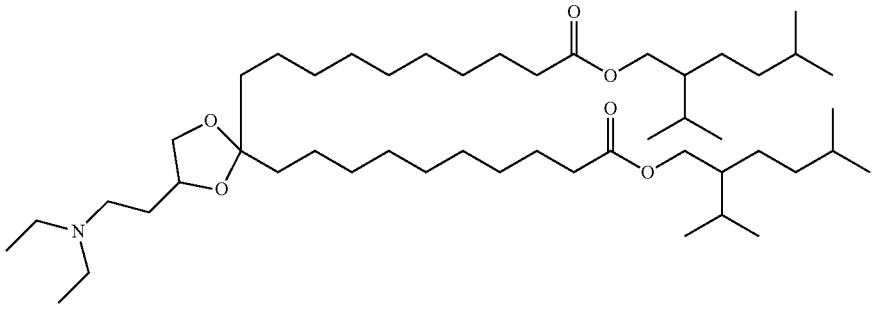

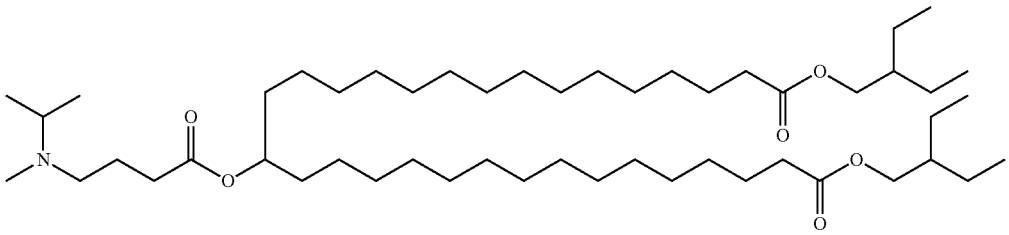

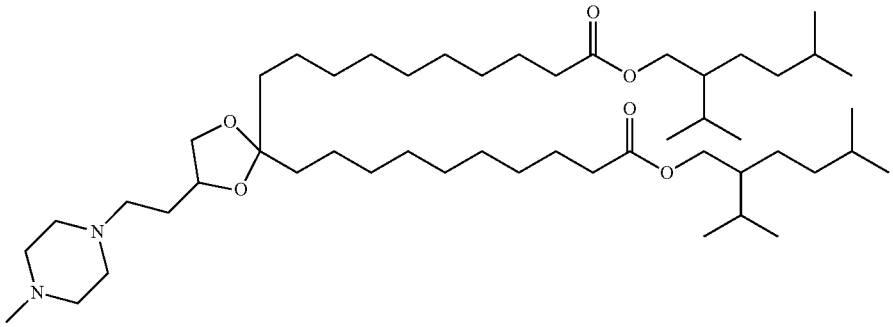

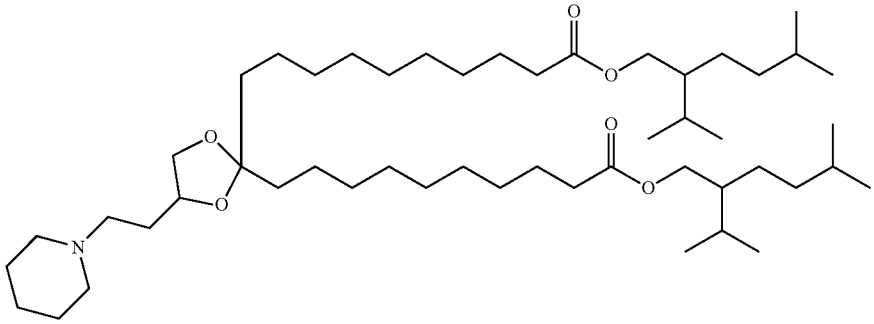

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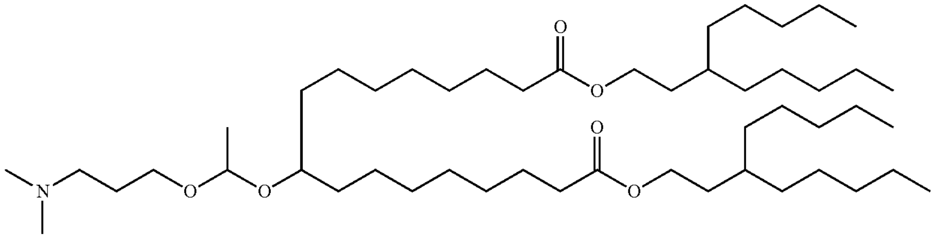

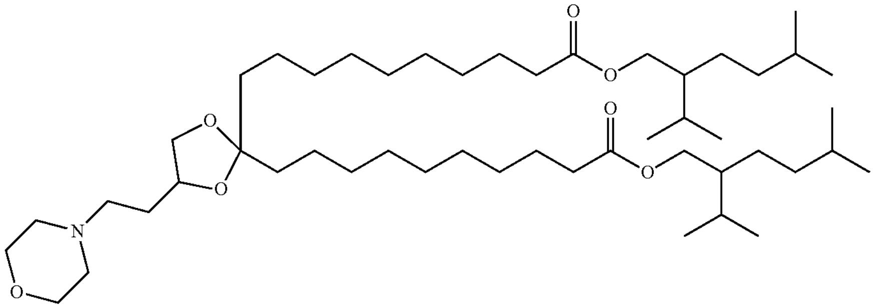

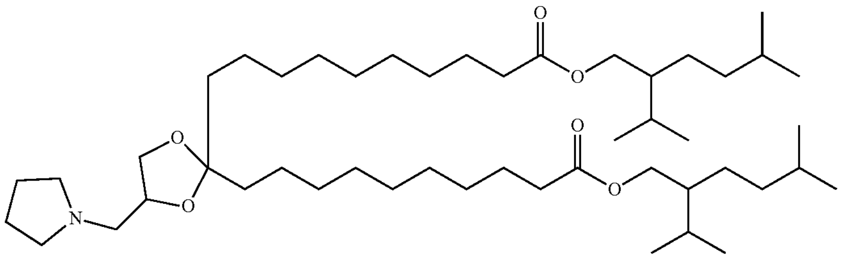

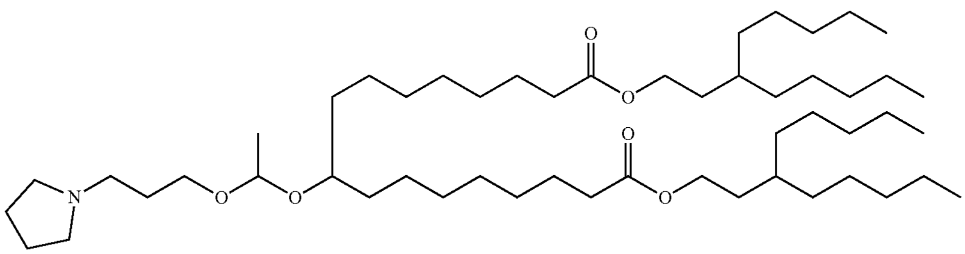

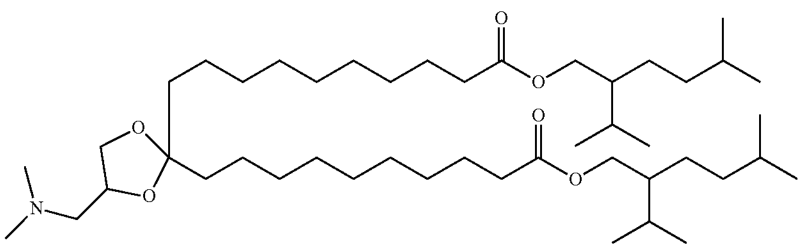

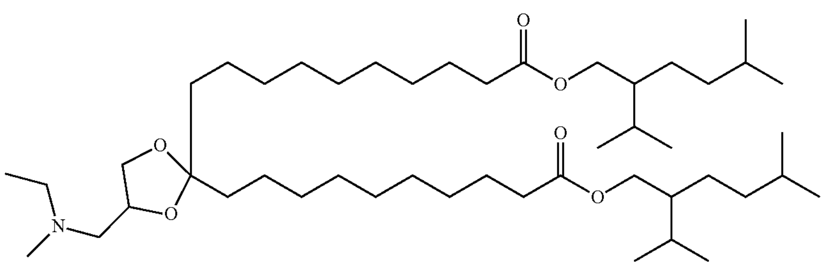

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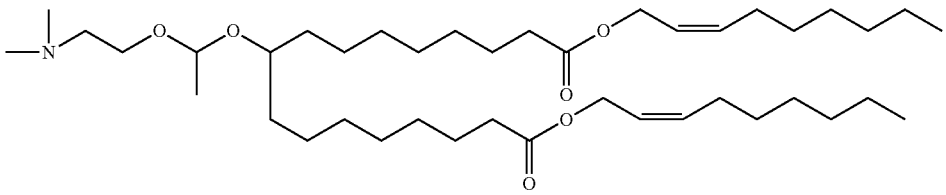

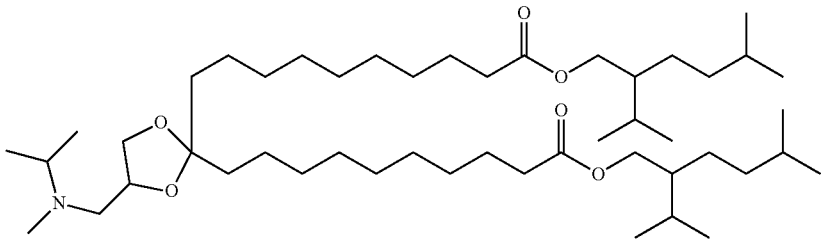

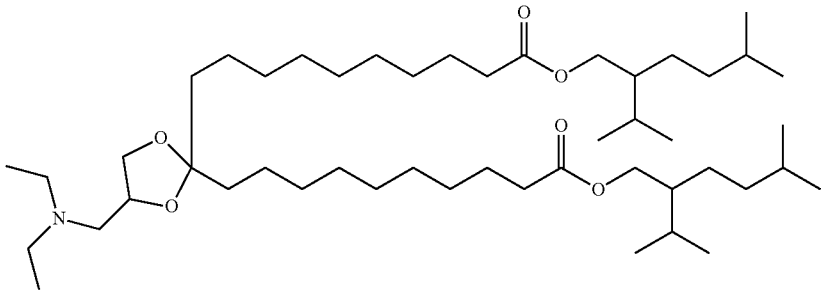

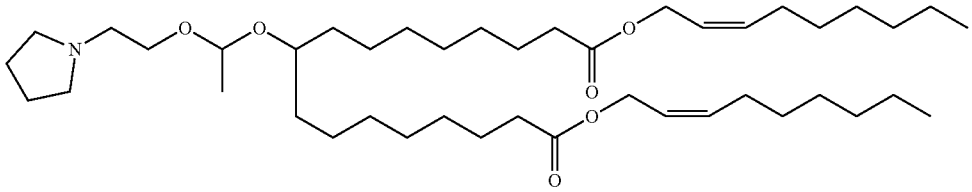

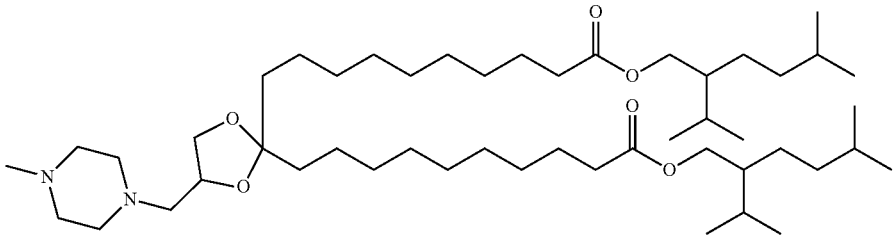

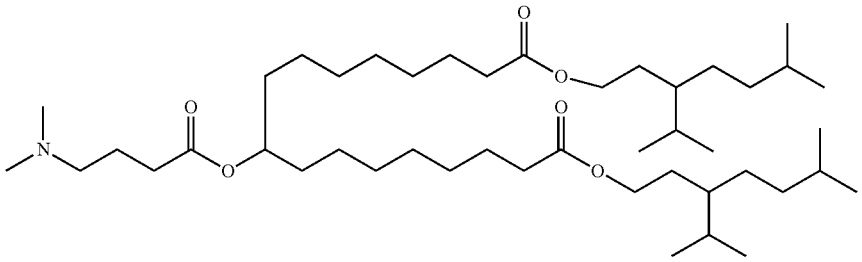

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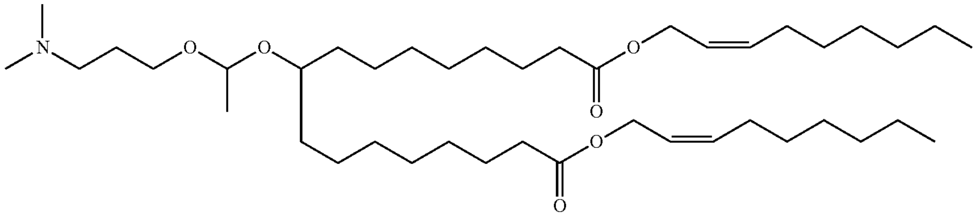

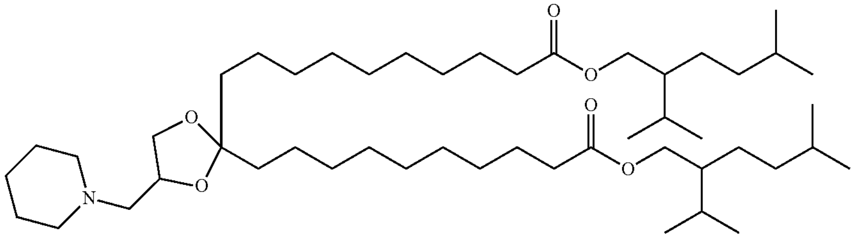

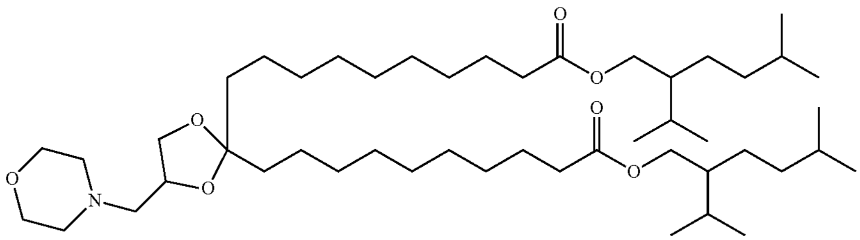

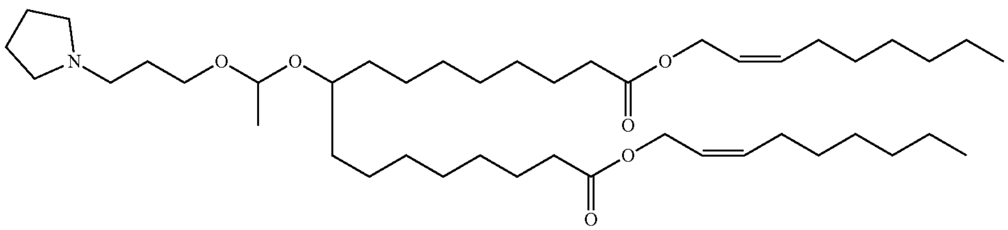

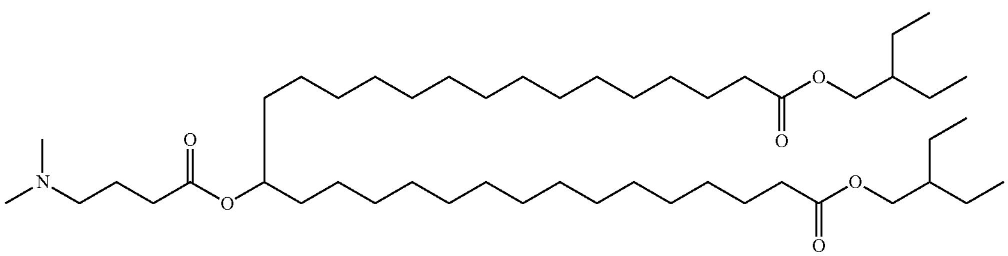

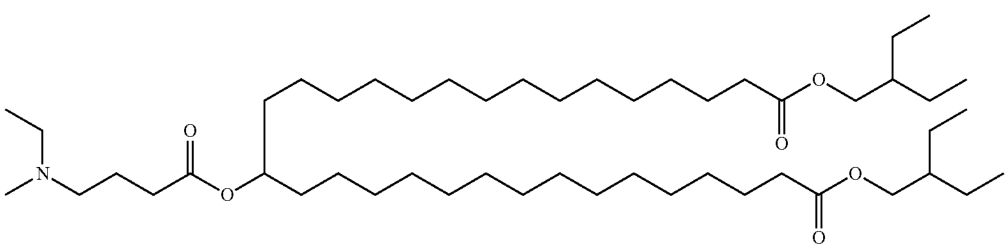

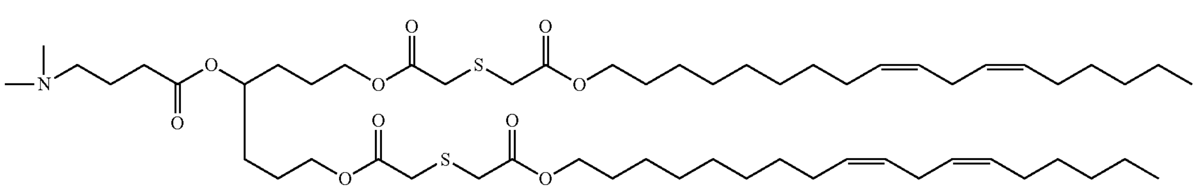

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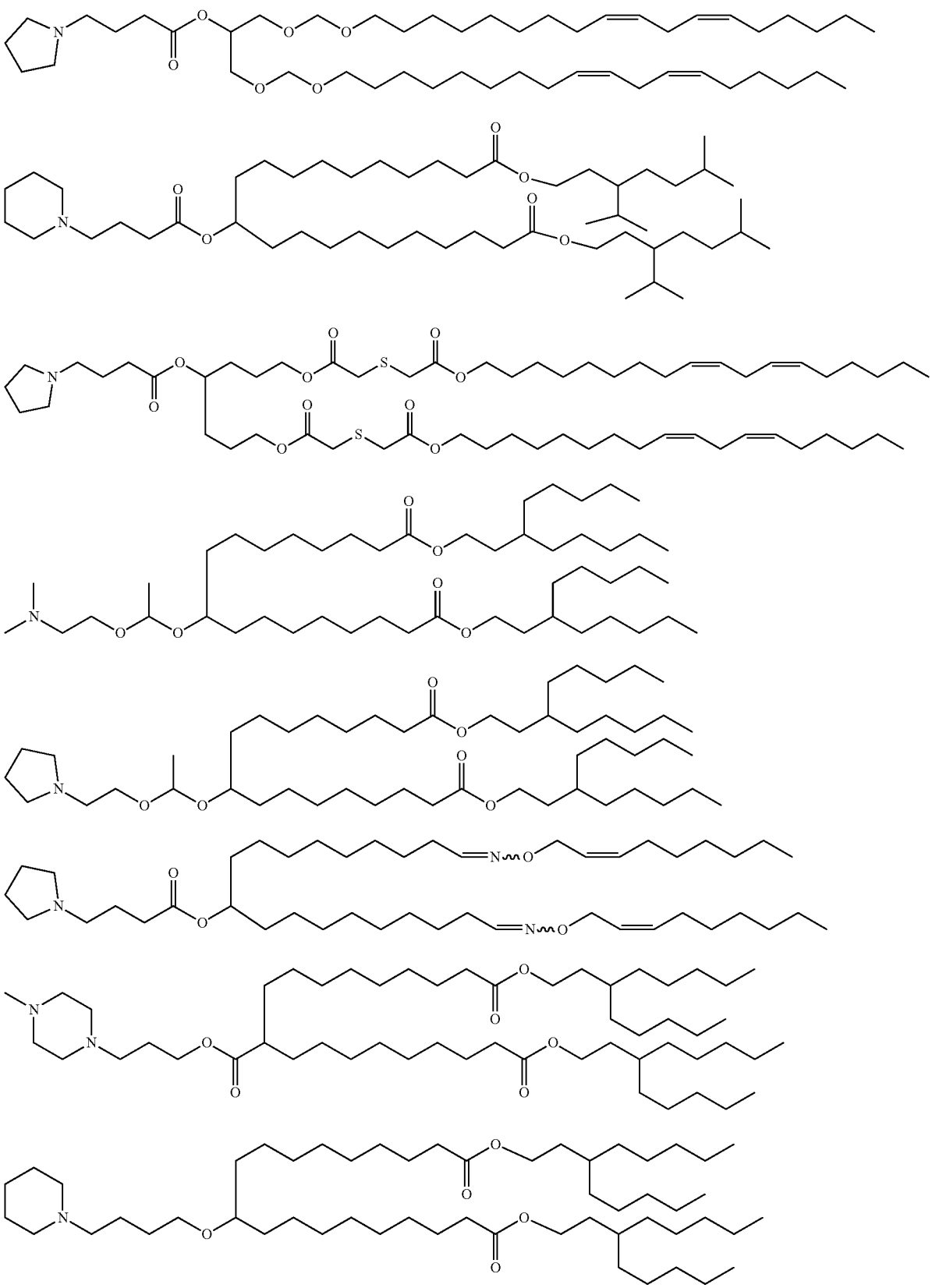

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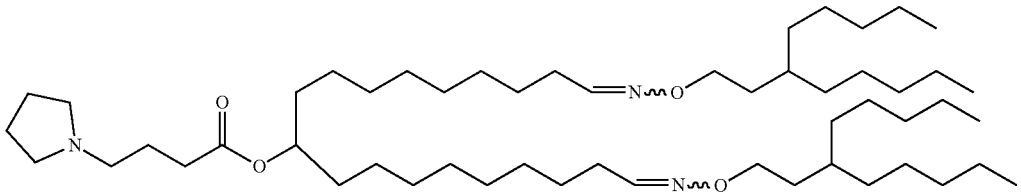

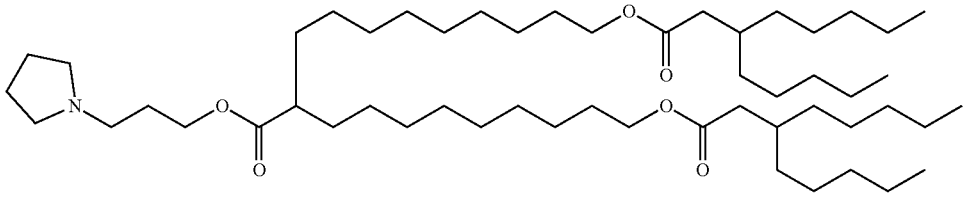

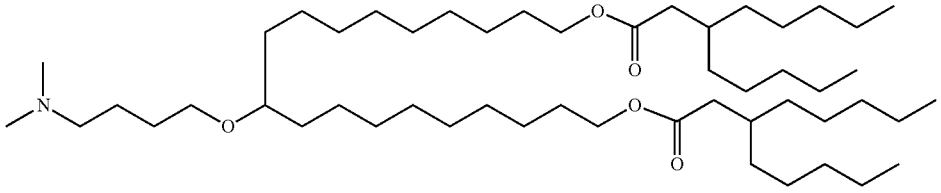

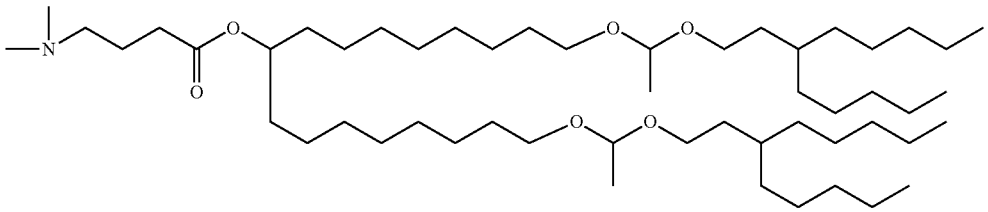

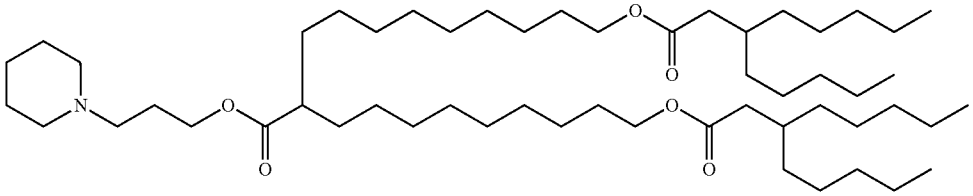

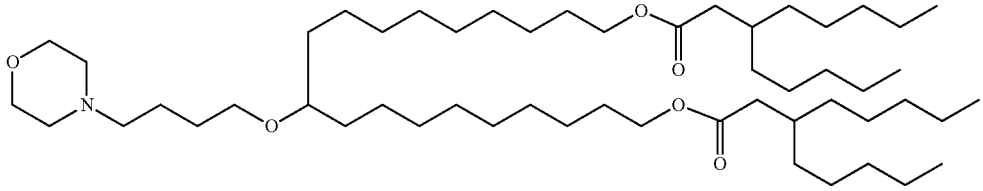

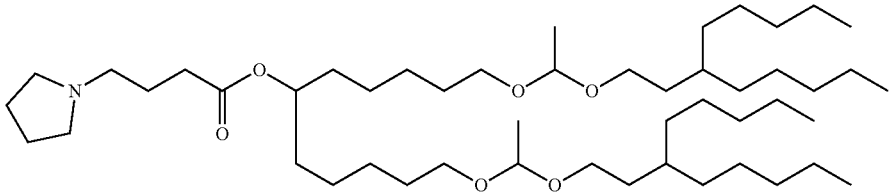

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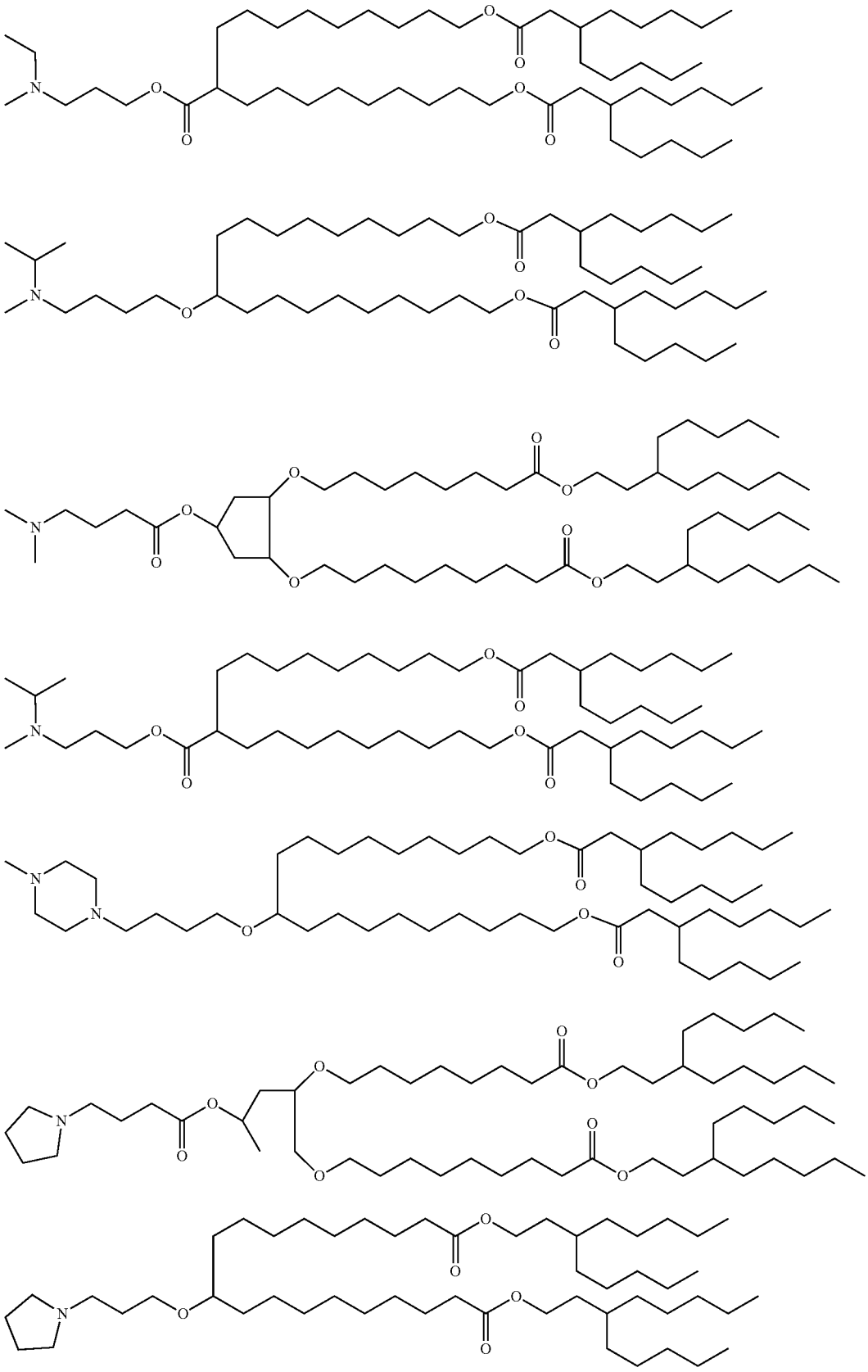

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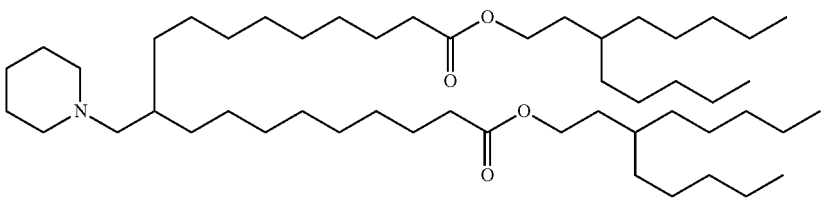

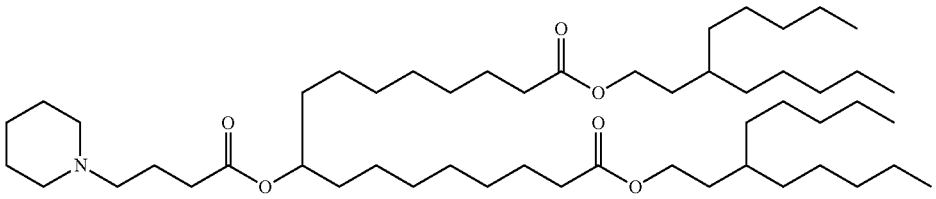

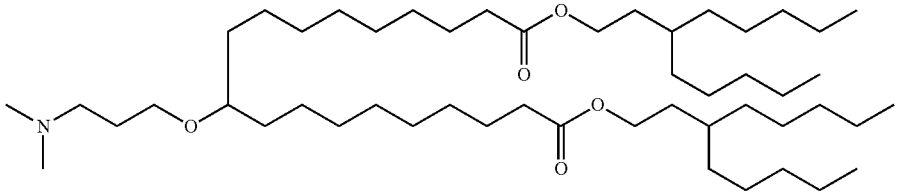

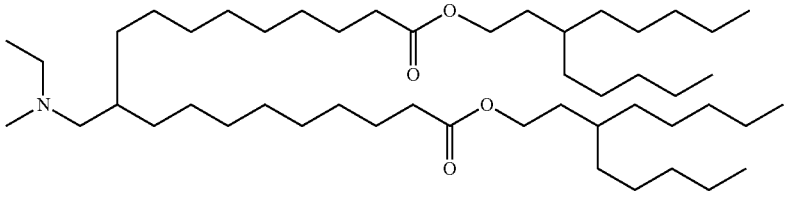

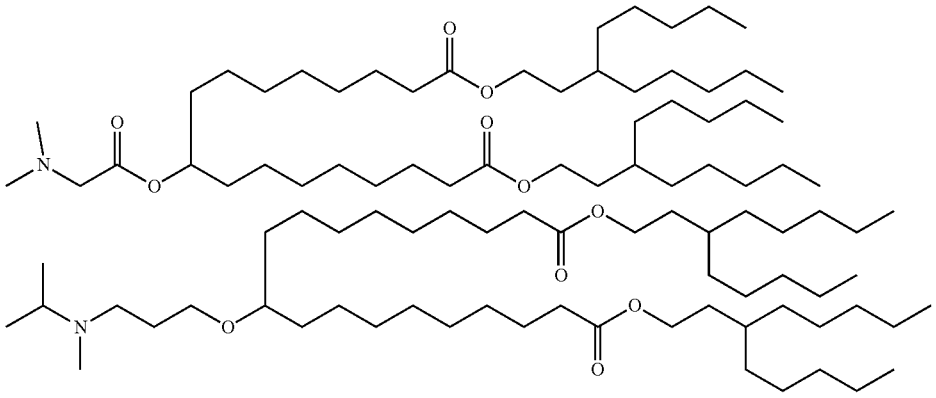

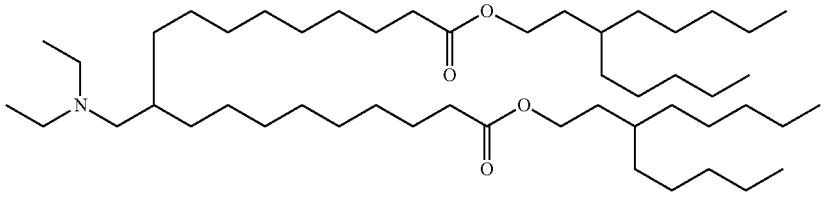

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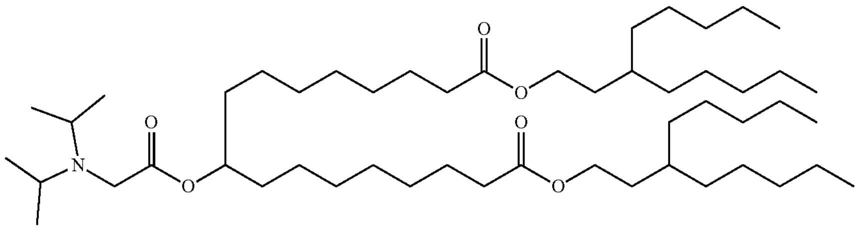

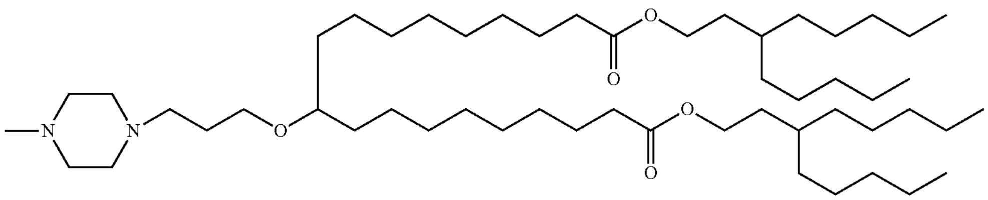

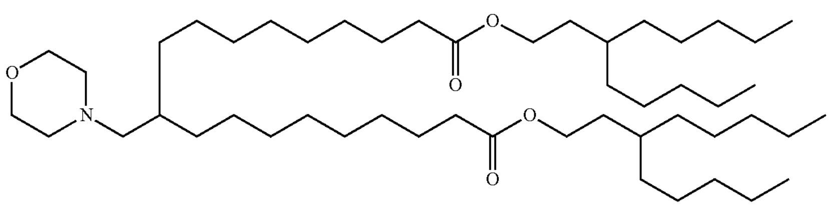

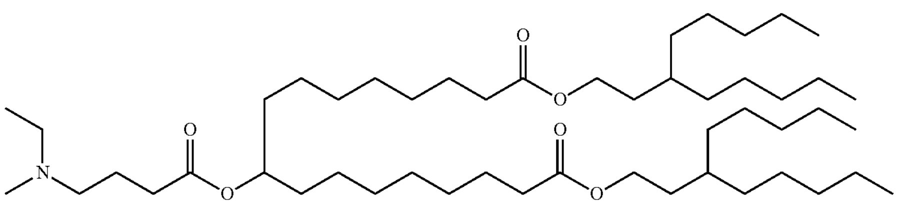

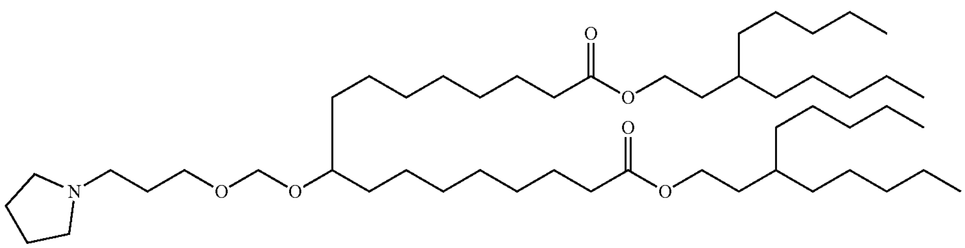

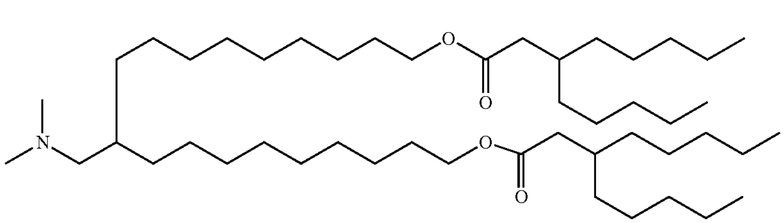

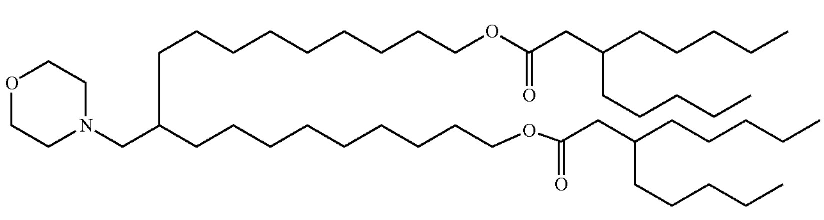

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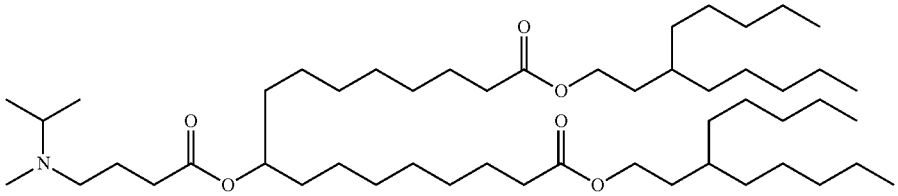

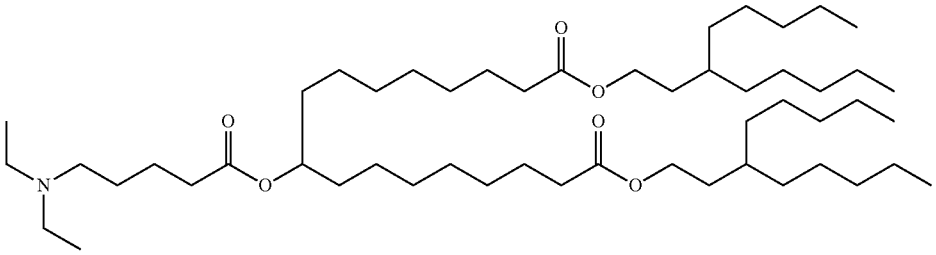

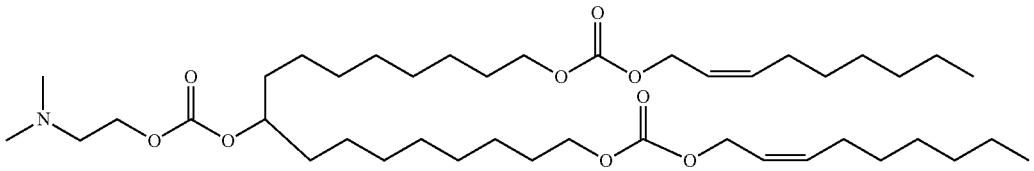

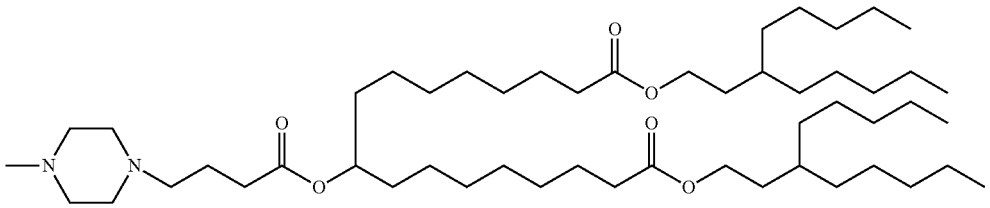

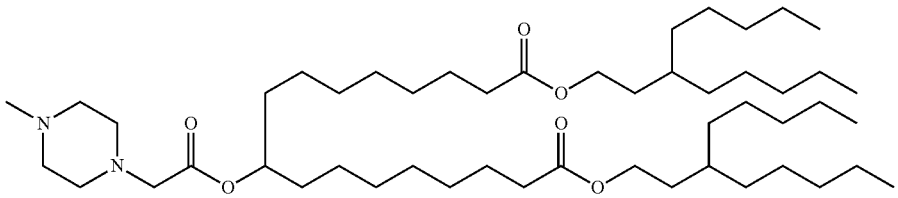

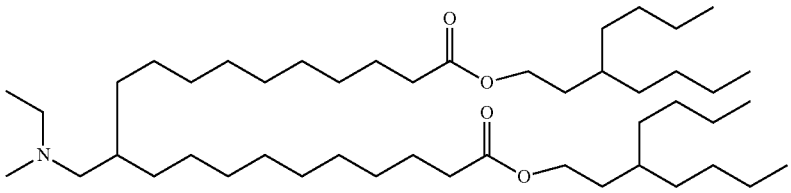

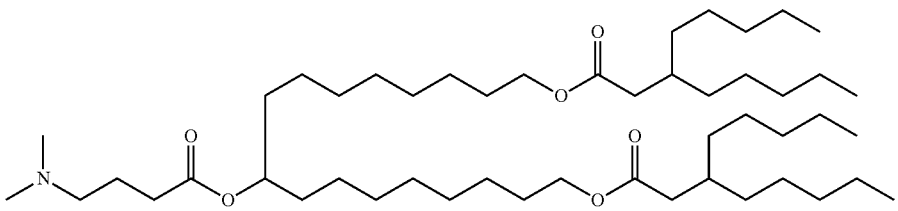

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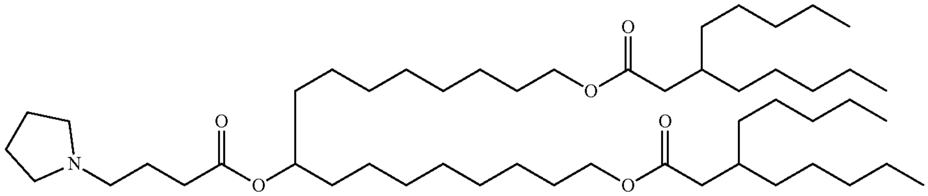

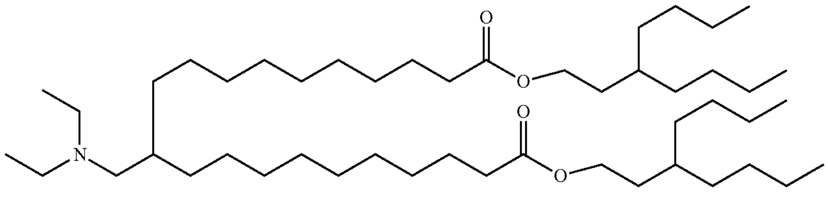

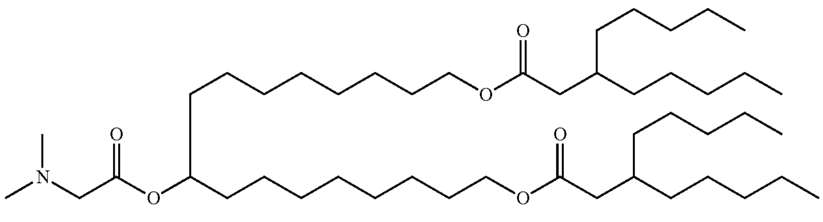

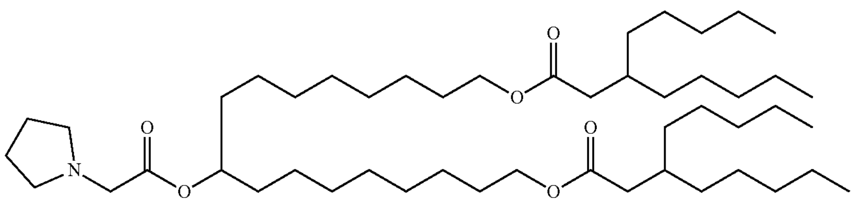

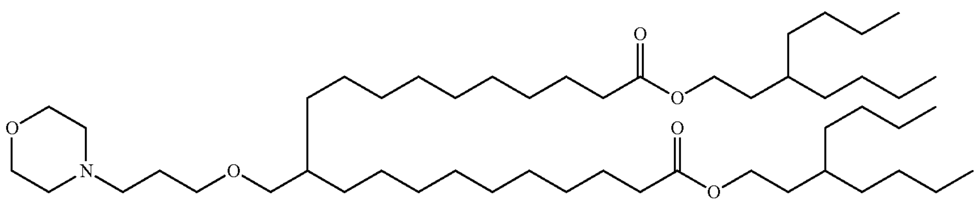

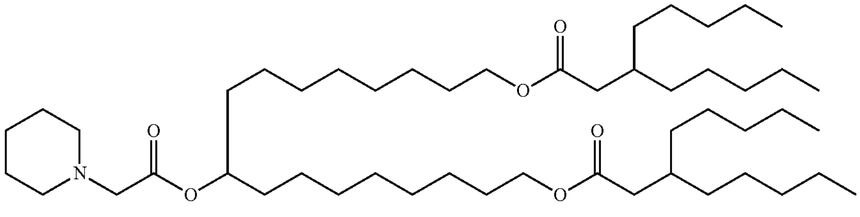

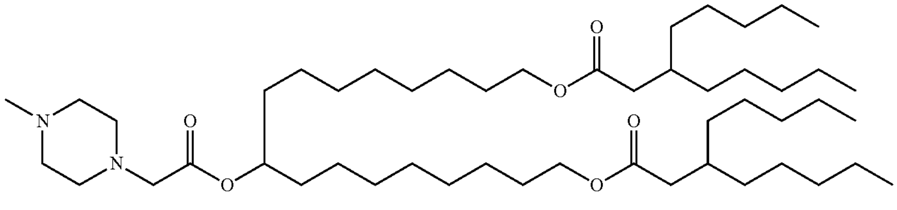

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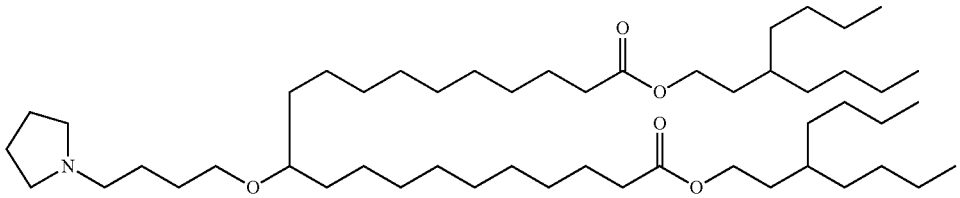

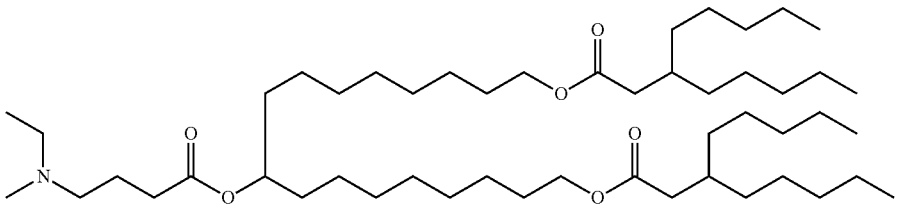

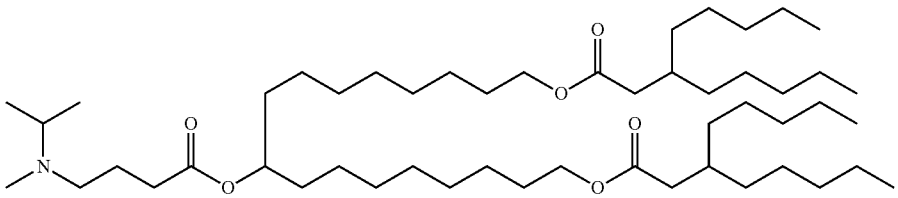

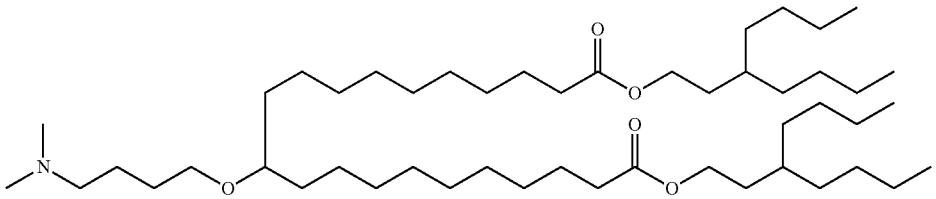

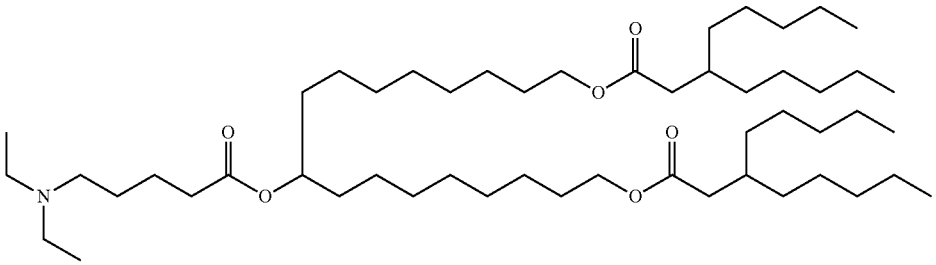

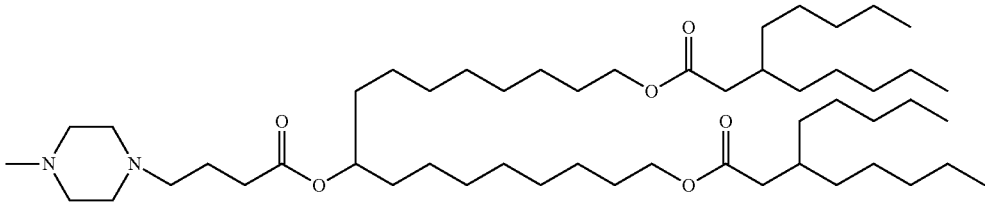

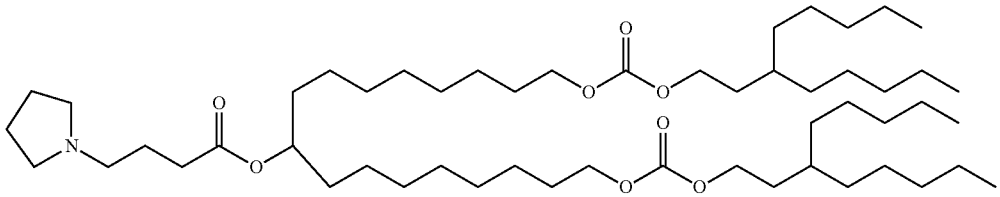

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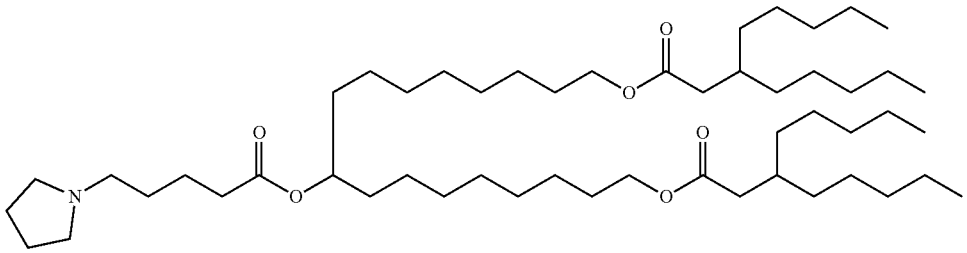

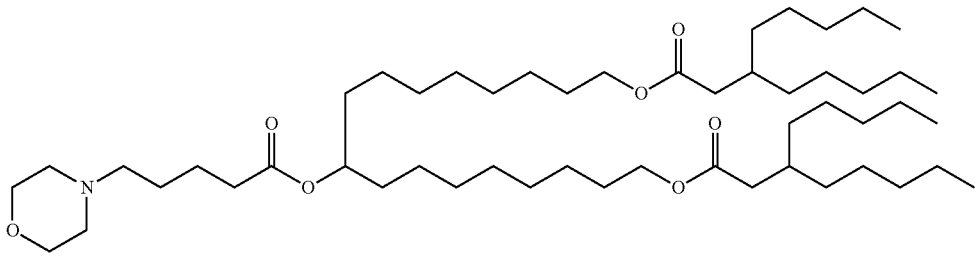

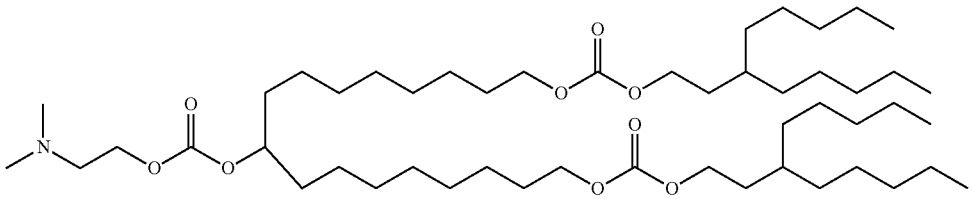

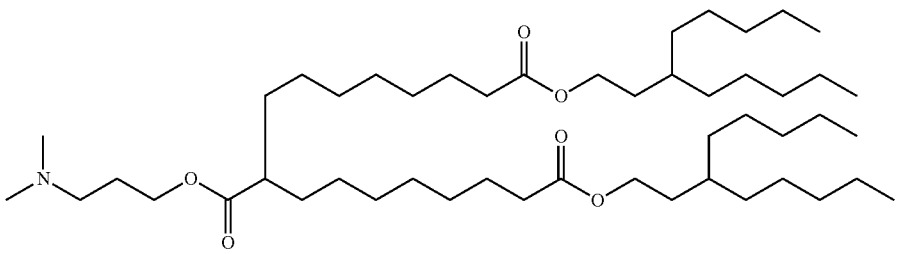

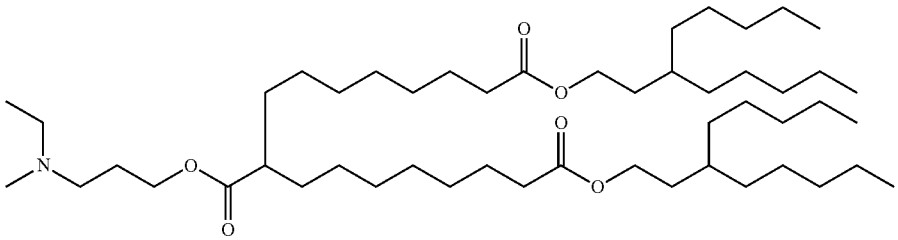

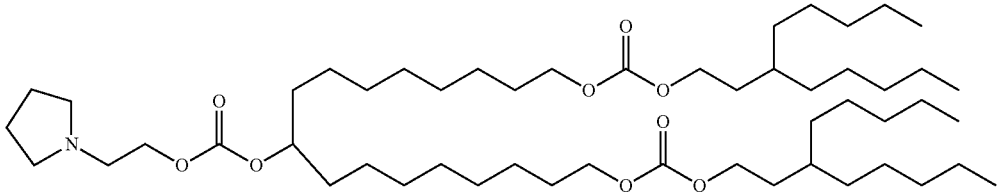

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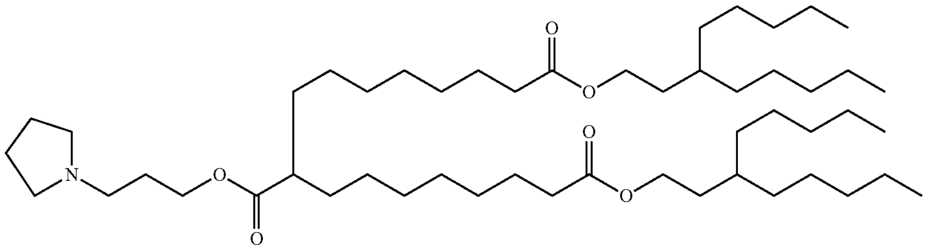

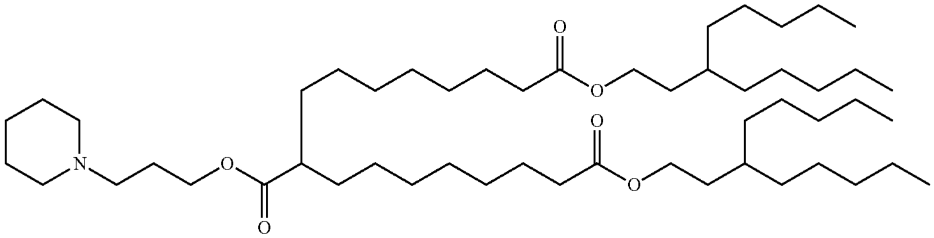

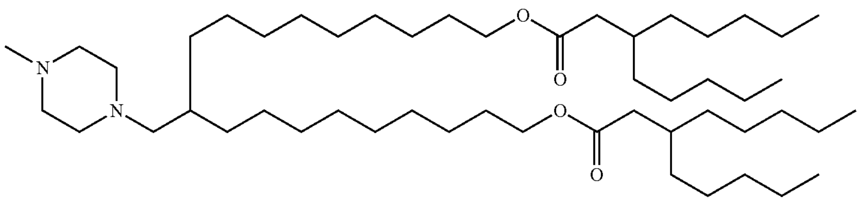

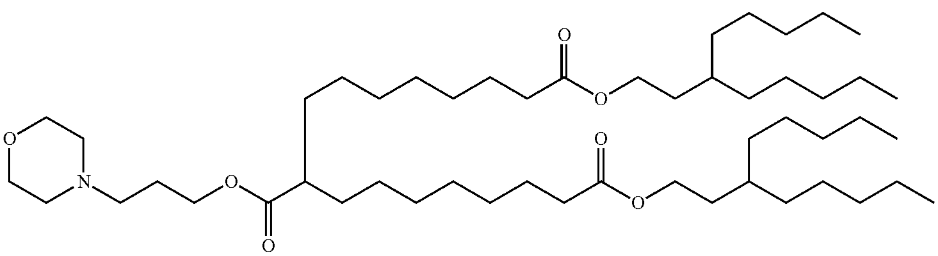

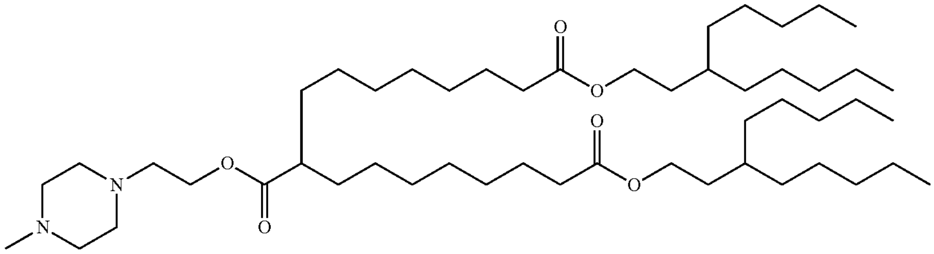

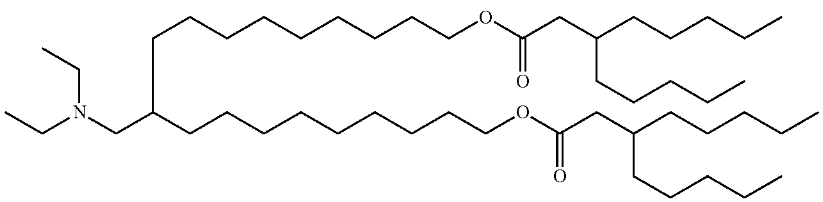

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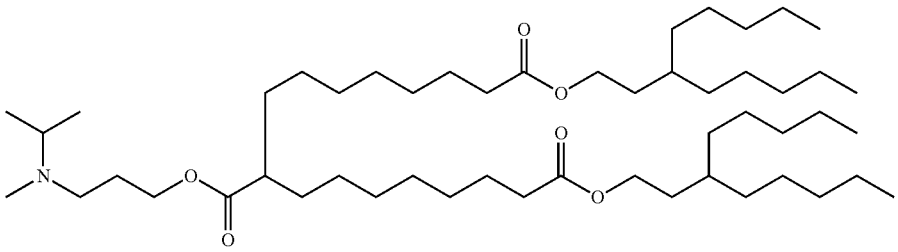

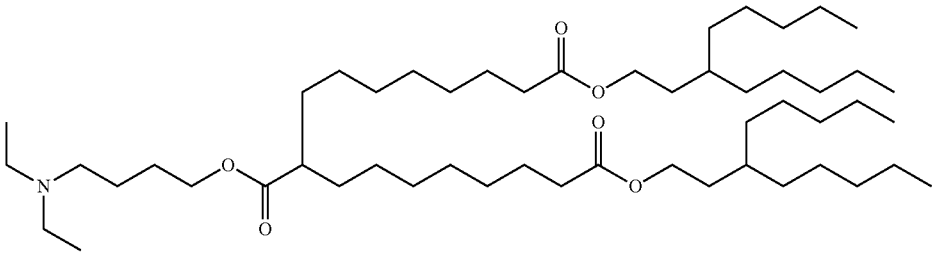

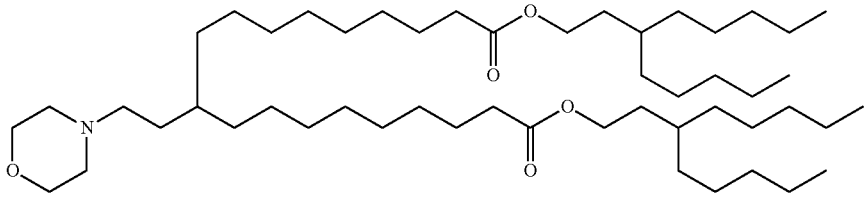

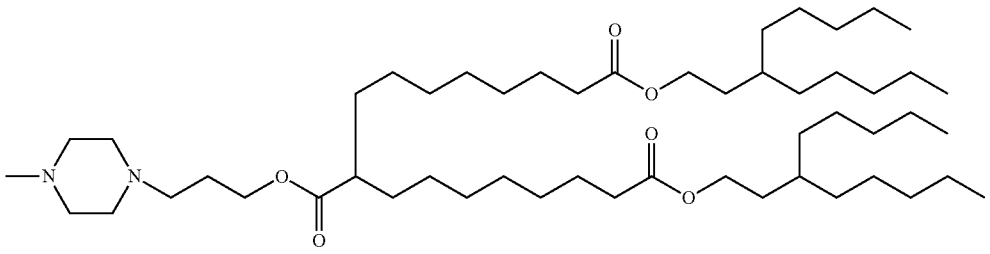

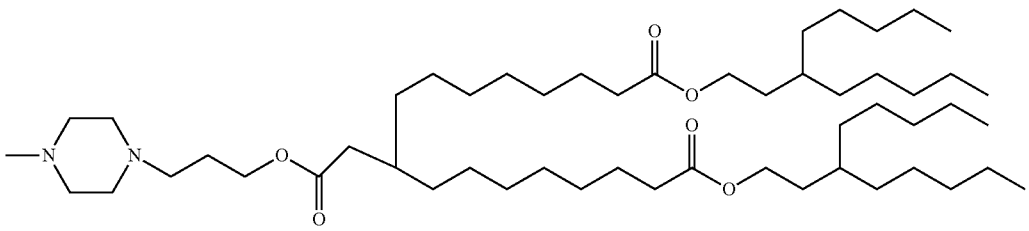

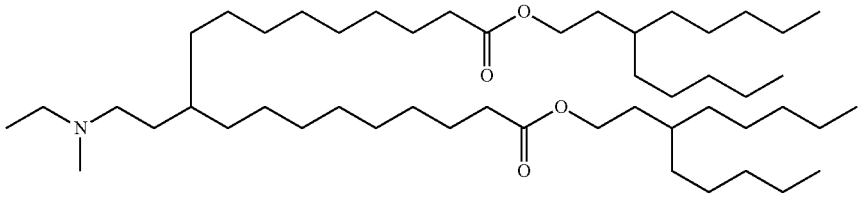

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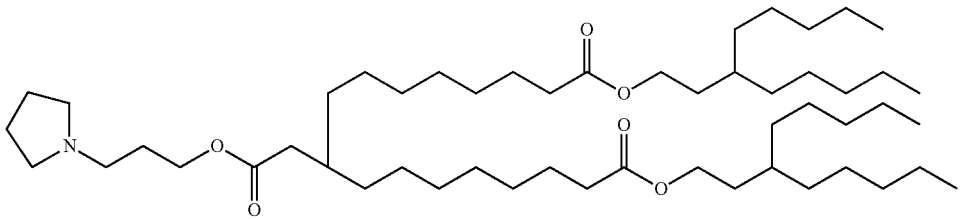

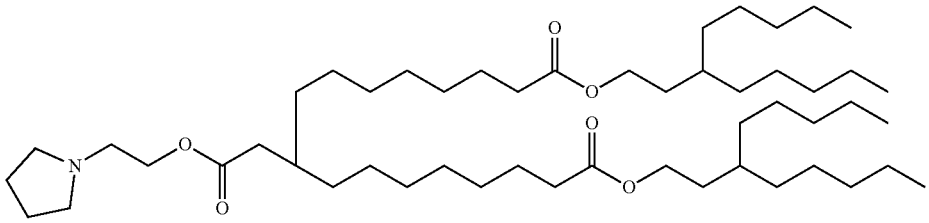

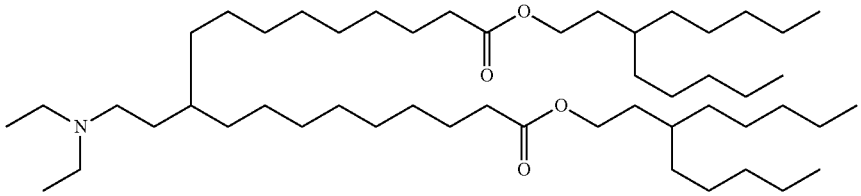

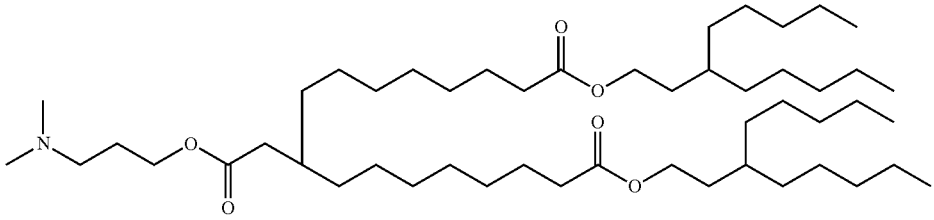

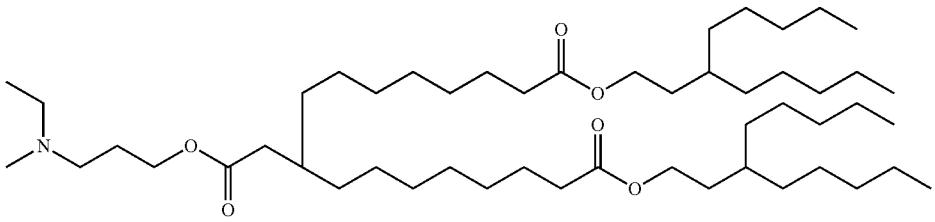

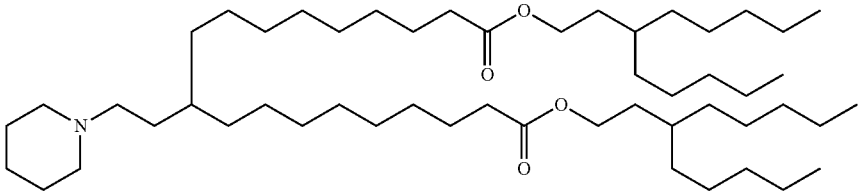

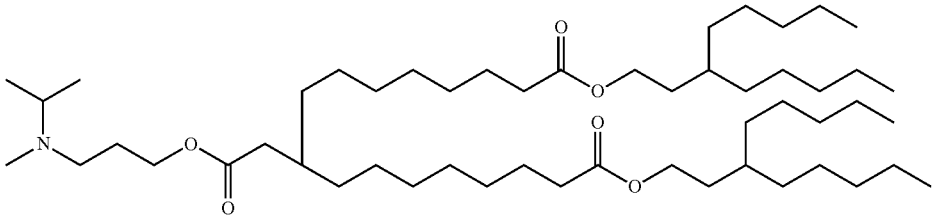

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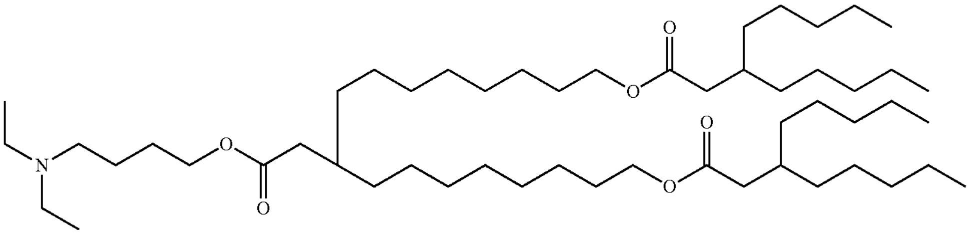

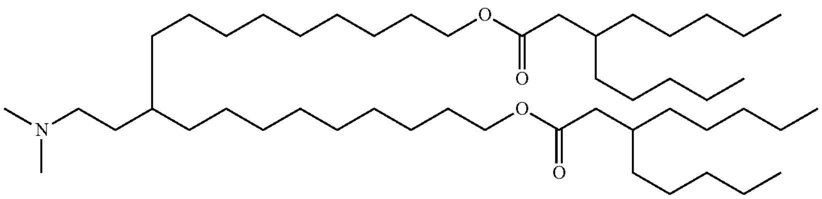

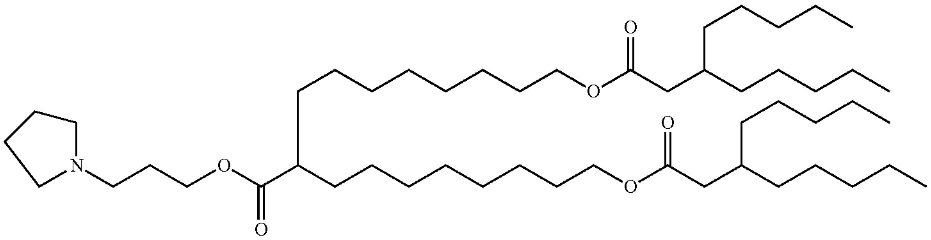

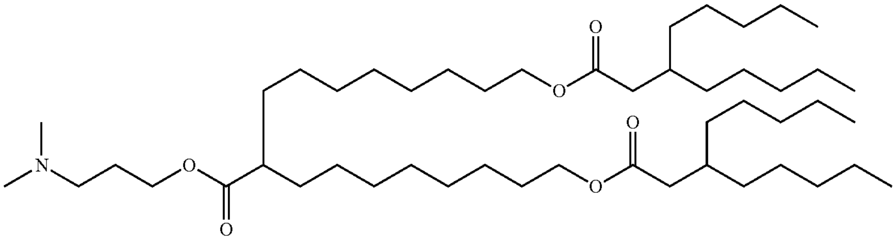

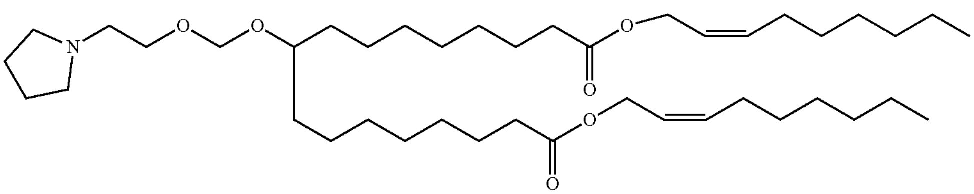

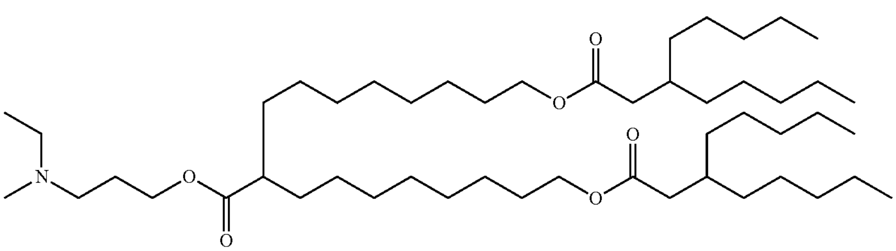

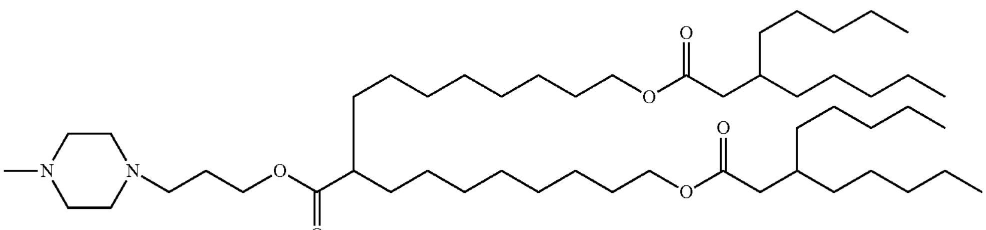

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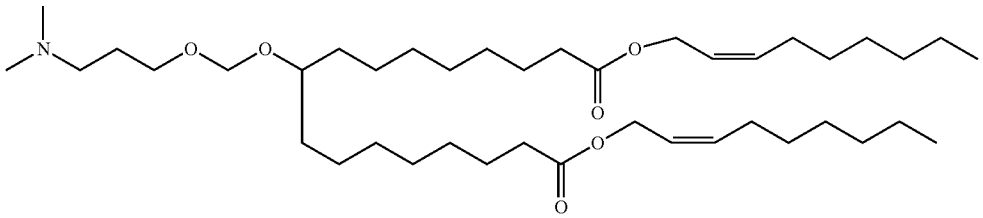

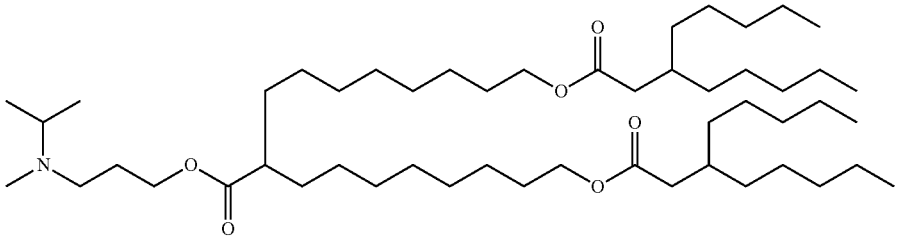

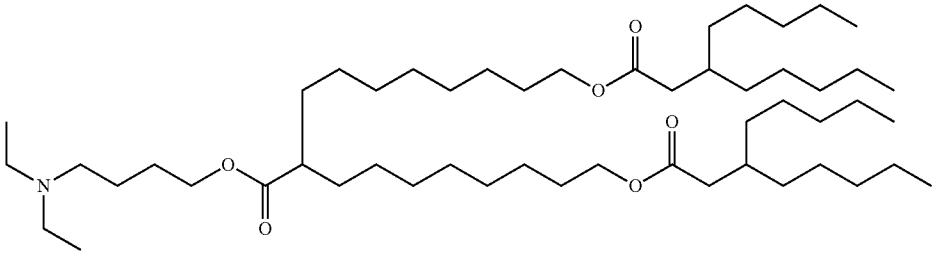

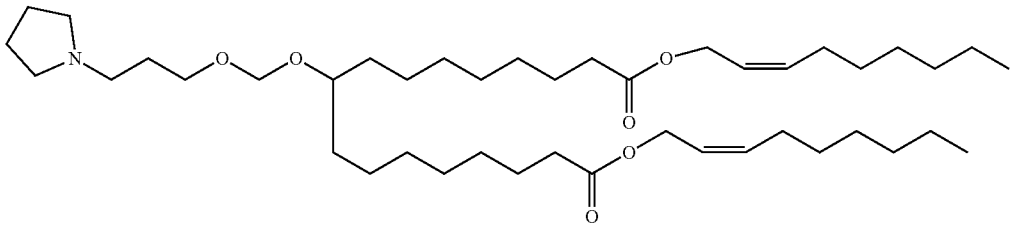

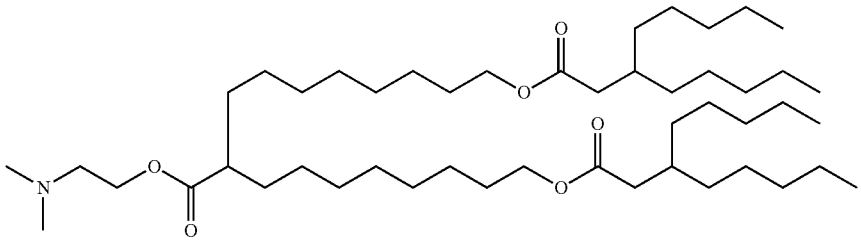

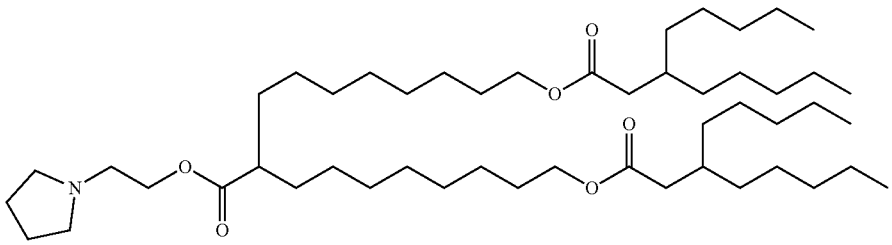

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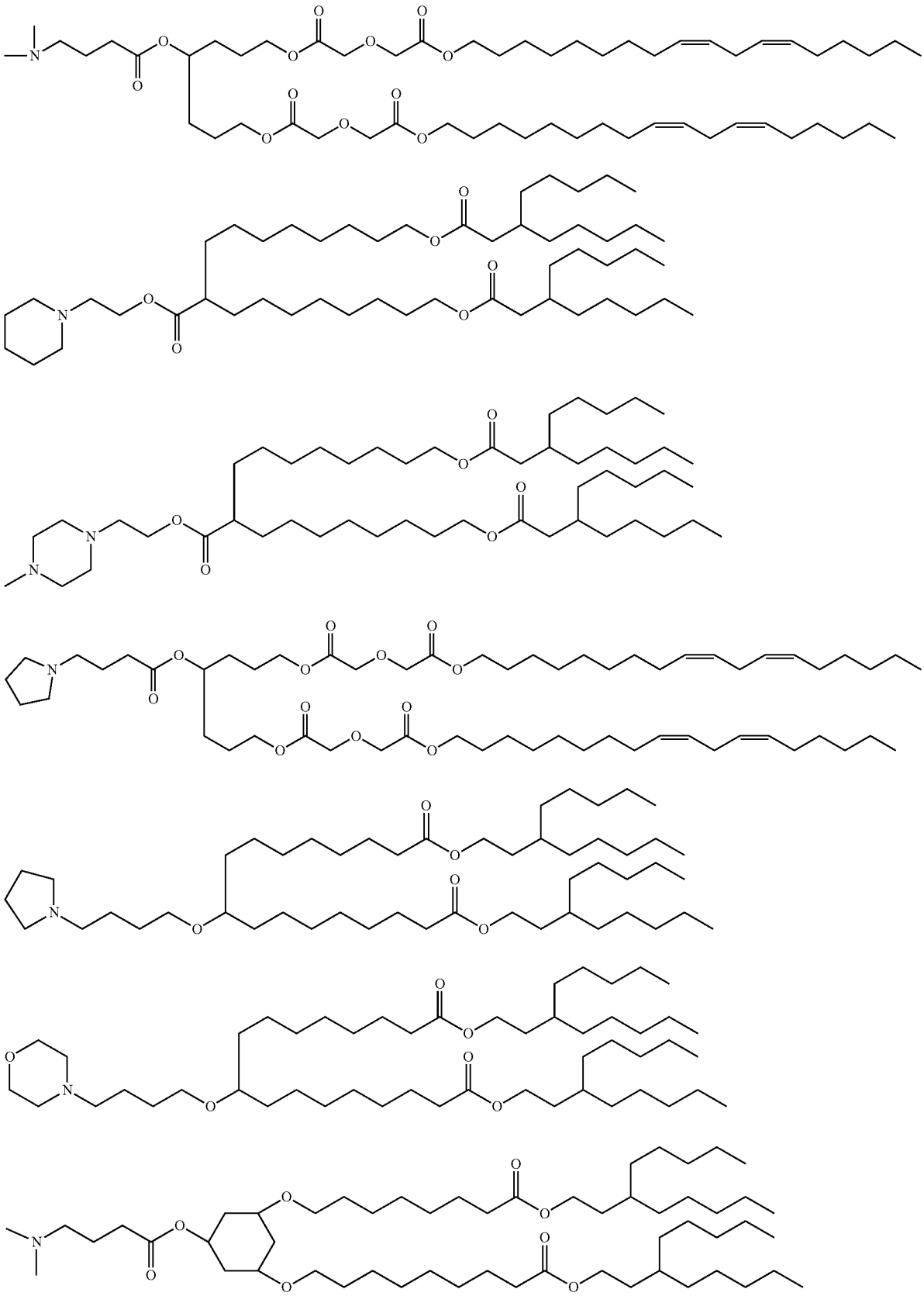

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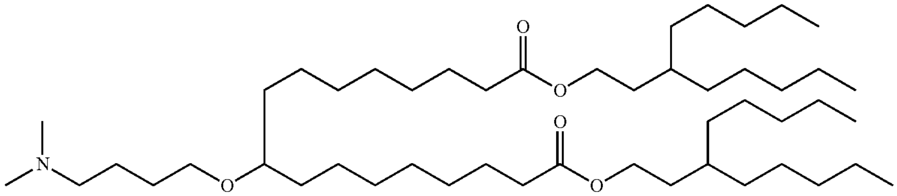

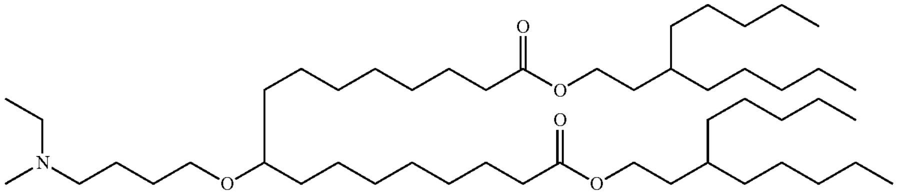

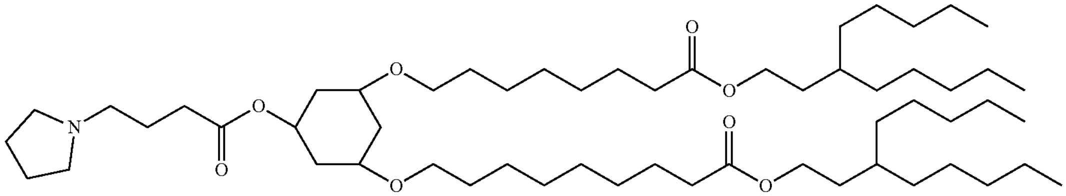

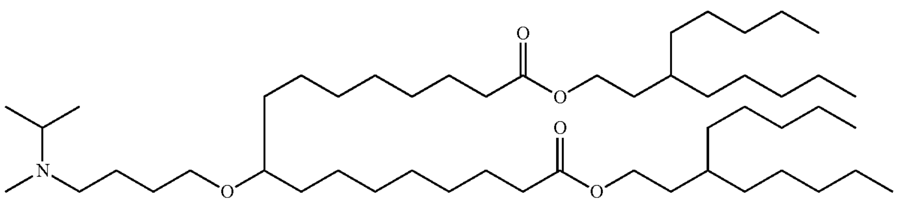

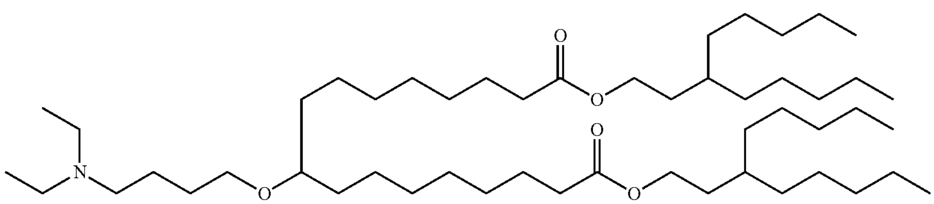

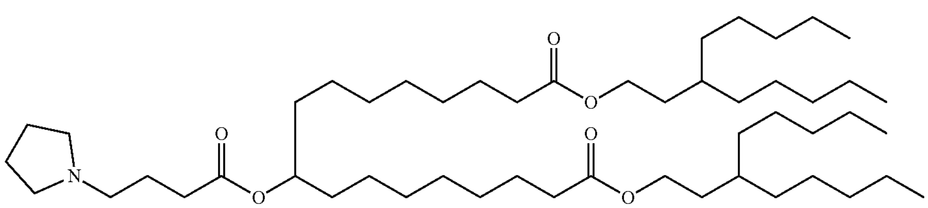

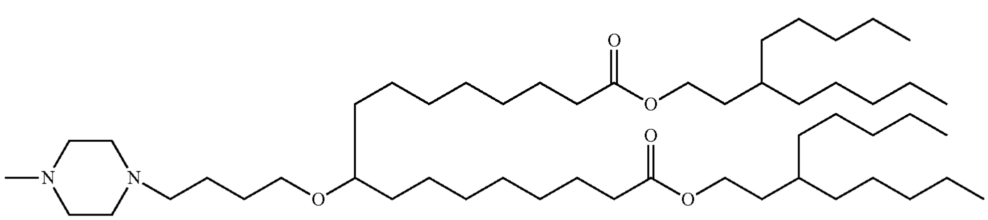

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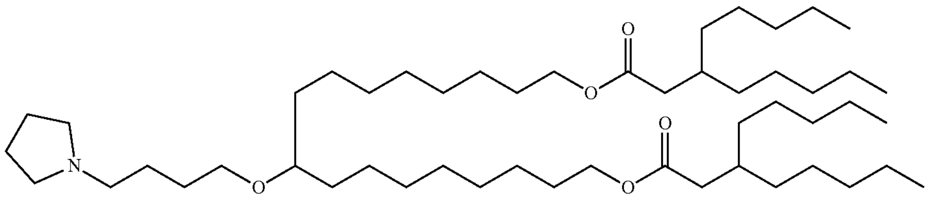

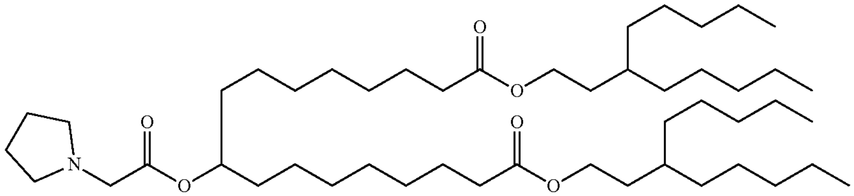

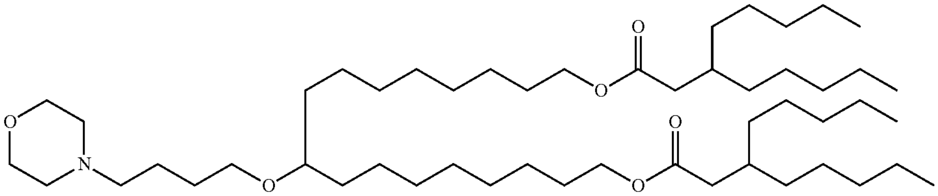

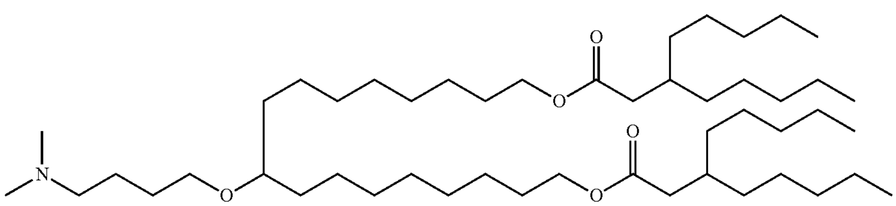

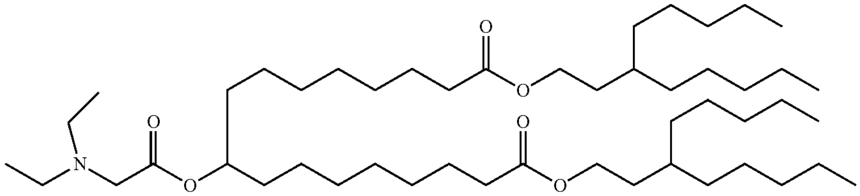

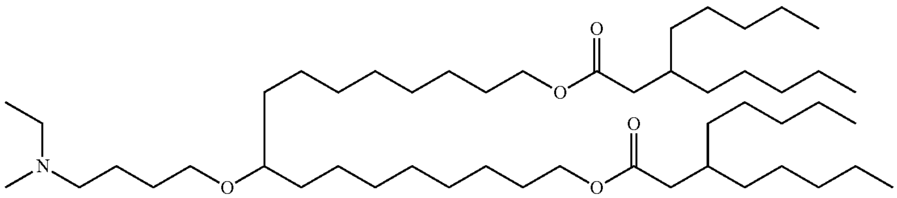

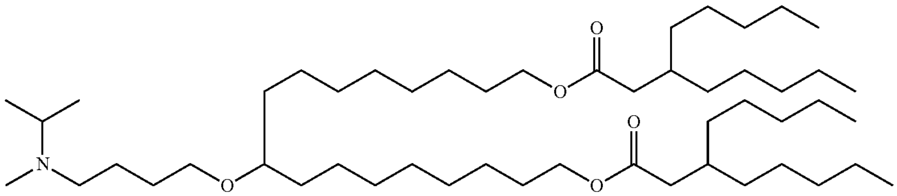

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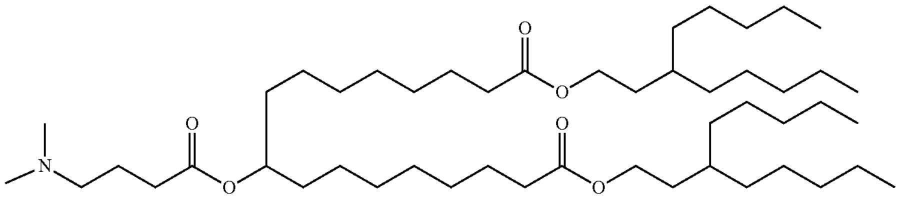

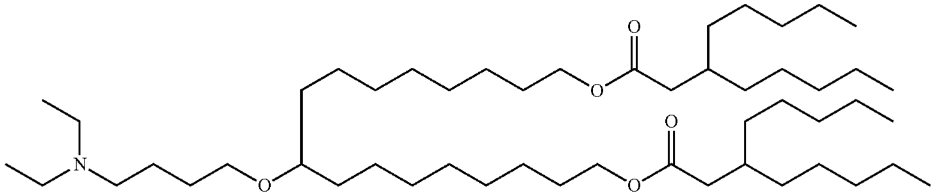

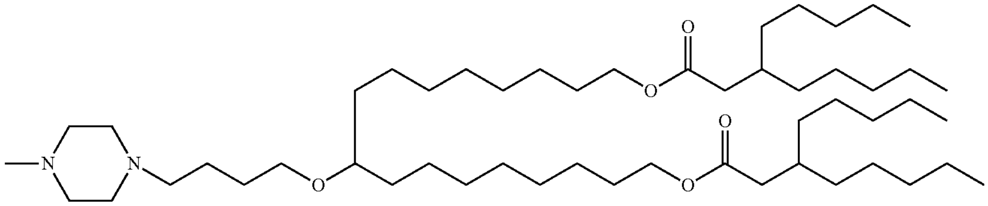

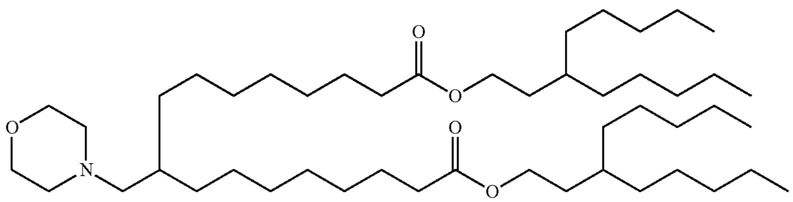

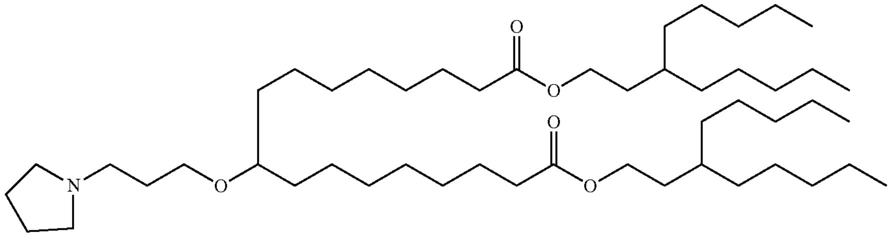

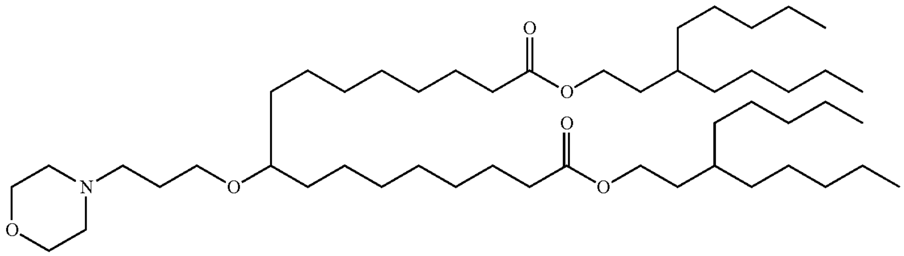

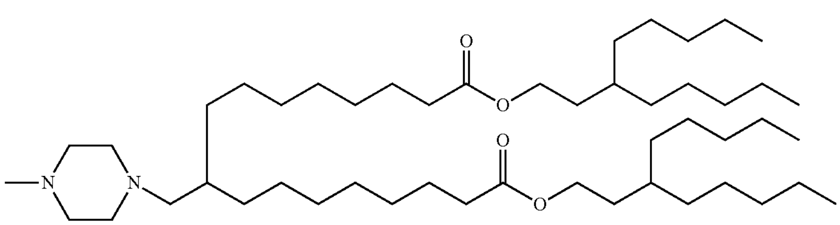

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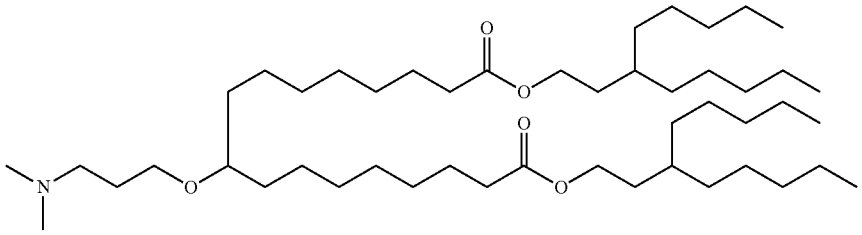

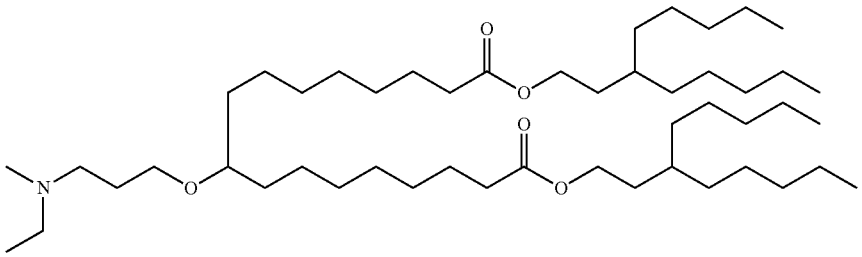

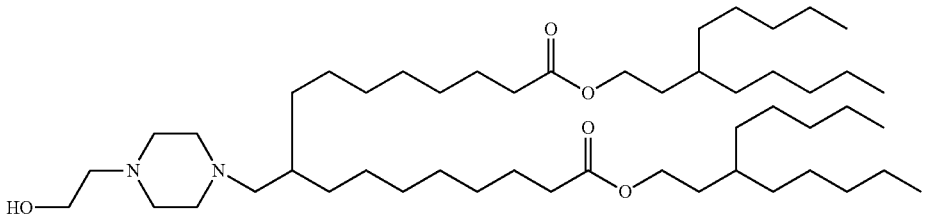

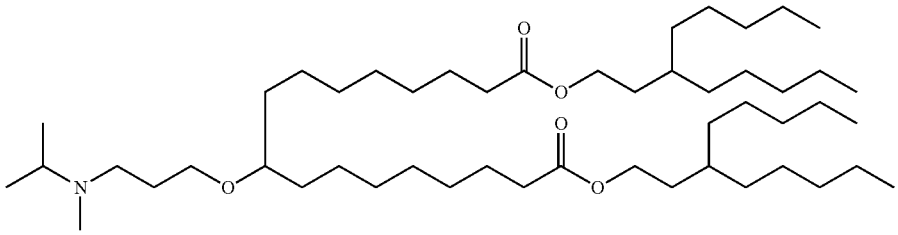

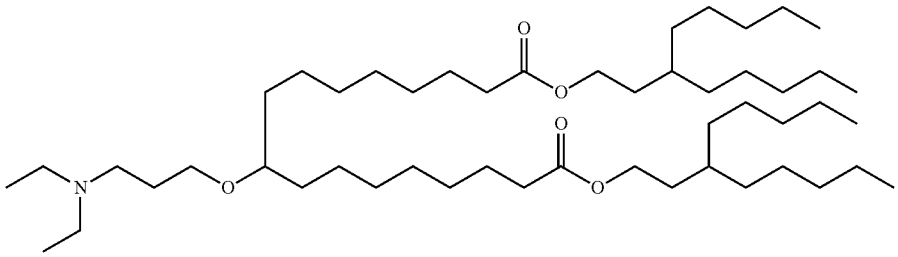

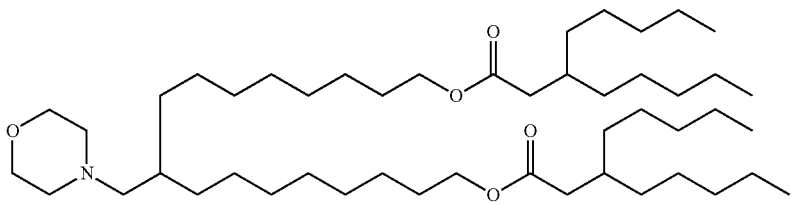

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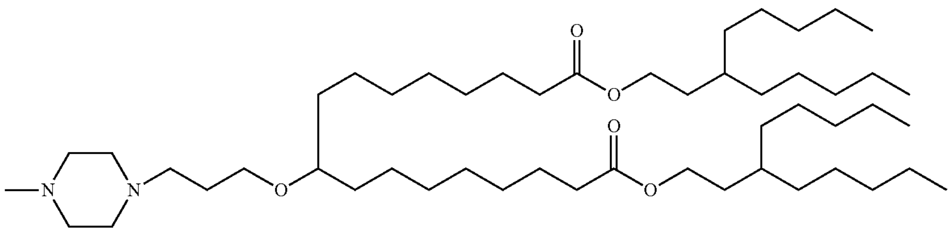

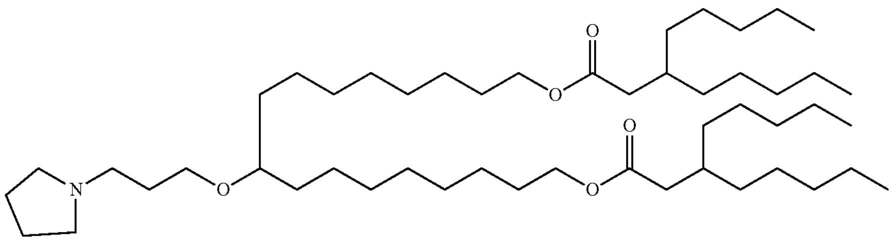

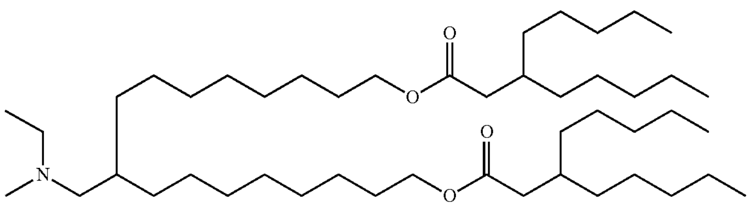

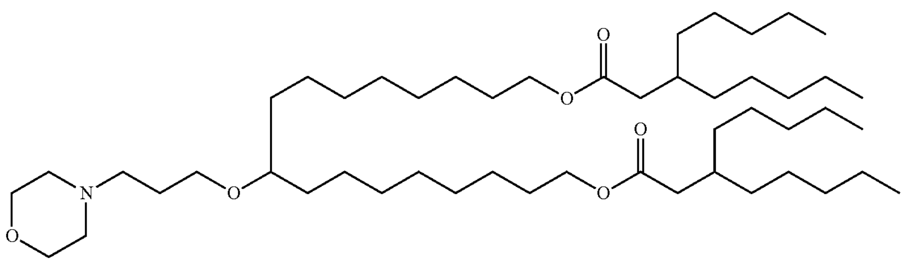

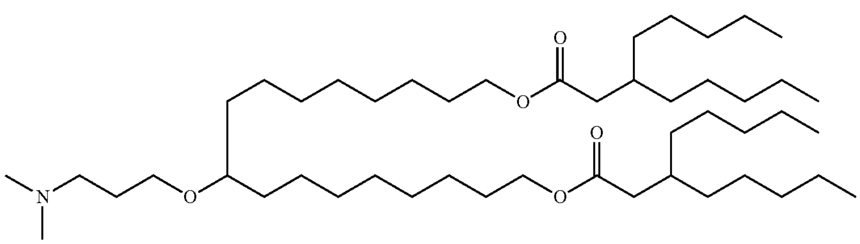

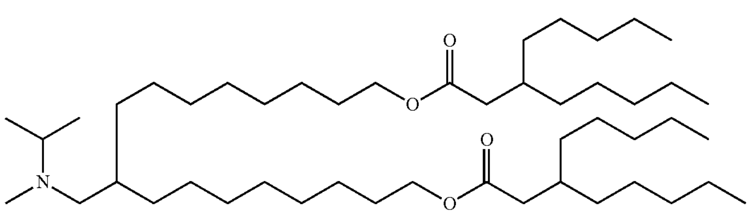

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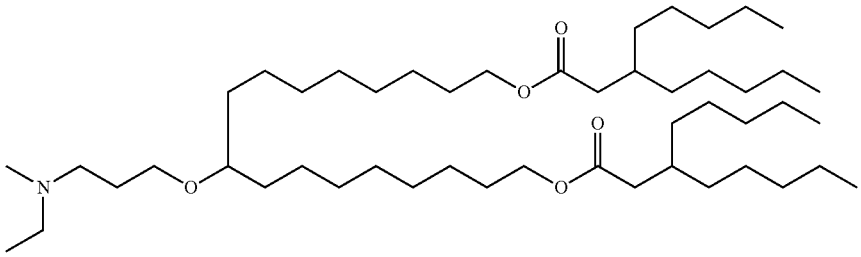

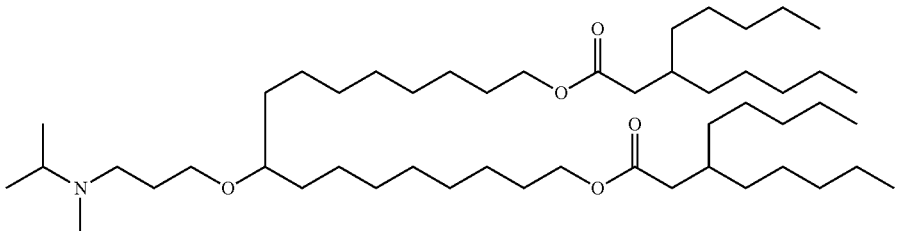

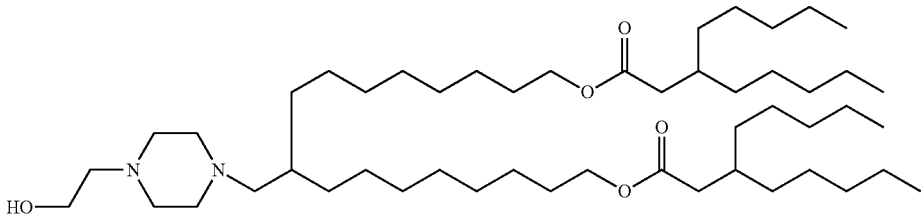

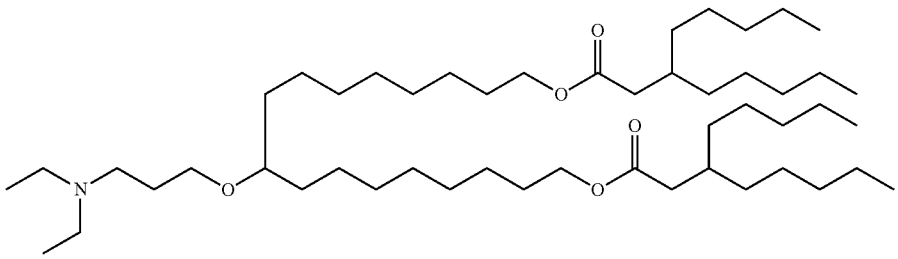

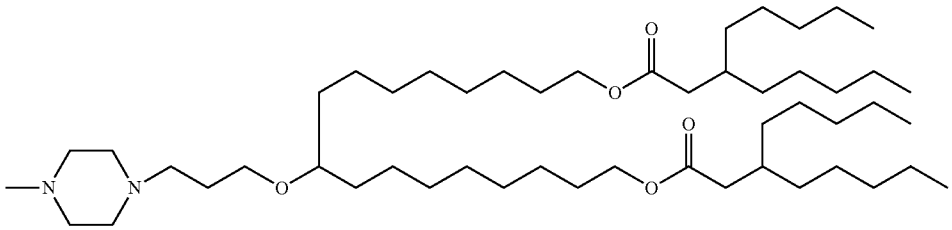

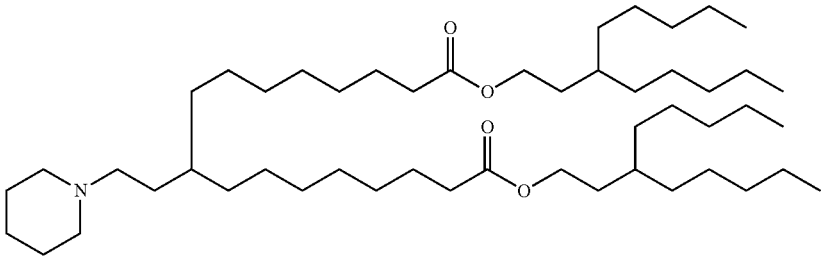

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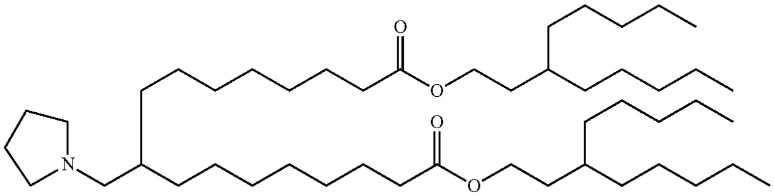

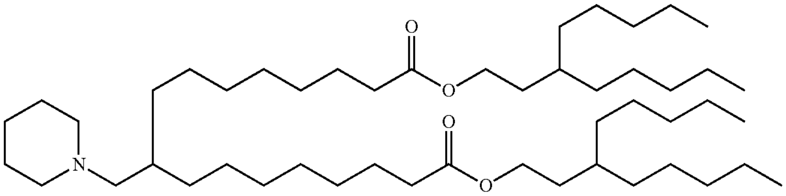

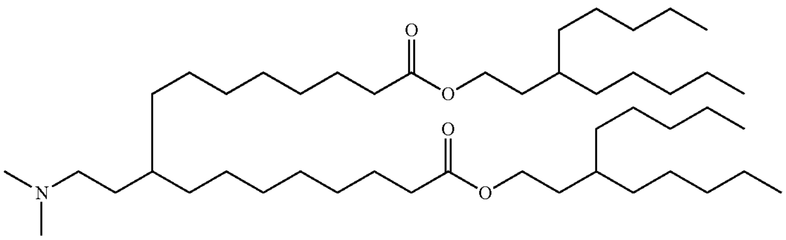

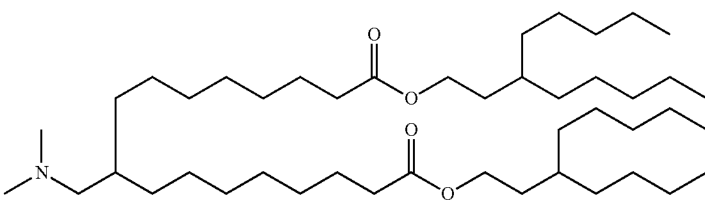

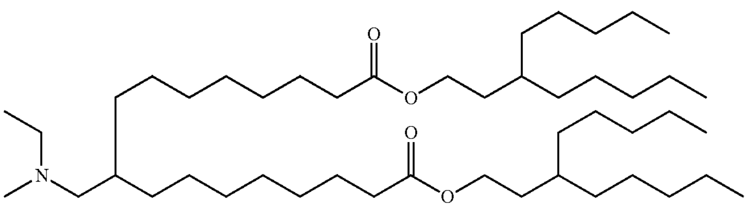

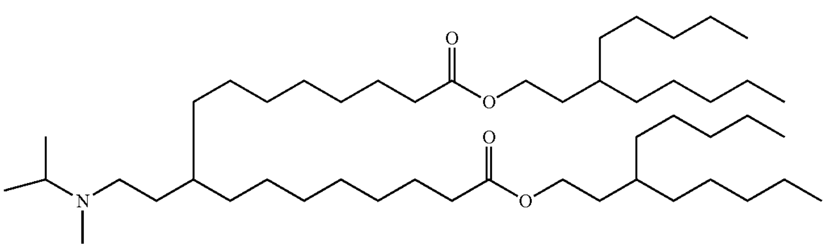

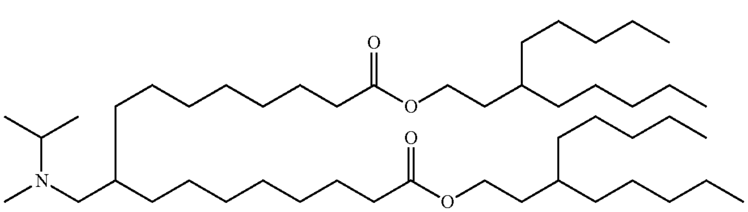

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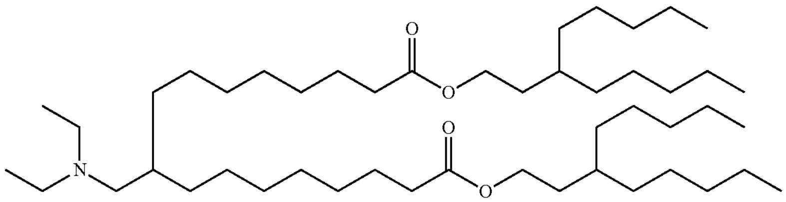

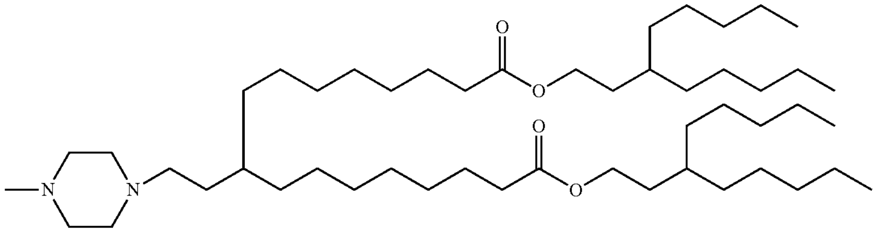

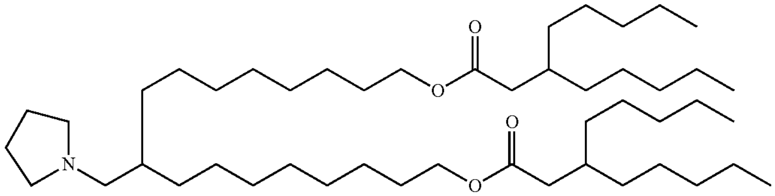

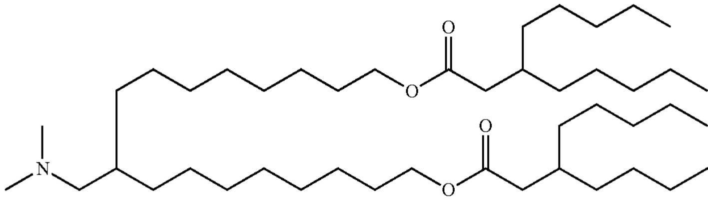

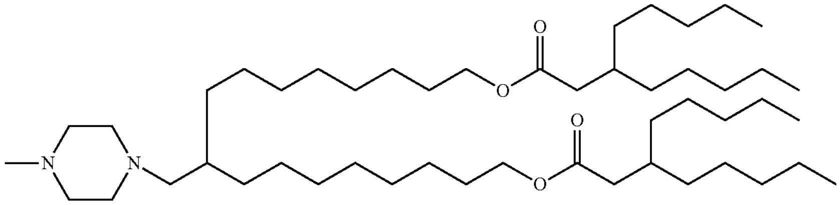

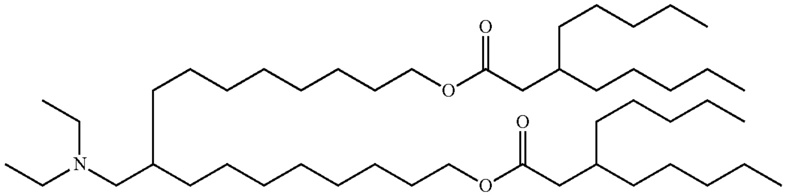

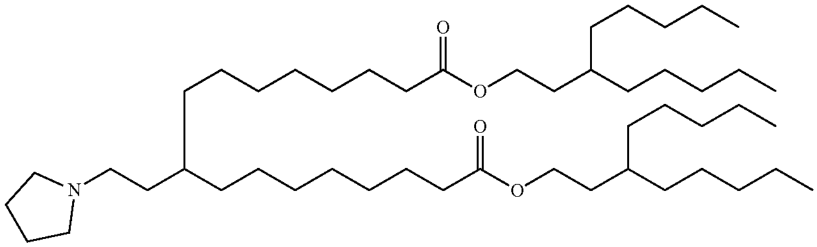

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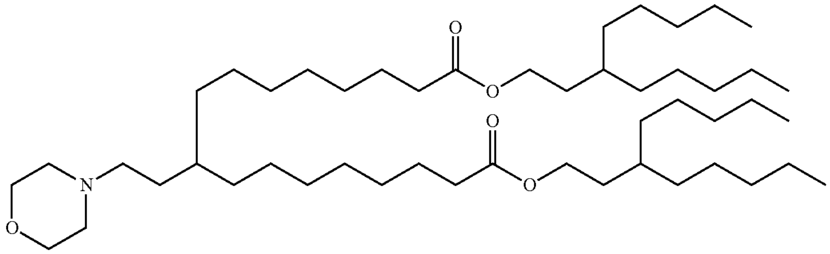

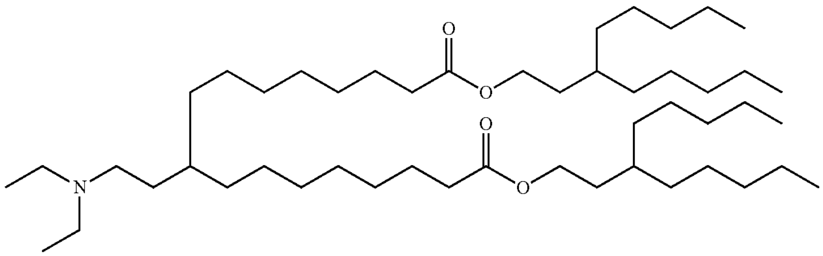

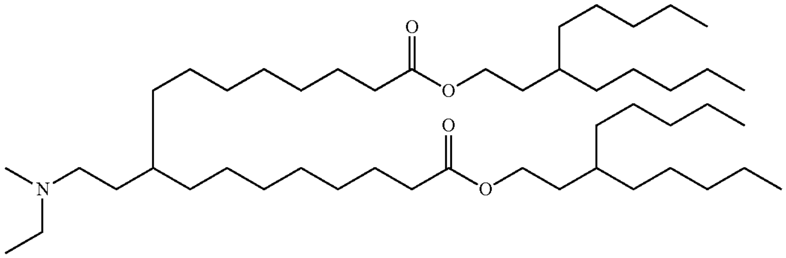

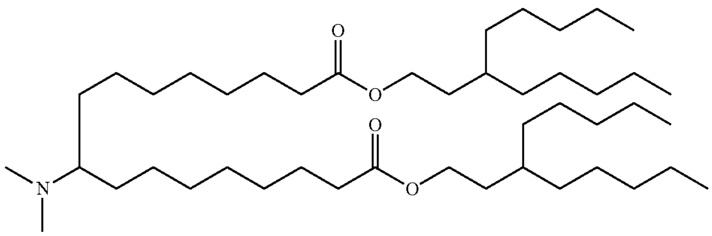

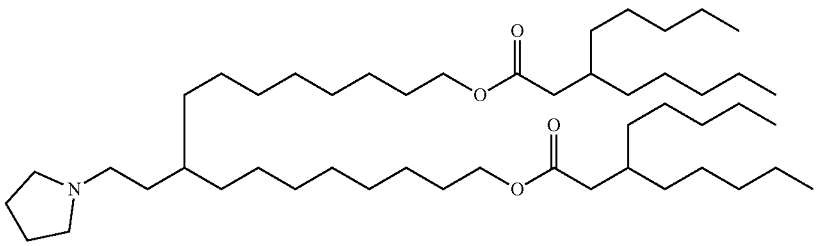

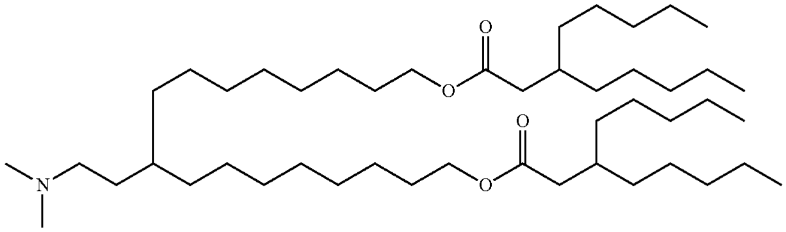

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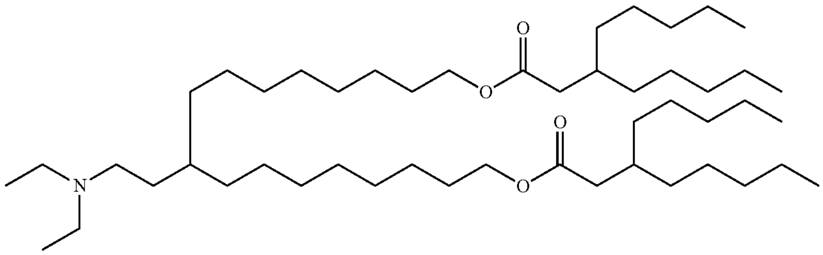

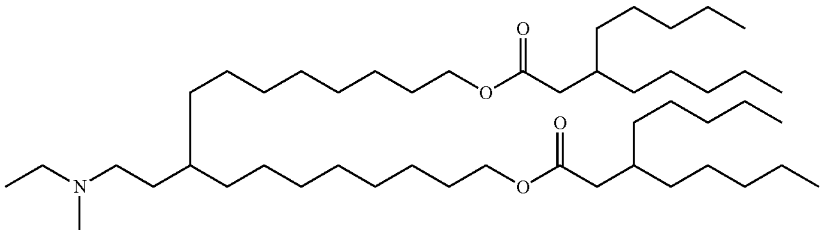

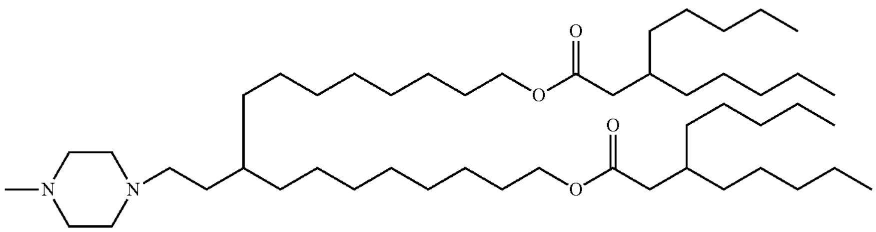

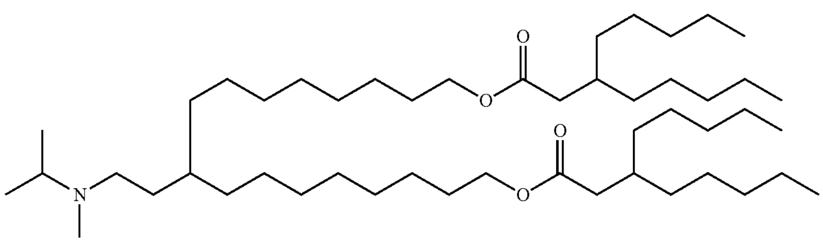

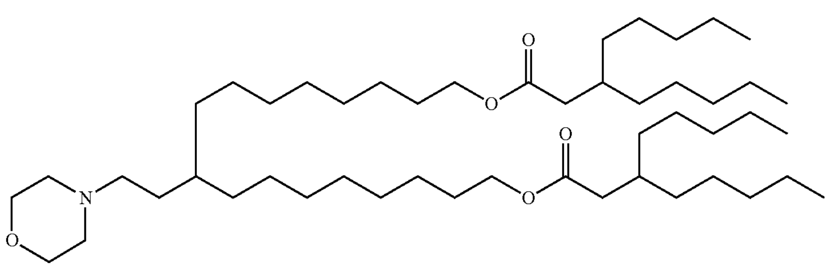

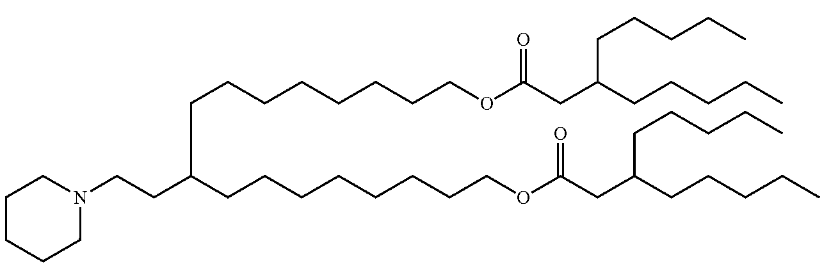

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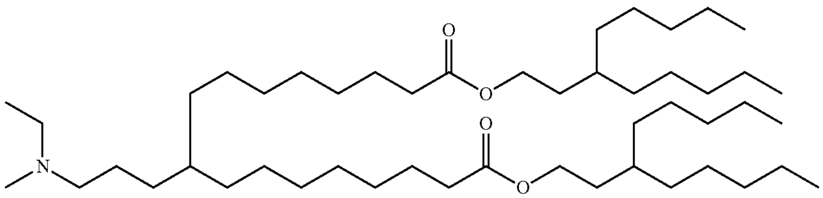

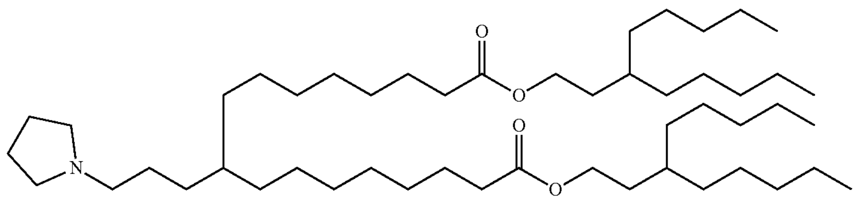

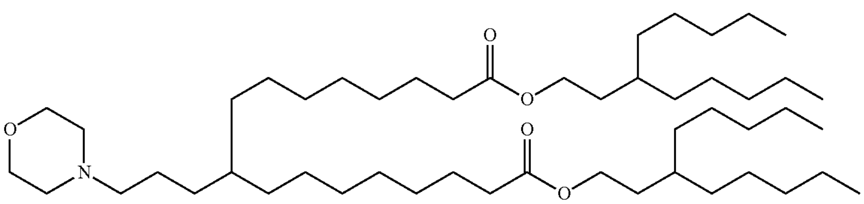

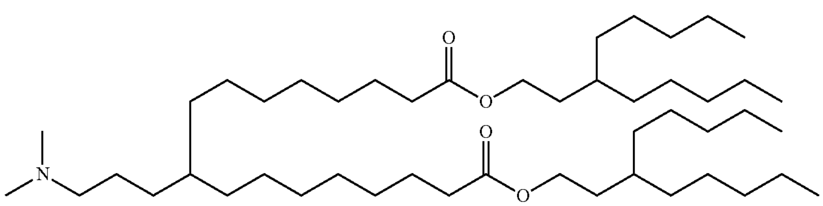

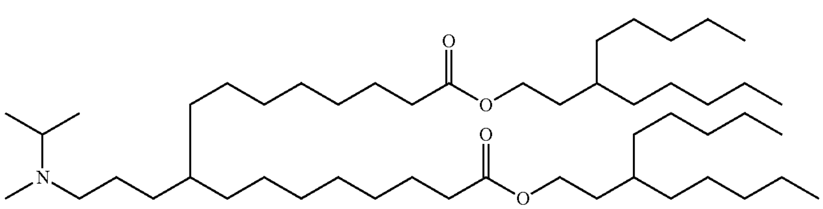

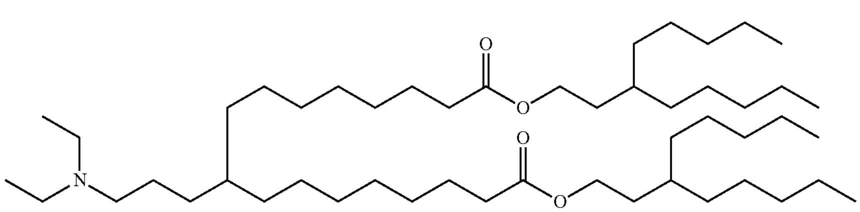

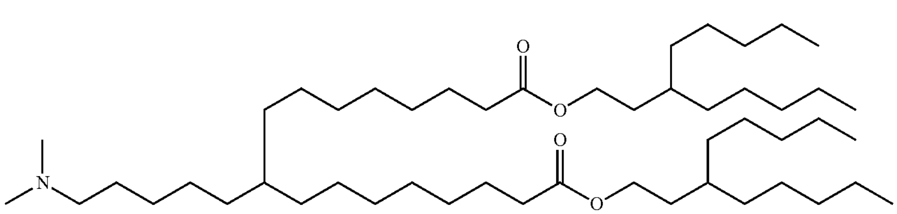

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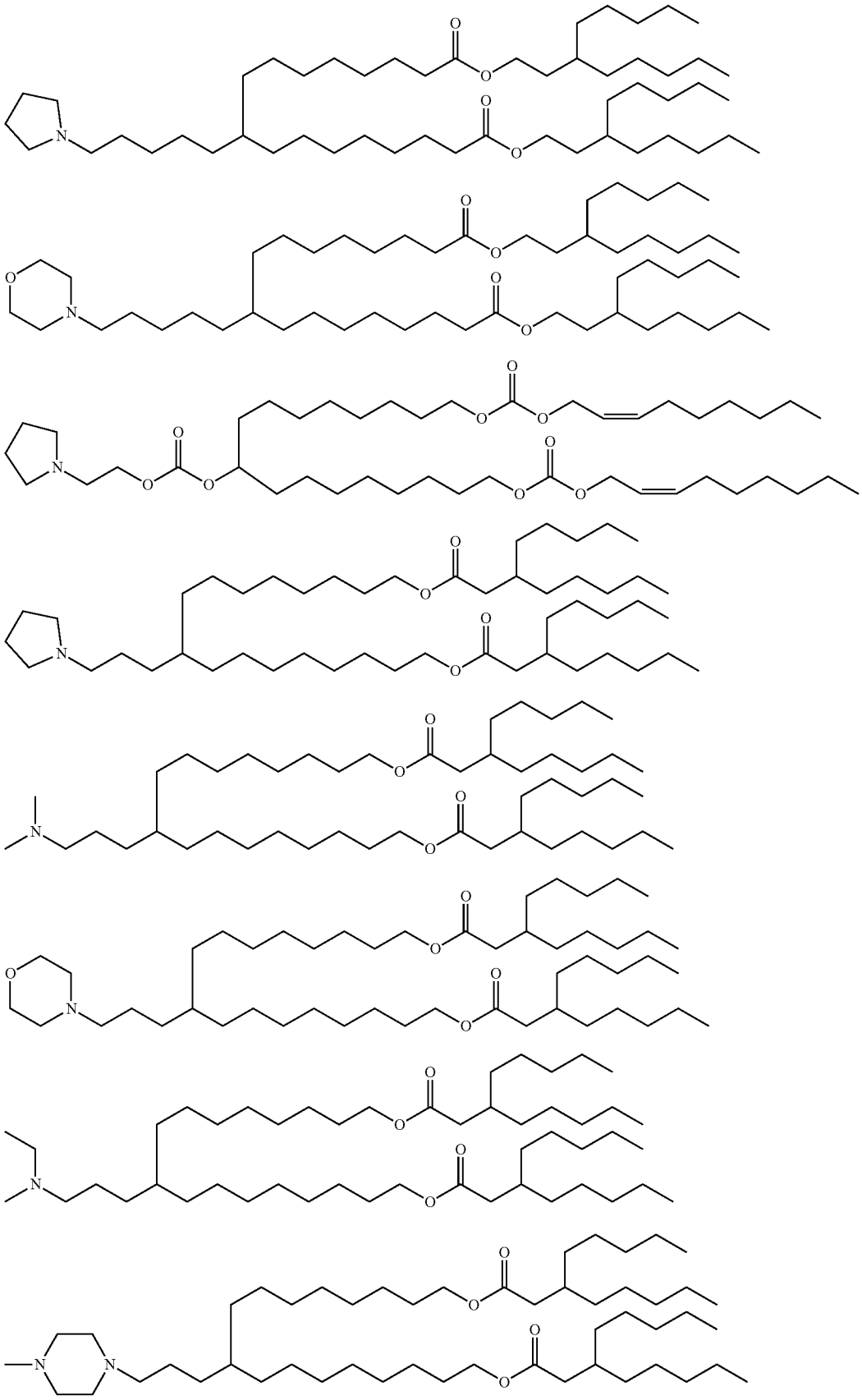

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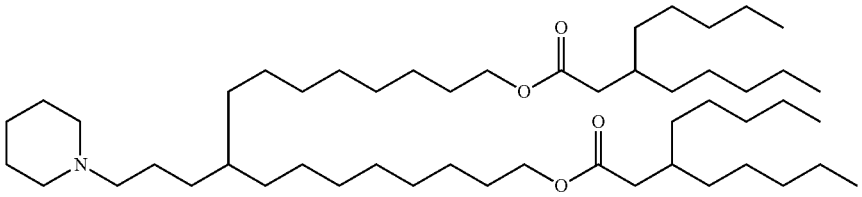

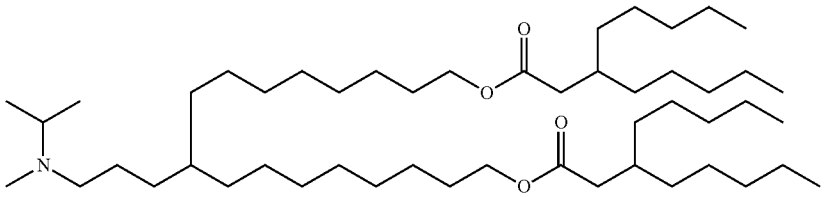

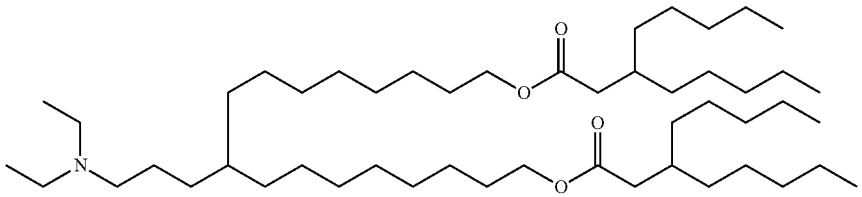

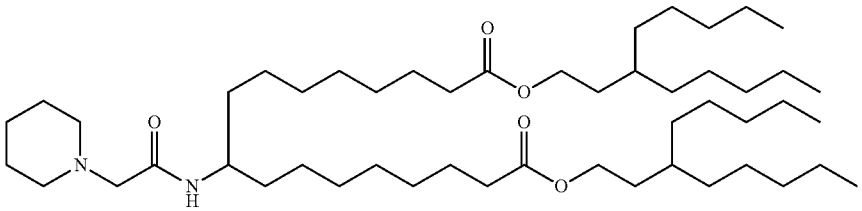

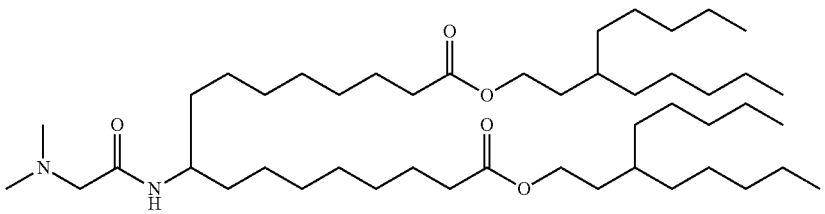

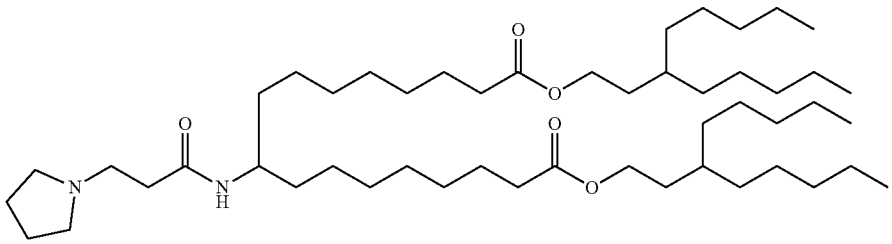

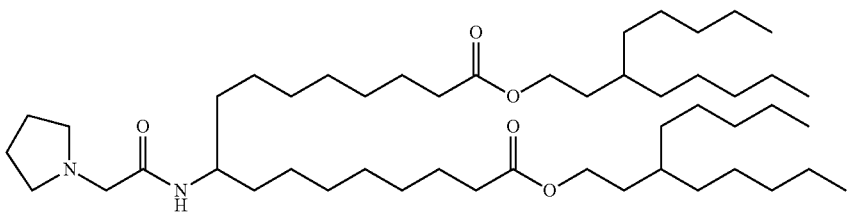

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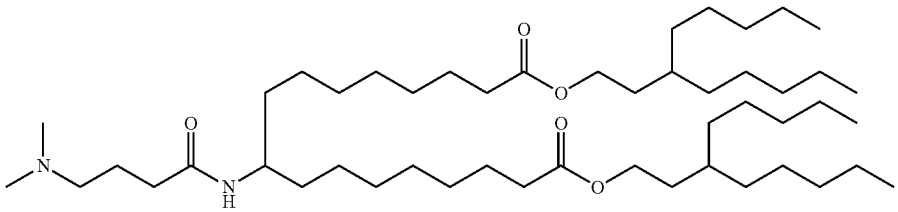

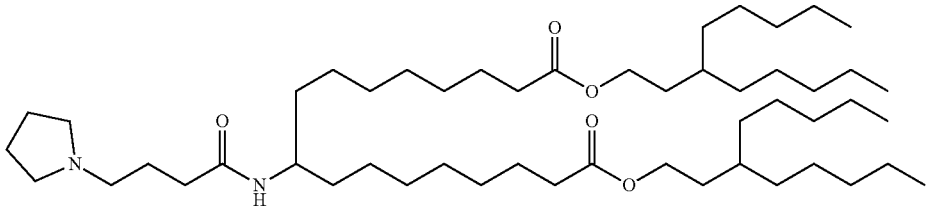

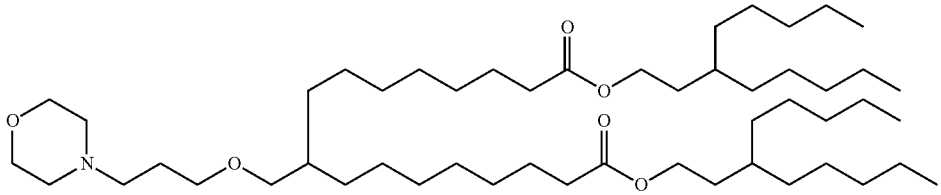

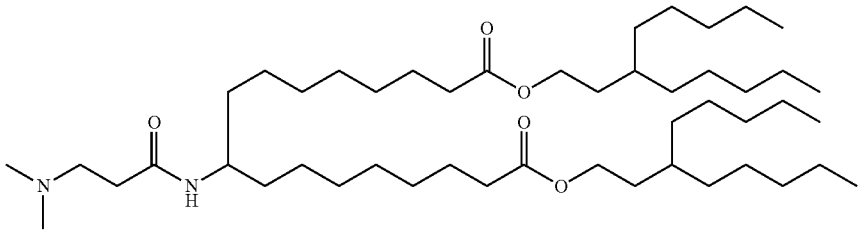

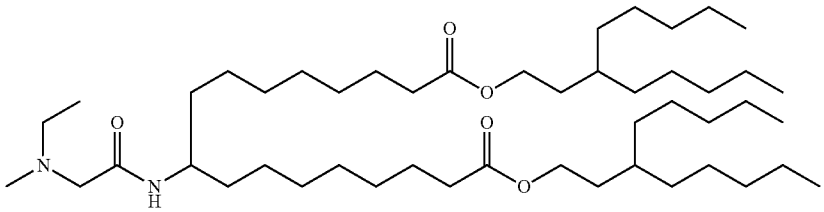

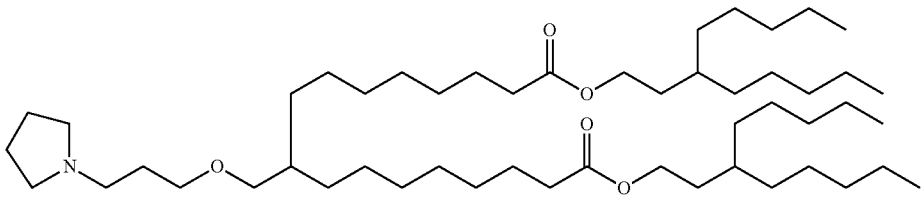

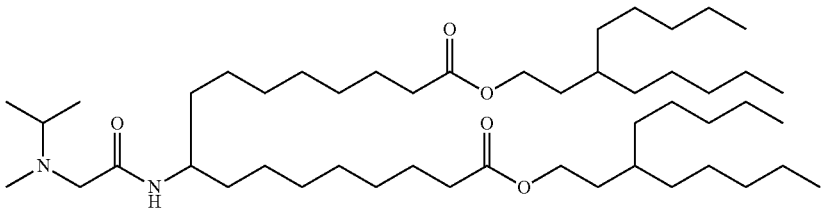

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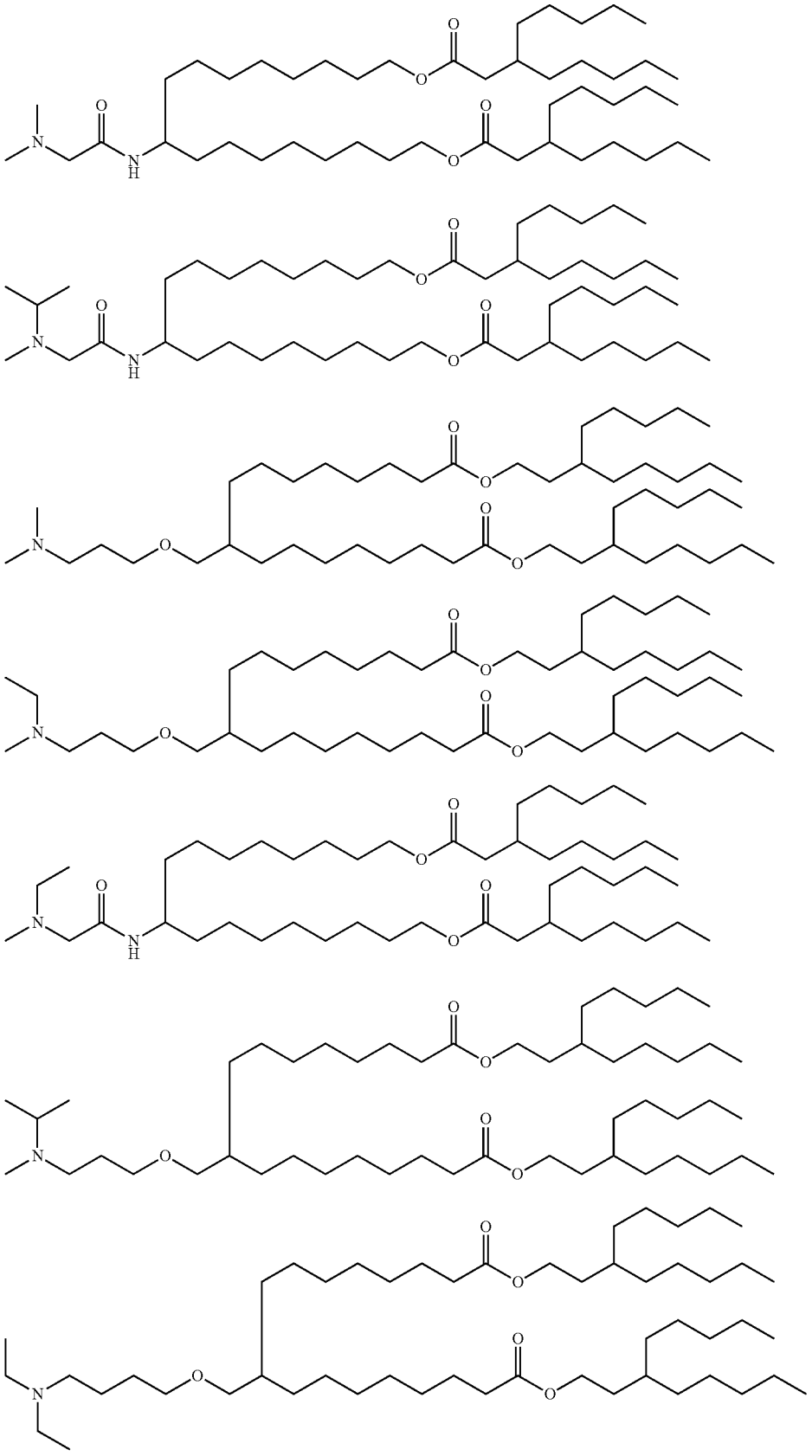

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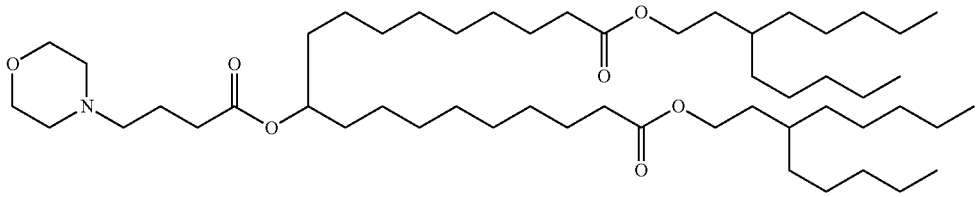

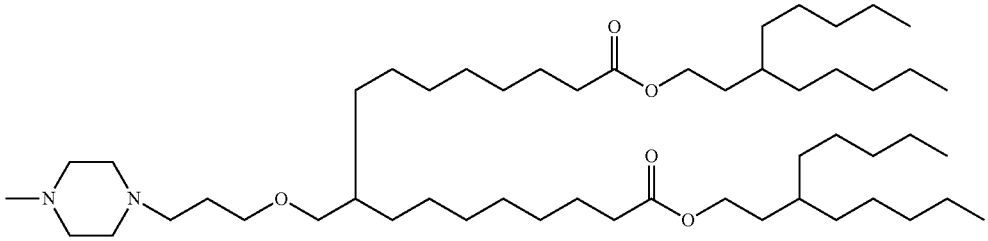

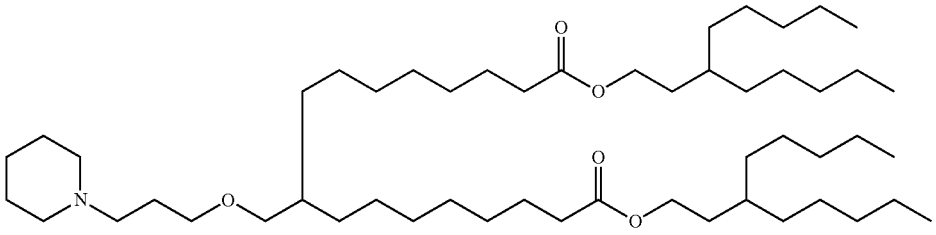

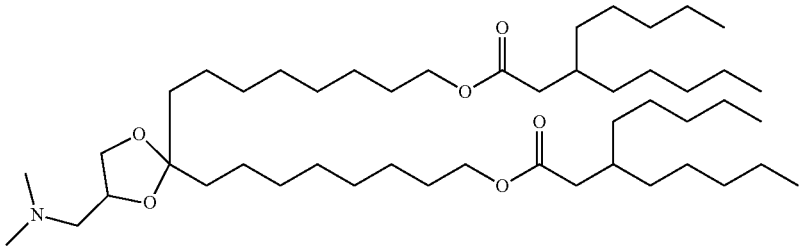

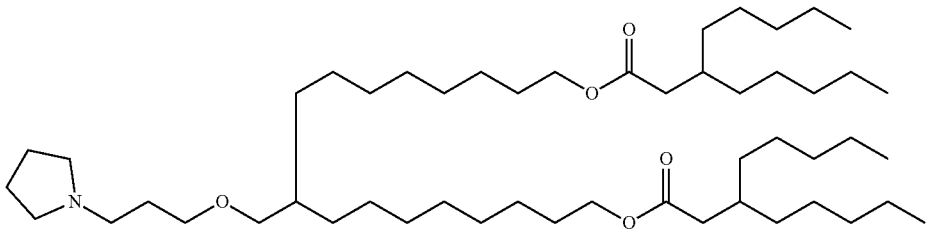

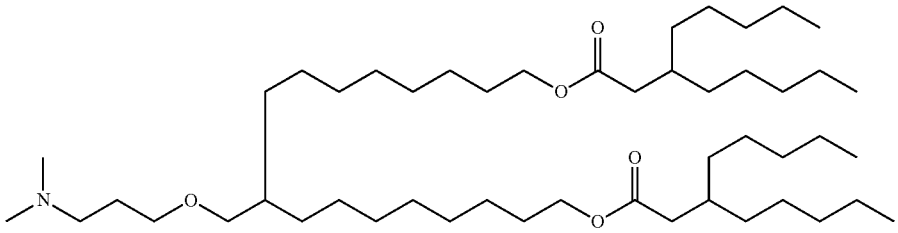

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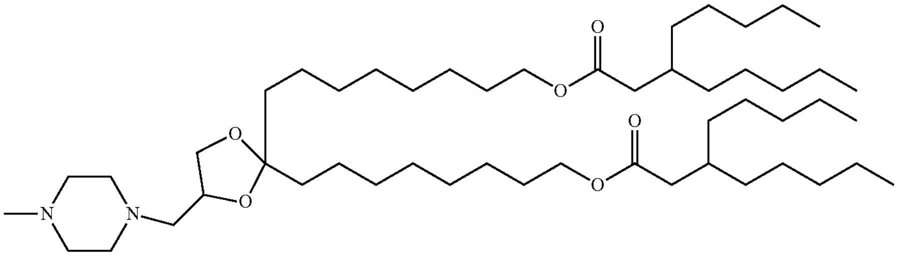

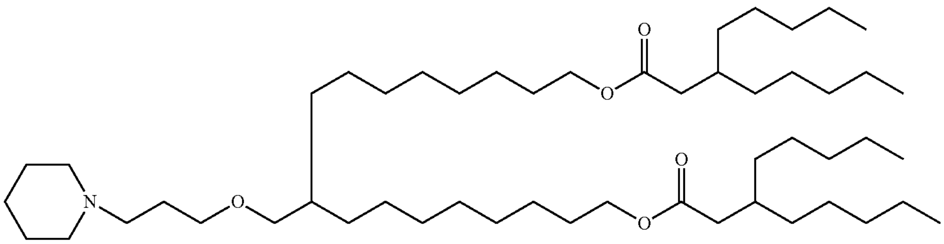

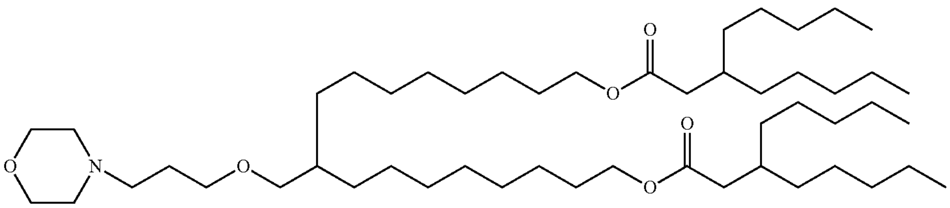

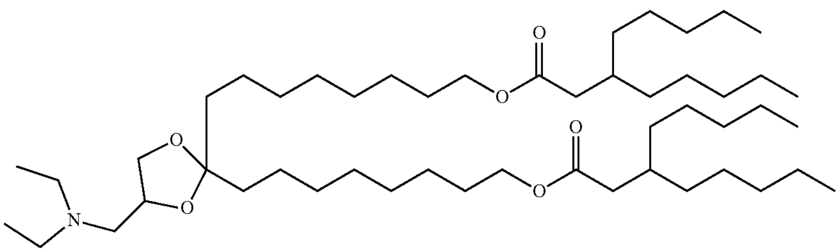

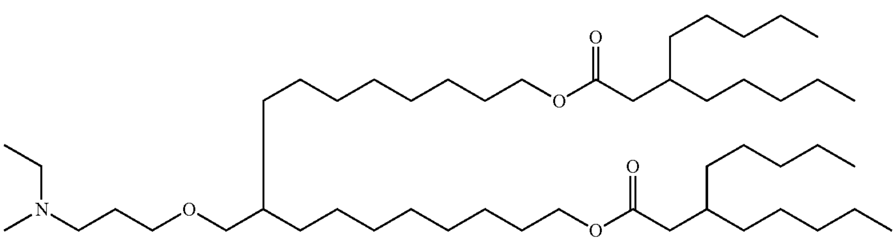

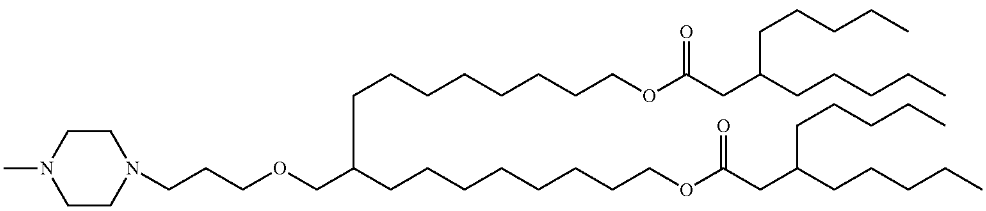

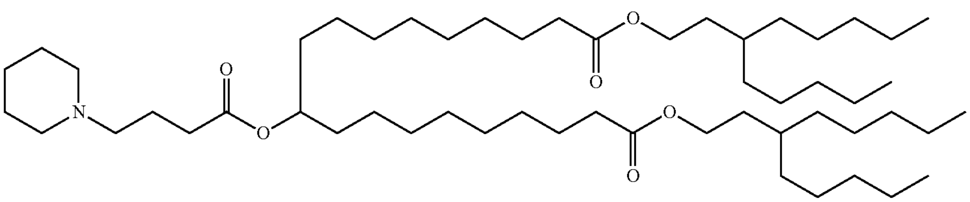

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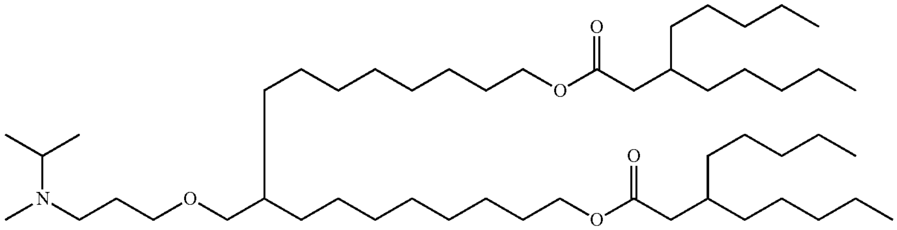

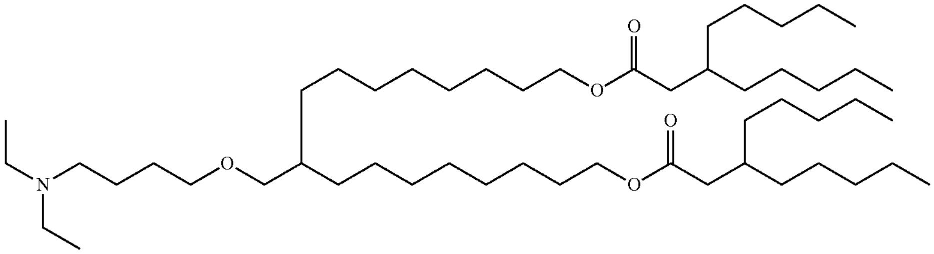

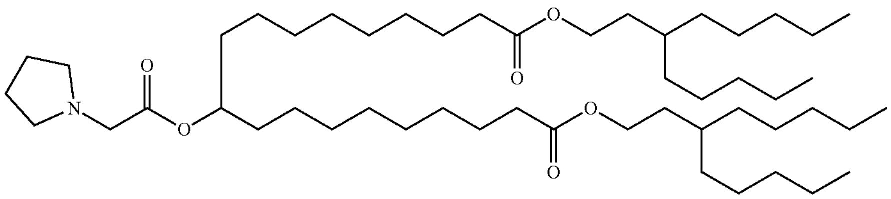

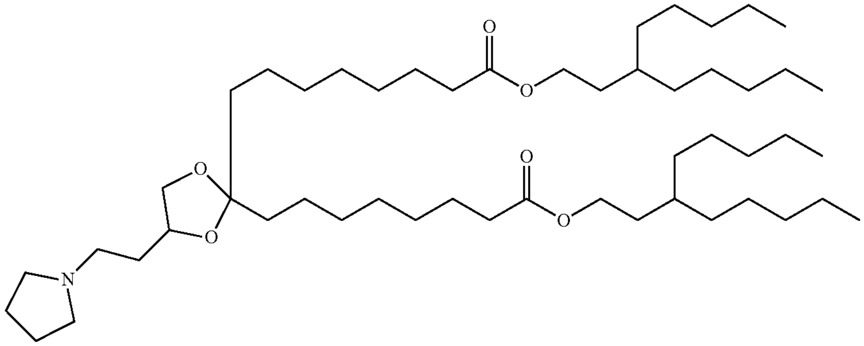

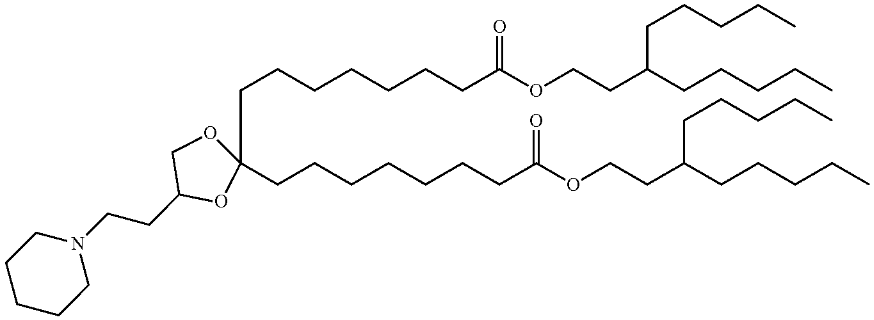

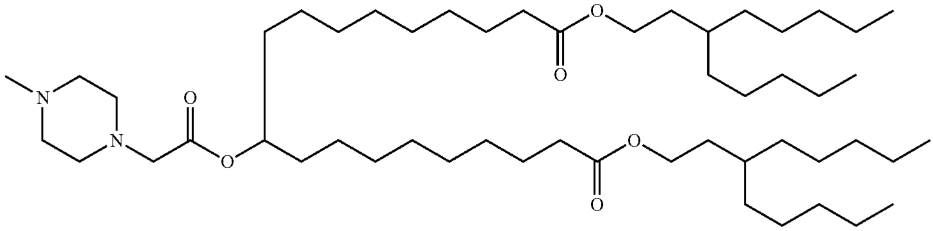

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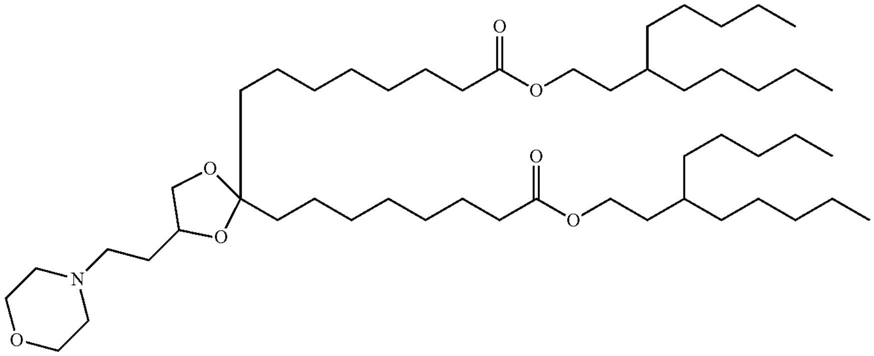

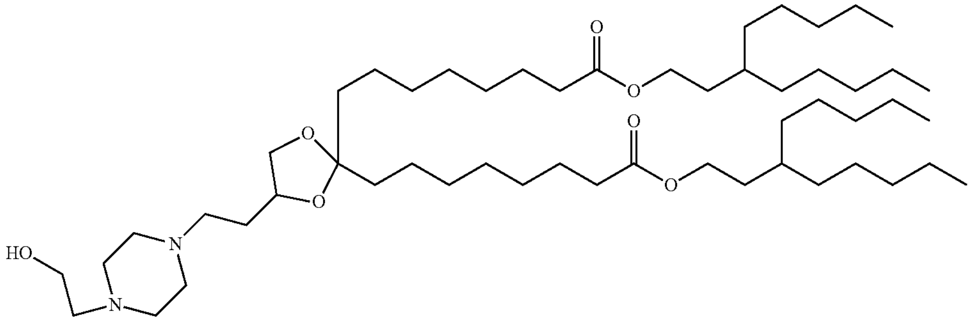

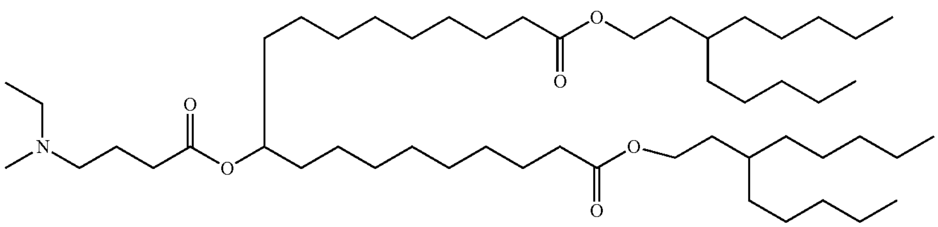

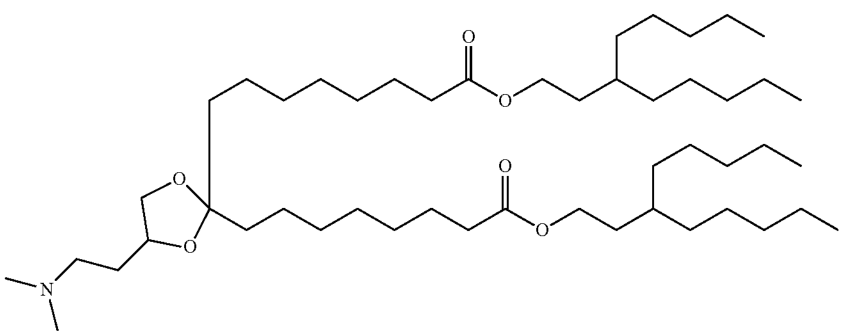

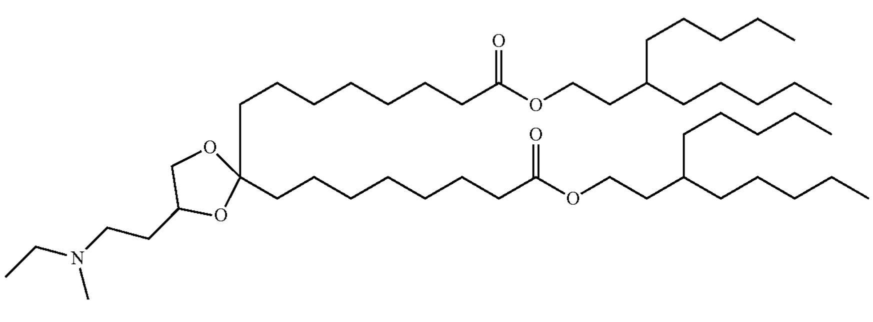

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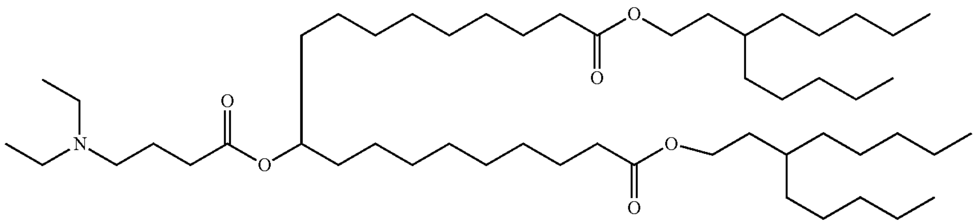

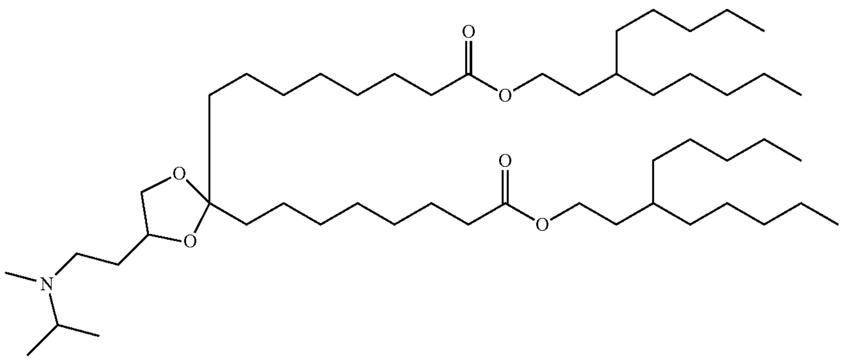

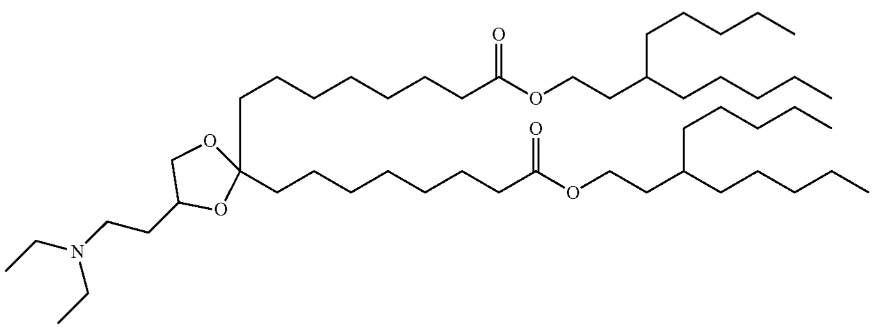

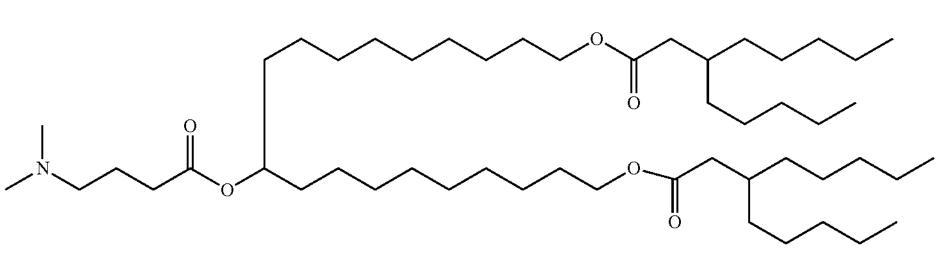

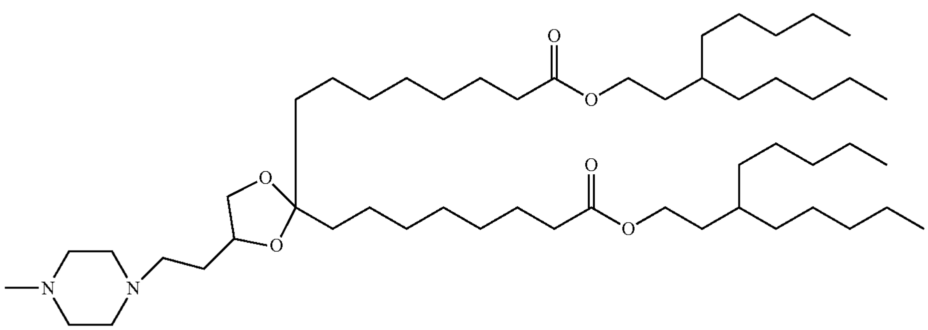

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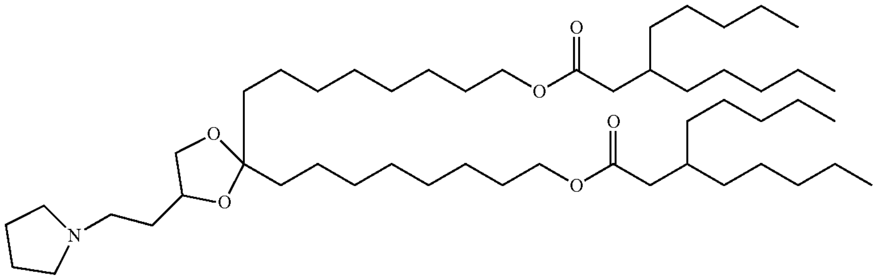

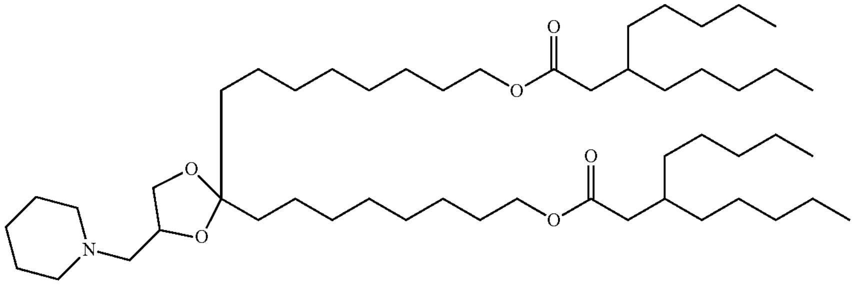

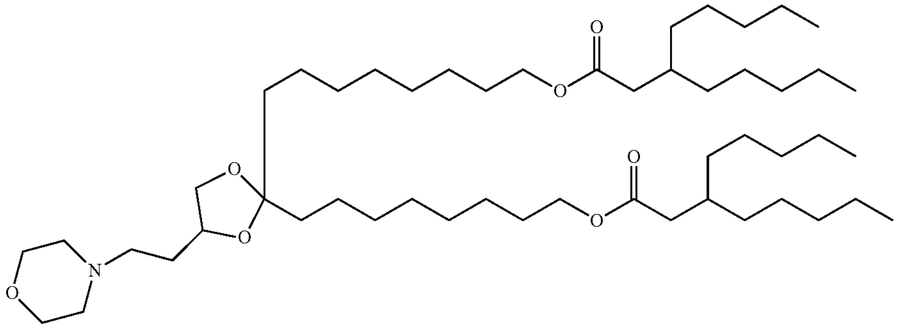

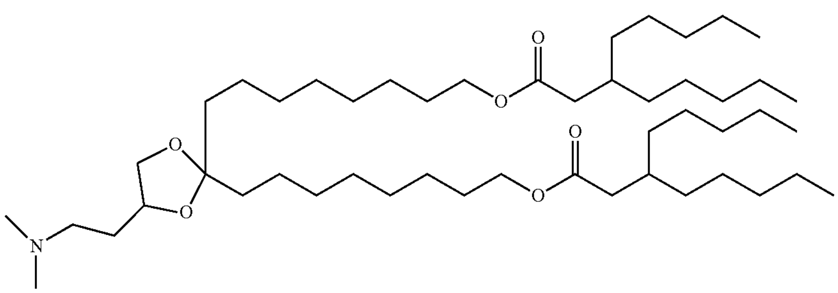

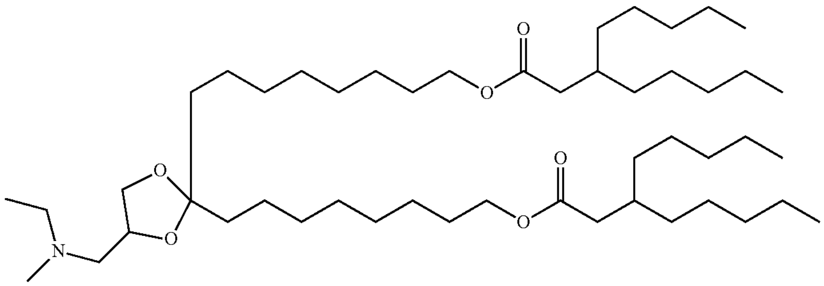

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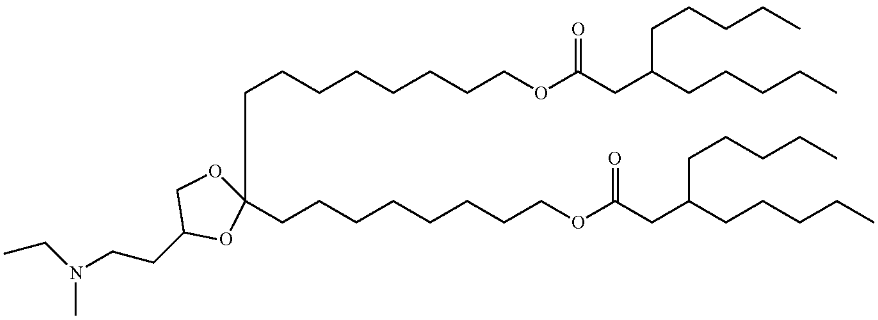

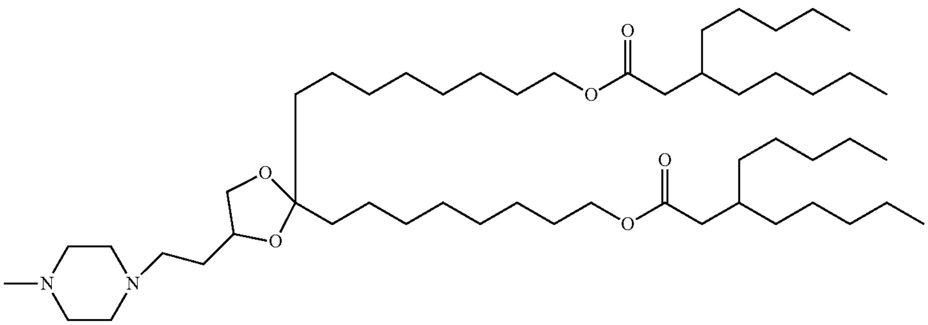

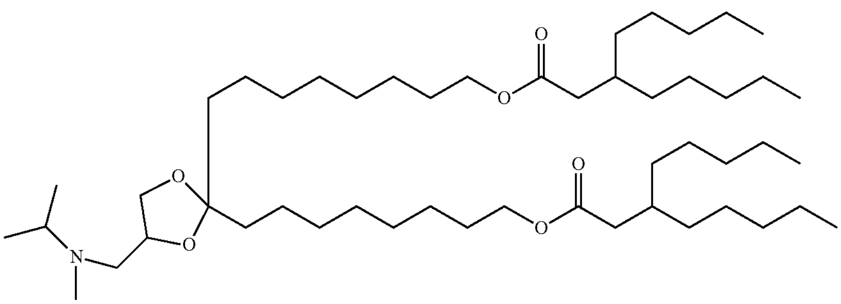

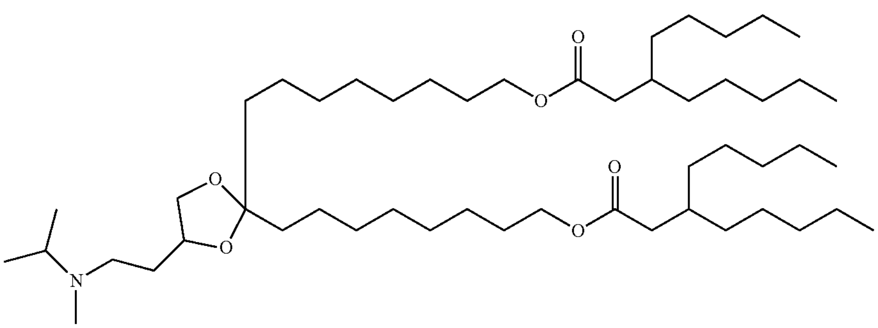

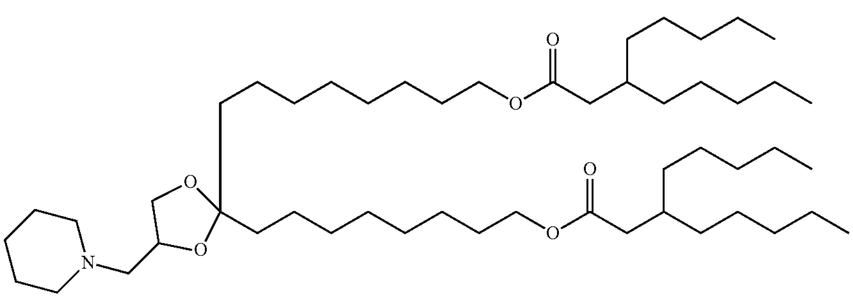

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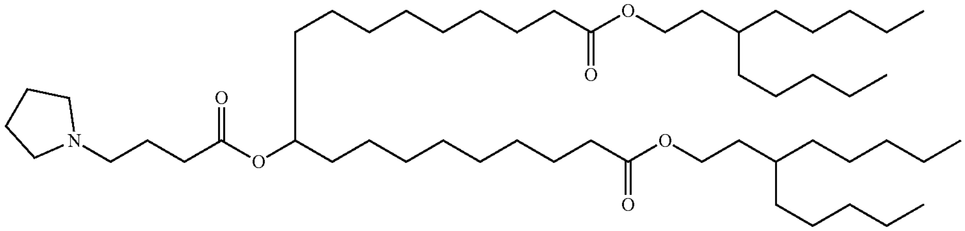

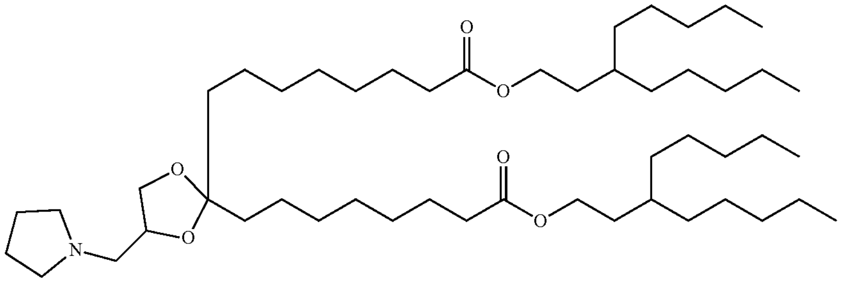

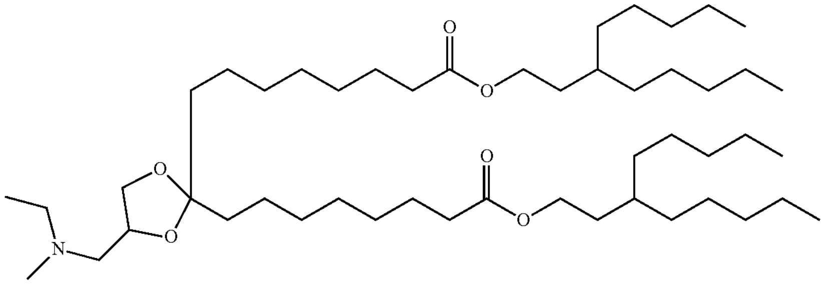

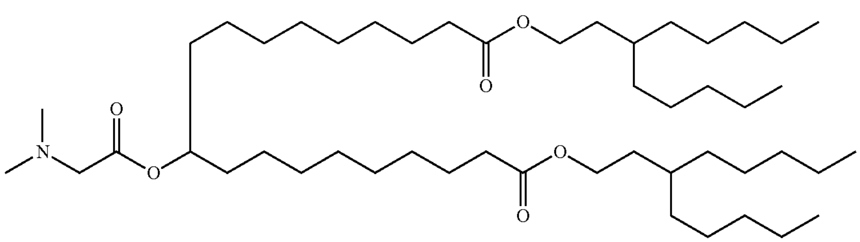

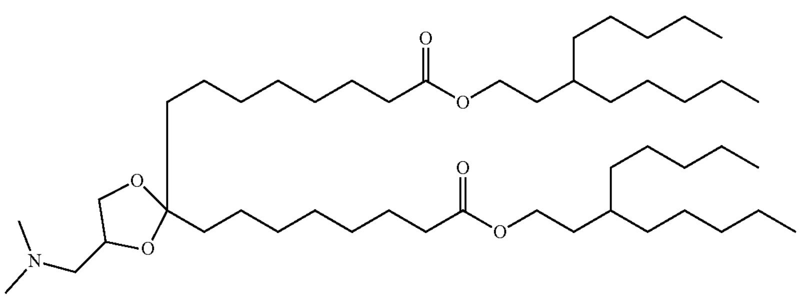

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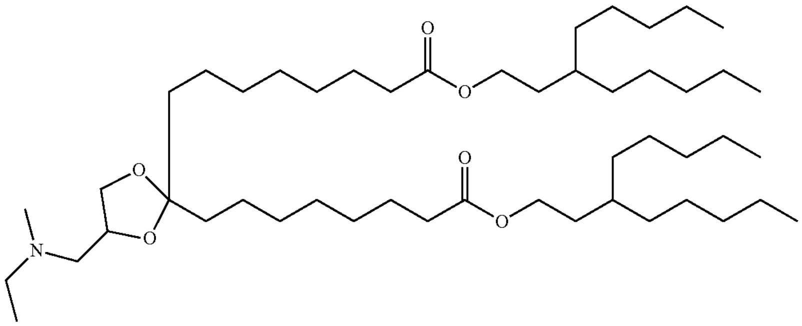

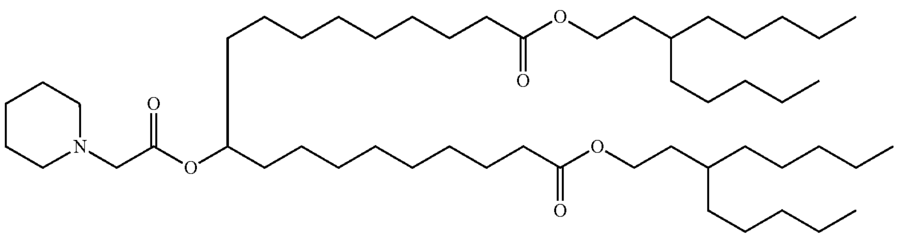

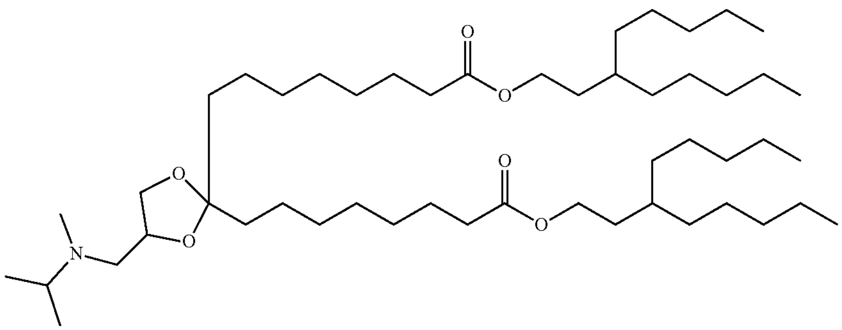

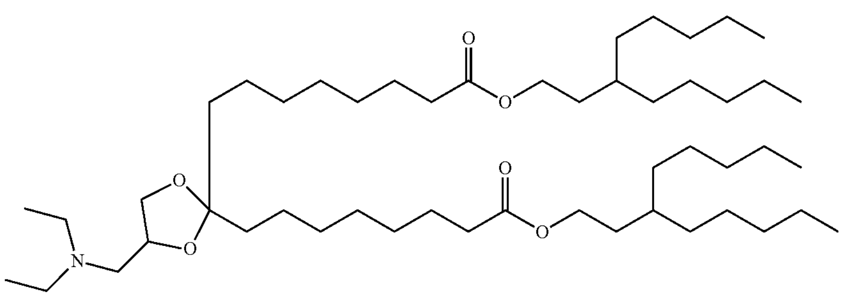

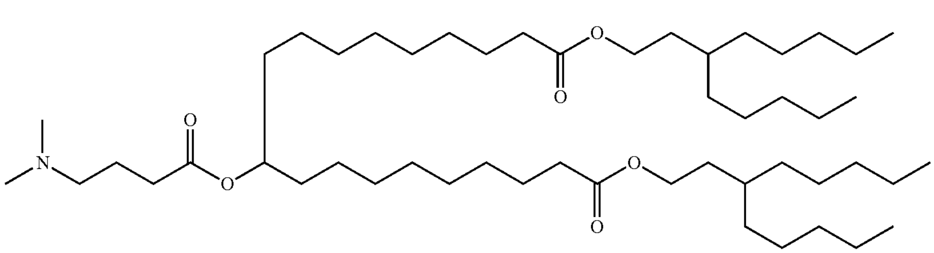

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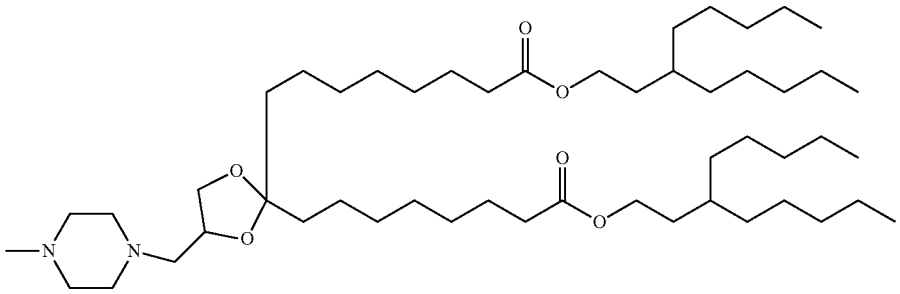

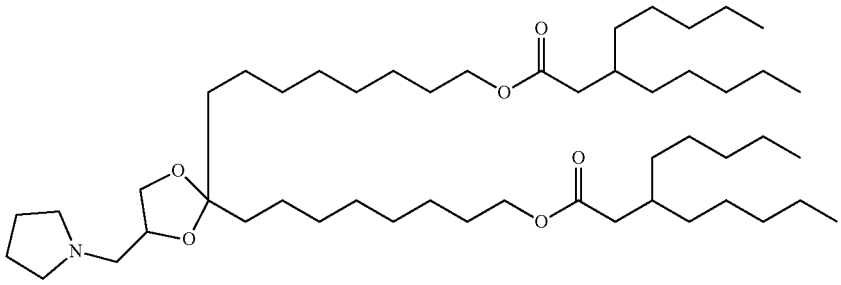

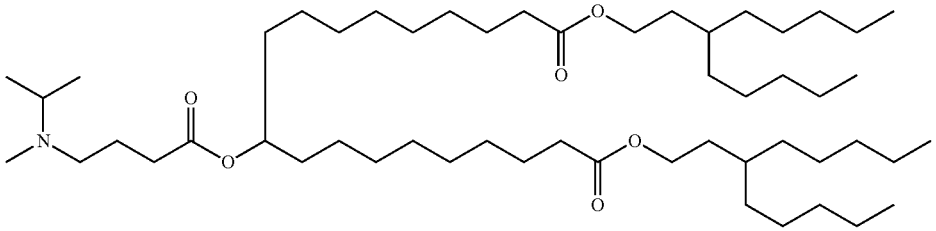

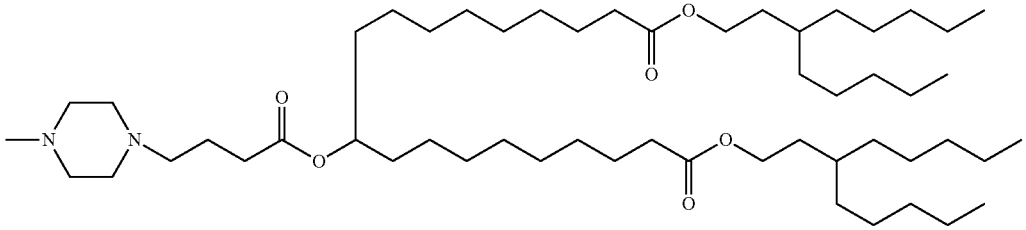

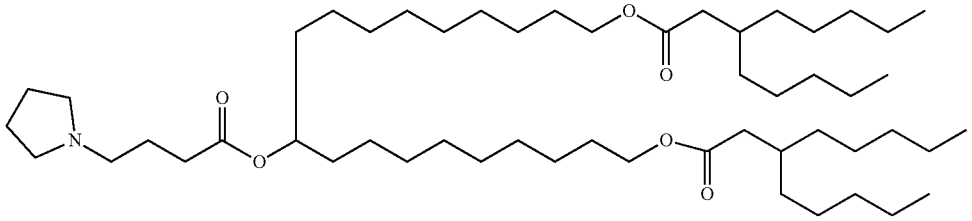

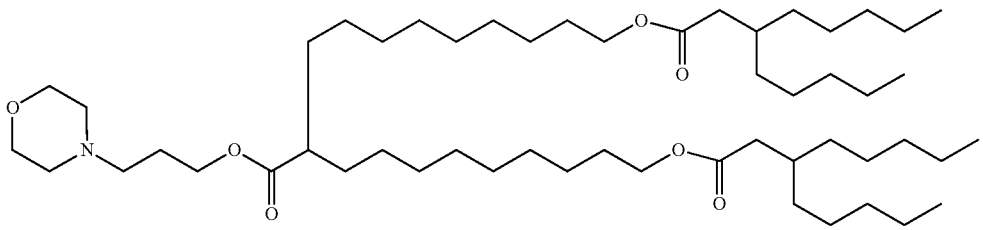

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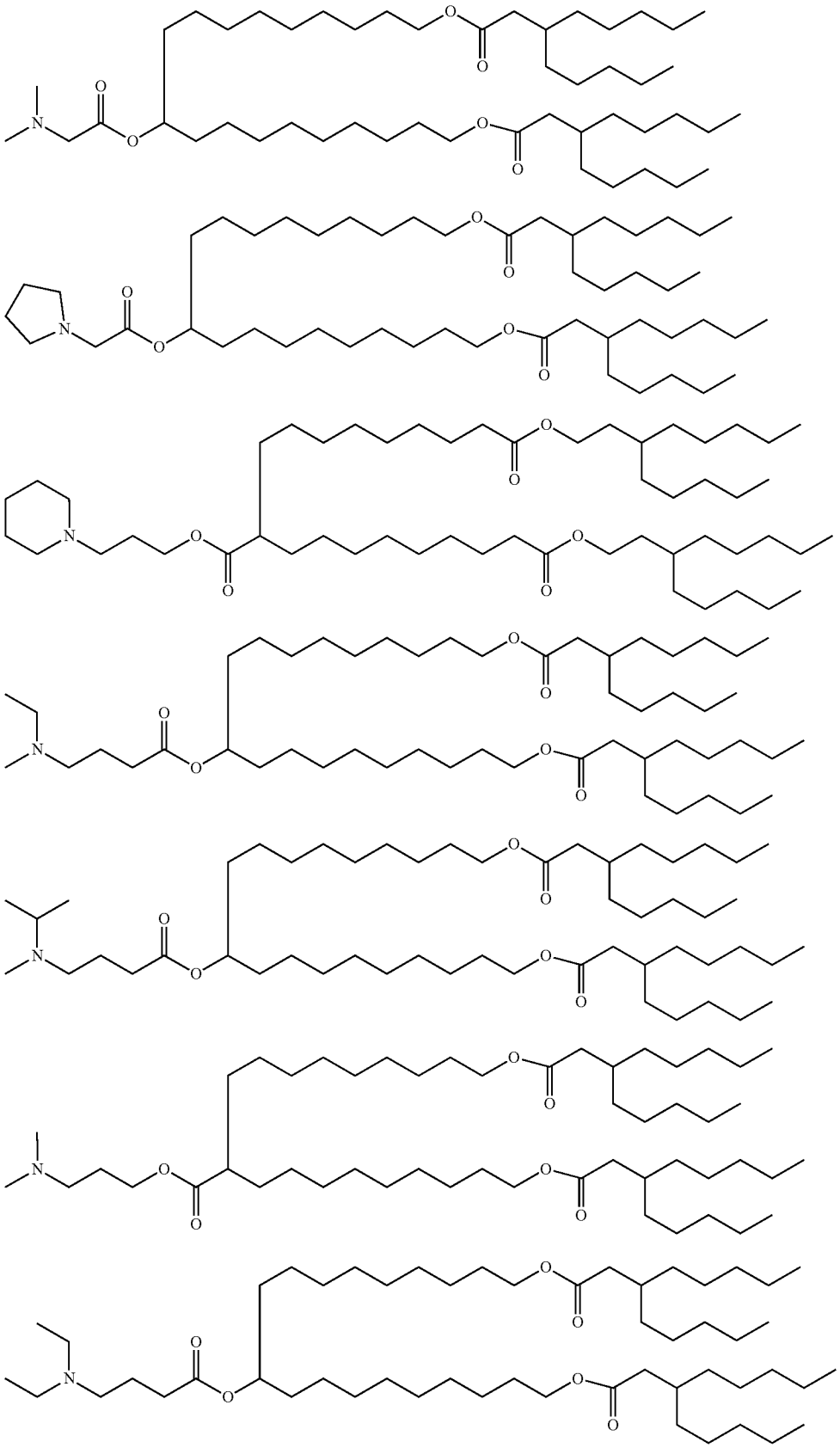

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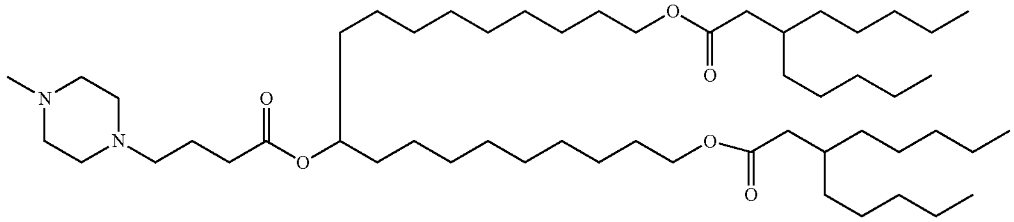

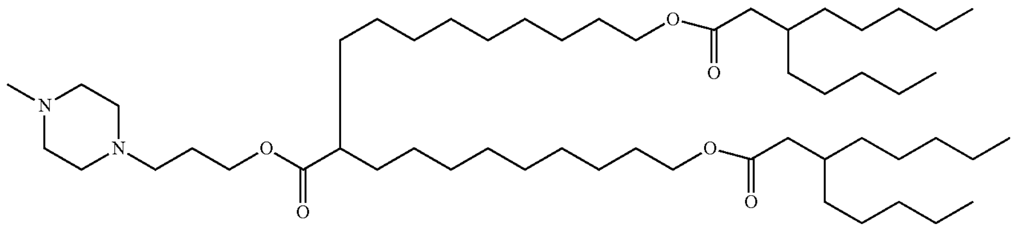

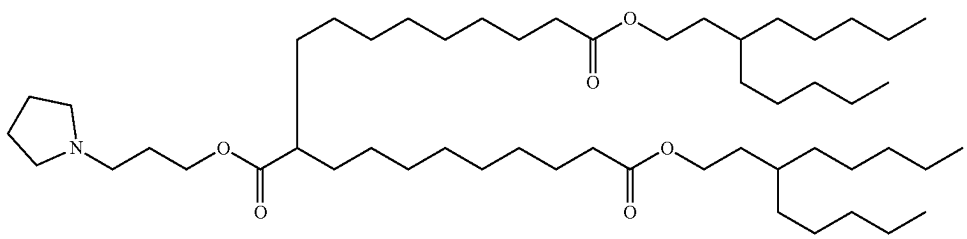

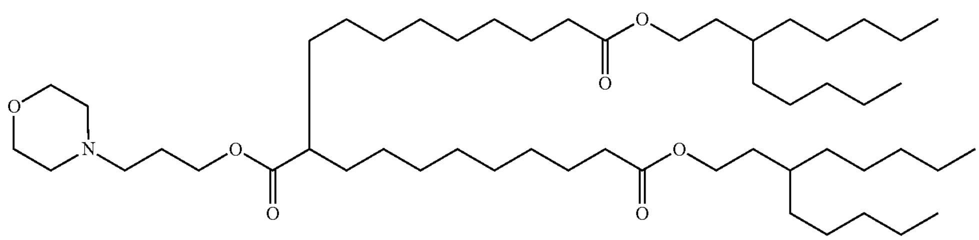

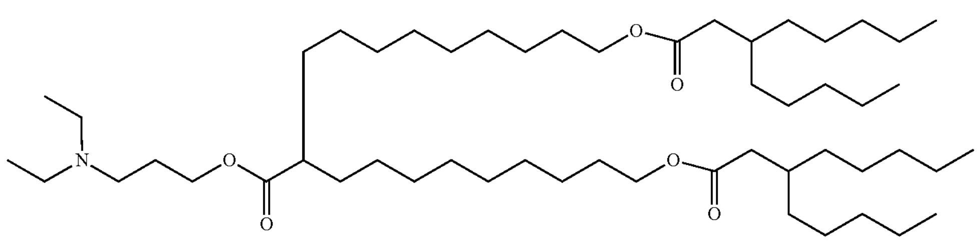

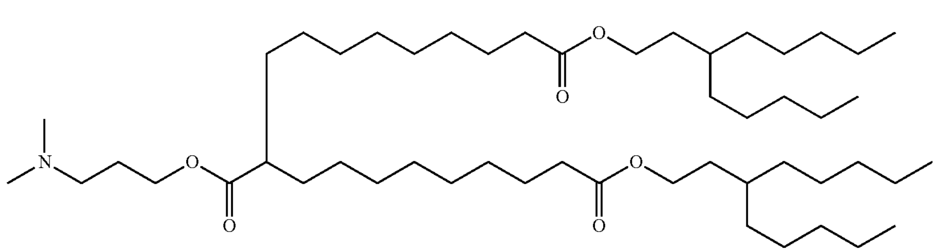

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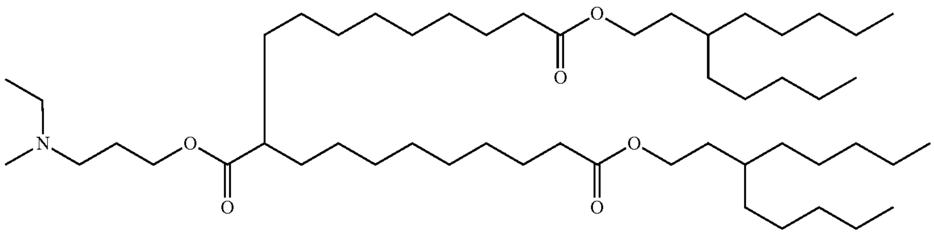

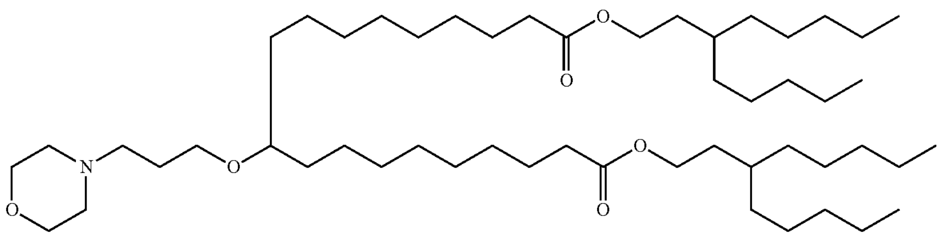

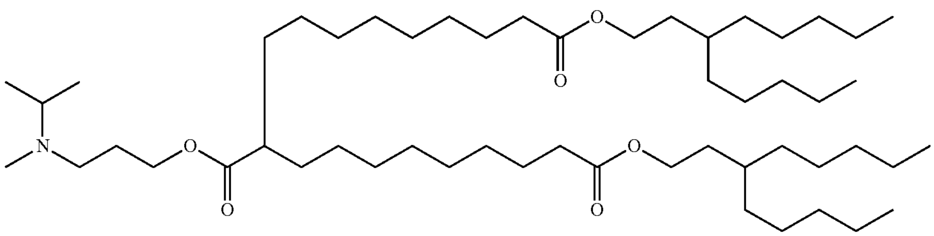

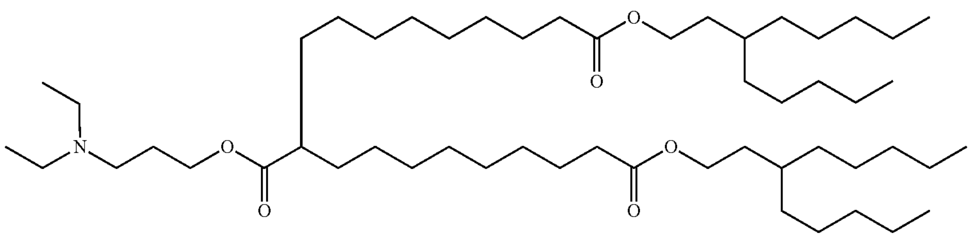

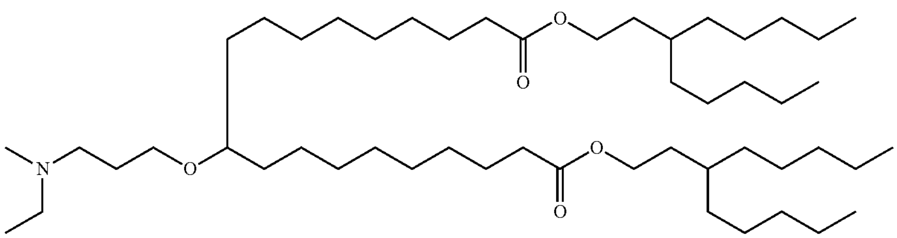

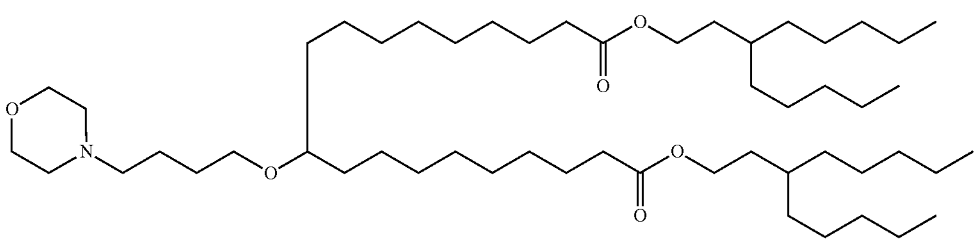

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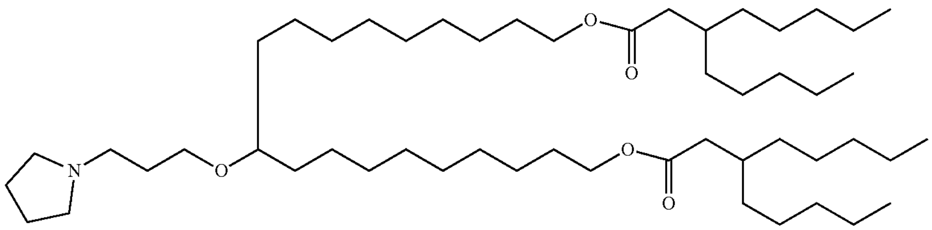

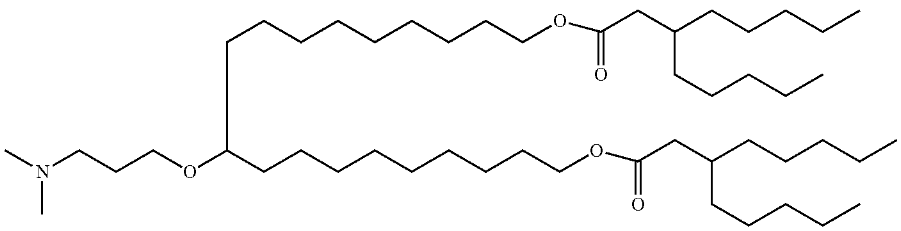

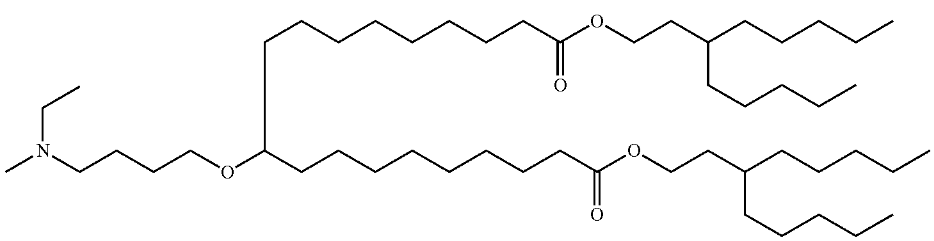

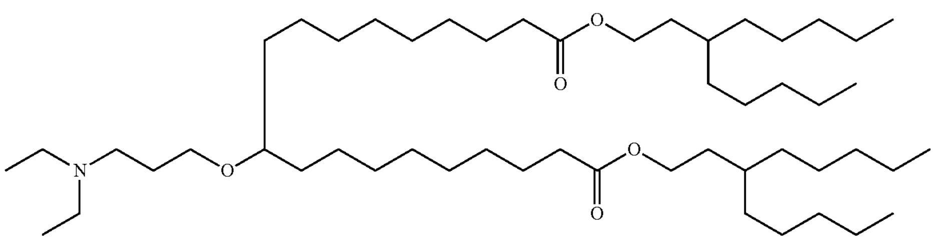

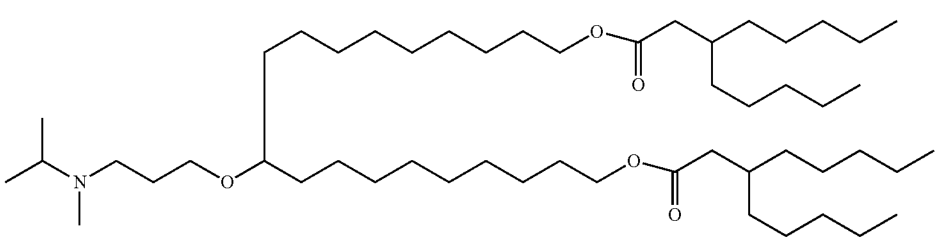

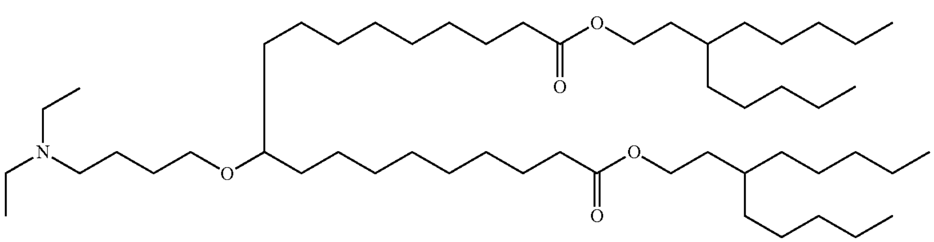

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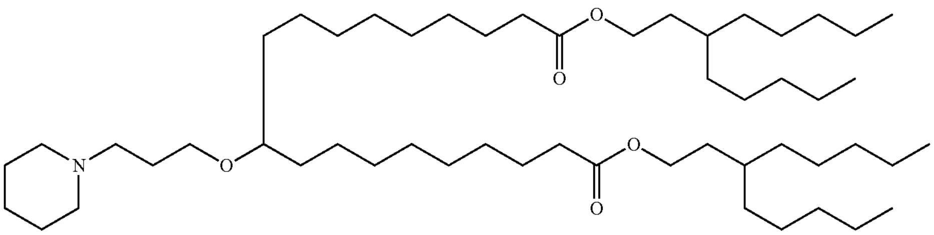

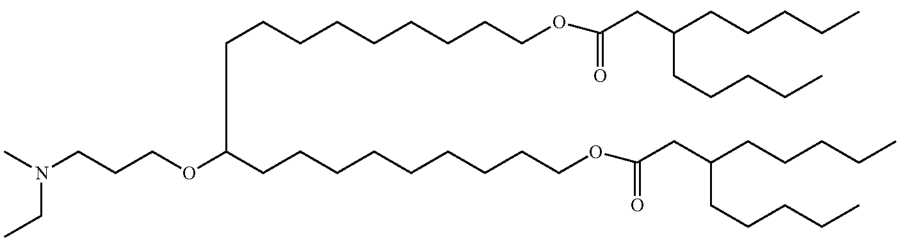

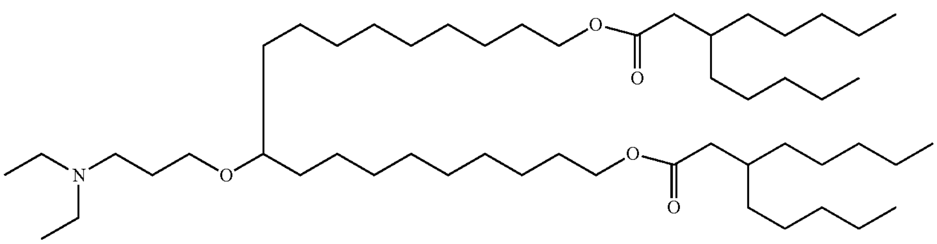

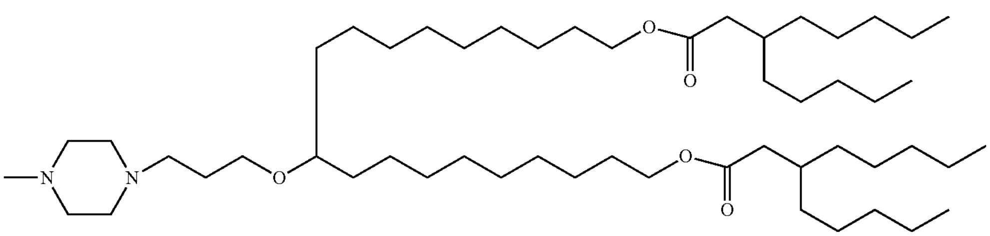

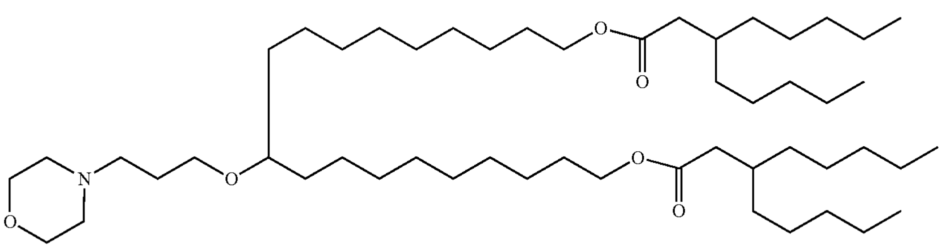

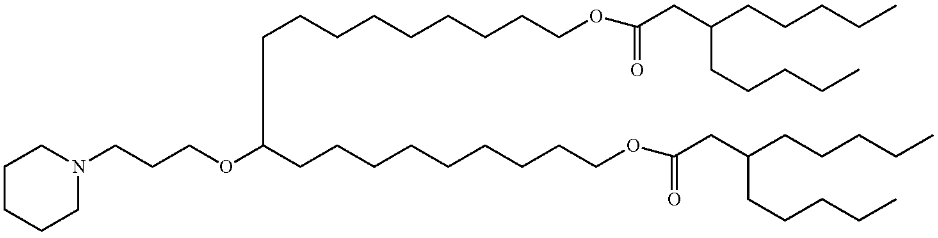

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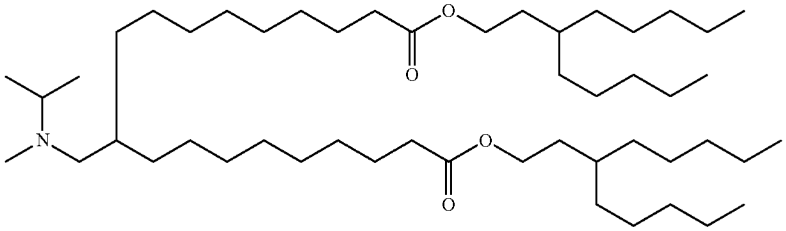

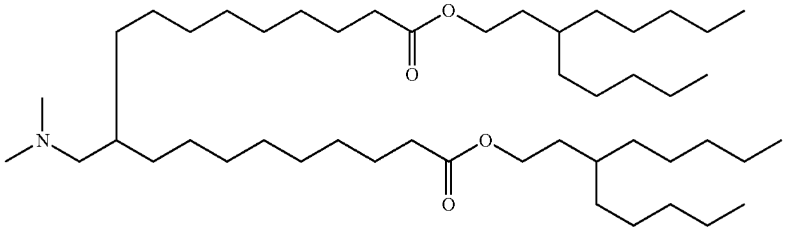

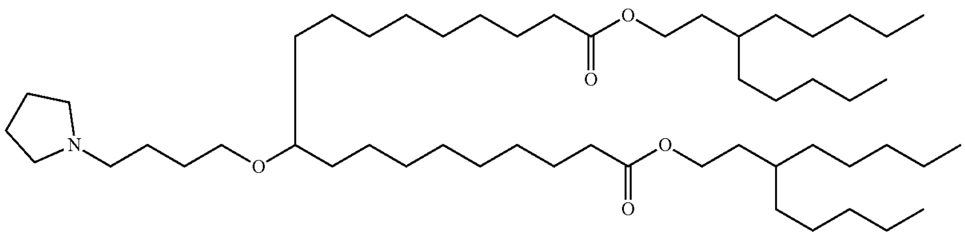

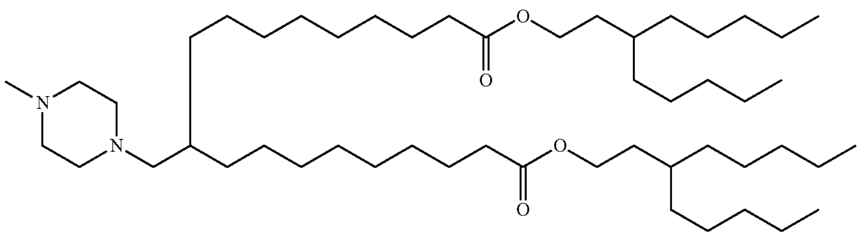

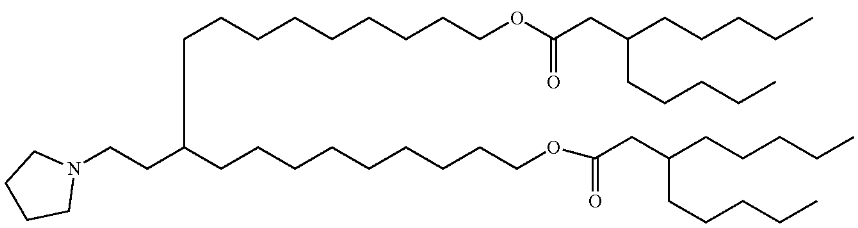

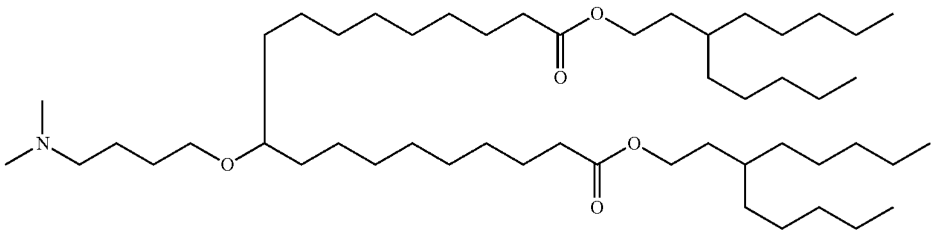

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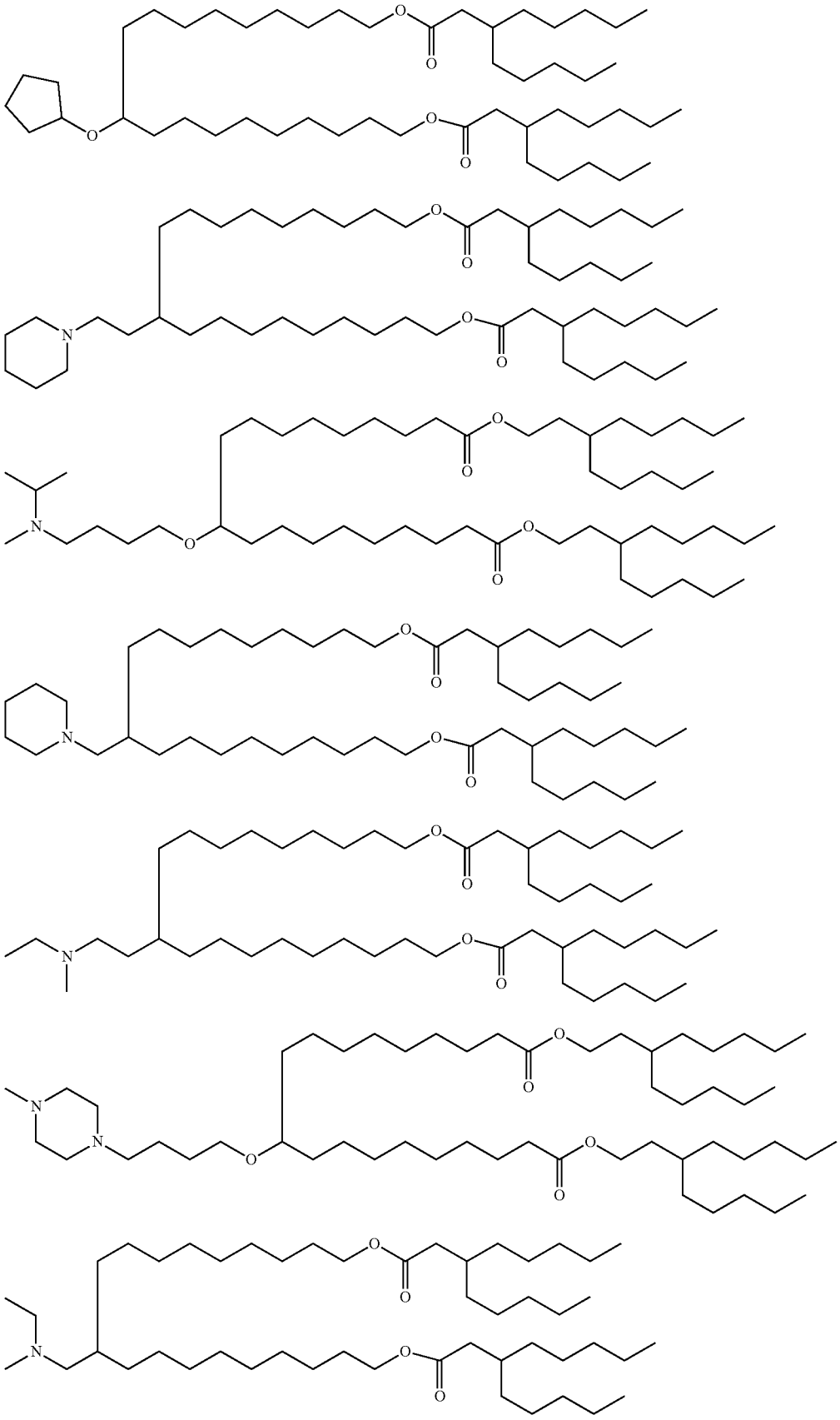

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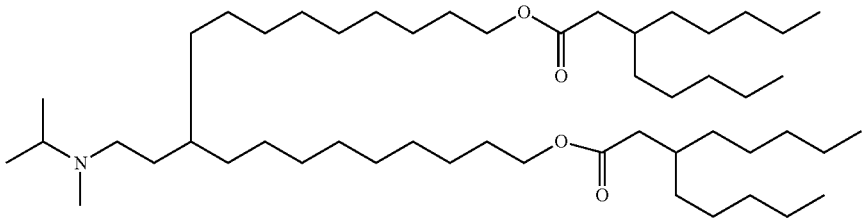

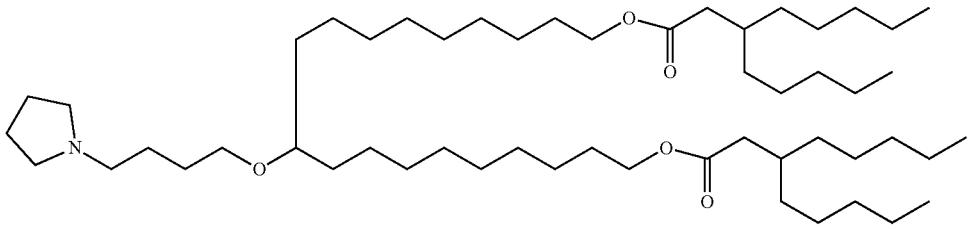

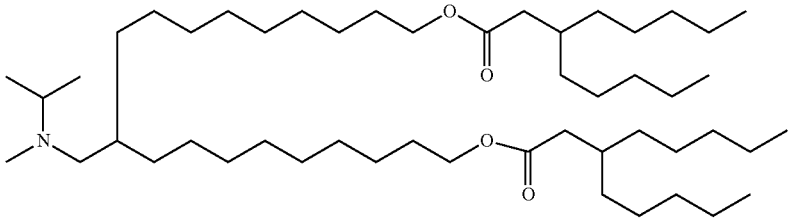

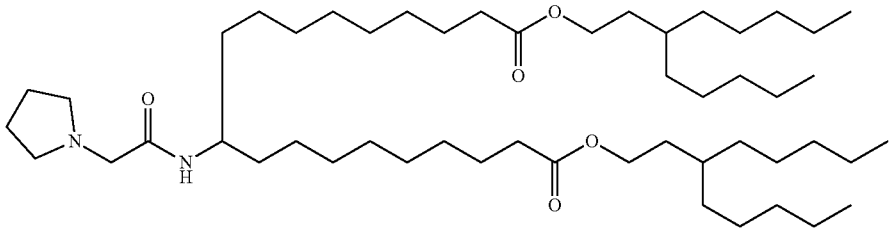

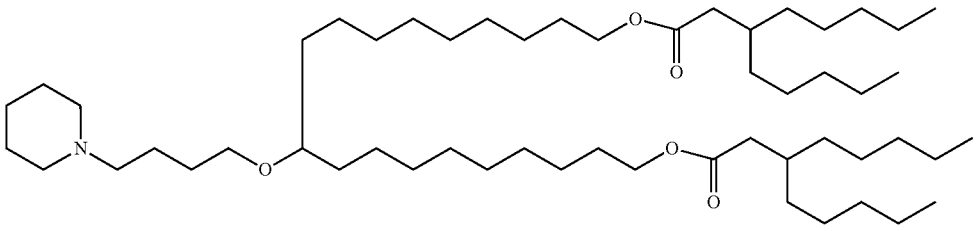

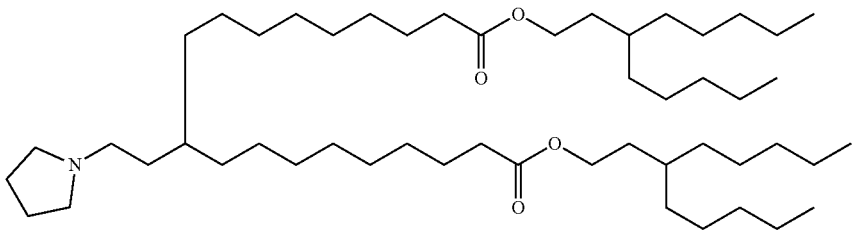

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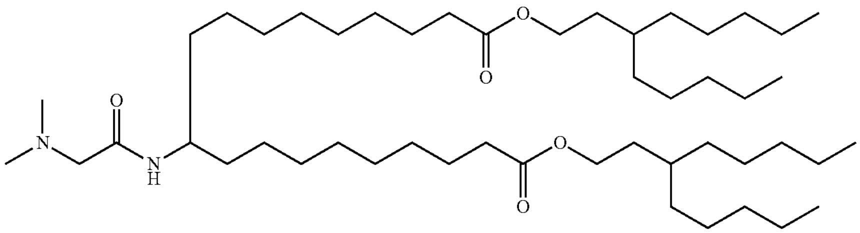

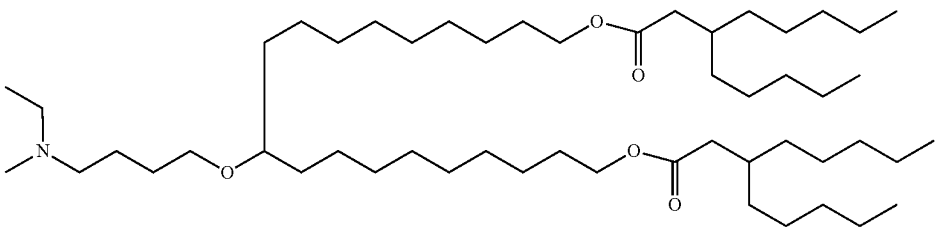

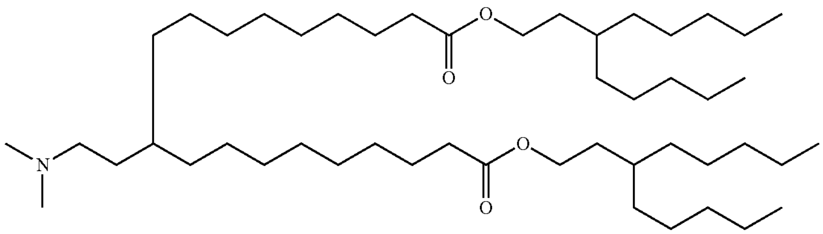

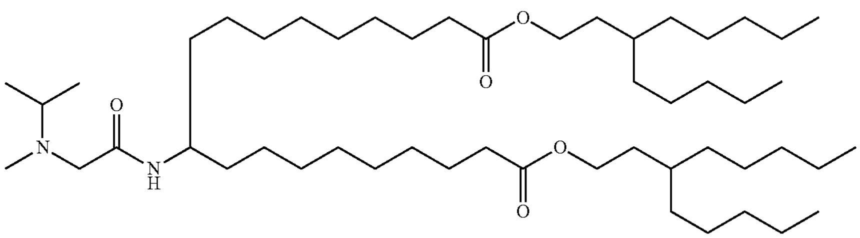

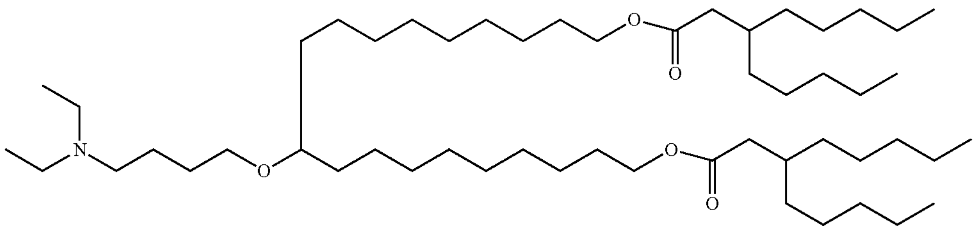

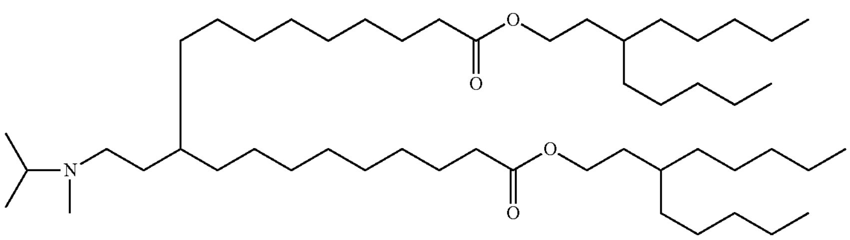

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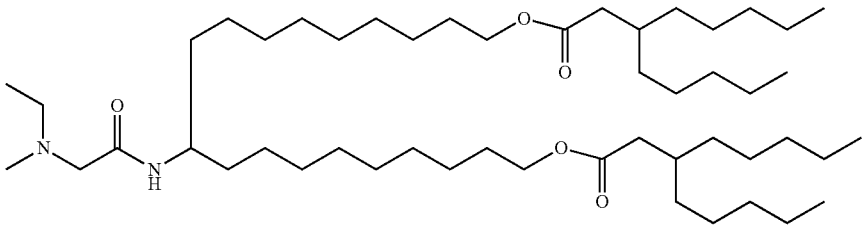

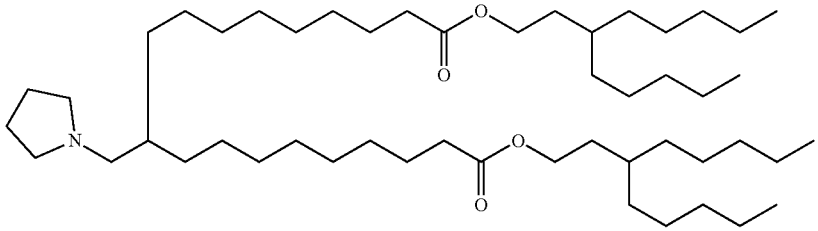

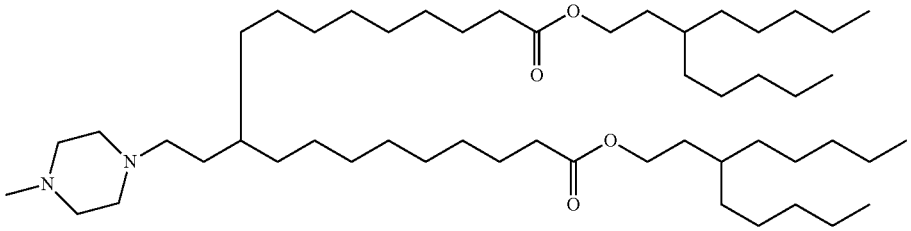

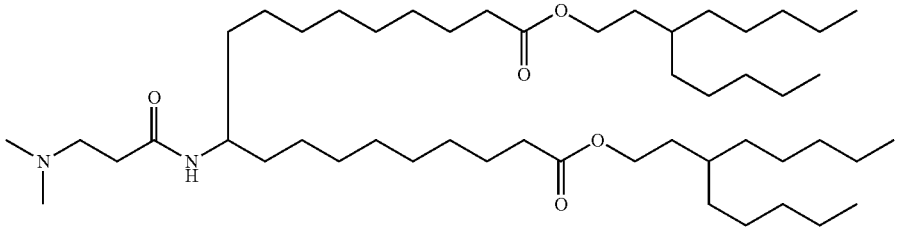

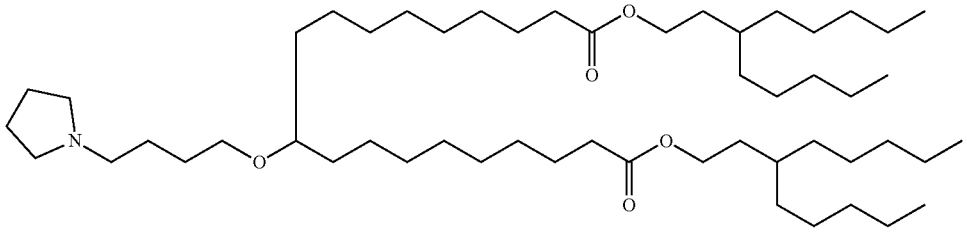

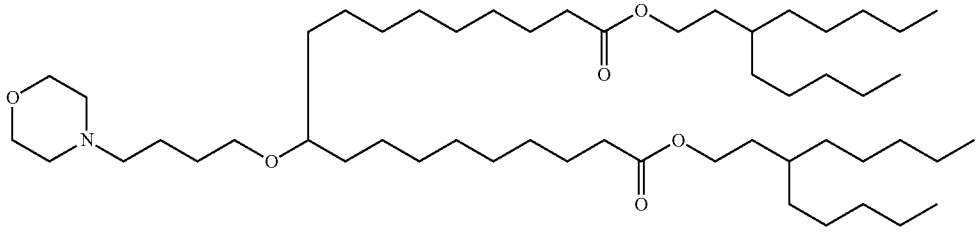

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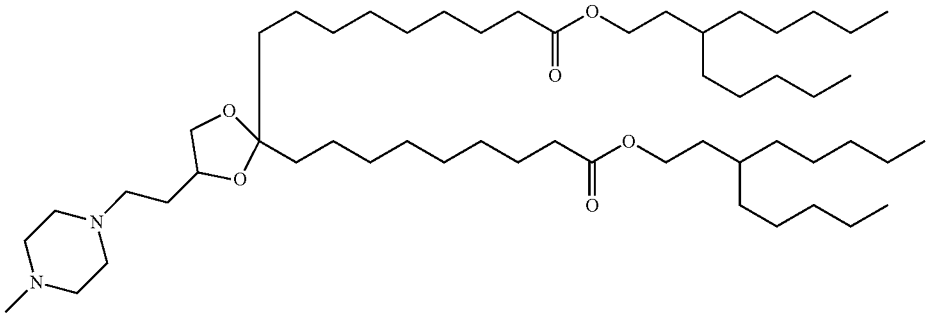

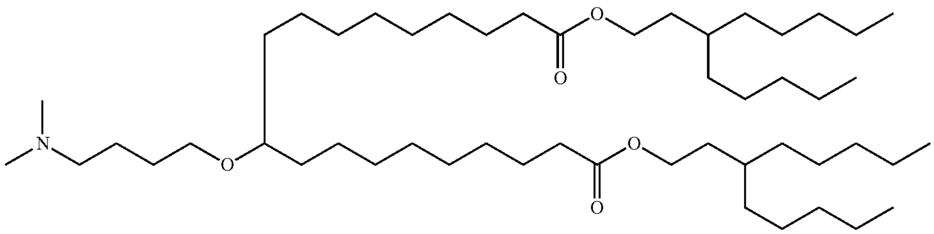

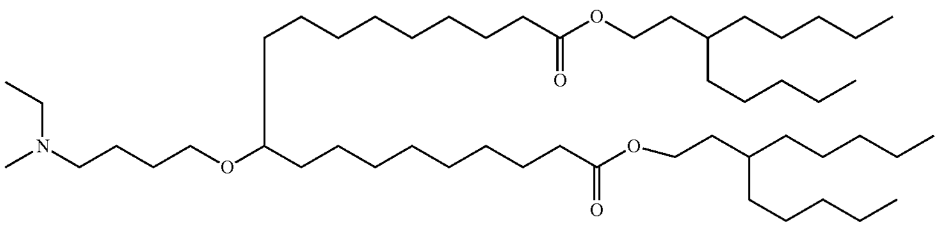

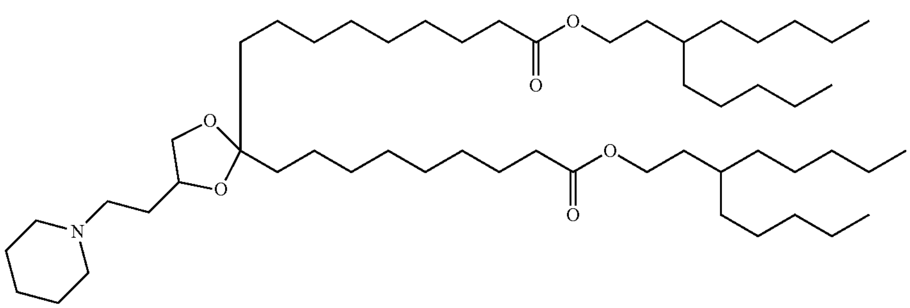

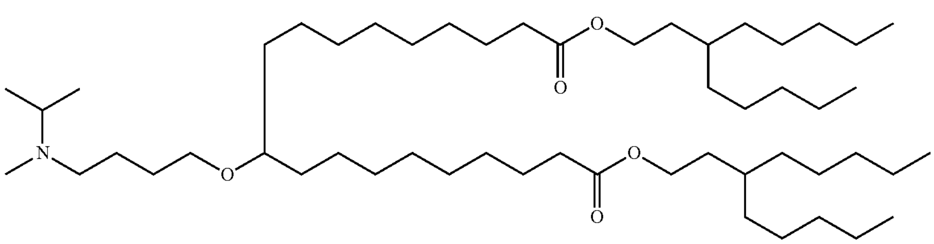

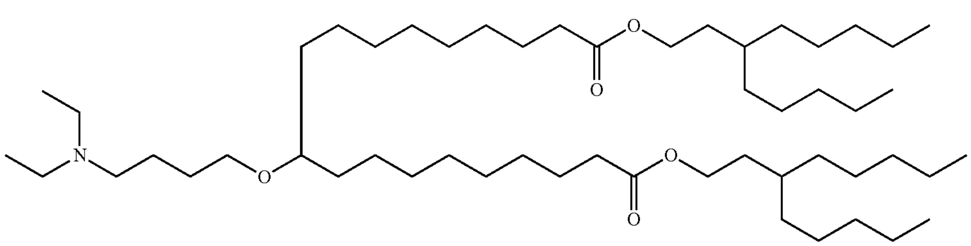

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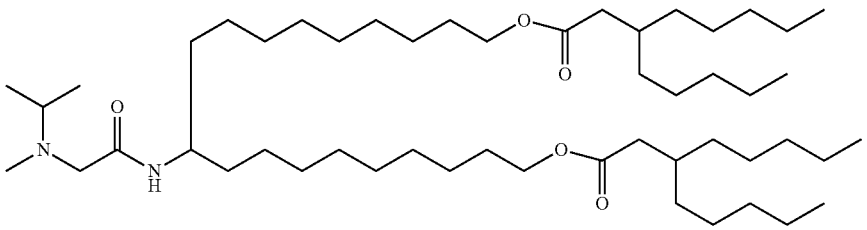

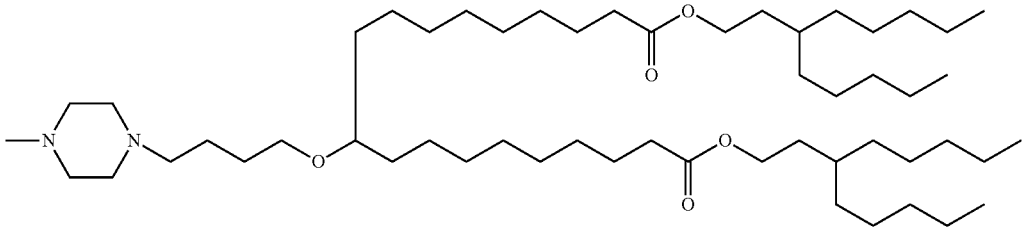

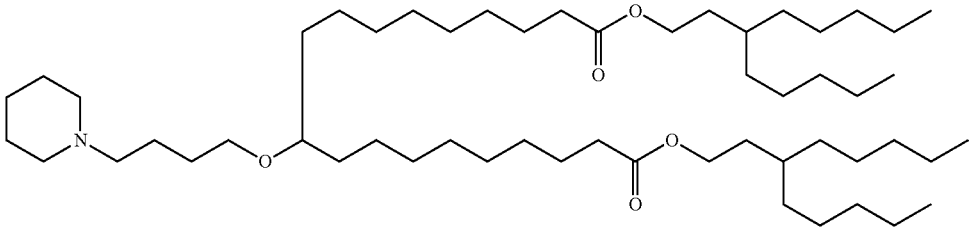

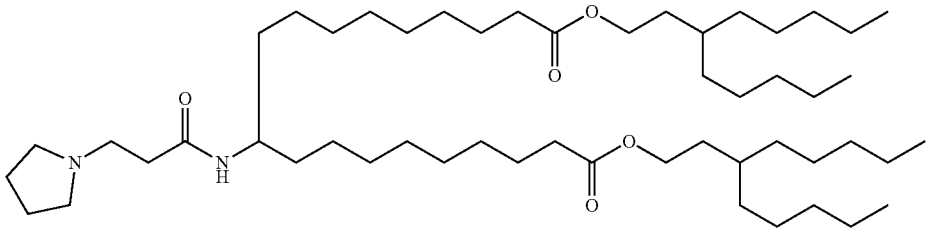

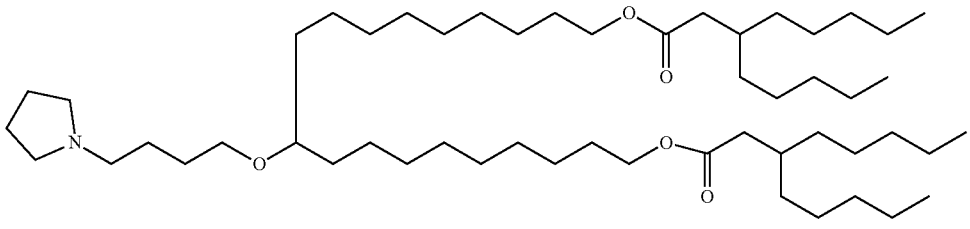

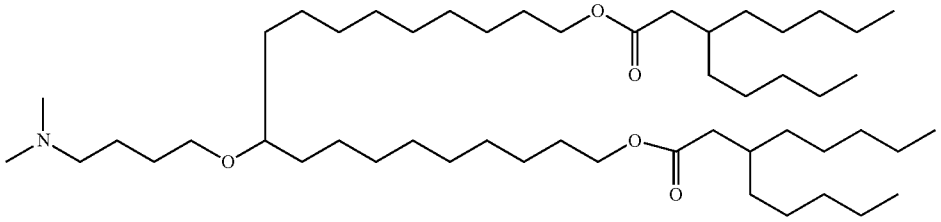

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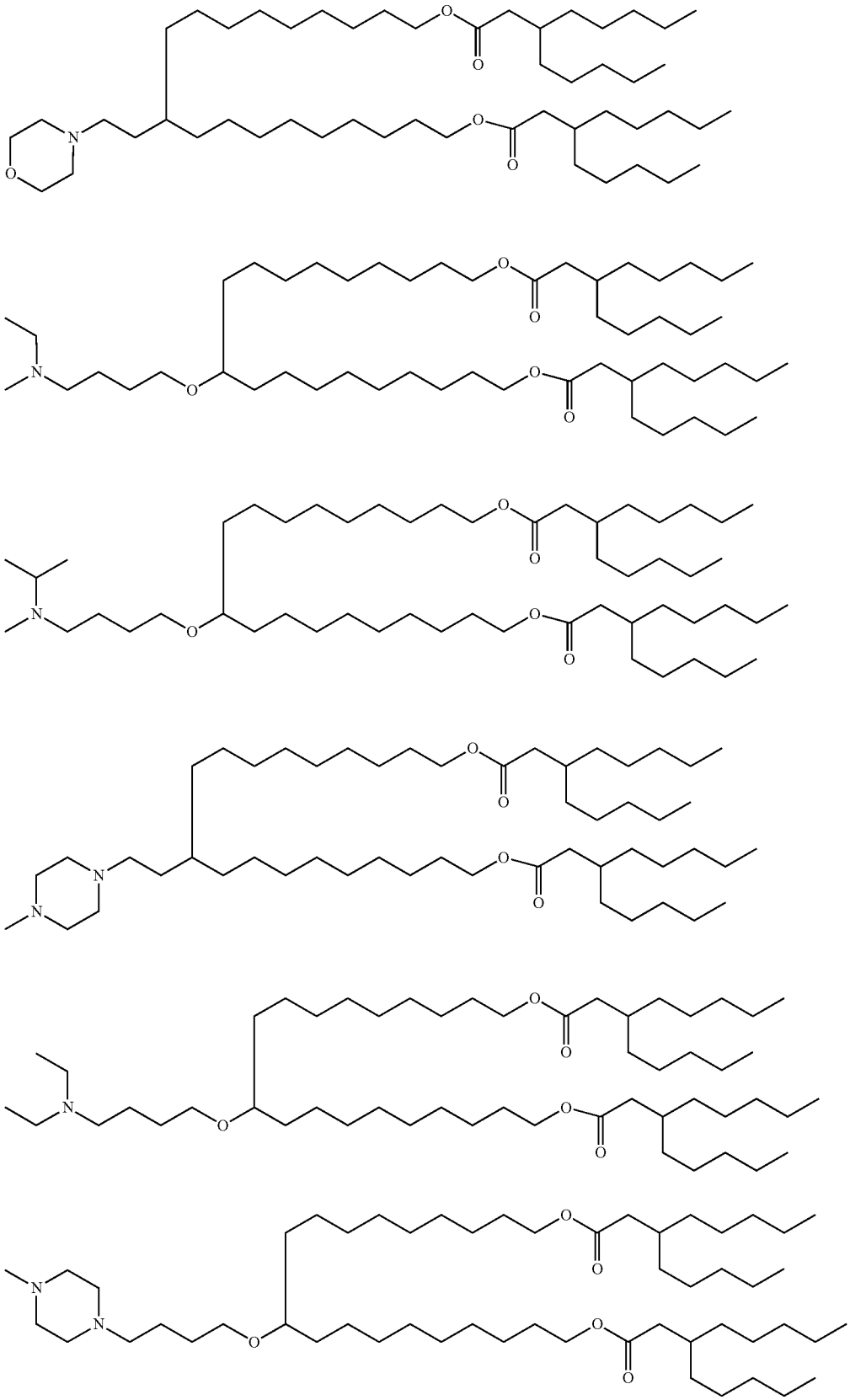

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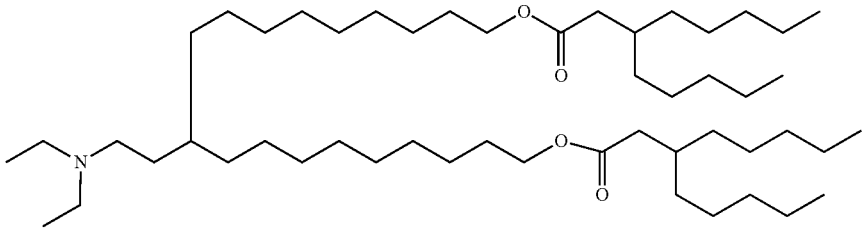

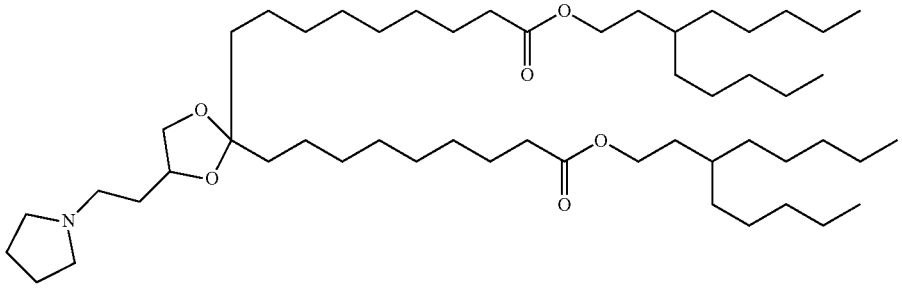

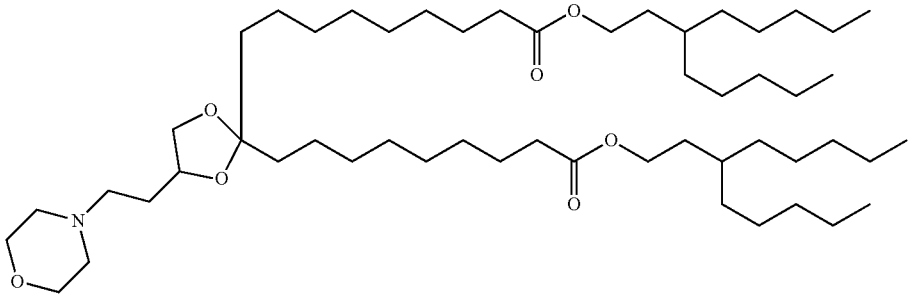

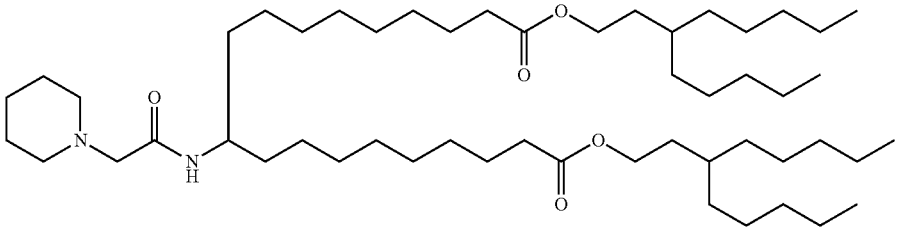

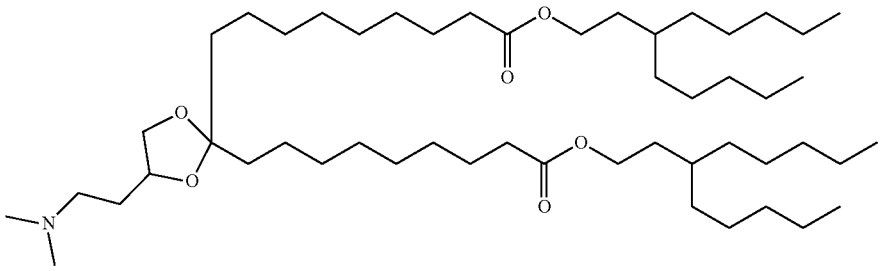

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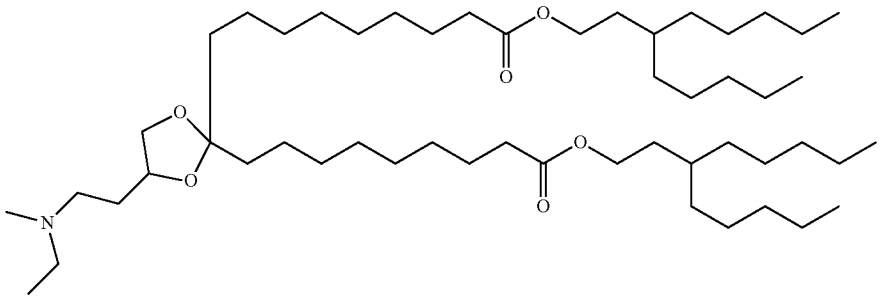

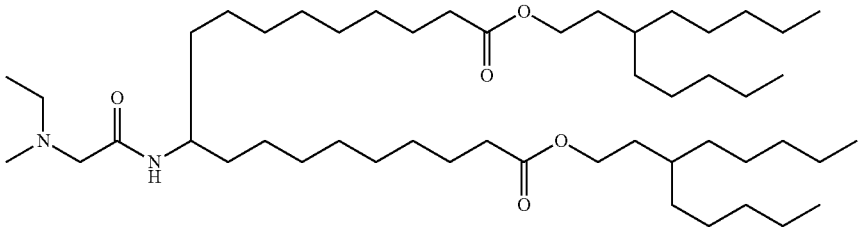

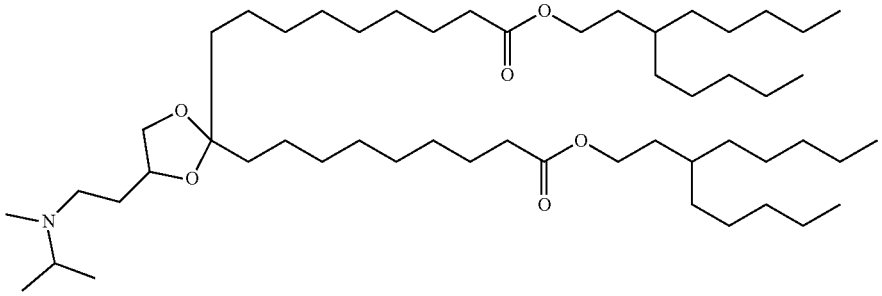

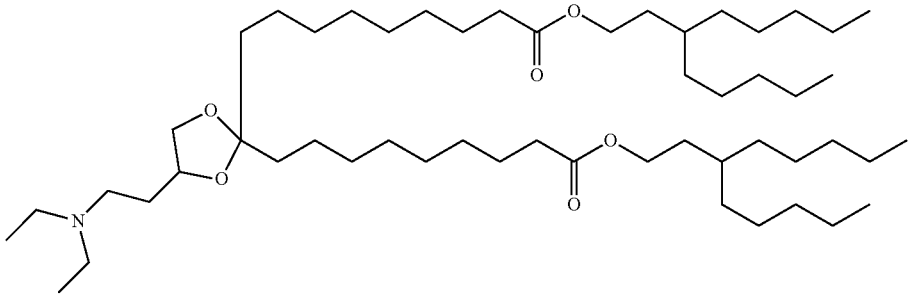

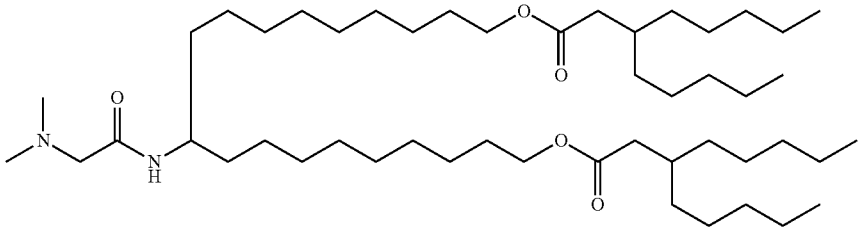

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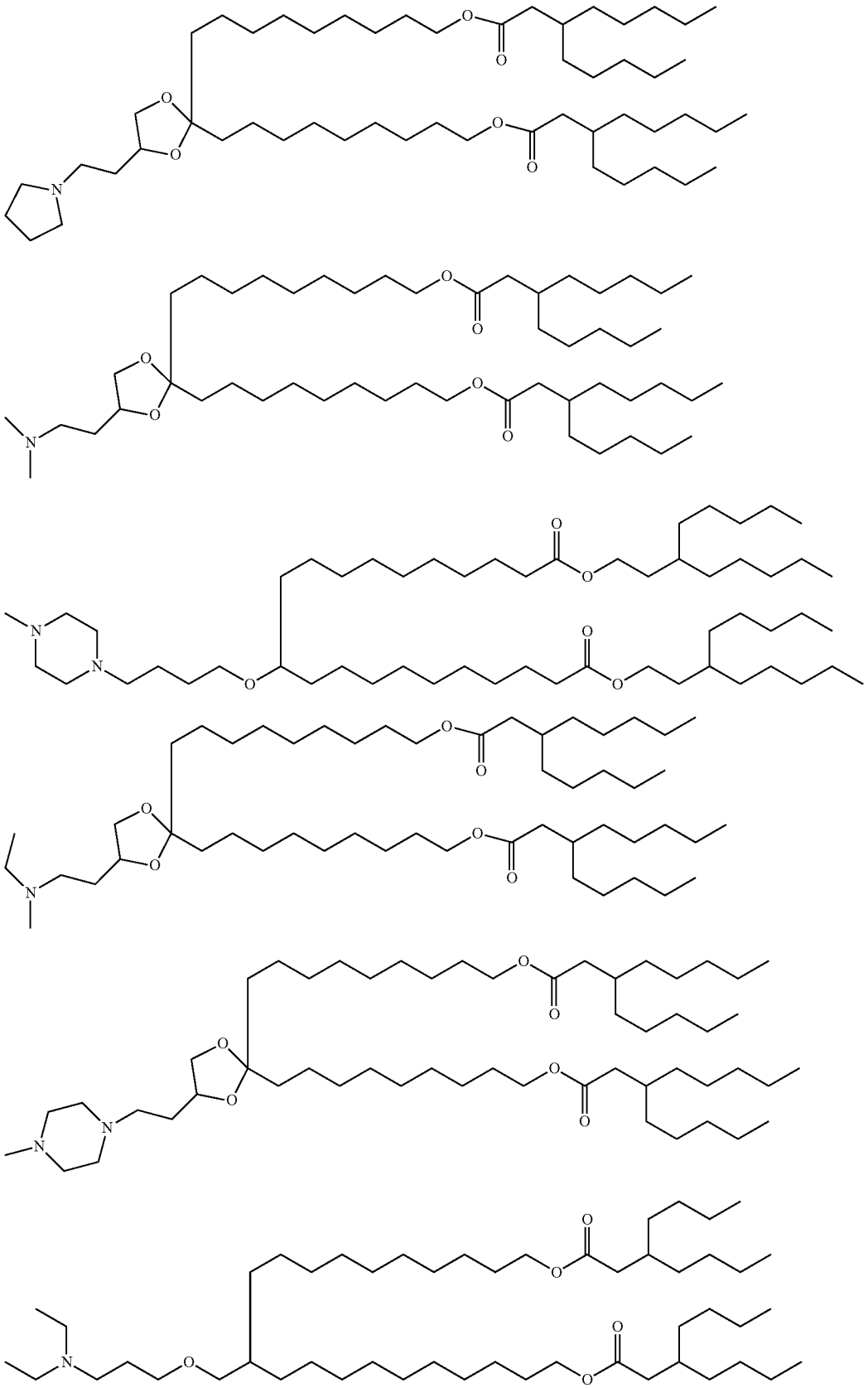

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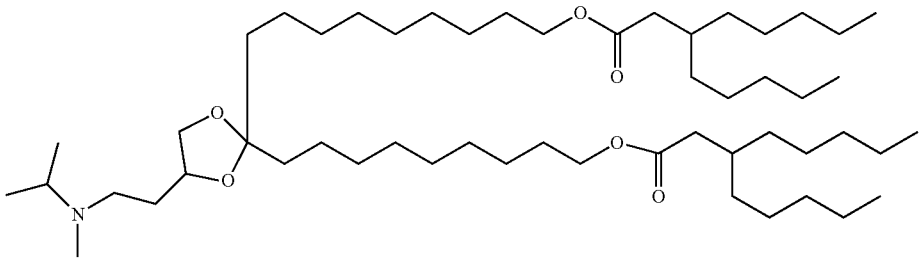

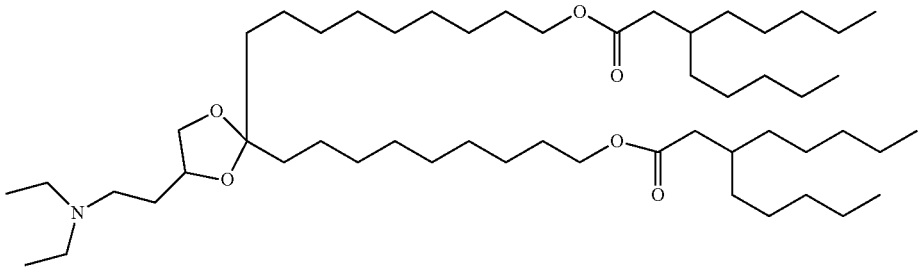

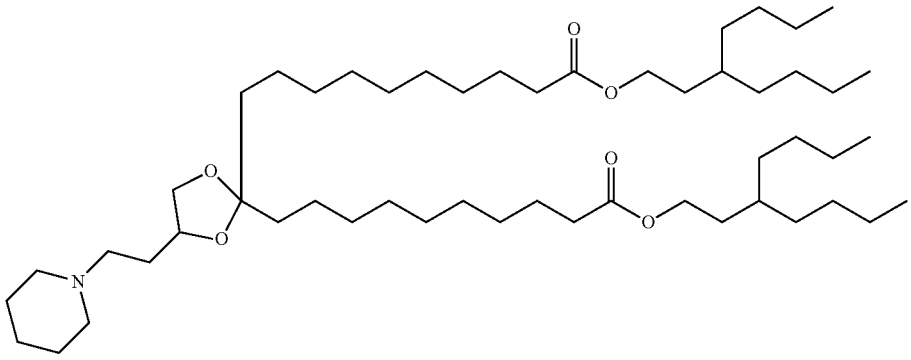

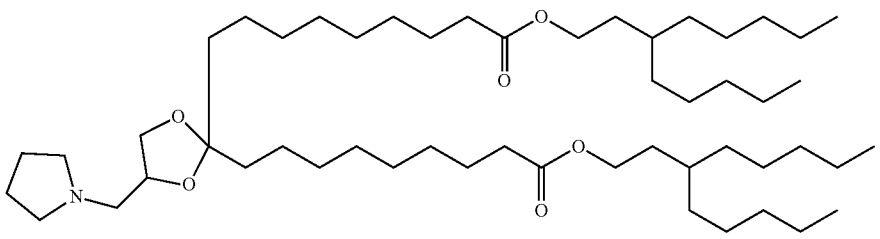

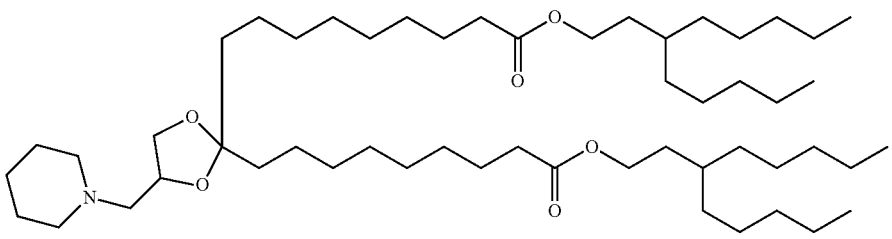

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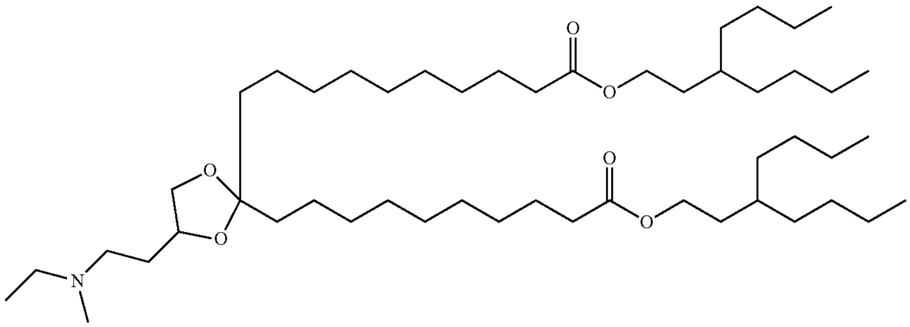

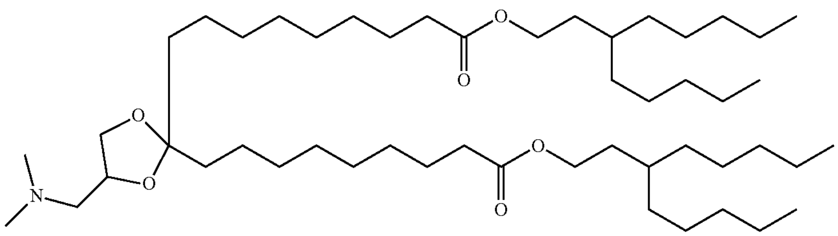

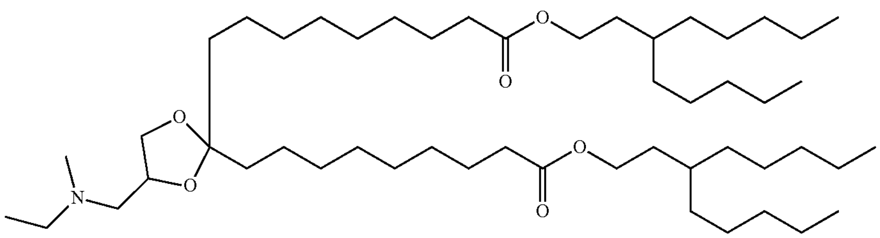

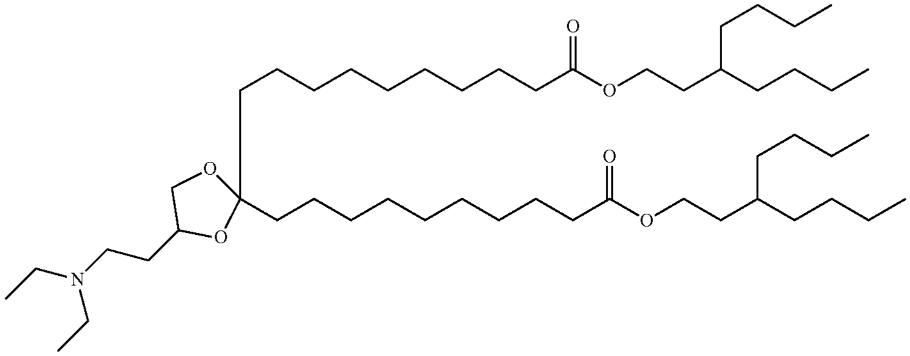

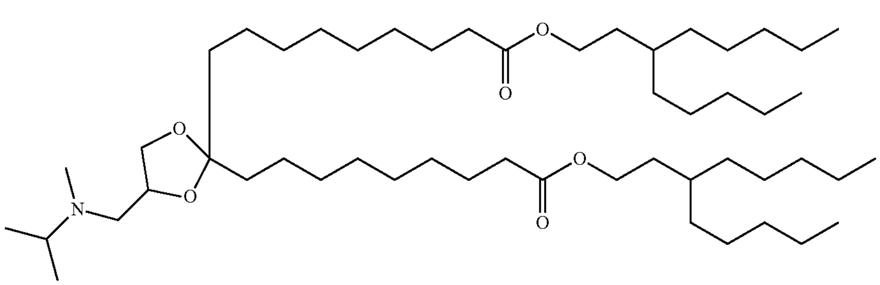

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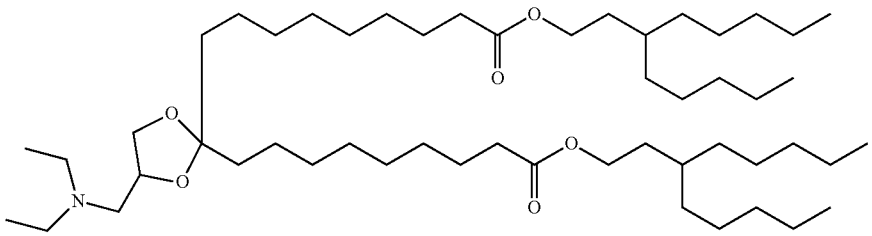

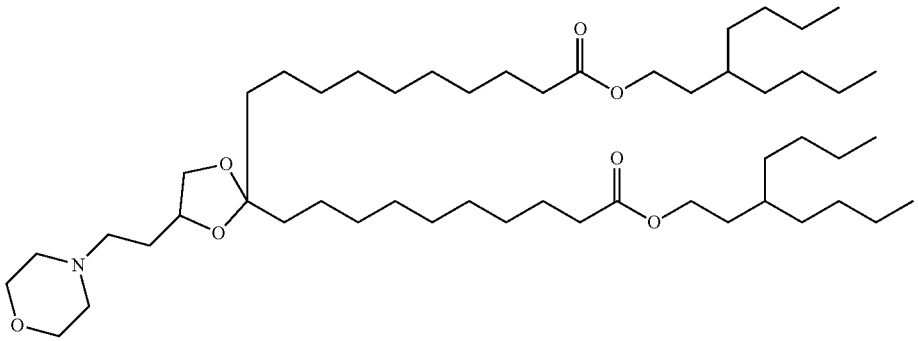

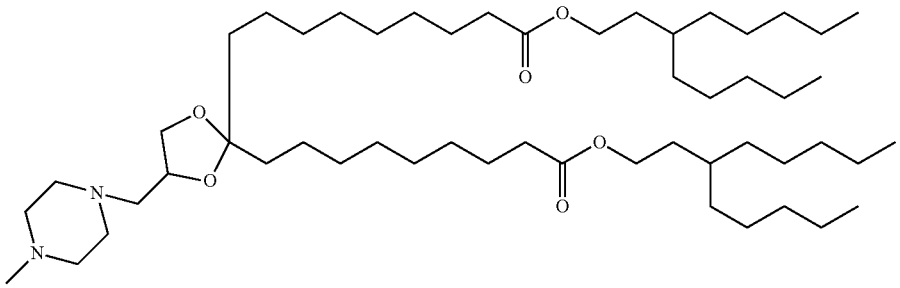

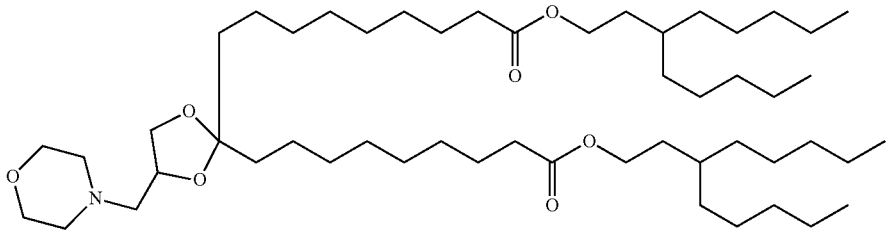

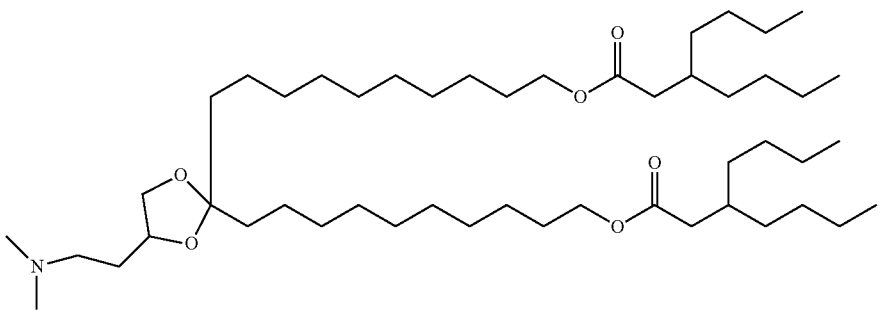

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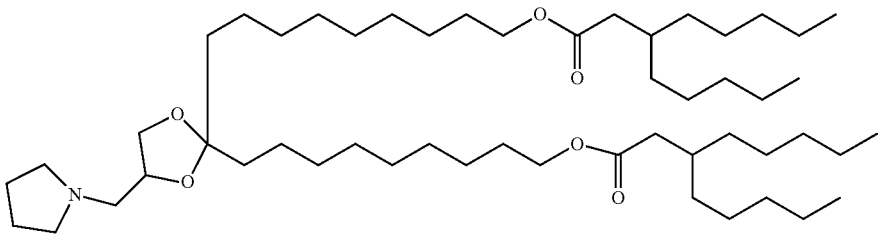

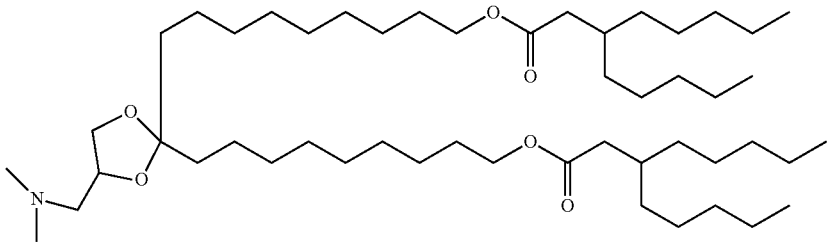

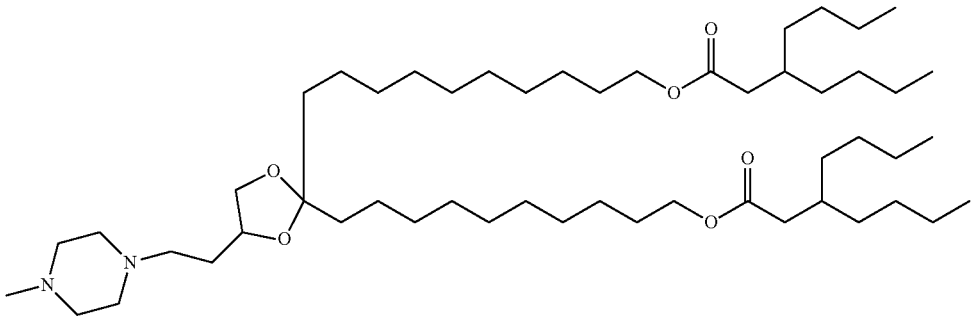

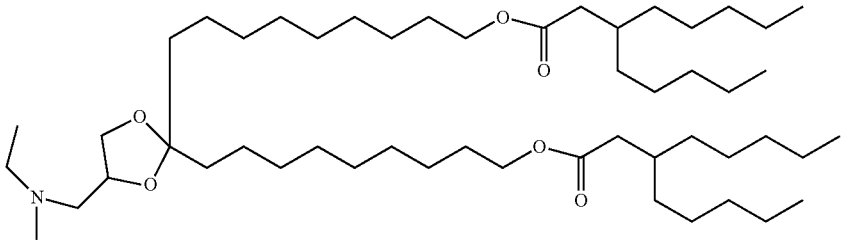

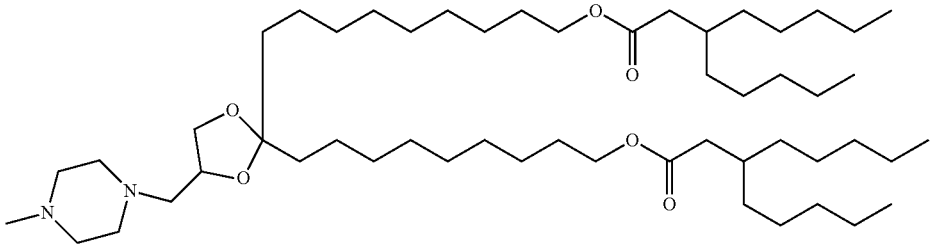

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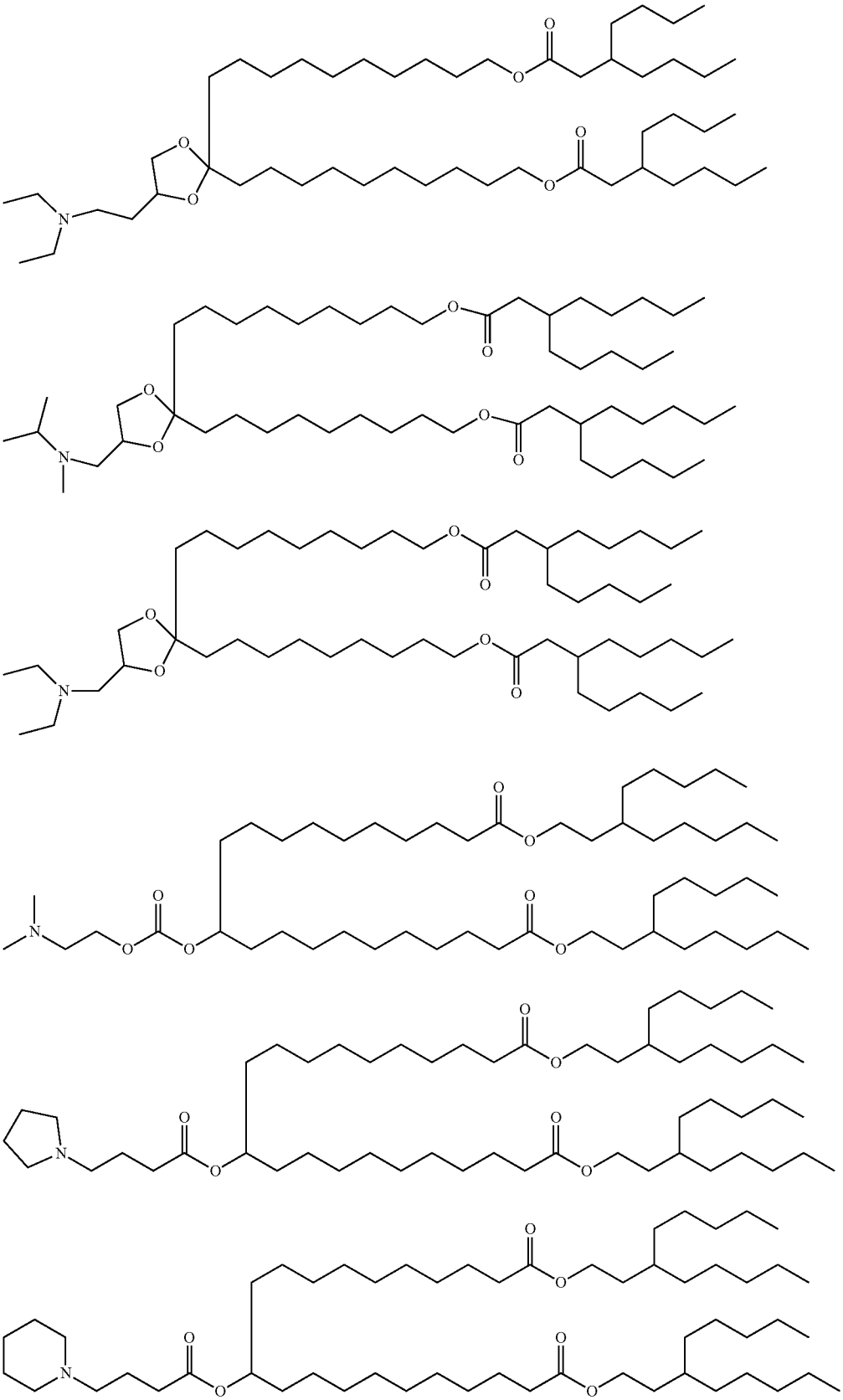

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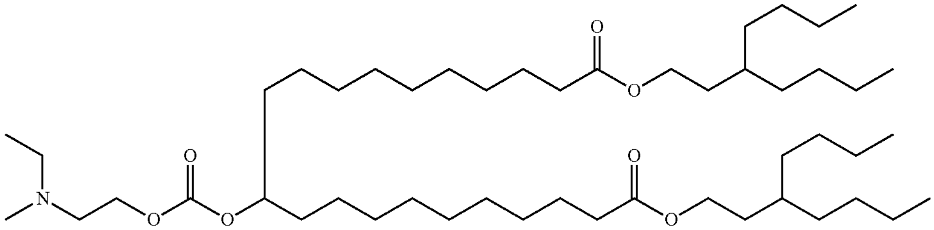

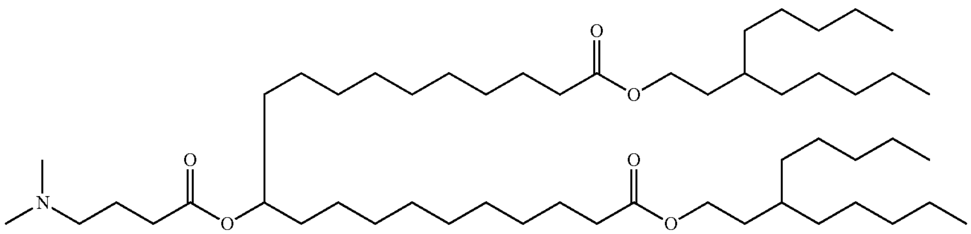

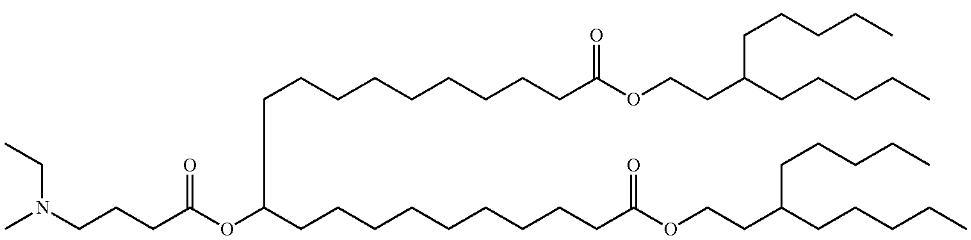

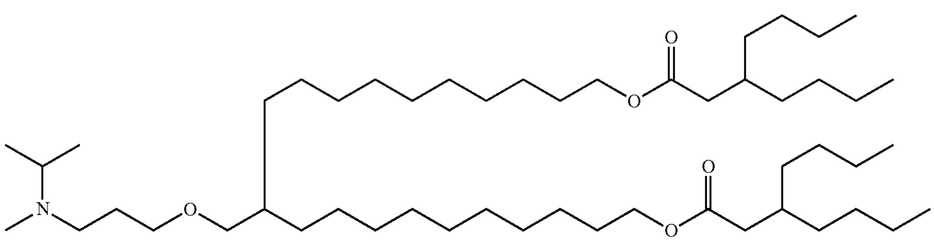

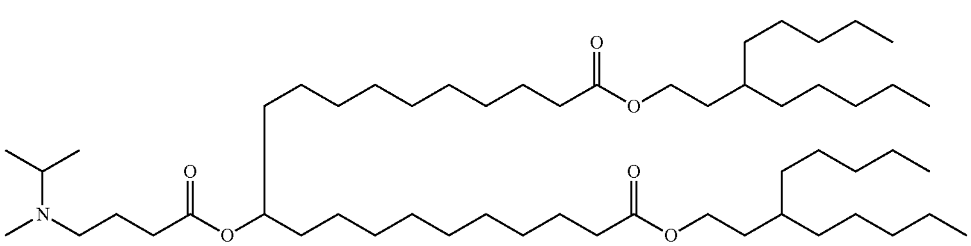

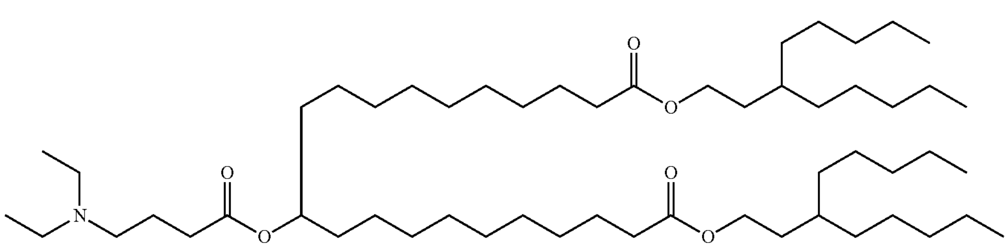

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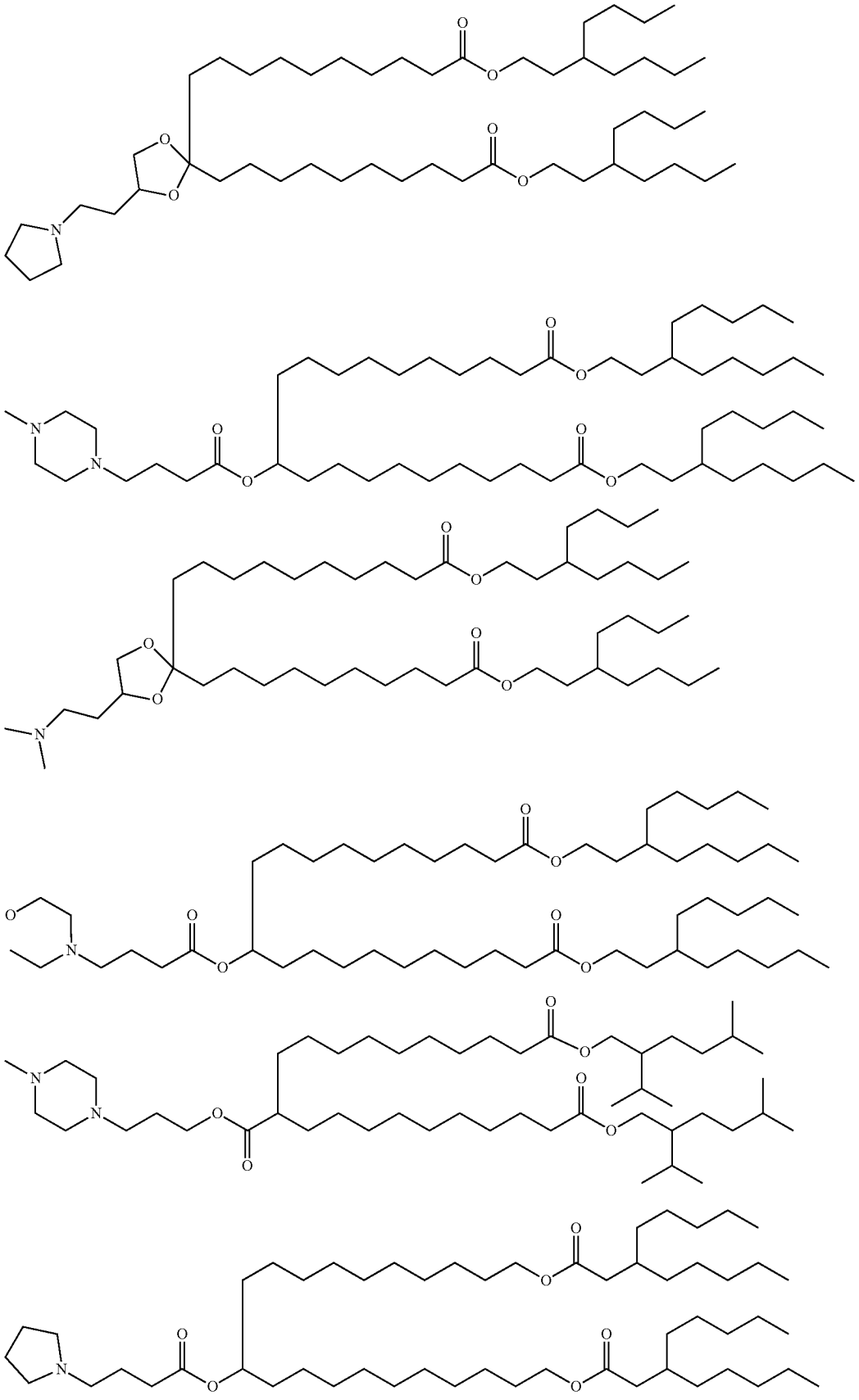

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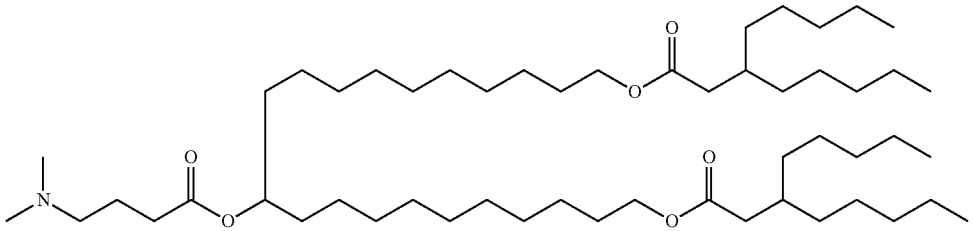

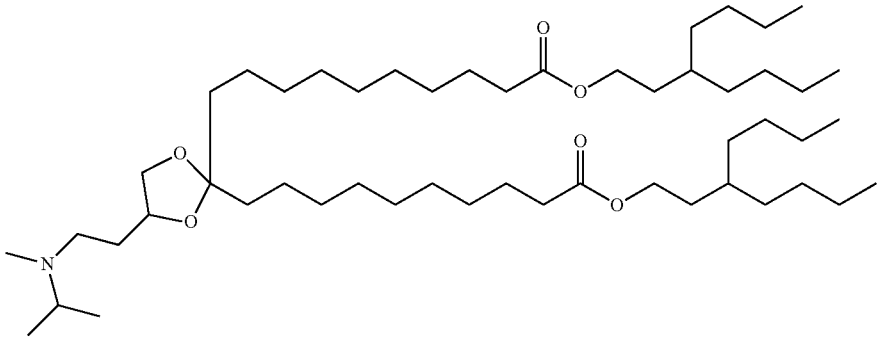

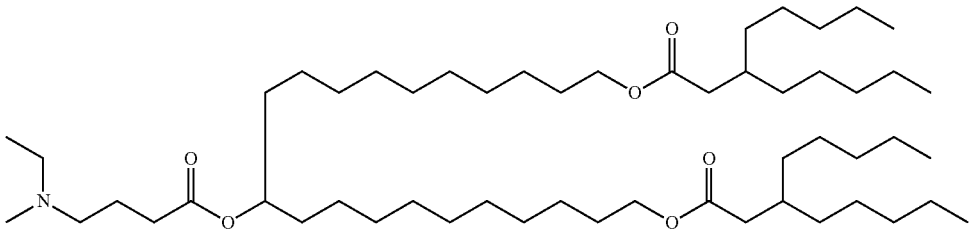

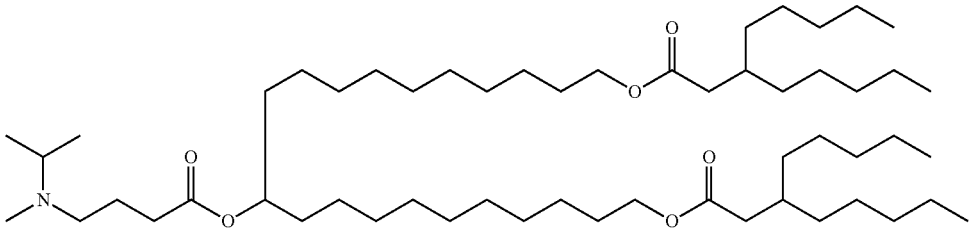

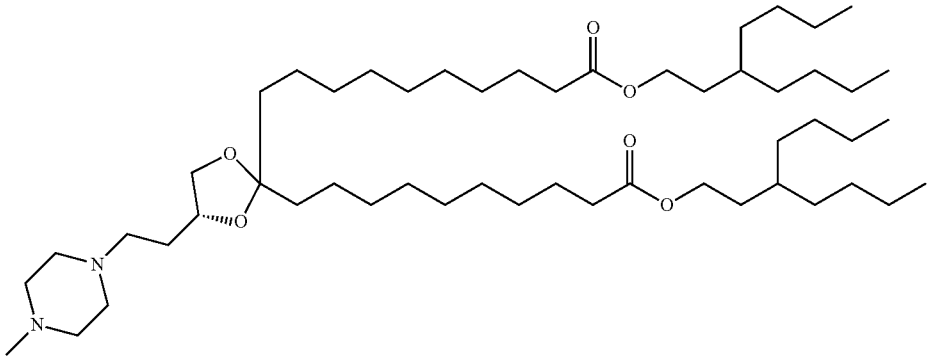

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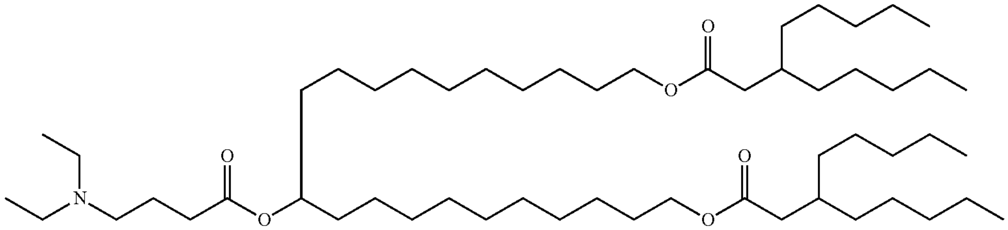

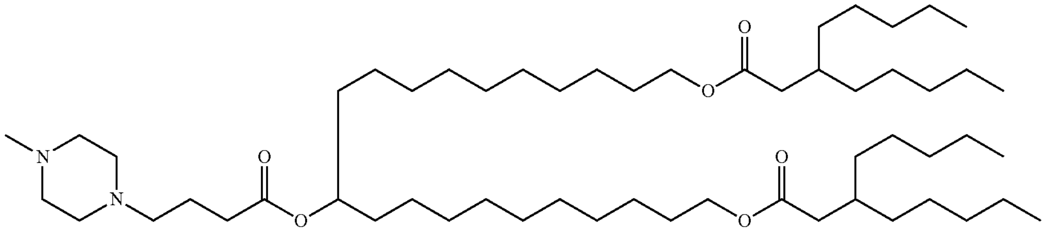

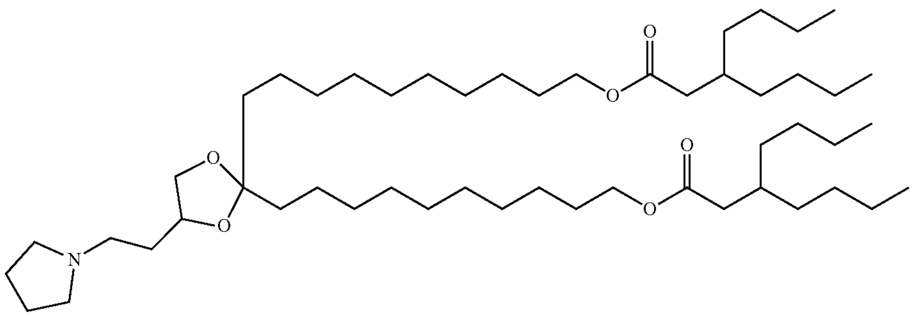

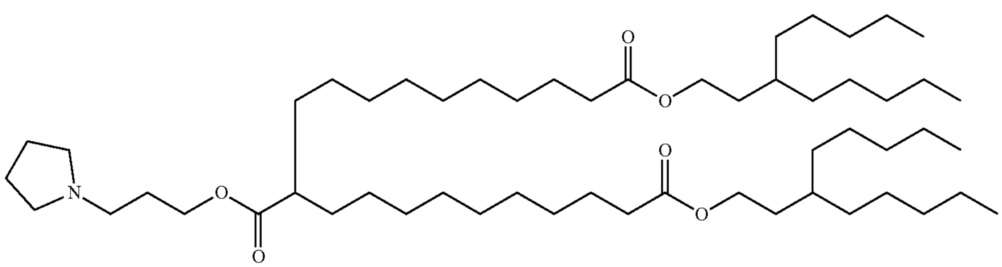

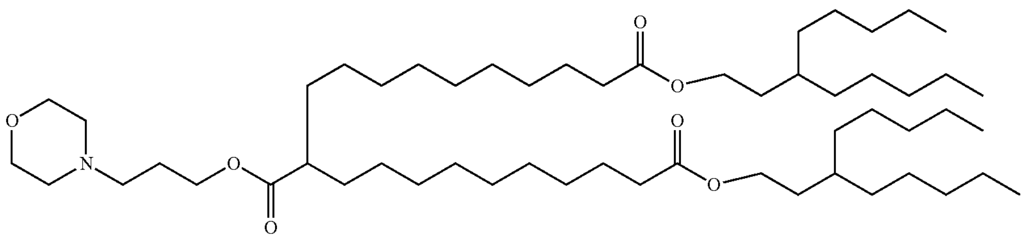

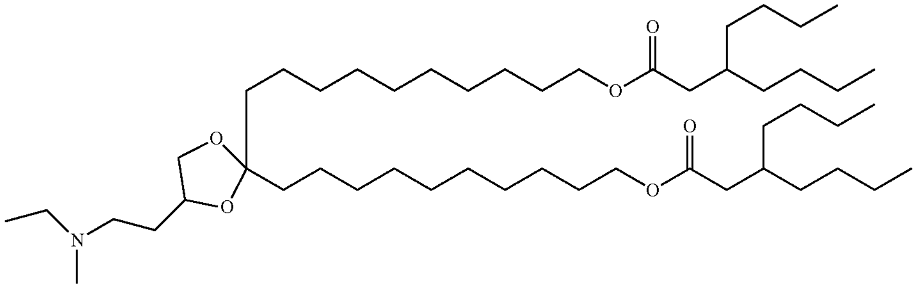

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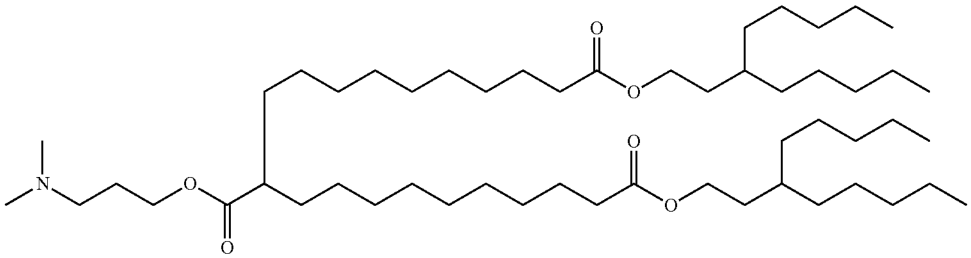

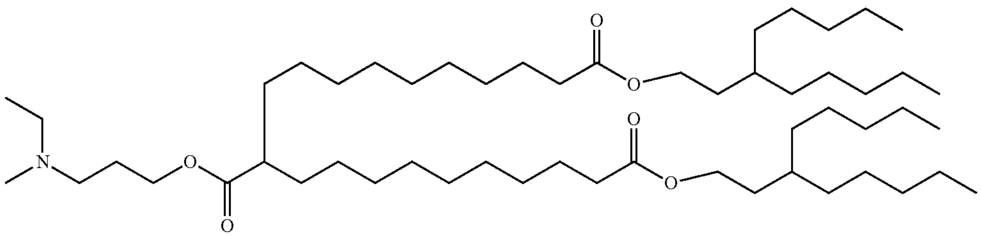

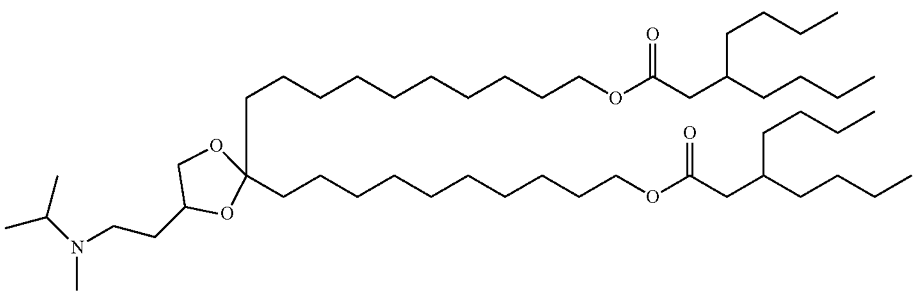

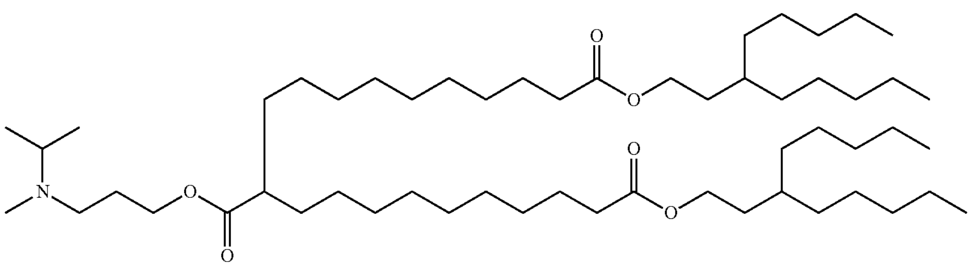

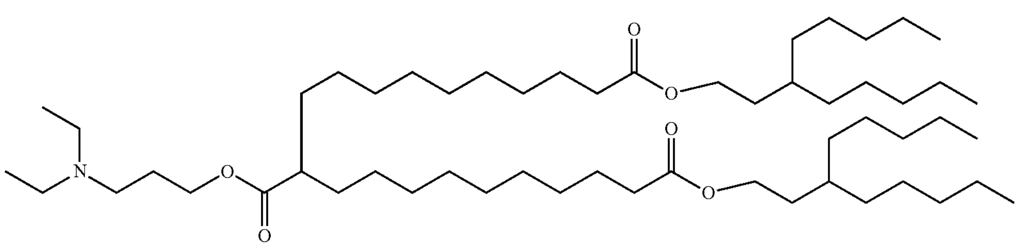

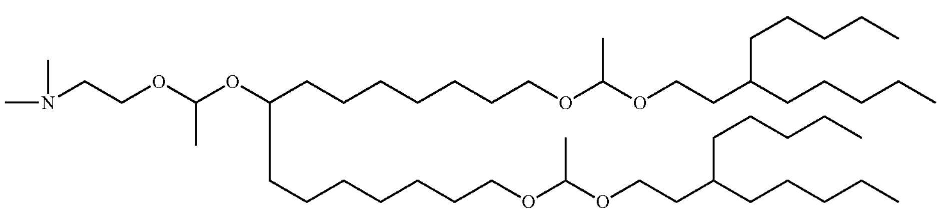

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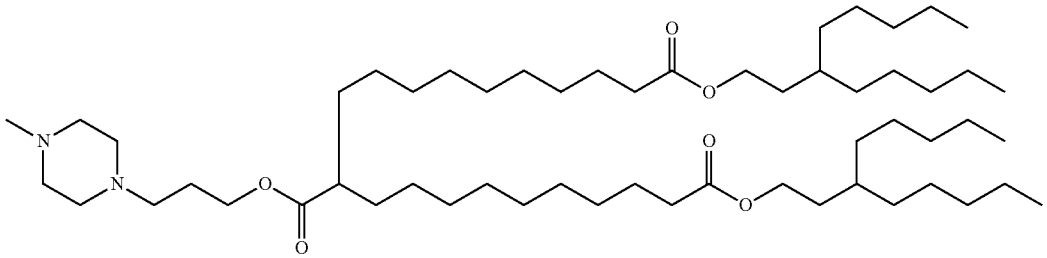

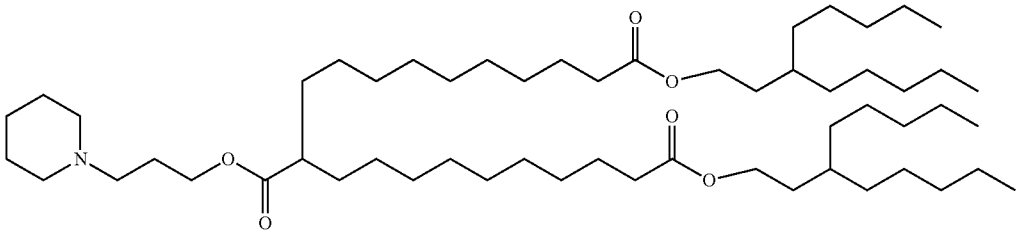

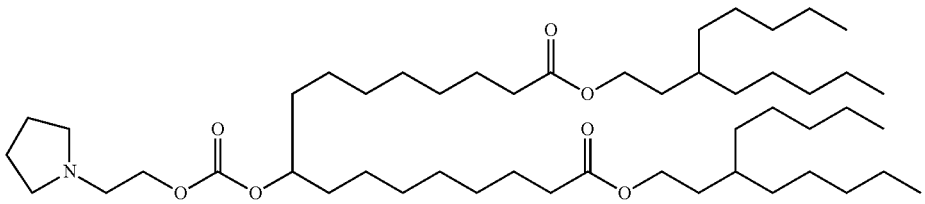

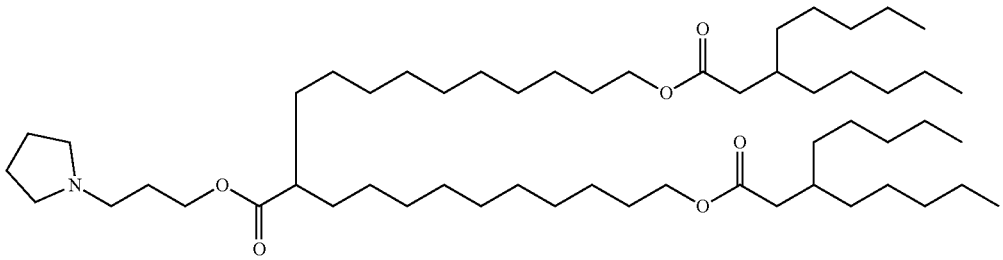

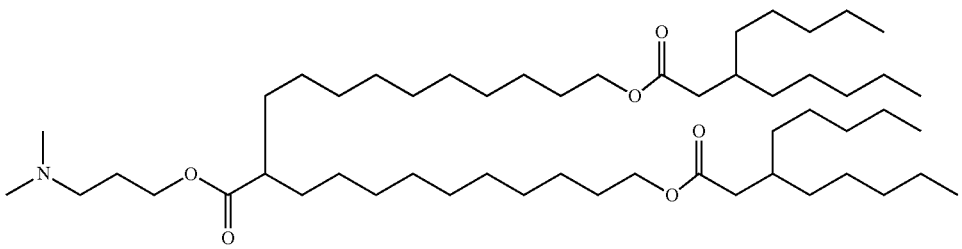

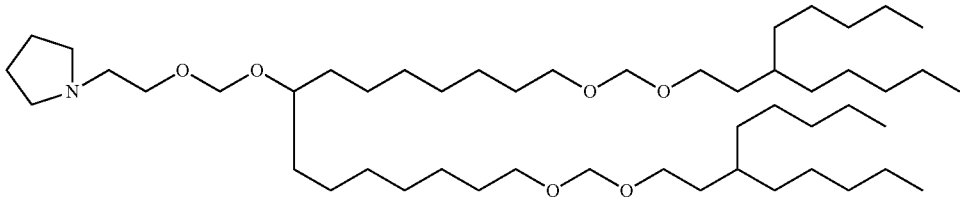

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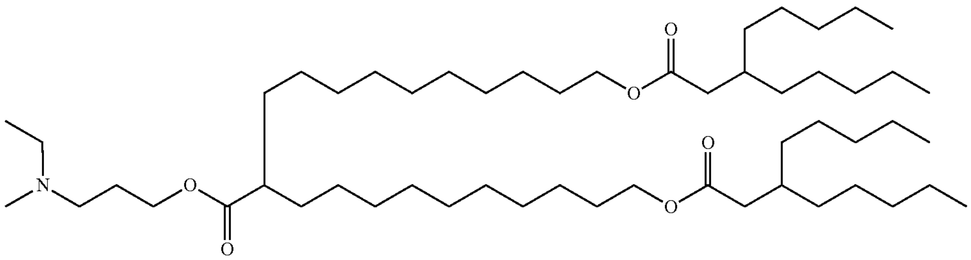

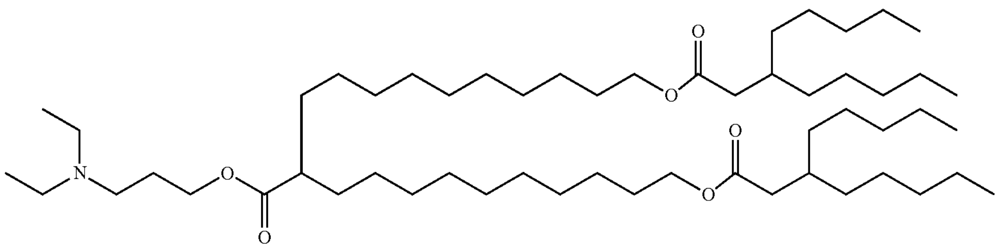

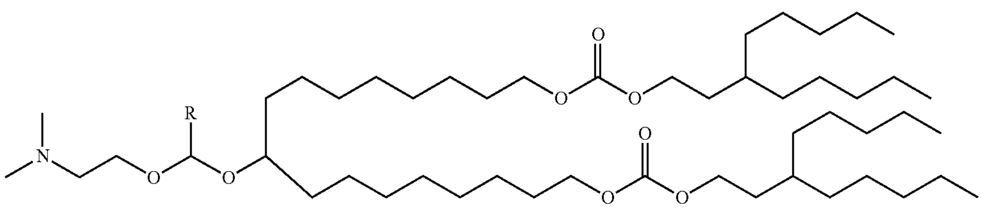

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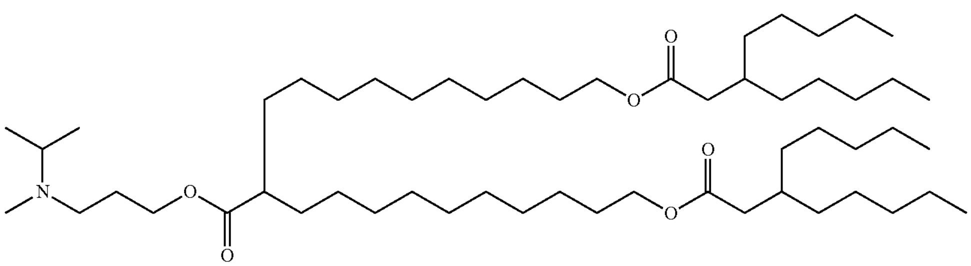

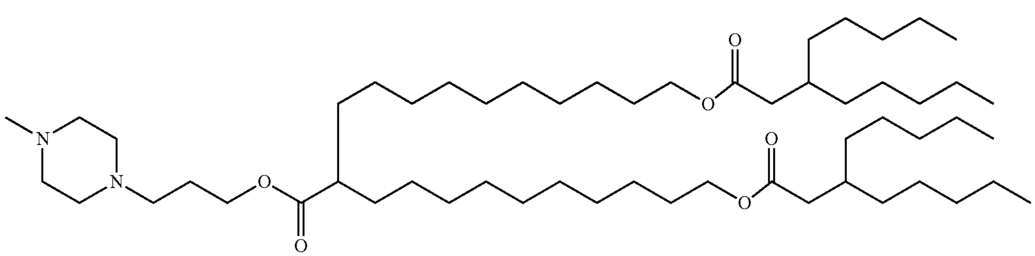

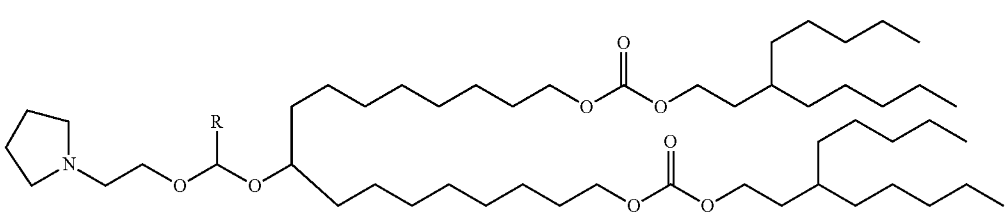

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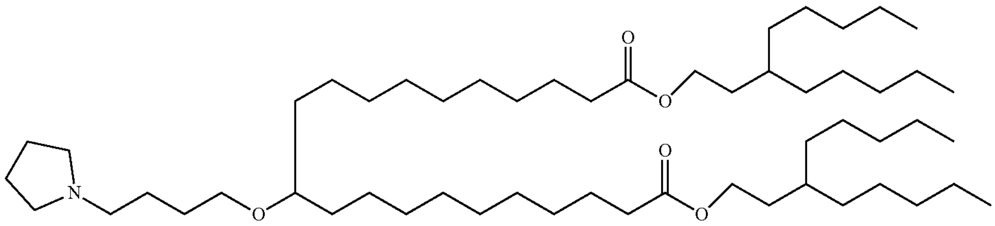

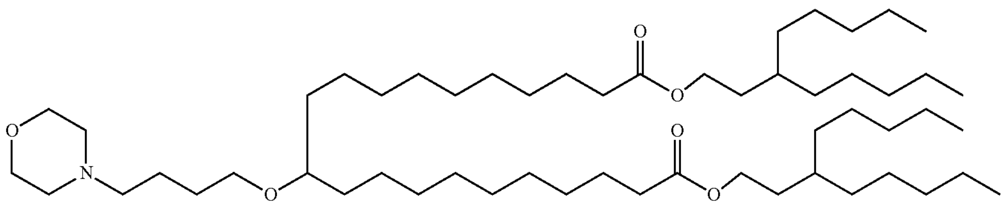

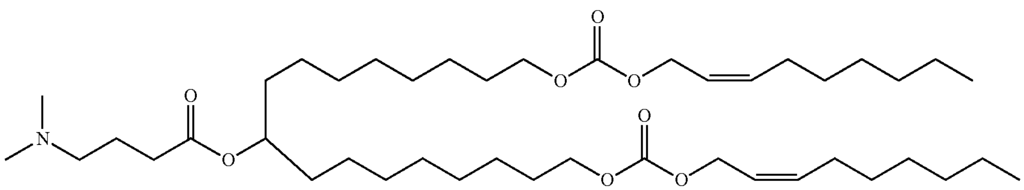

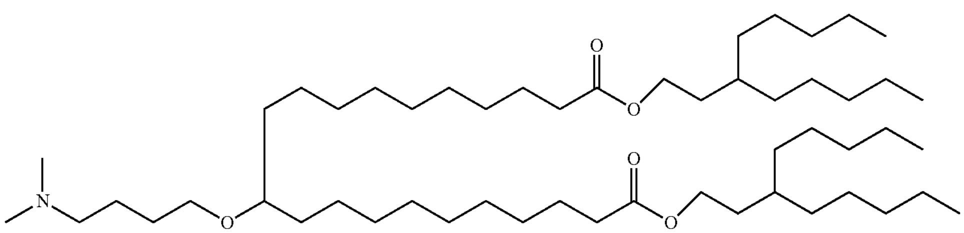

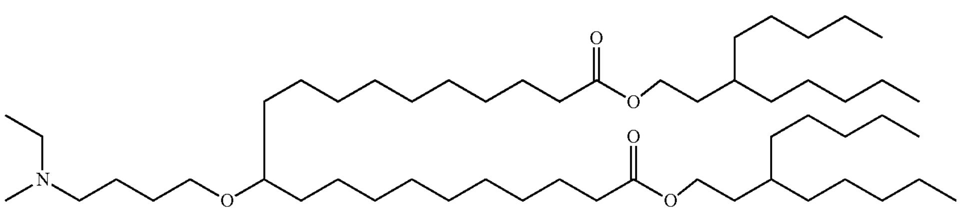

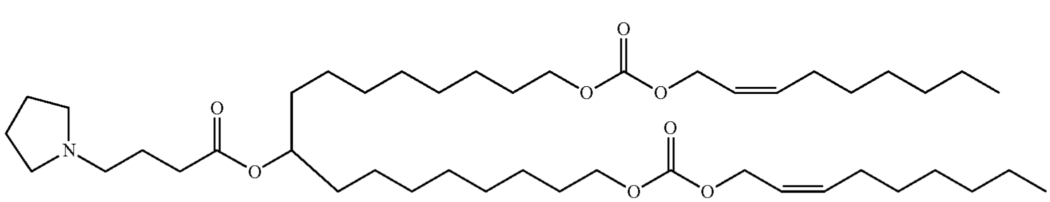

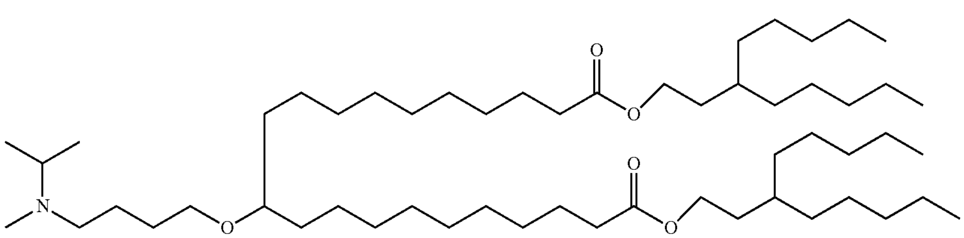

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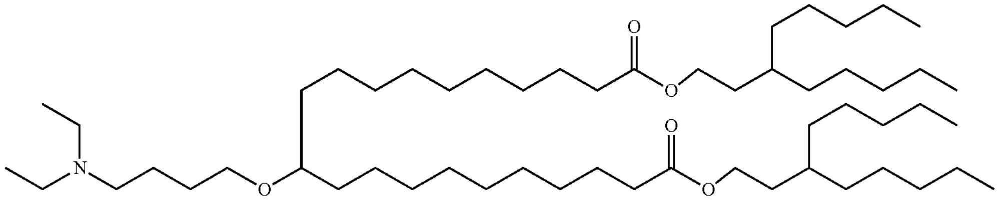

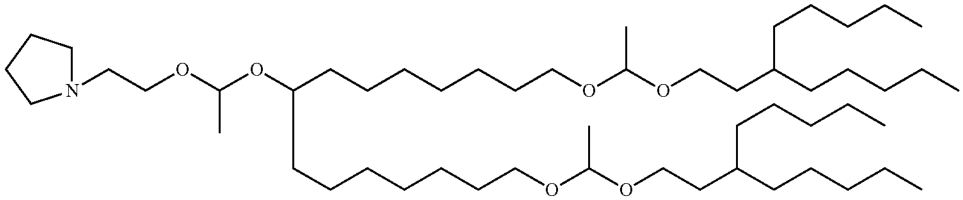

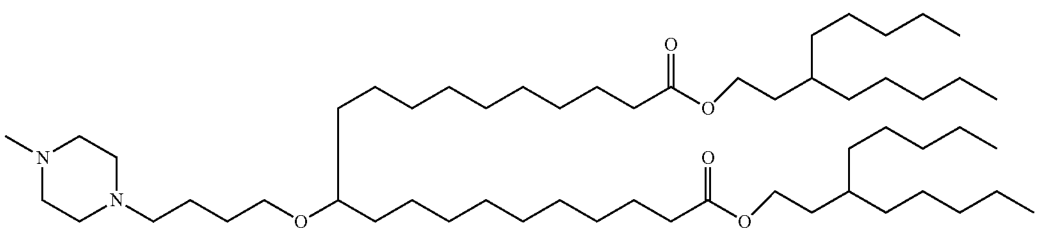

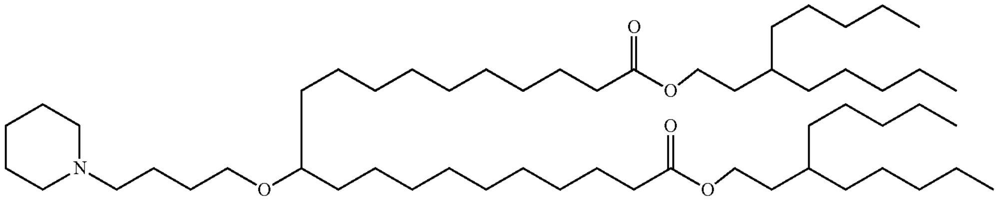

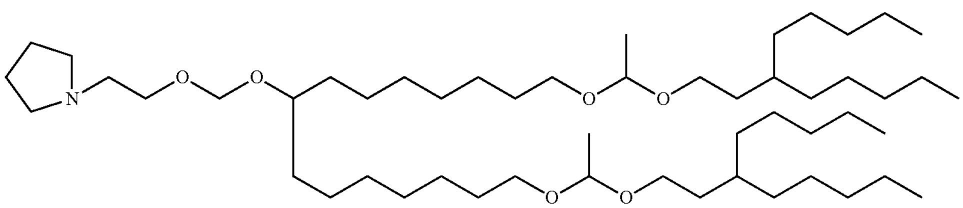

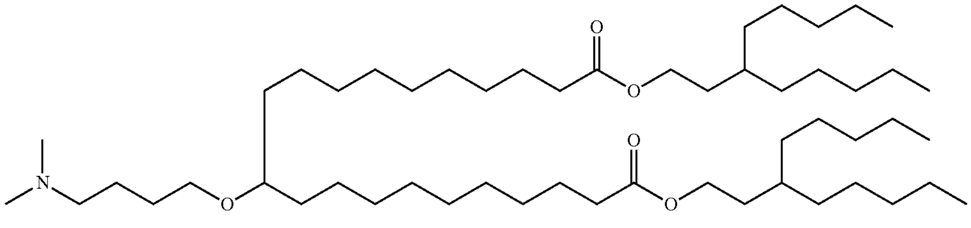

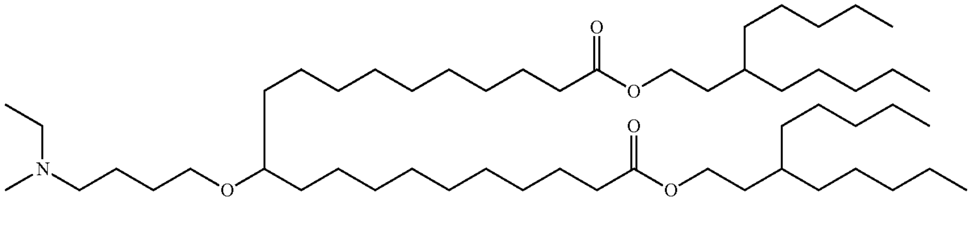

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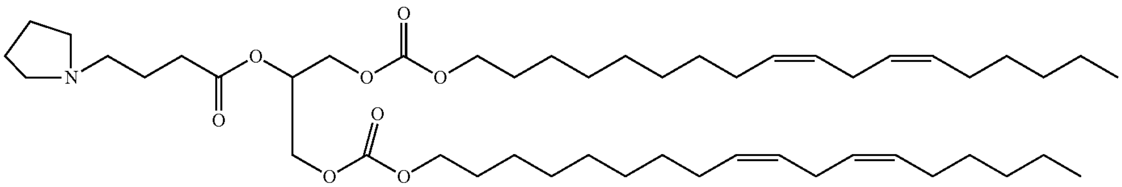

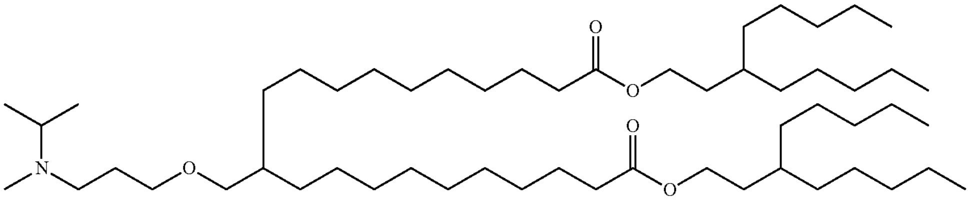

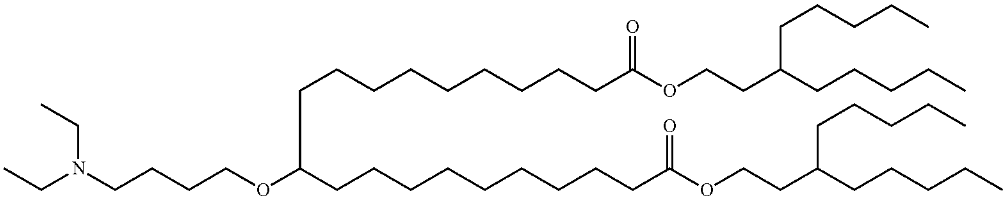

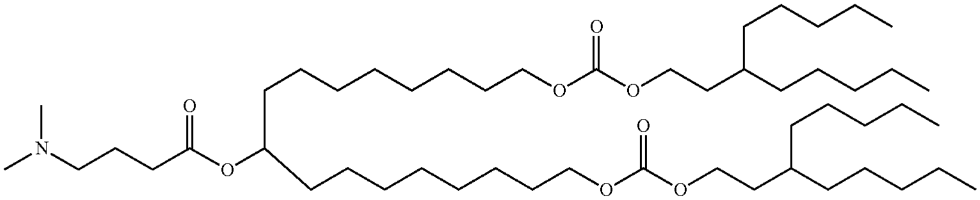

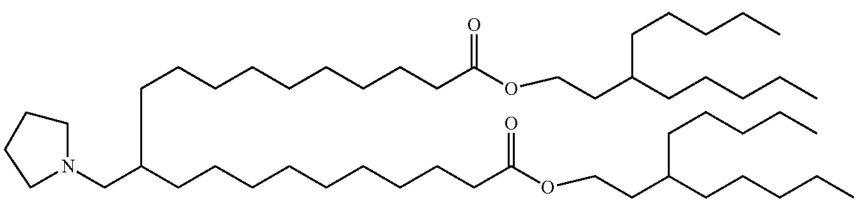

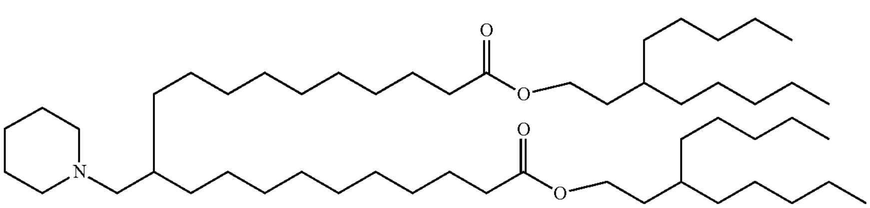

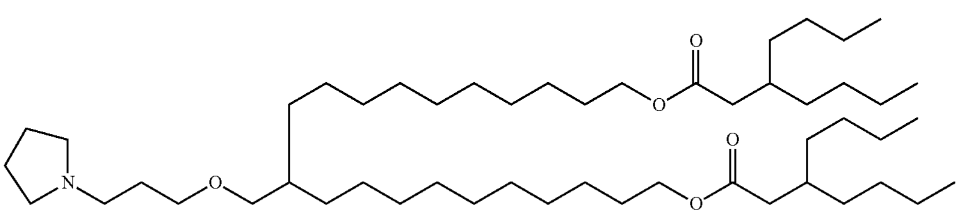

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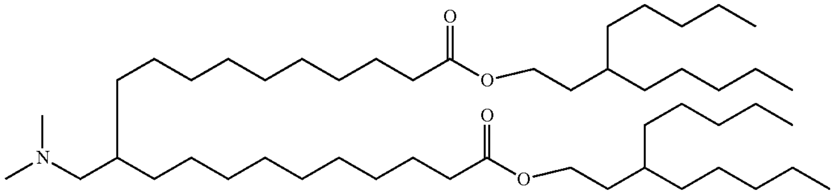

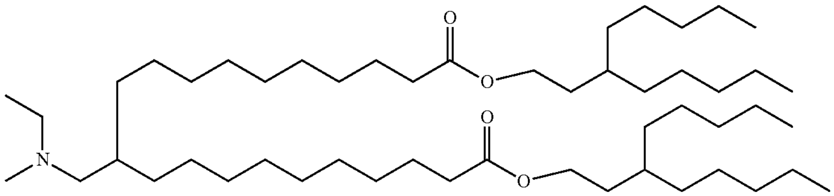

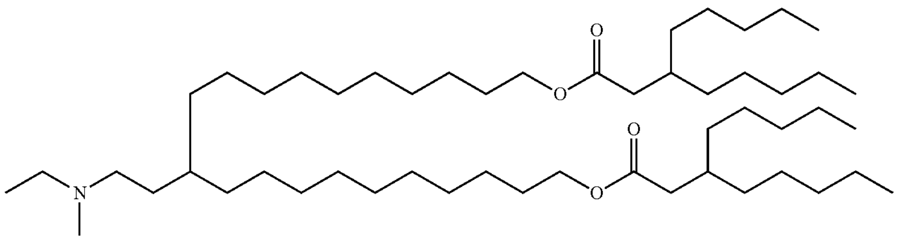

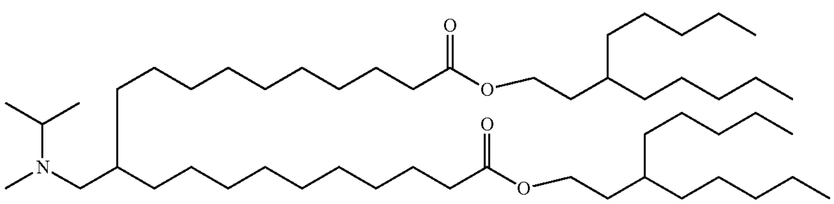

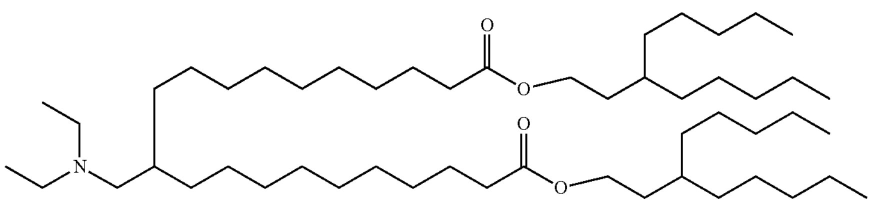

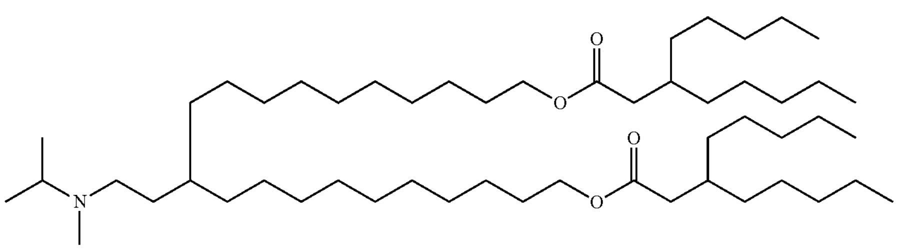

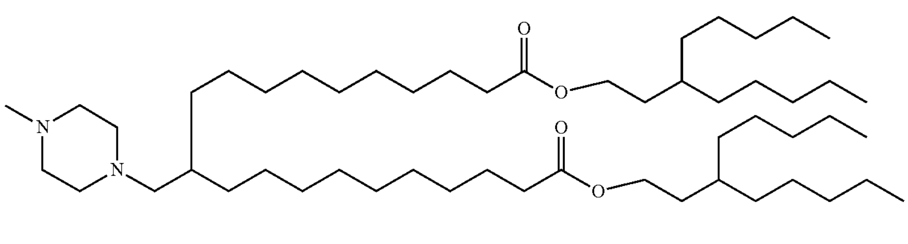

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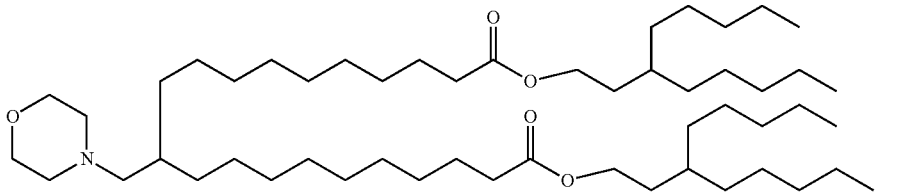

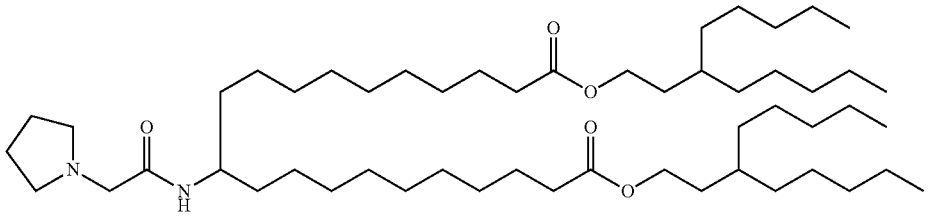

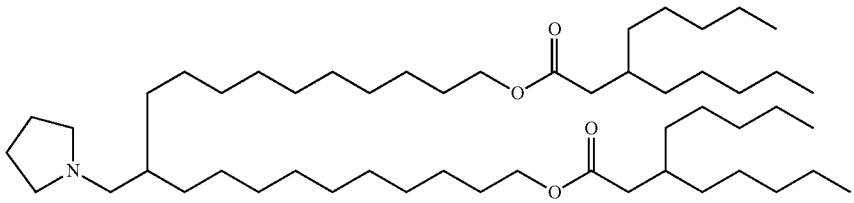

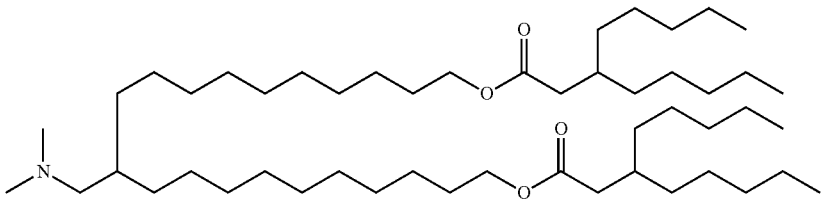

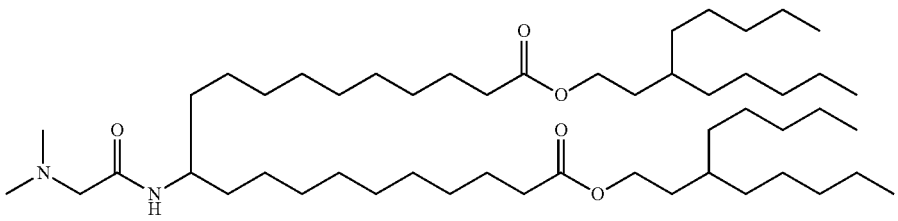

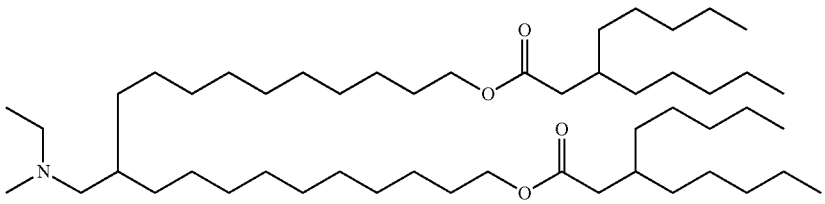

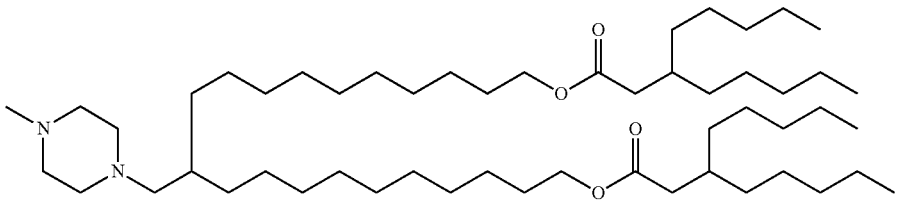

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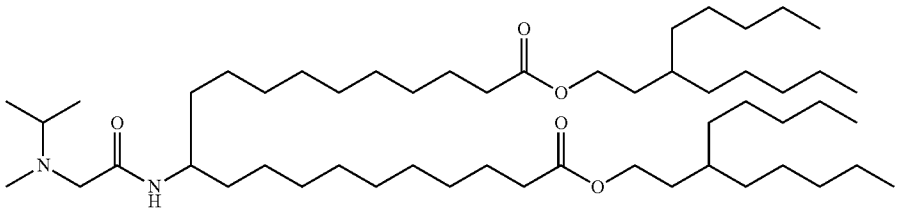

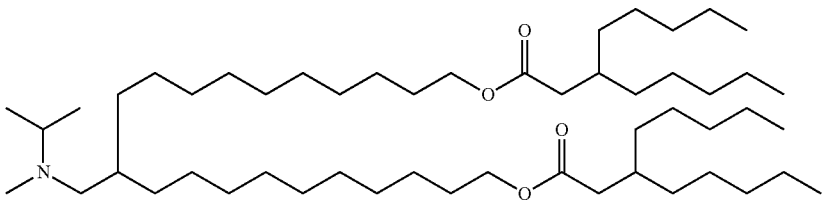

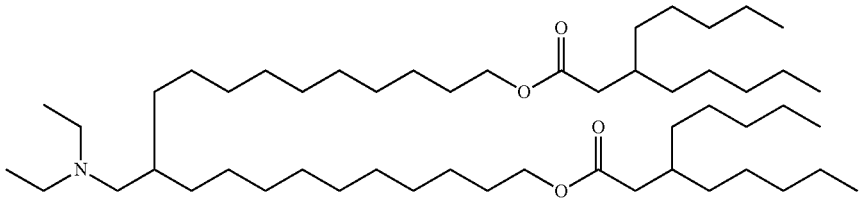

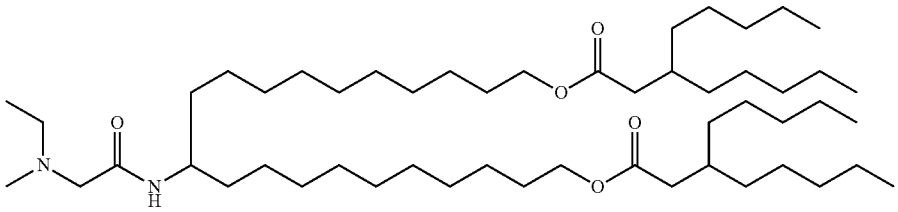

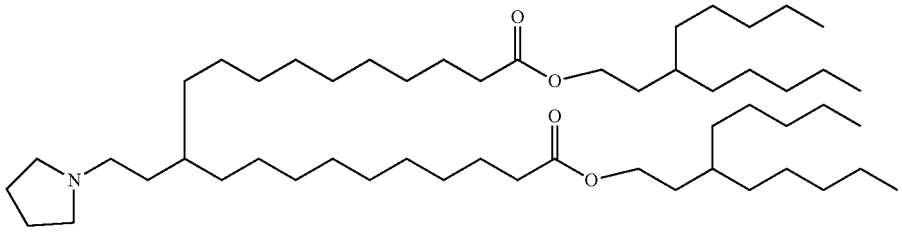

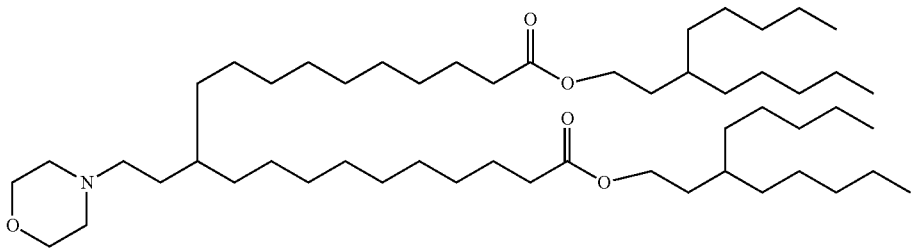

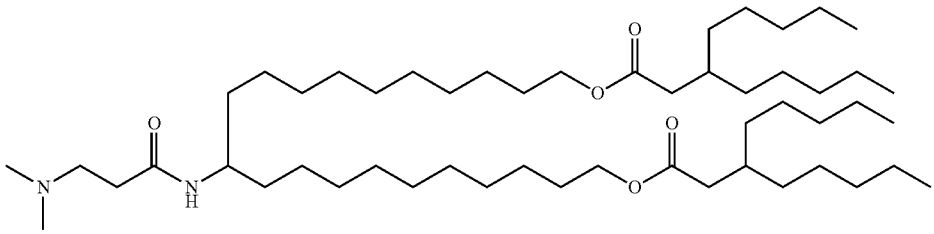

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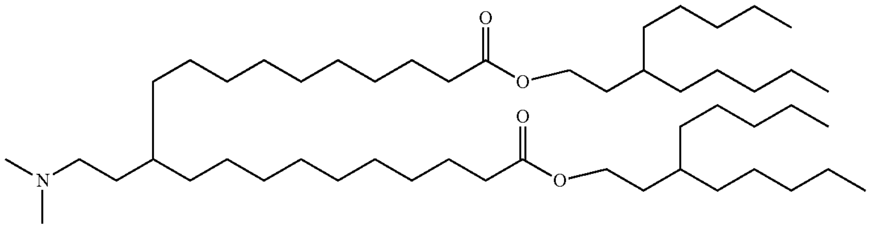

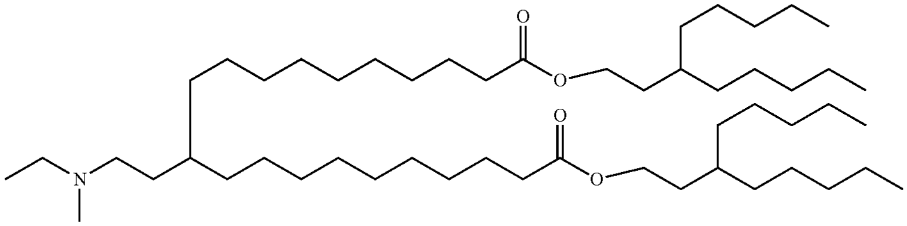

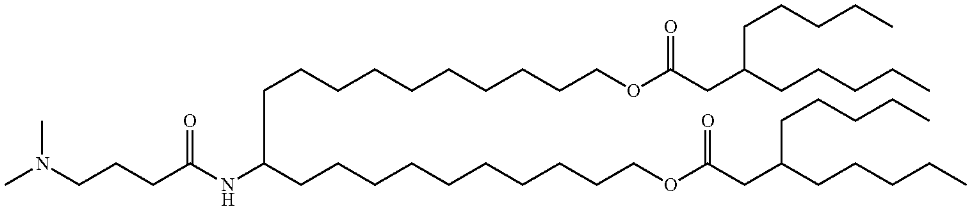

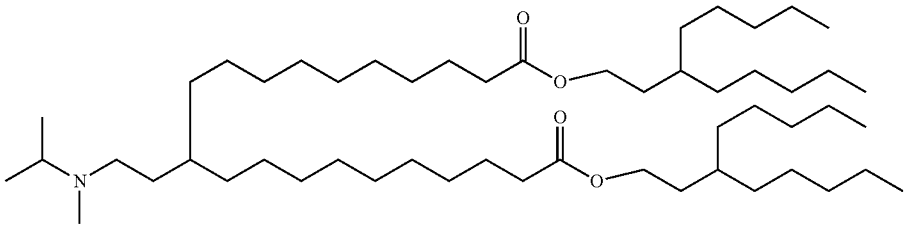

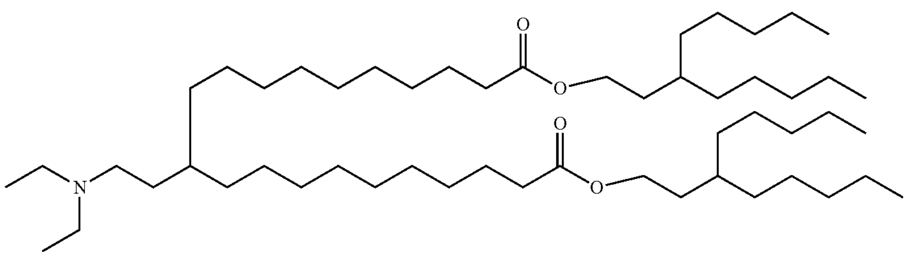

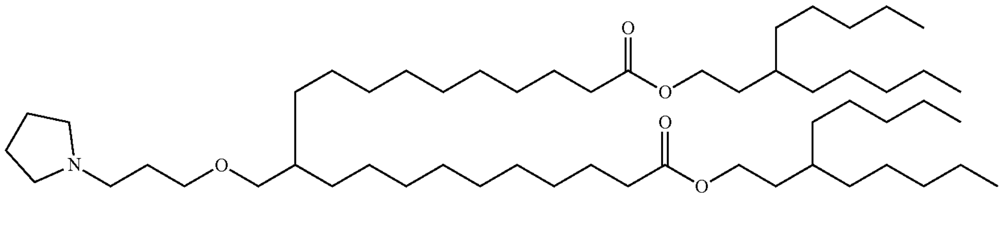

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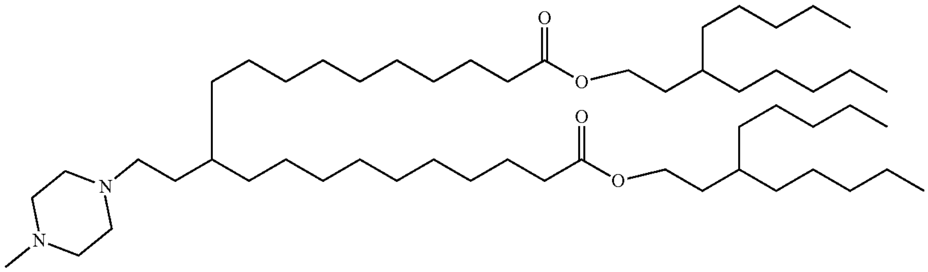

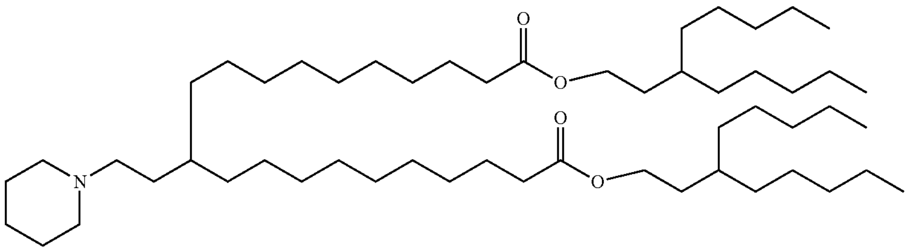

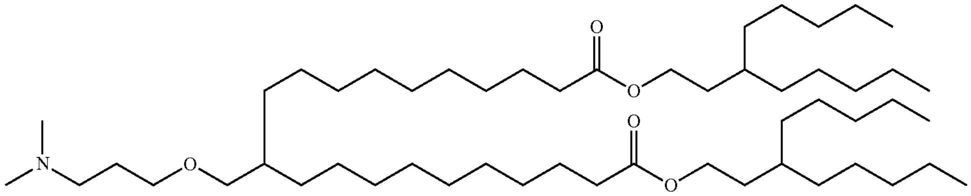

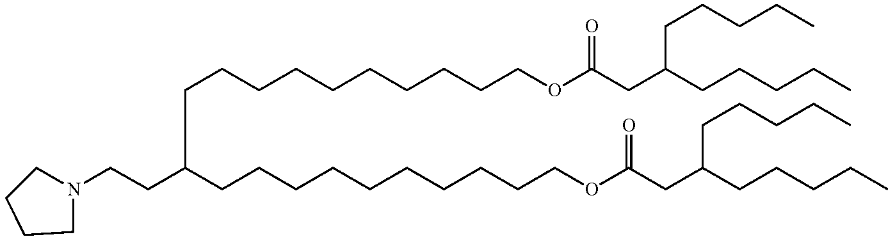

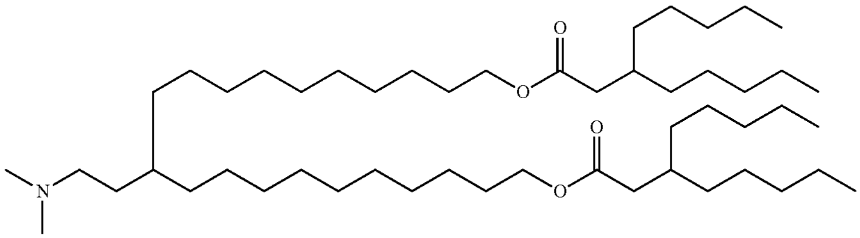

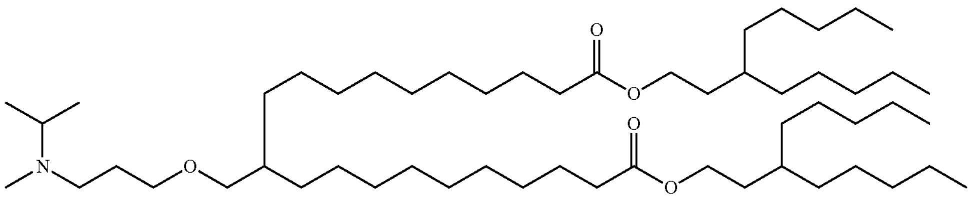

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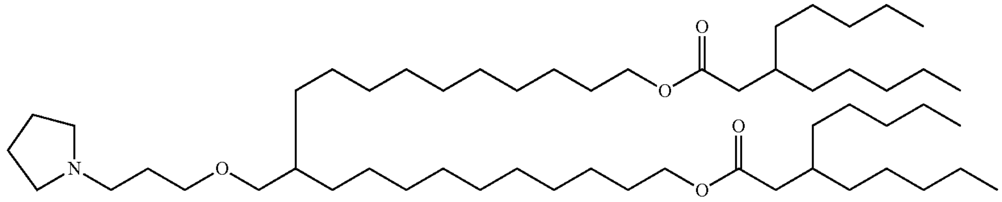

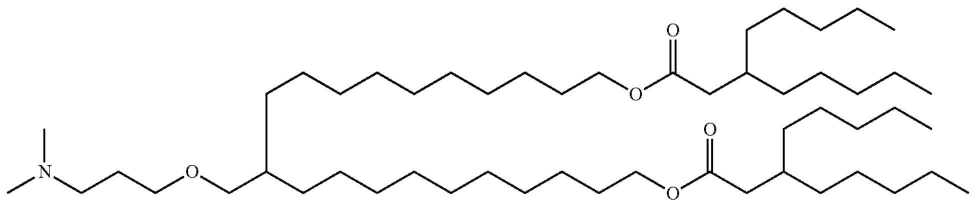

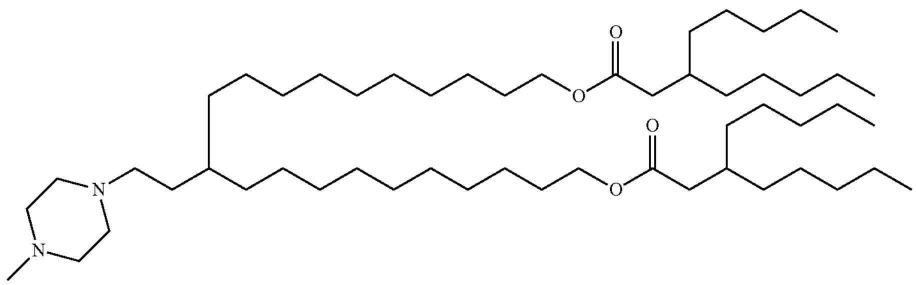

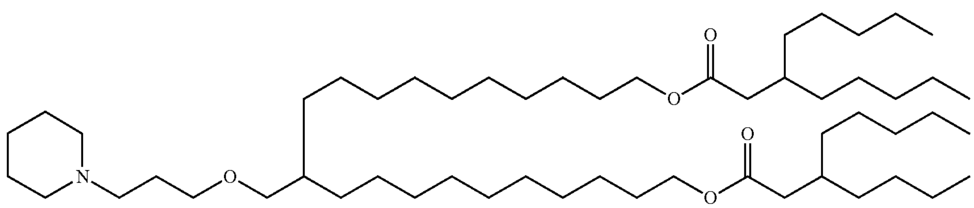

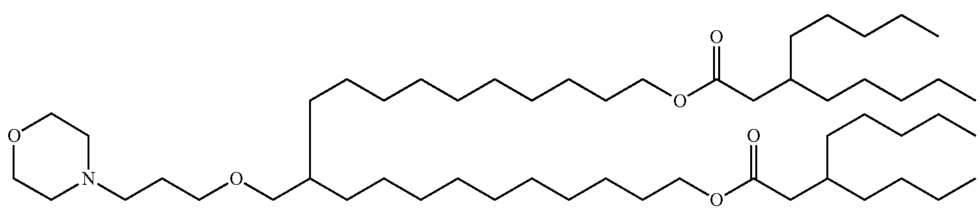

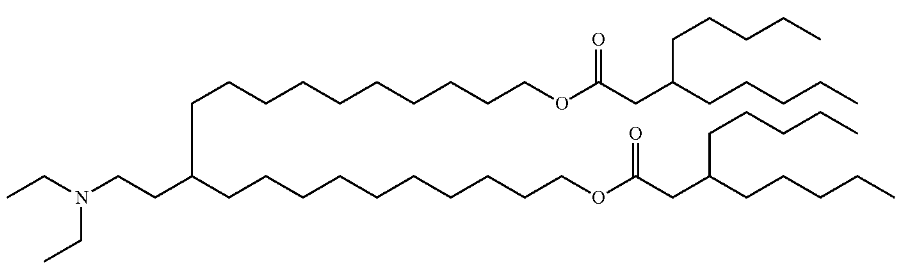

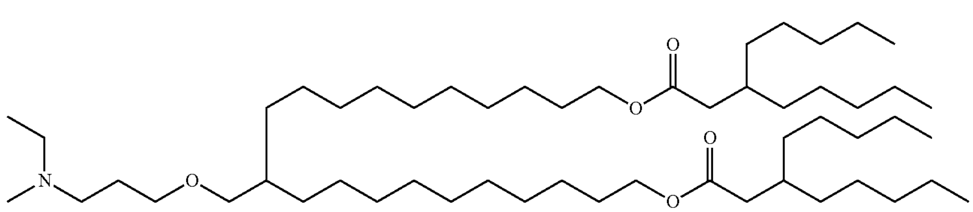

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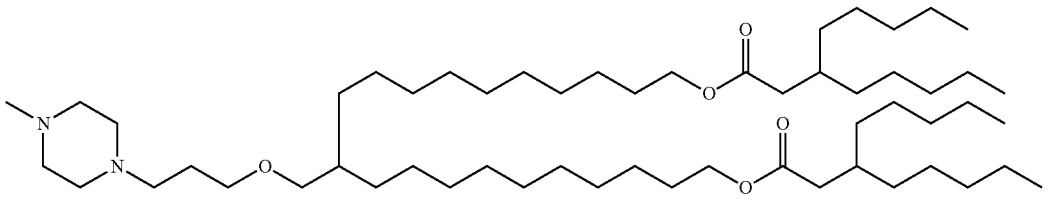

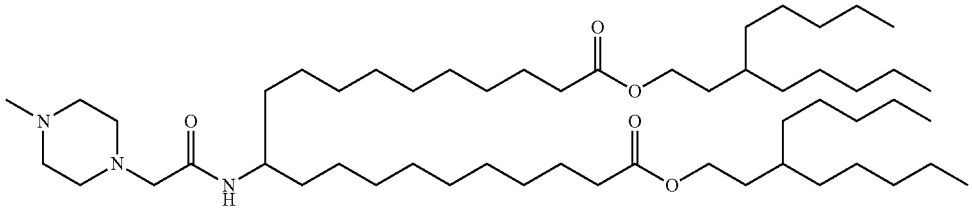

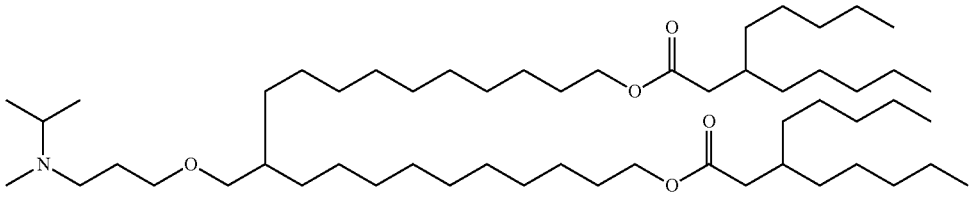

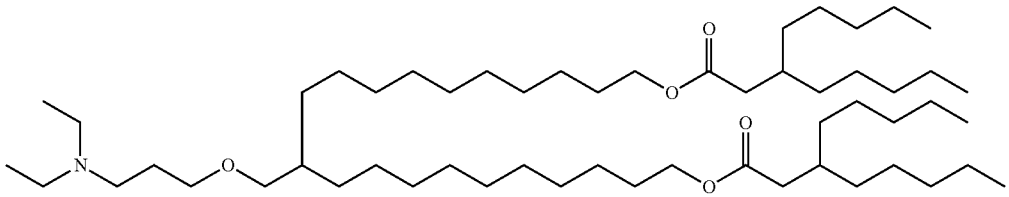

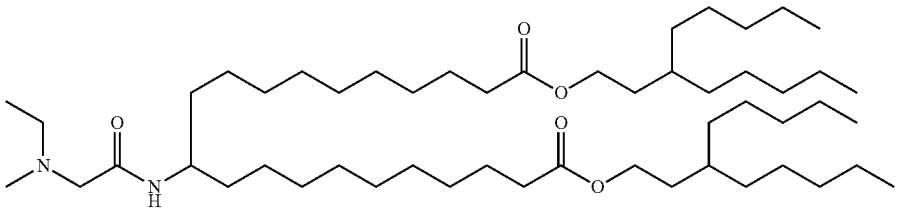

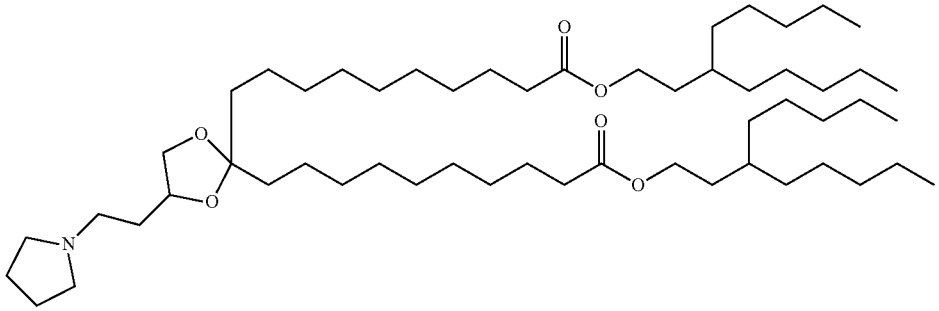

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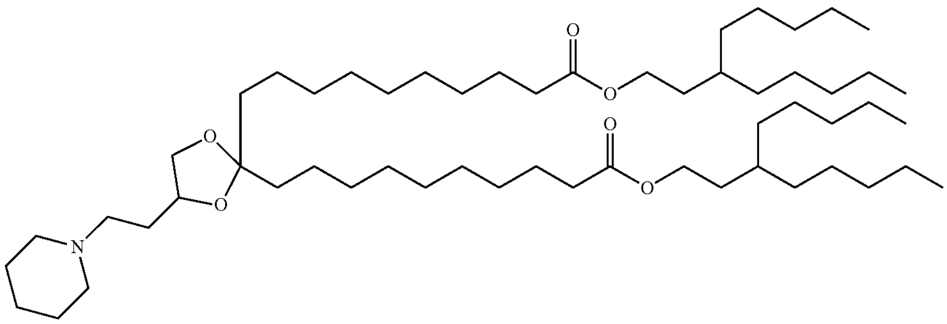

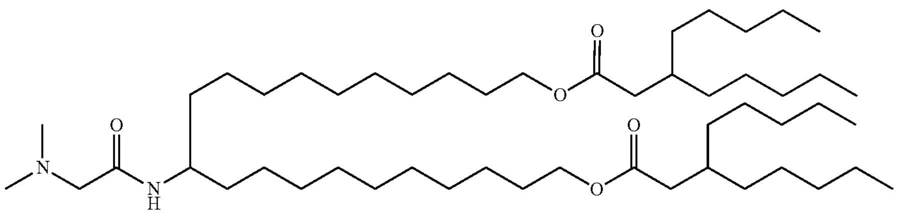

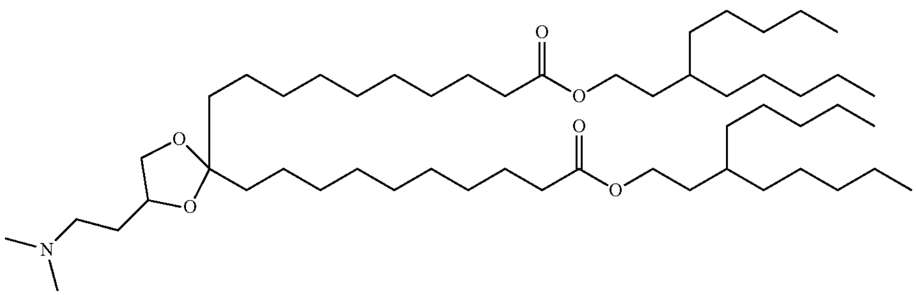

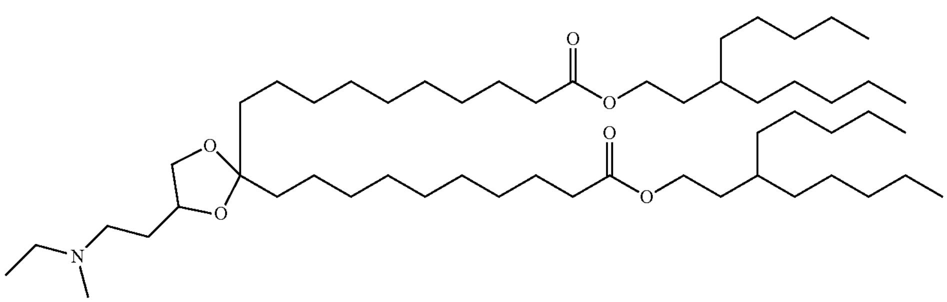

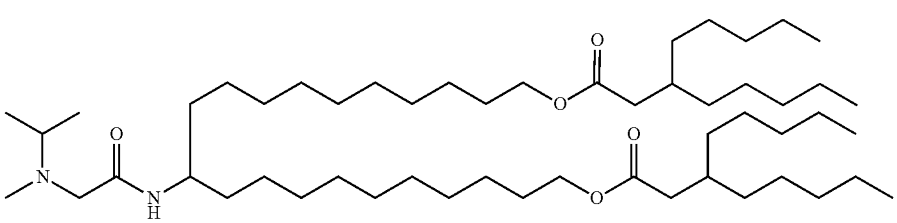

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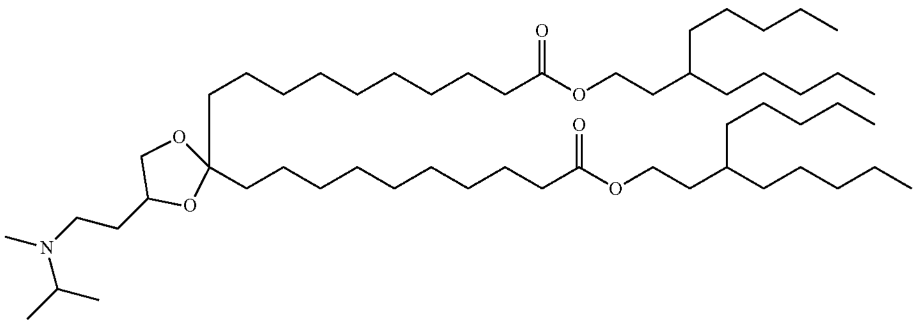

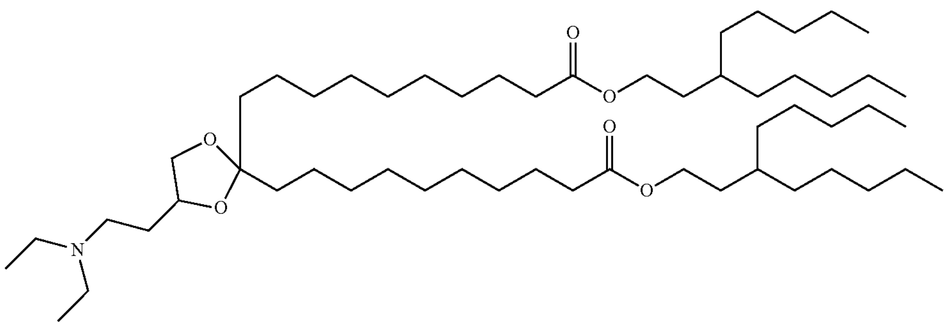

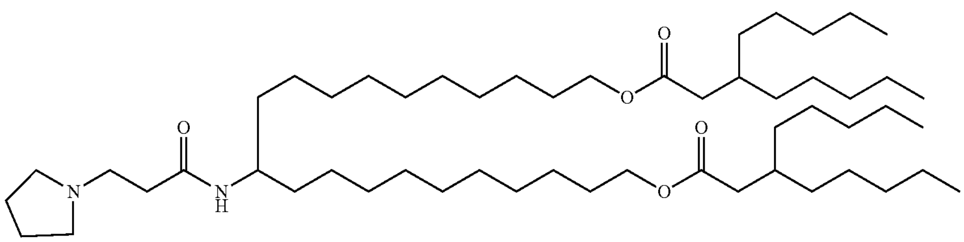

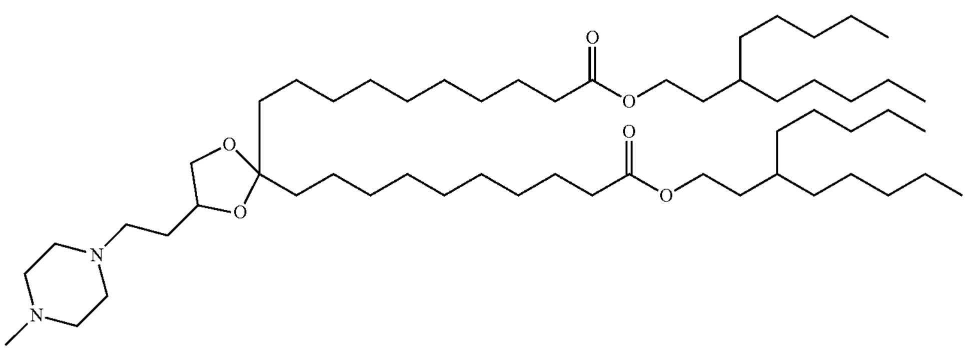

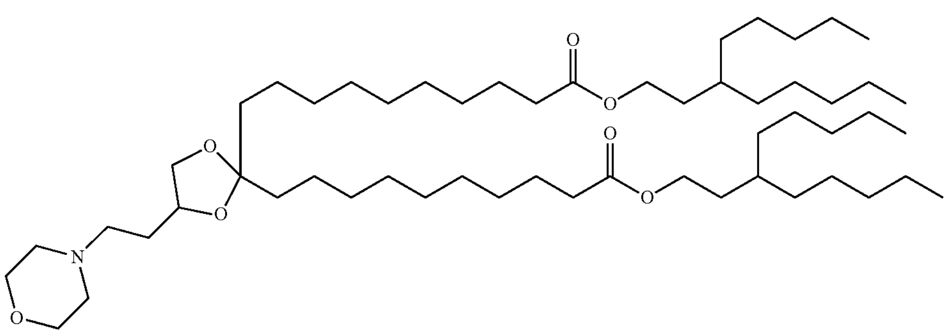

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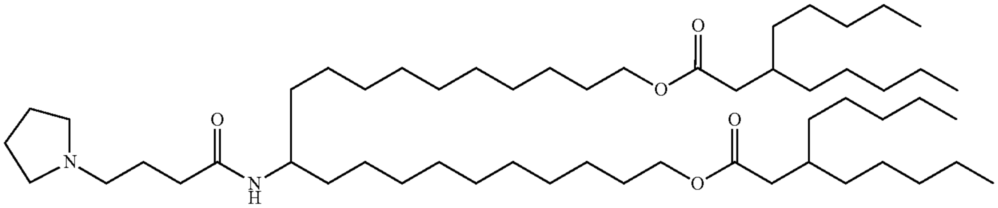

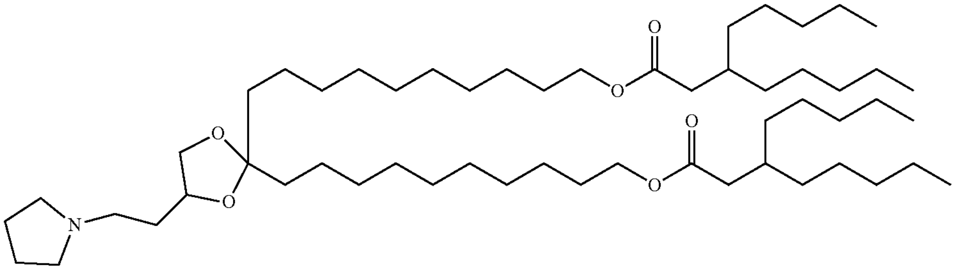

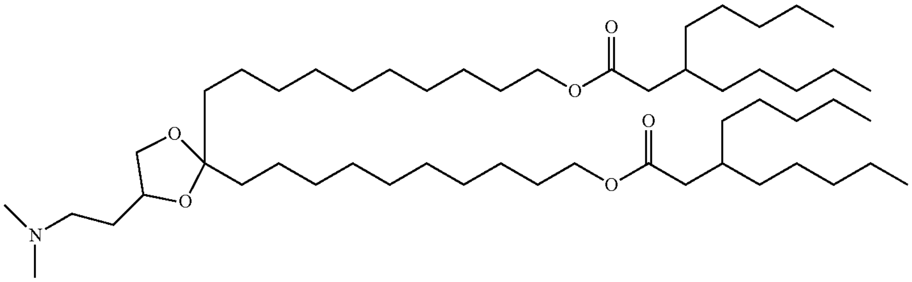

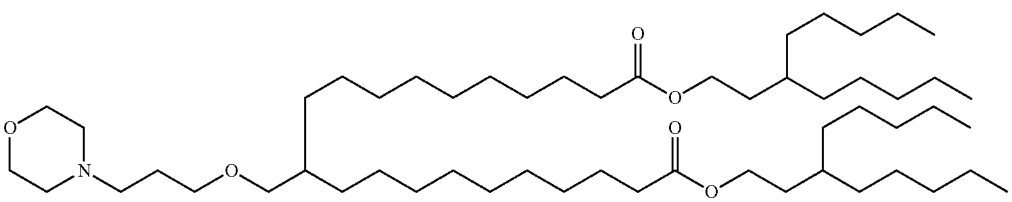

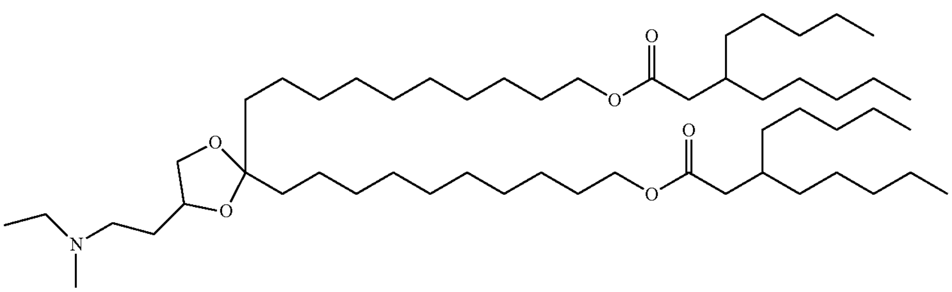

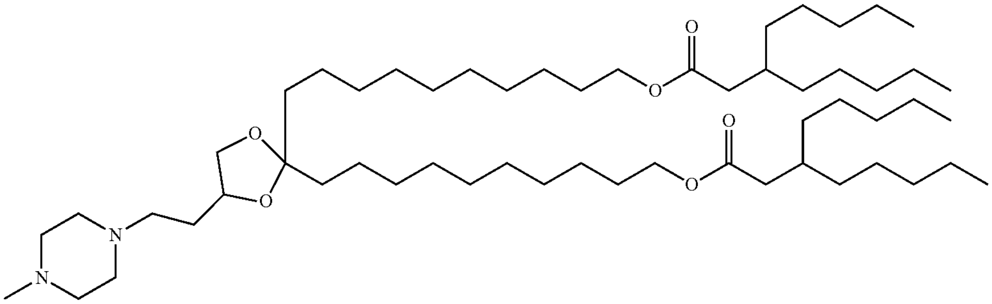

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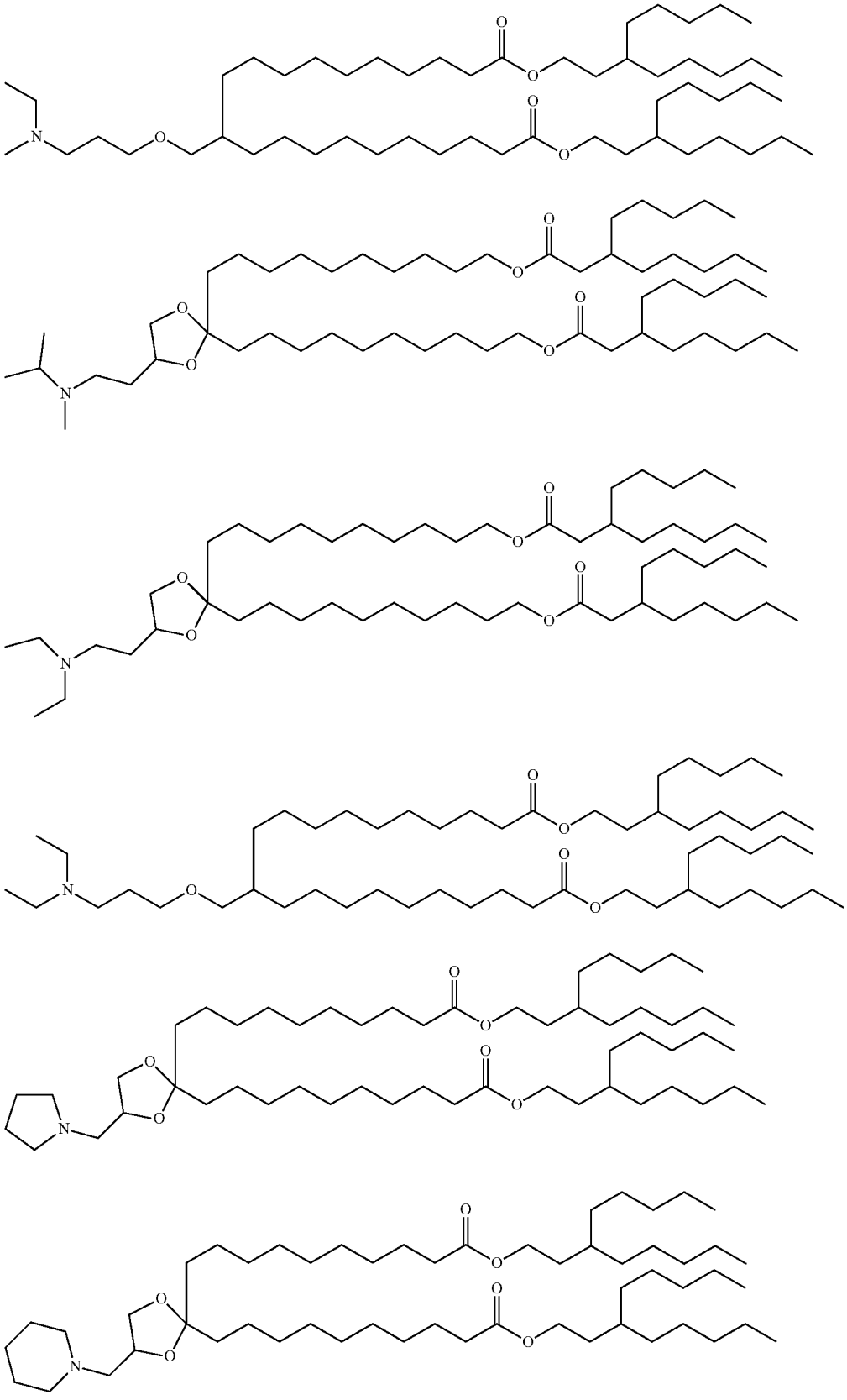

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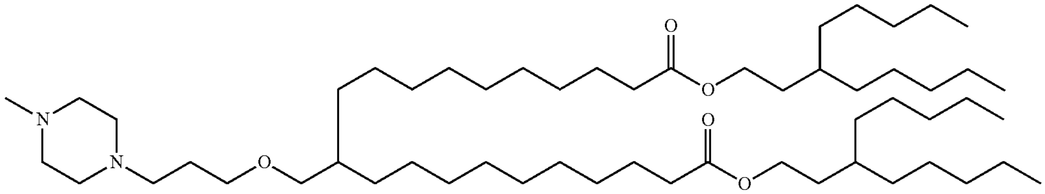

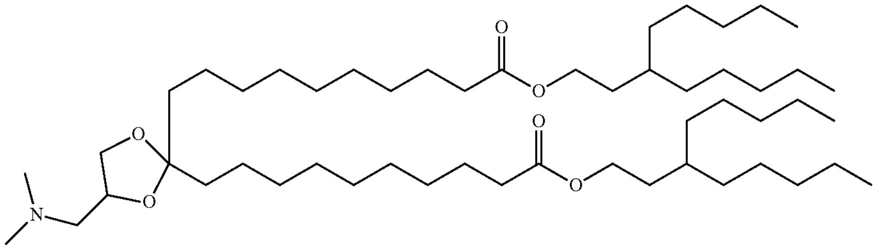

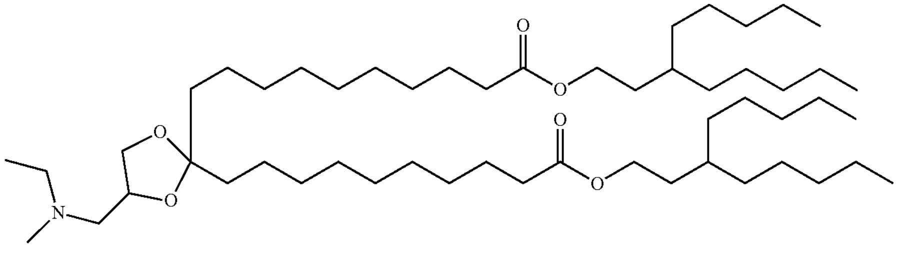

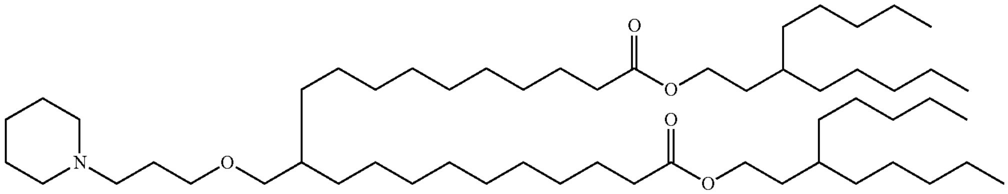

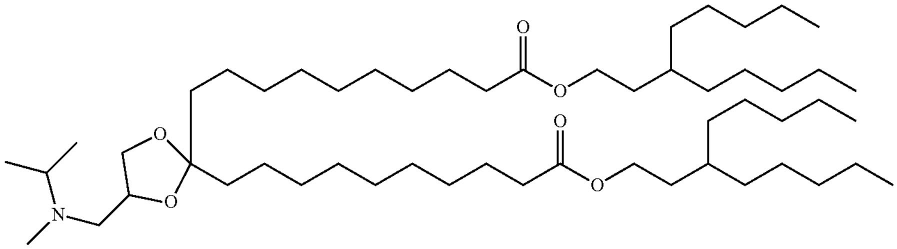

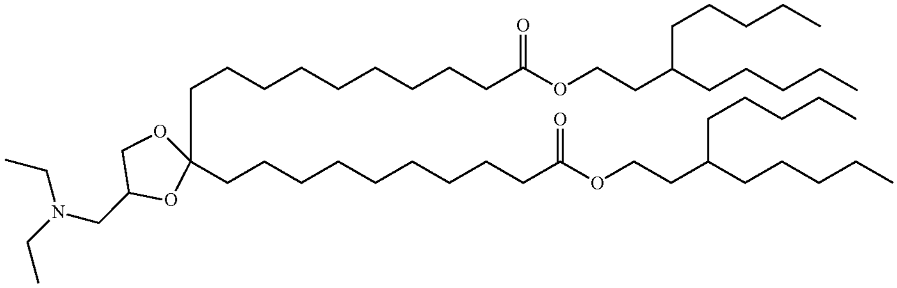

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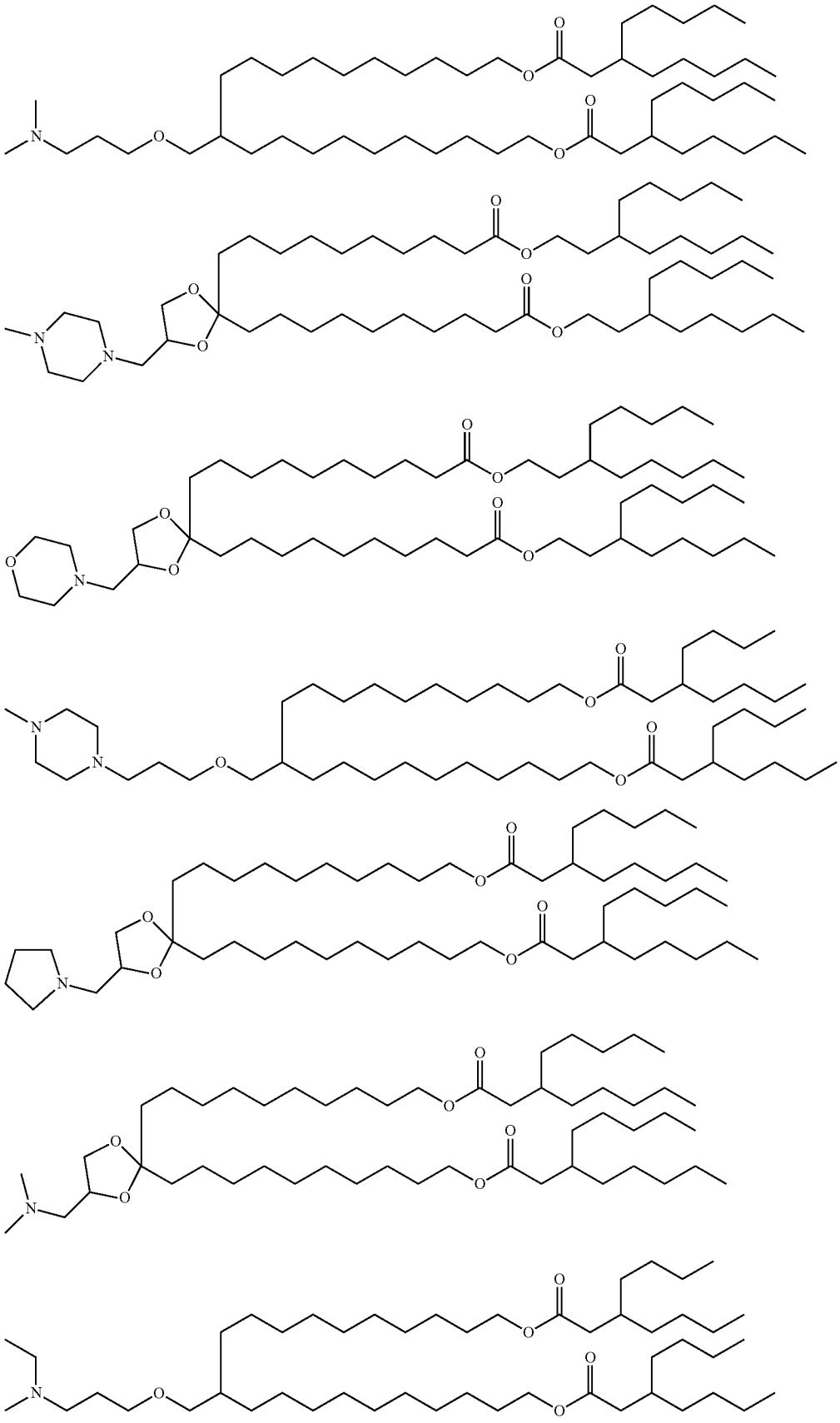

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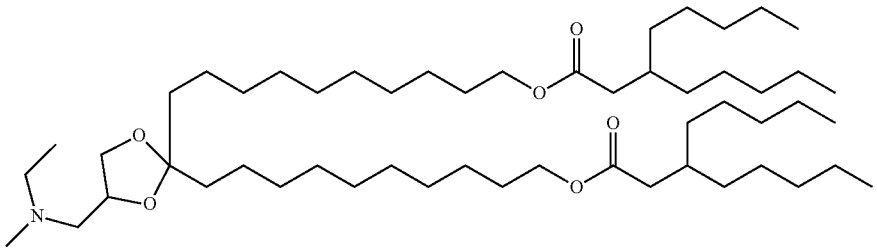

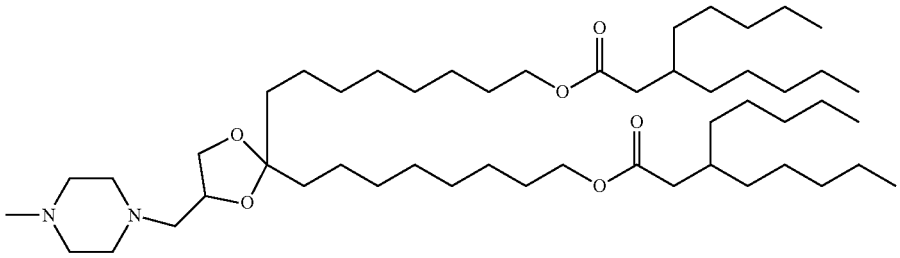

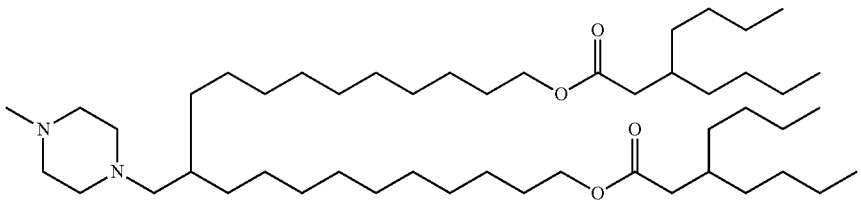

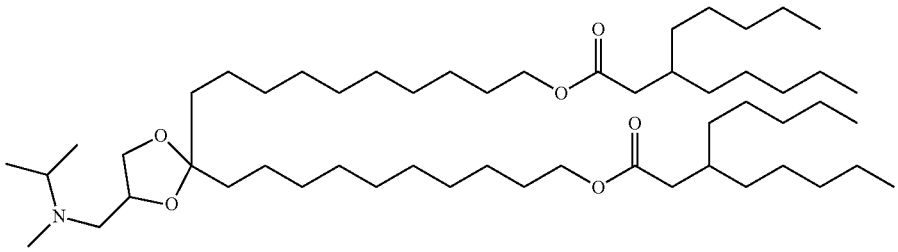

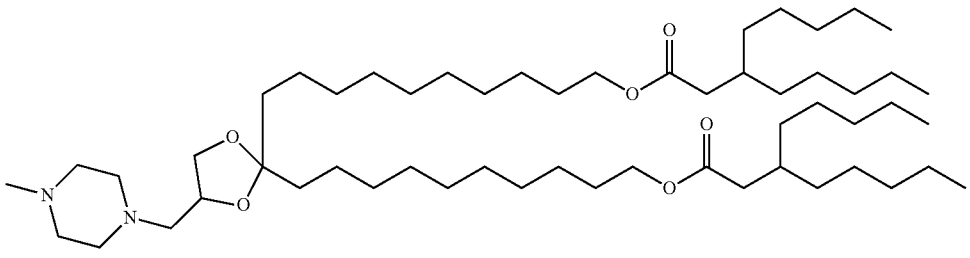

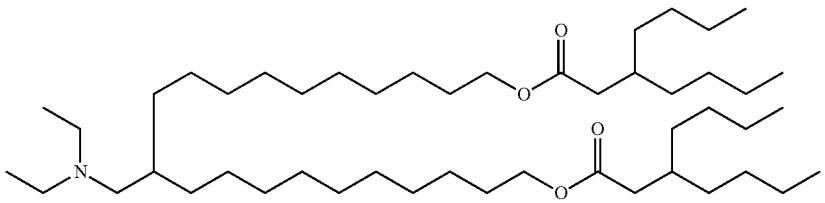

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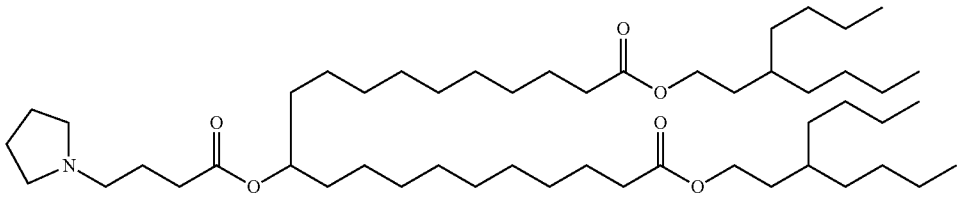

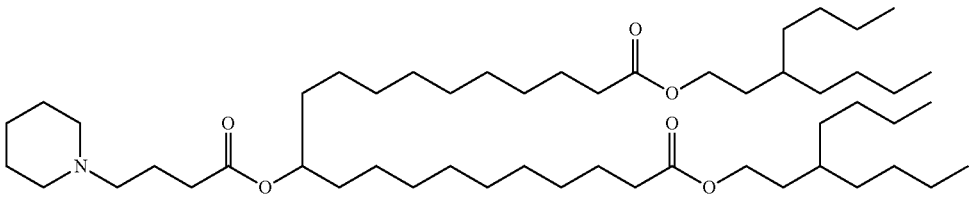

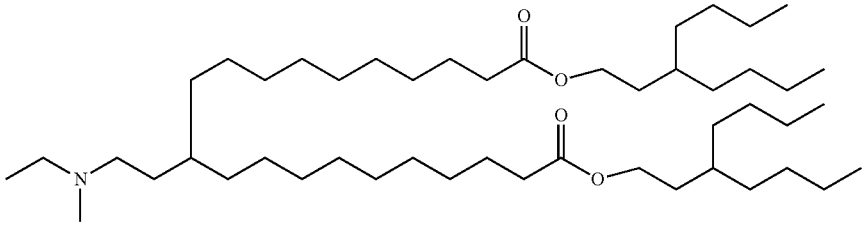

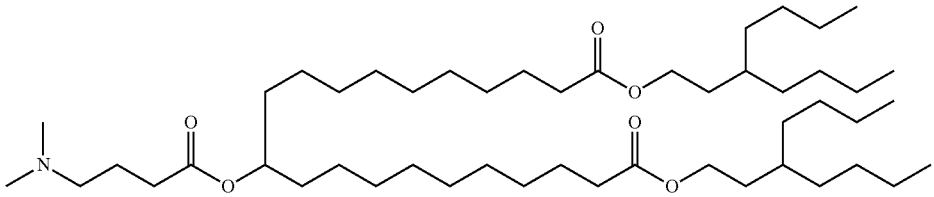

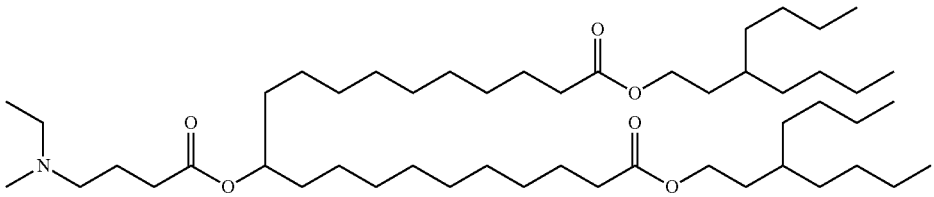

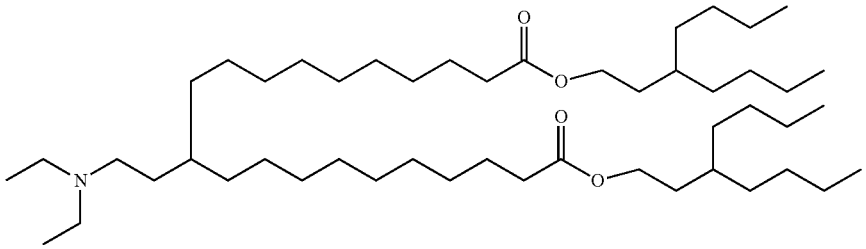

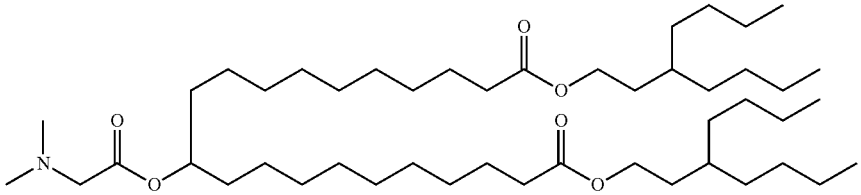

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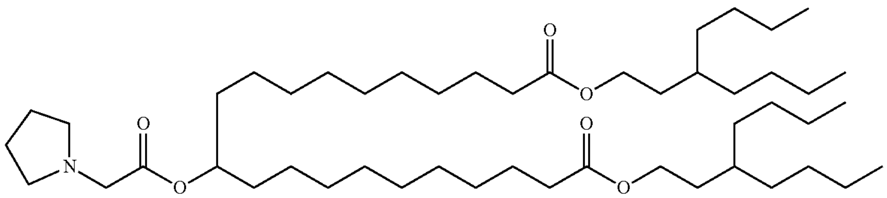

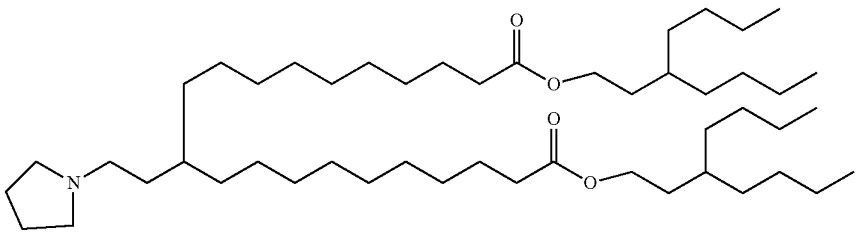

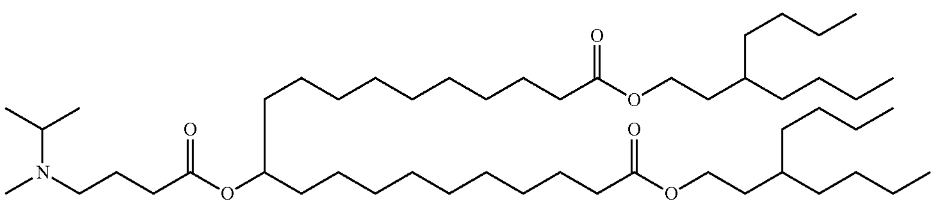

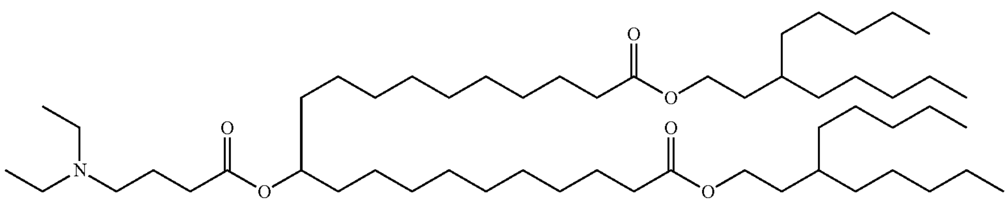

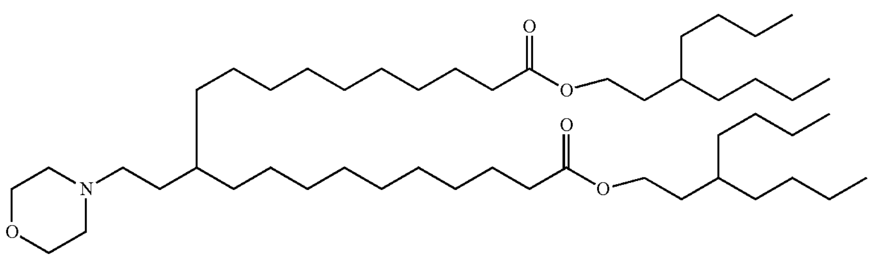

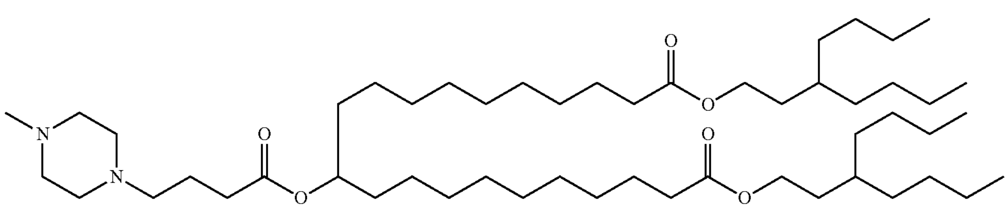

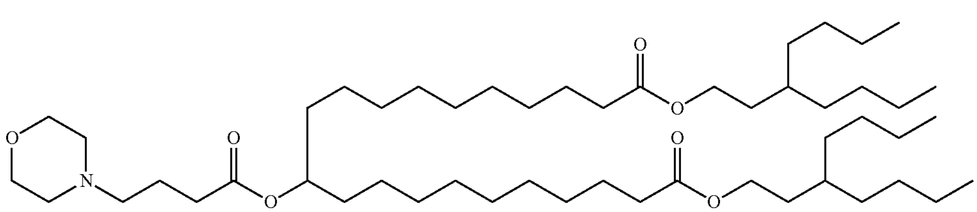

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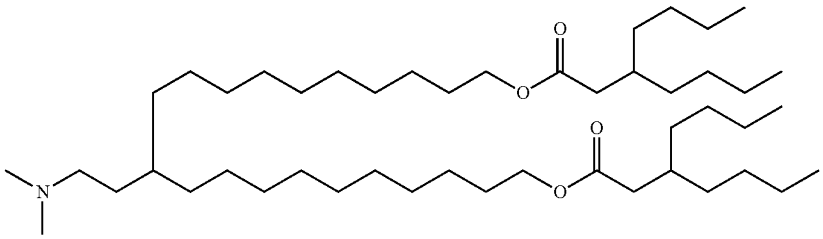

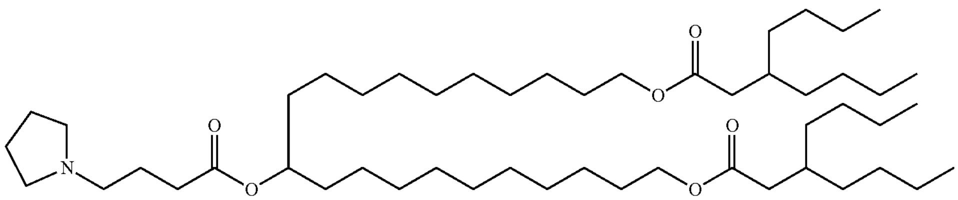

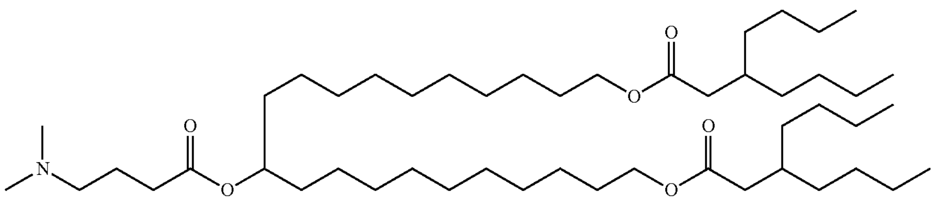

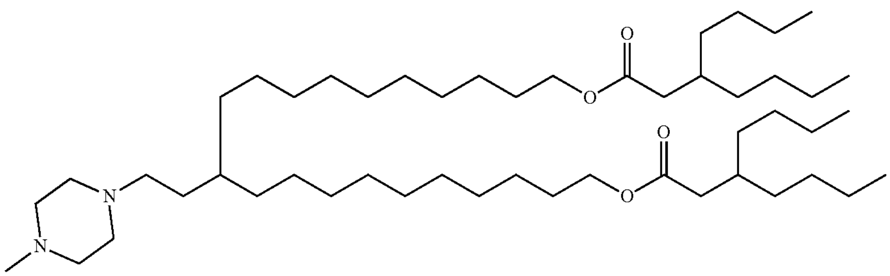

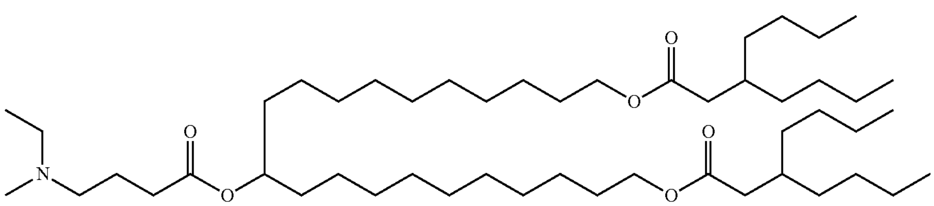

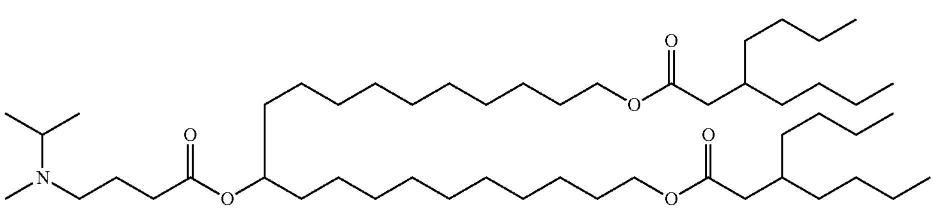

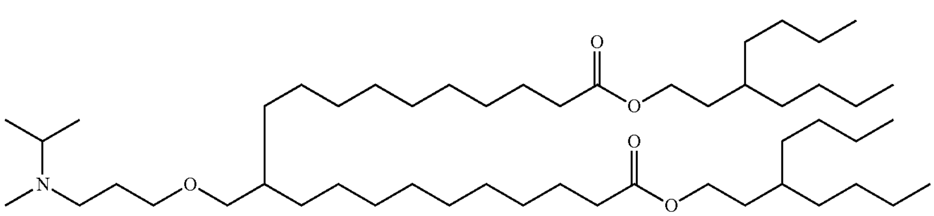

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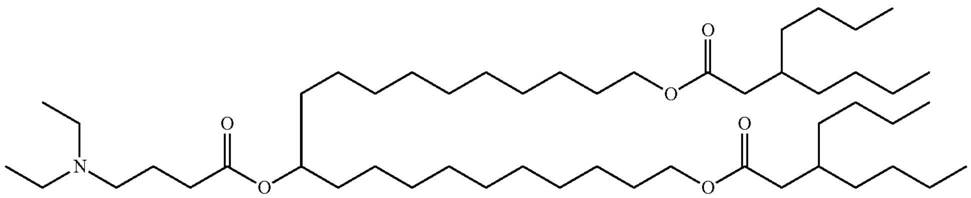

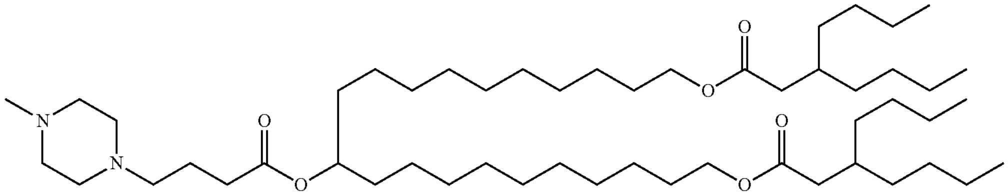

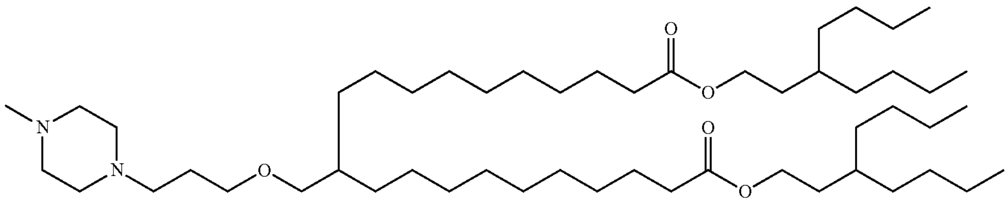

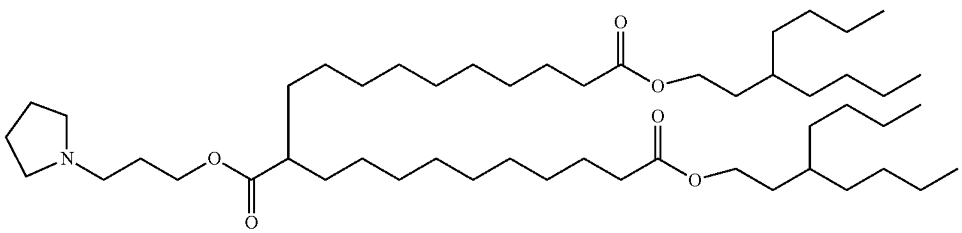

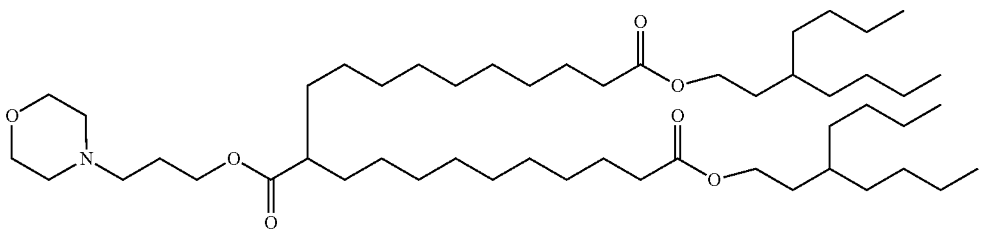

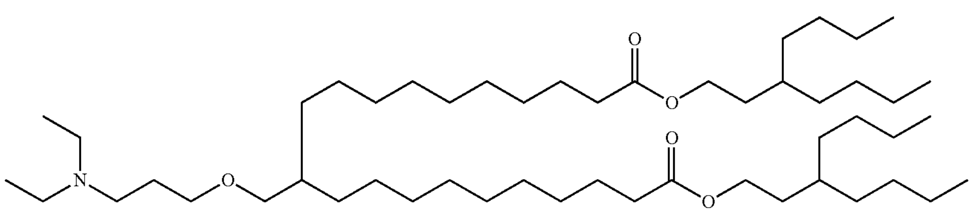

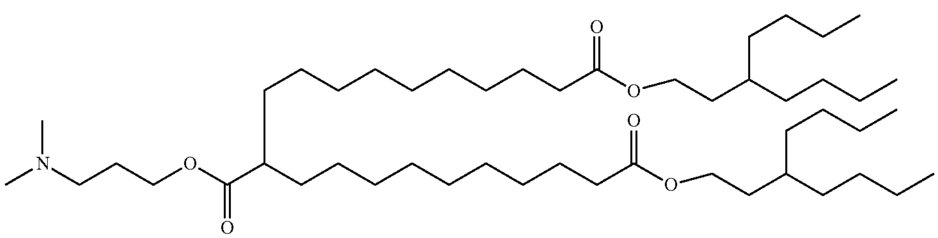

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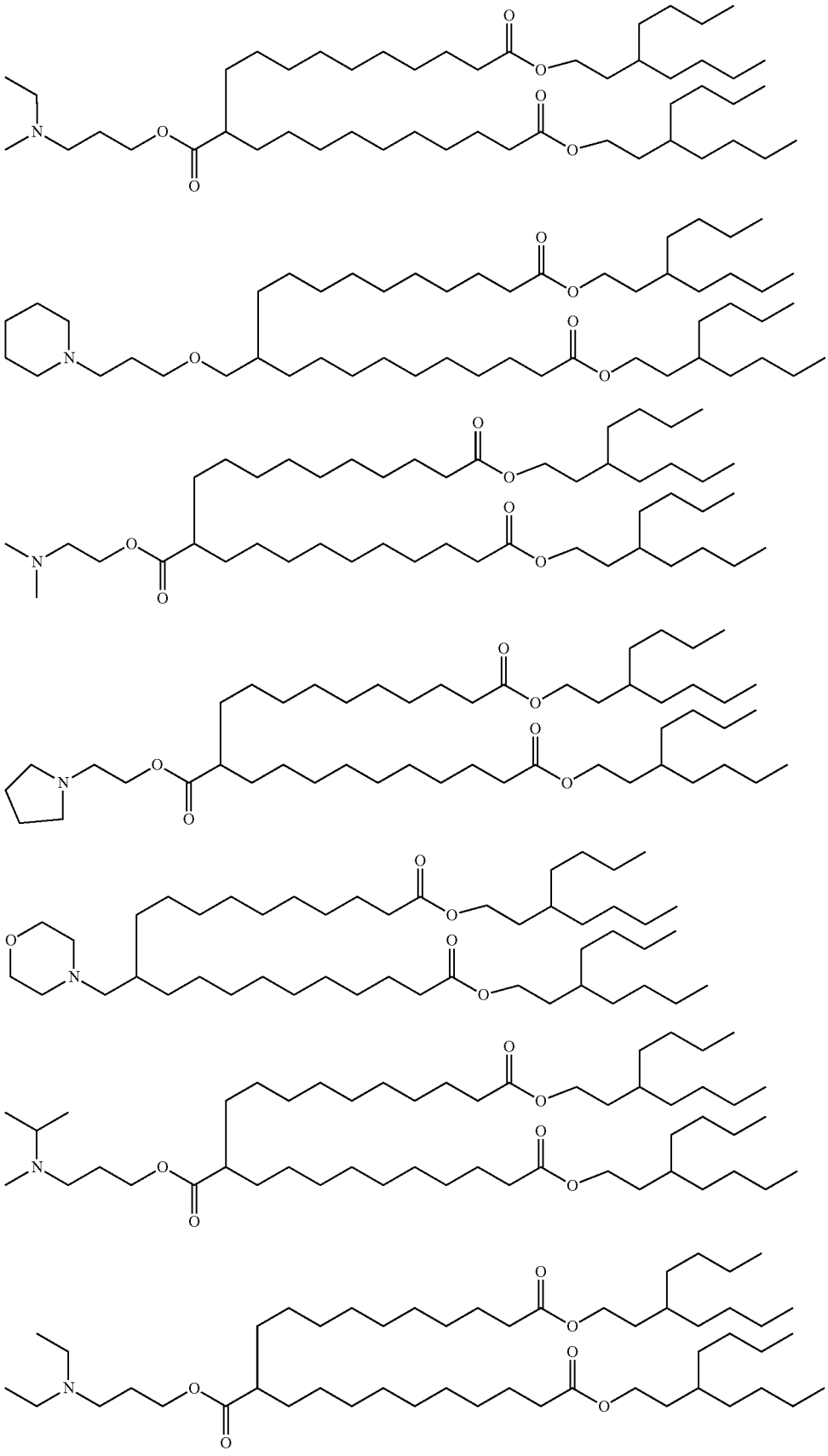

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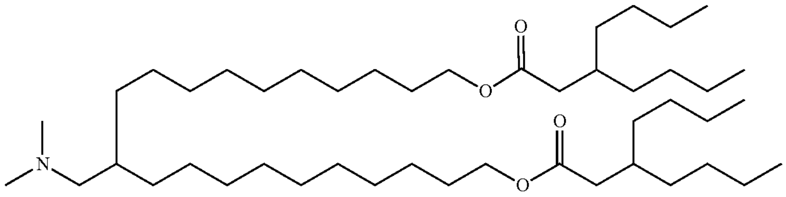

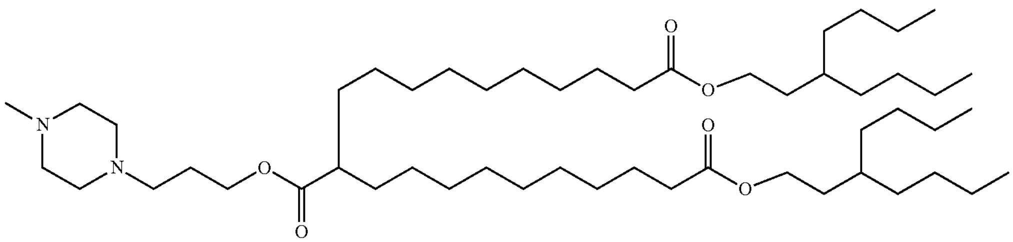

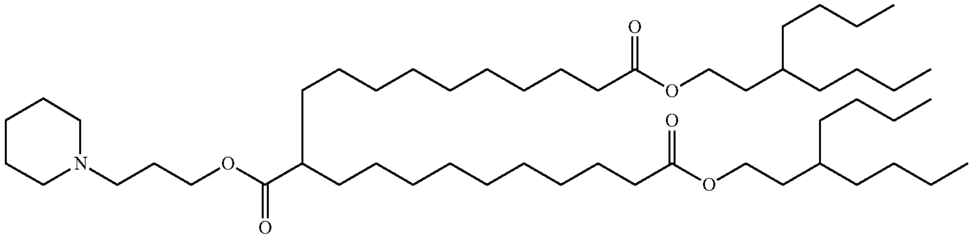

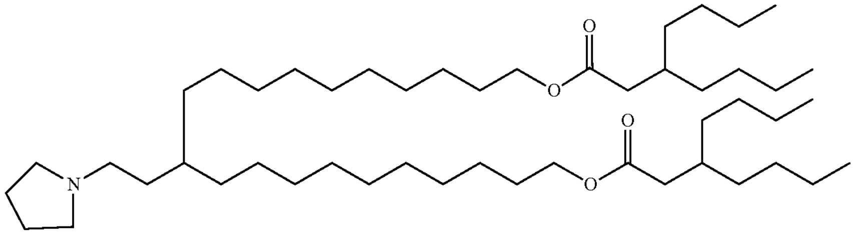

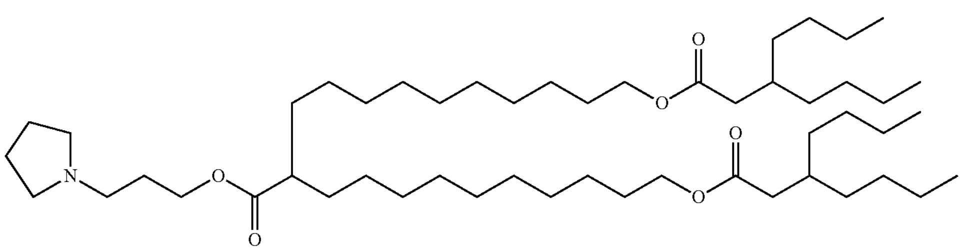

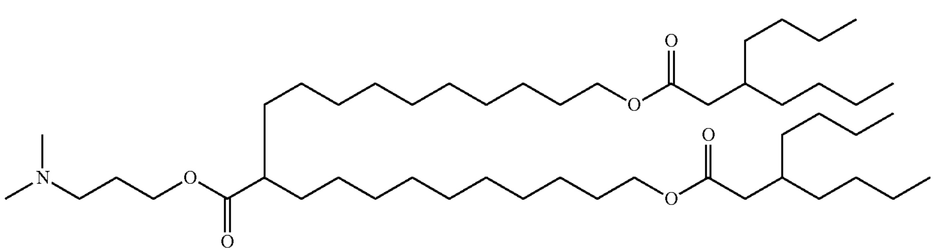

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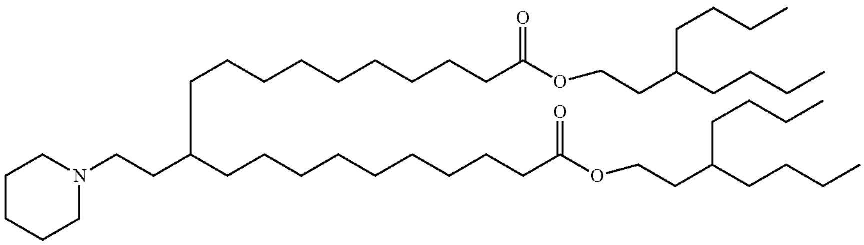

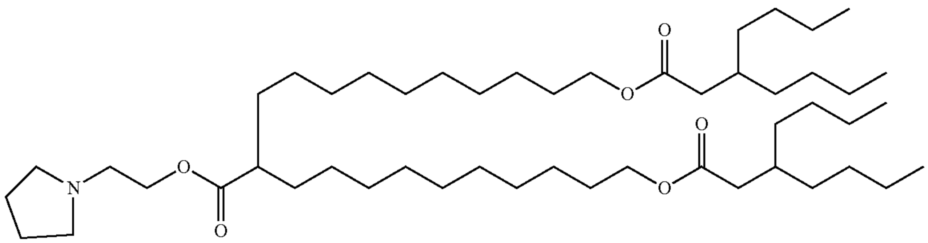

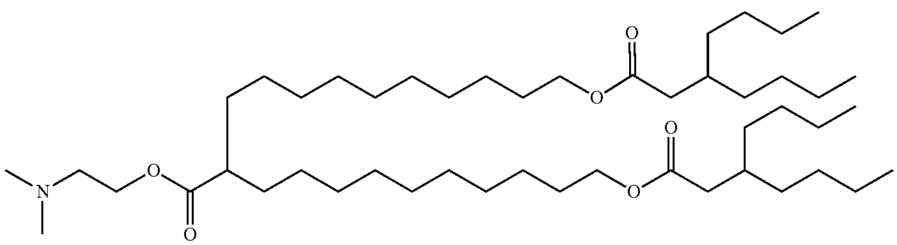

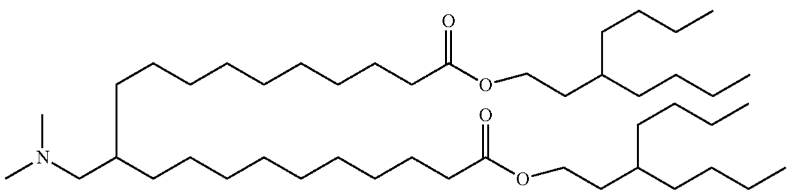

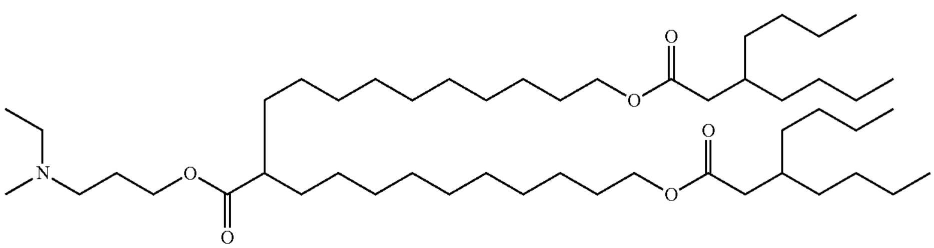

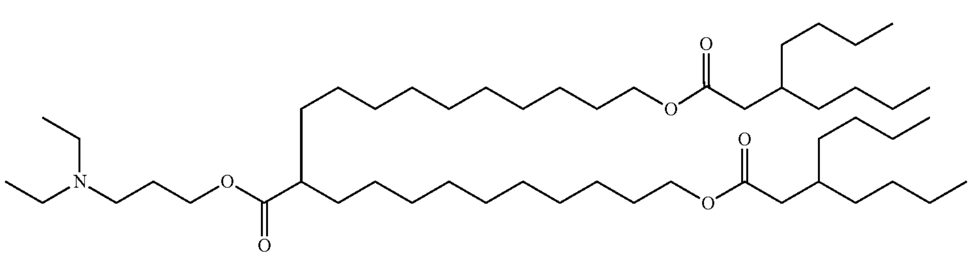

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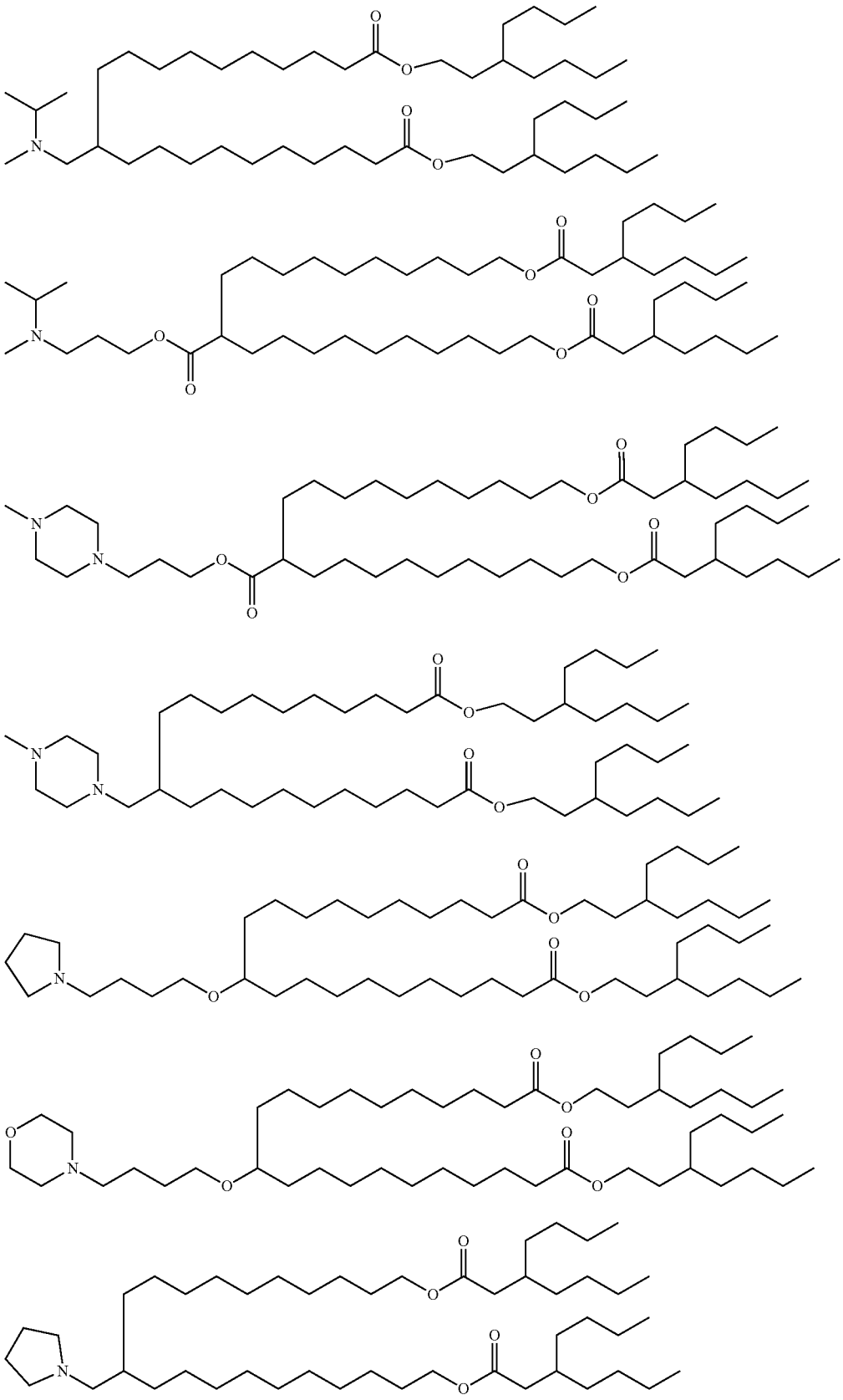

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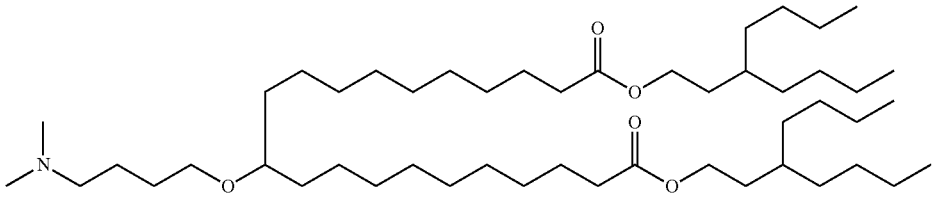

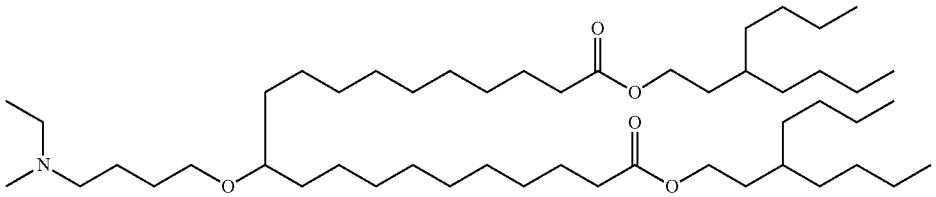

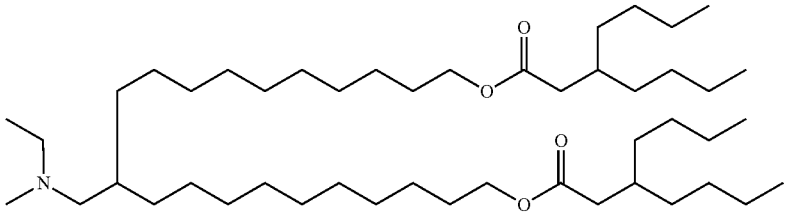

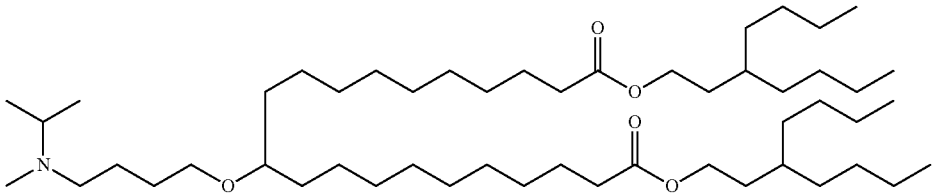

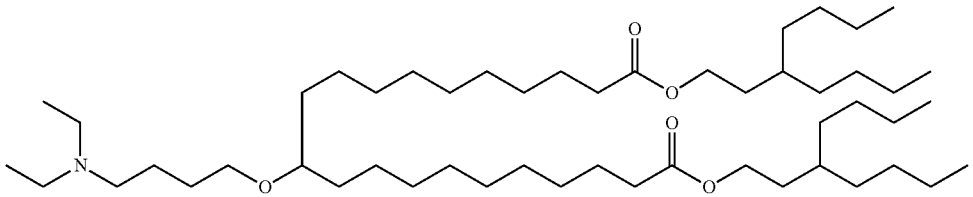

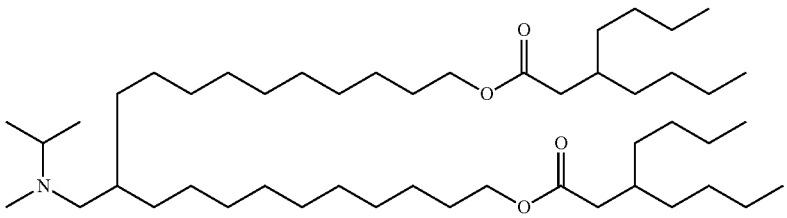

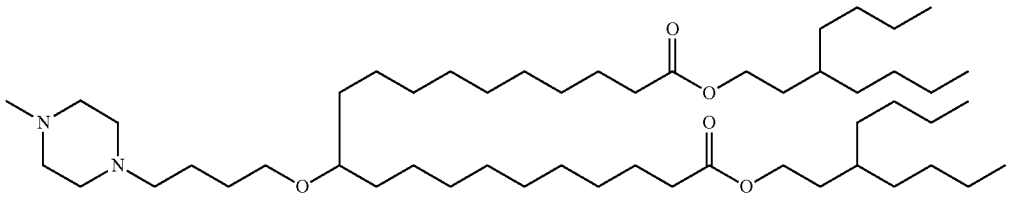

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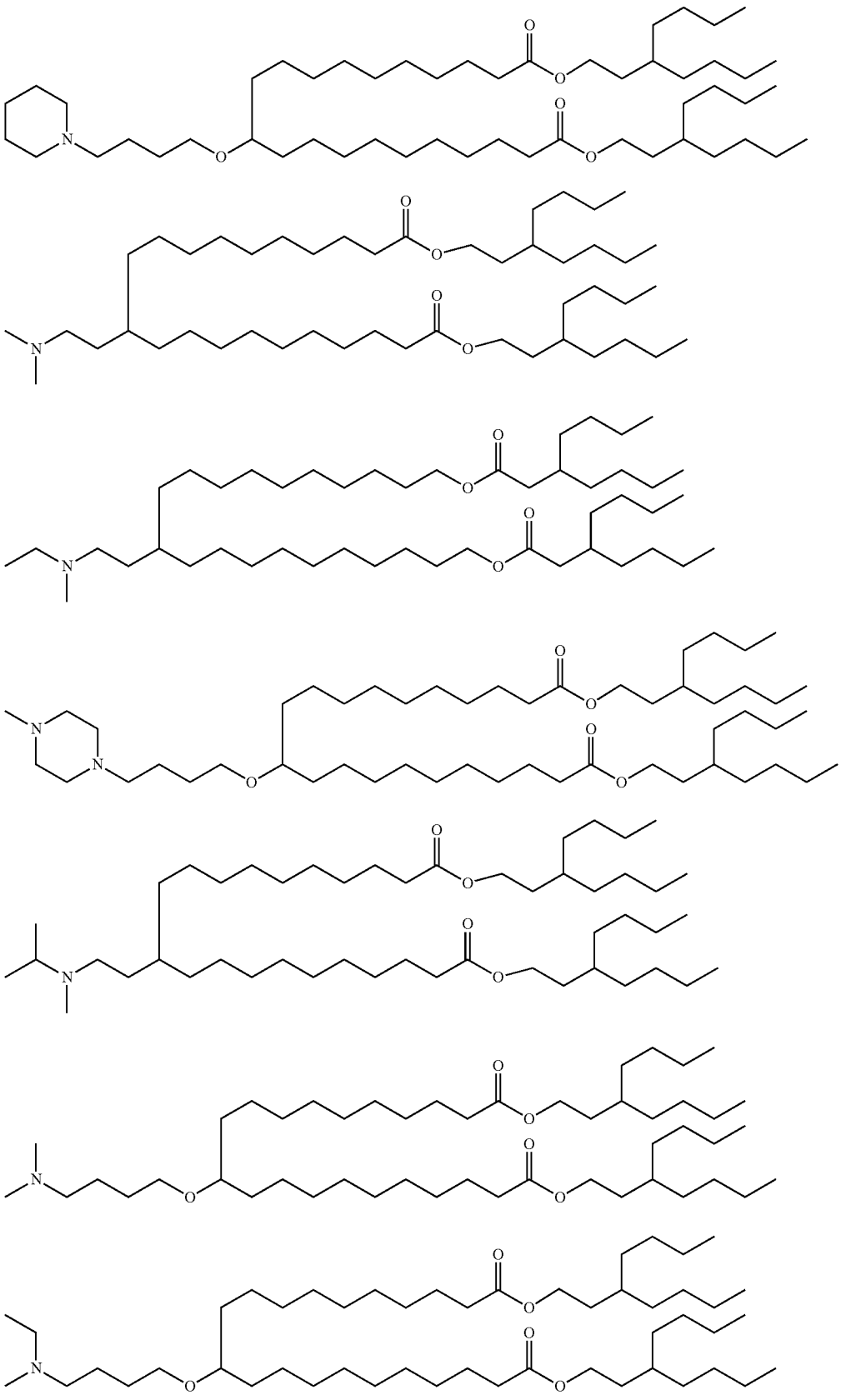

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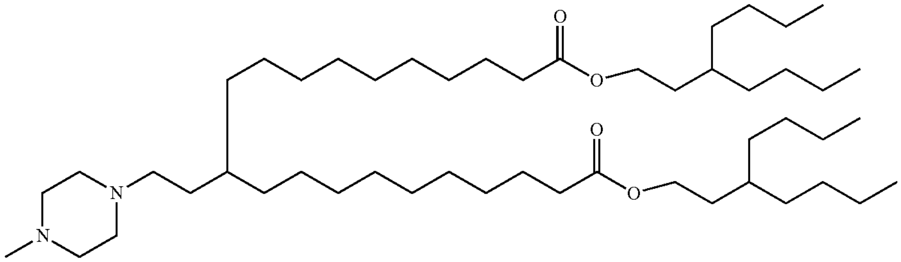

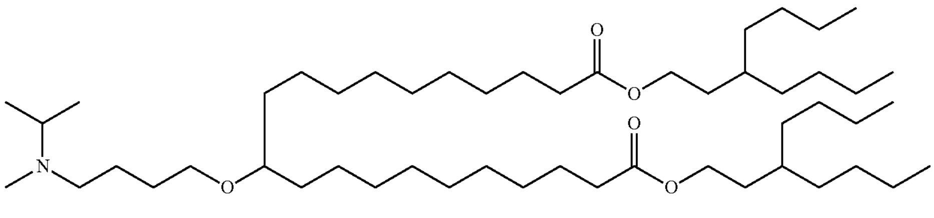

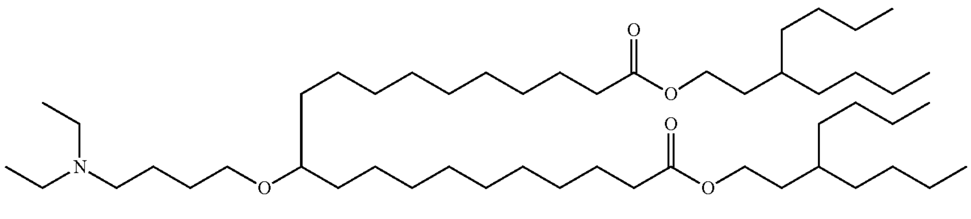

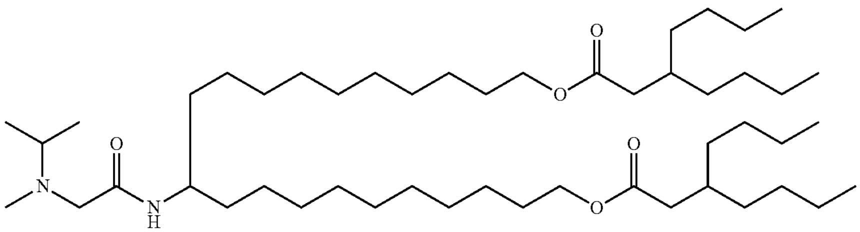

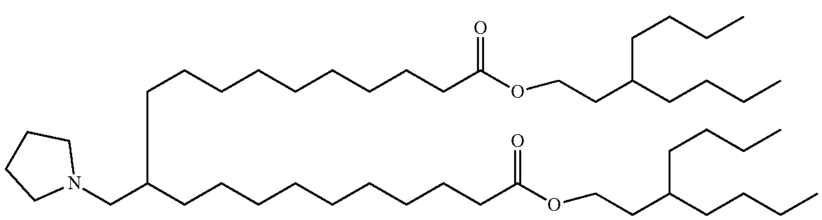

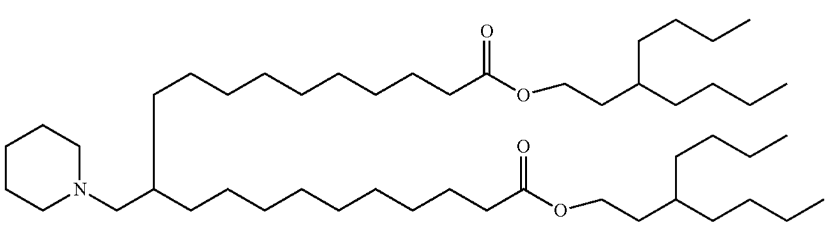

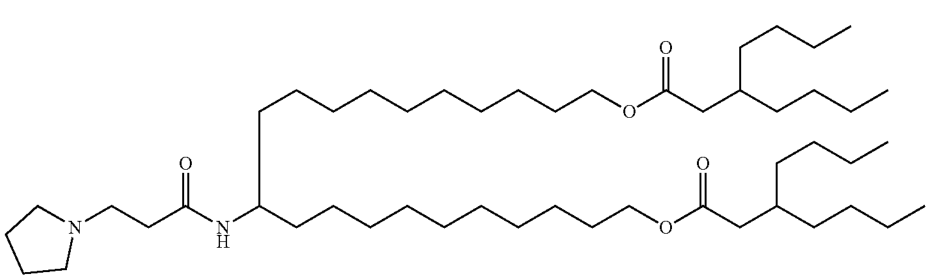

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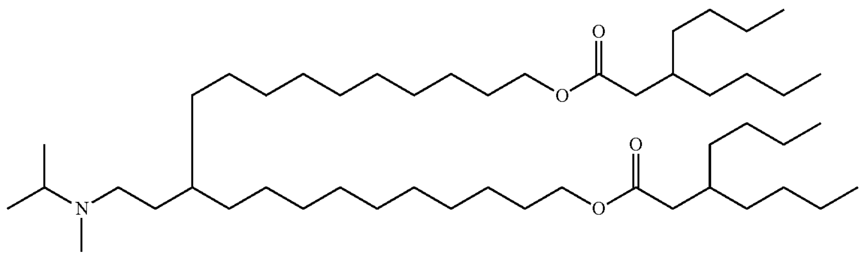

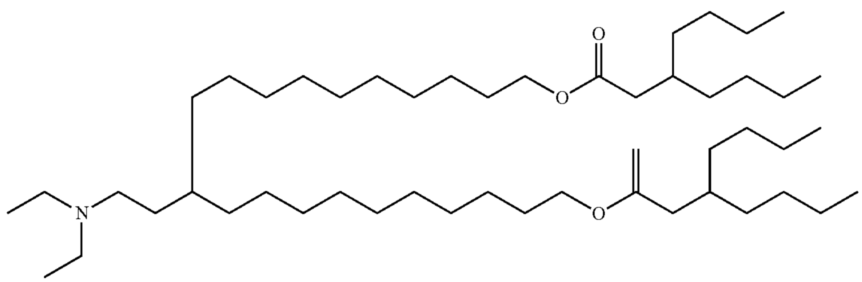

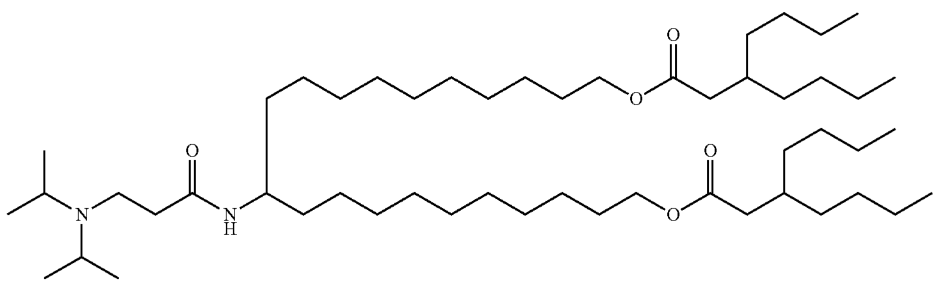

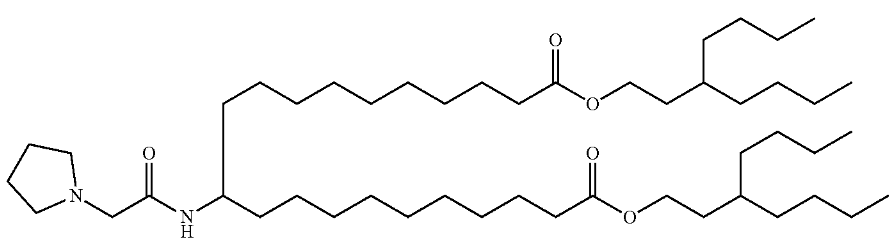

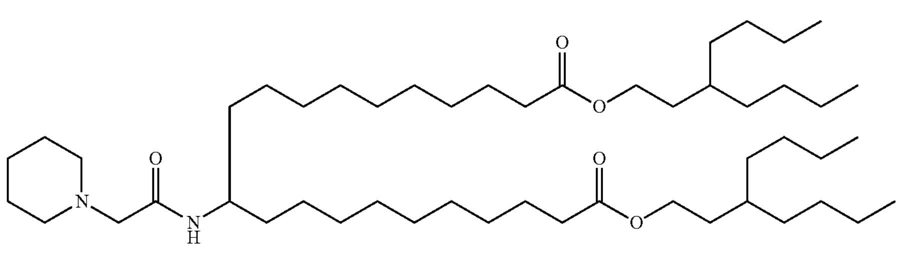

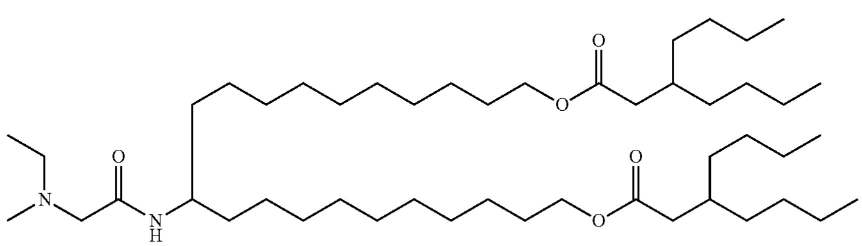

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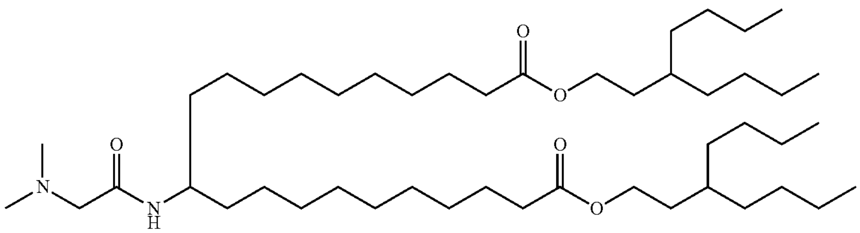

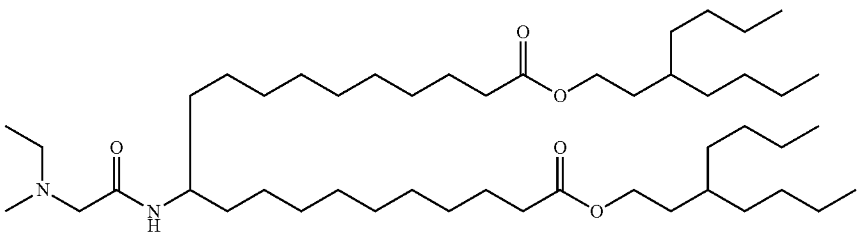

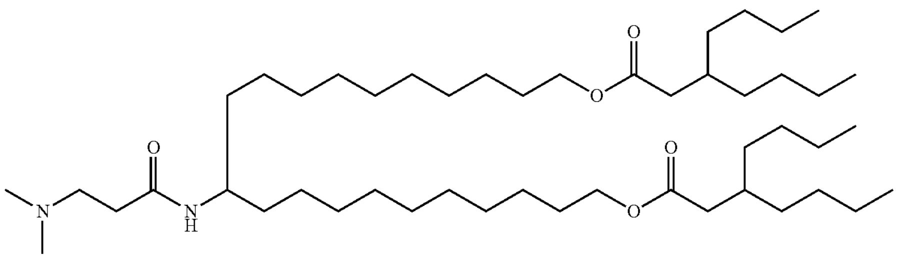

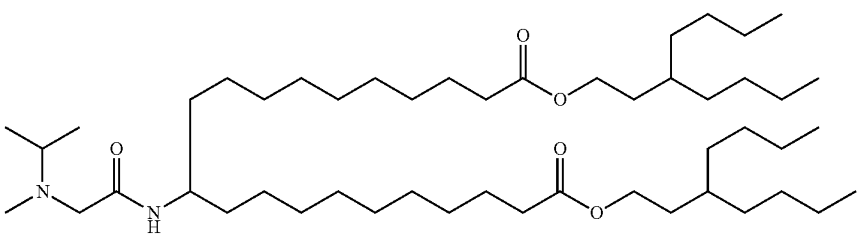

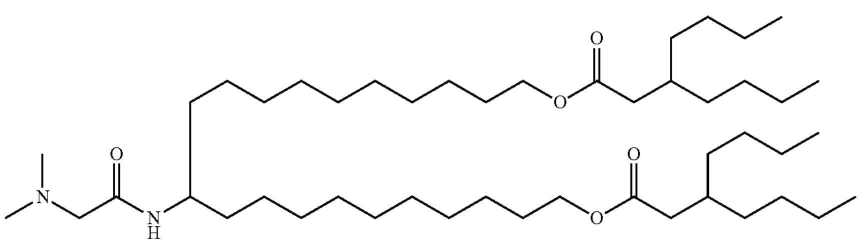

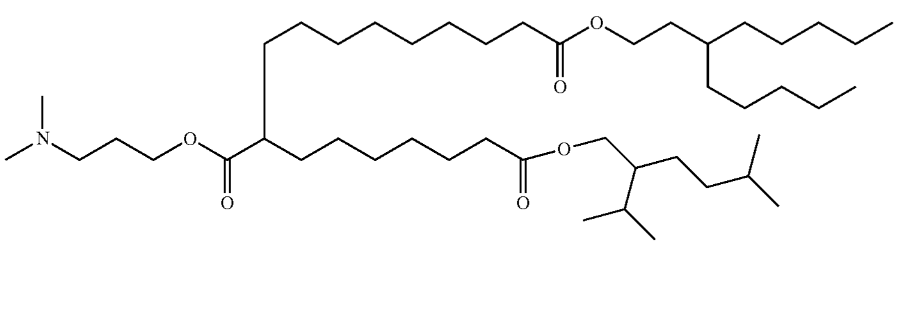

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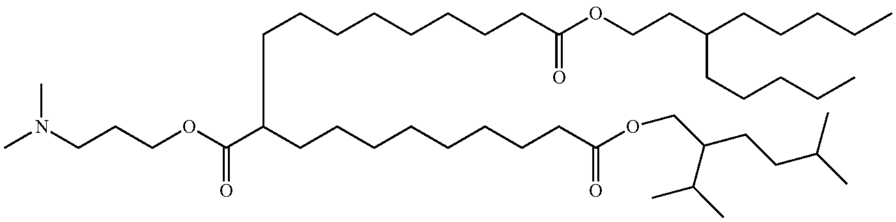
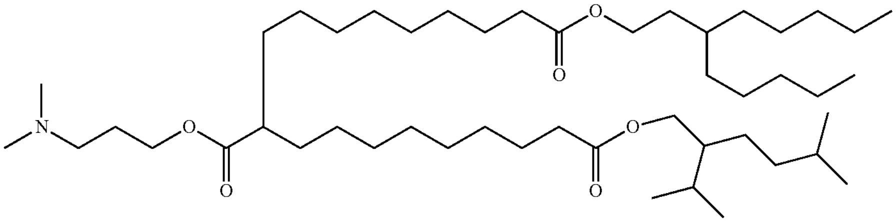
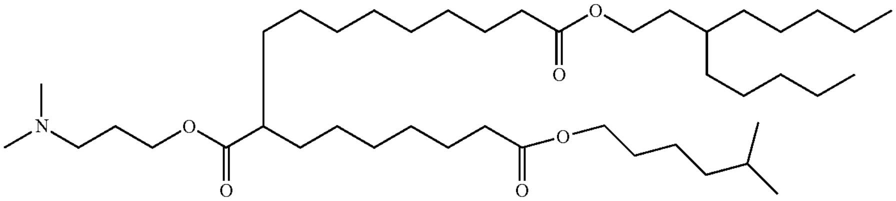
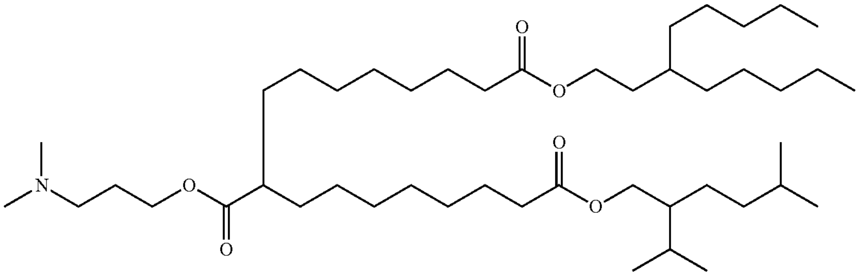
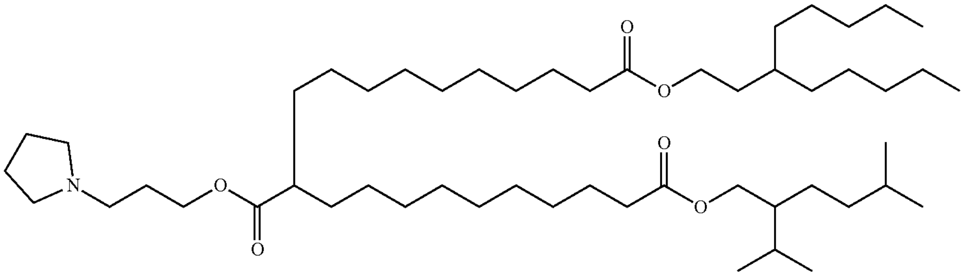
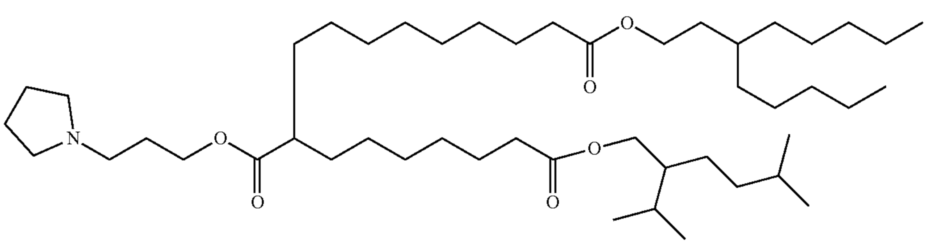






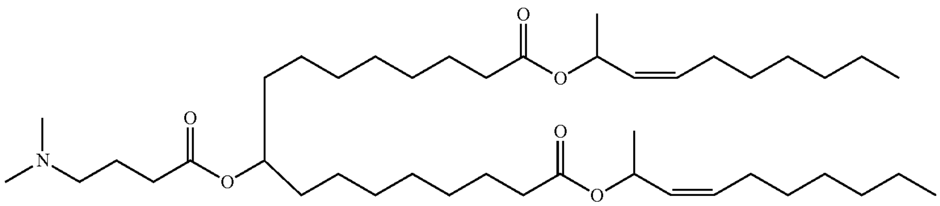


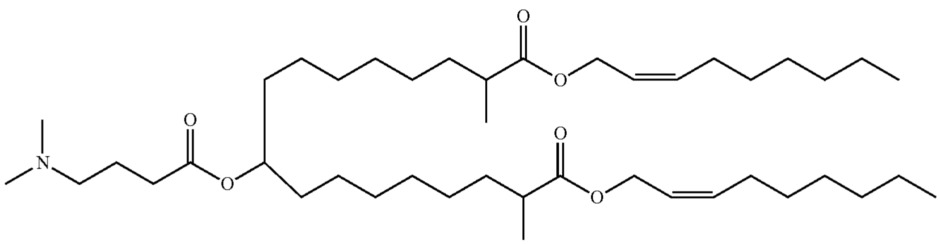


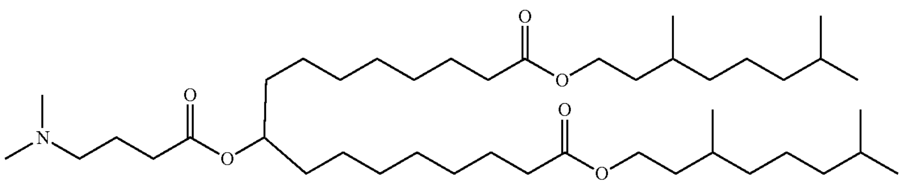


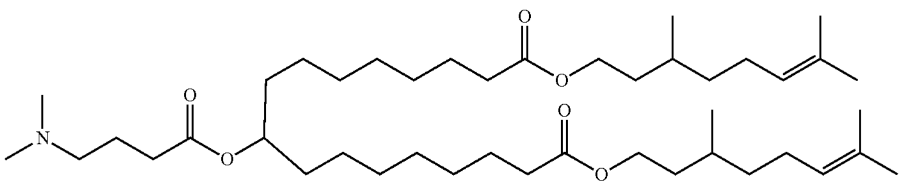
In another aspect, the present invention relates to a method of preparing a compound of Formula I-VII. Suitable exemplary synthetic methods are illustrated in Schemes 1-27 shown in the Examples section below.

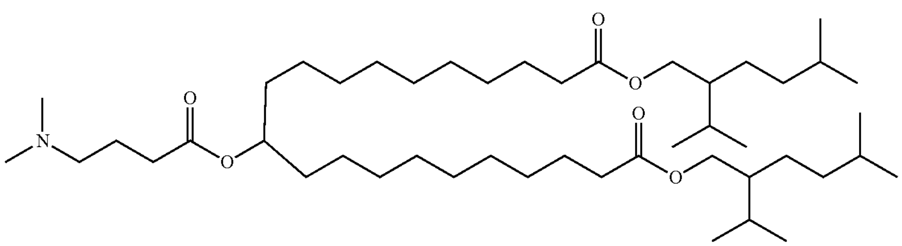
In one embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof). These cationic lipids are suitable for forming nucleic acid-lipid particles.

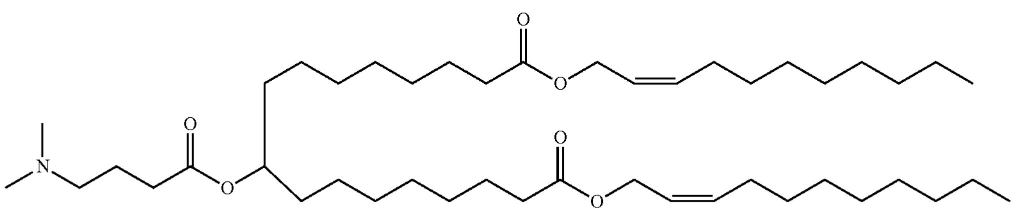


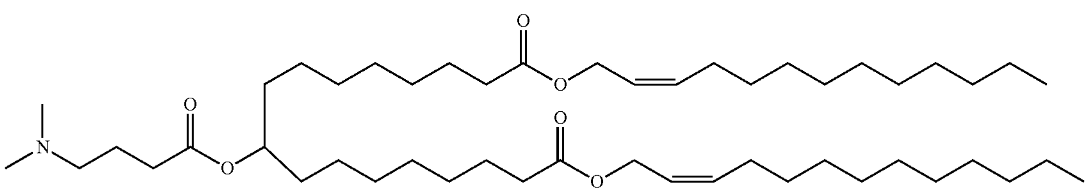


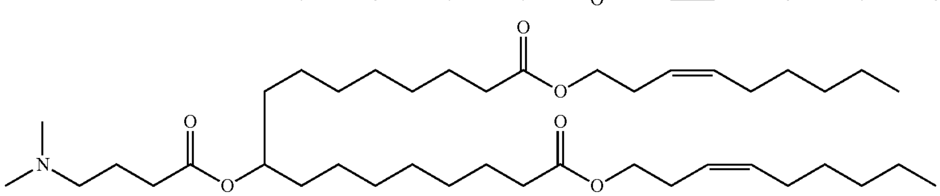












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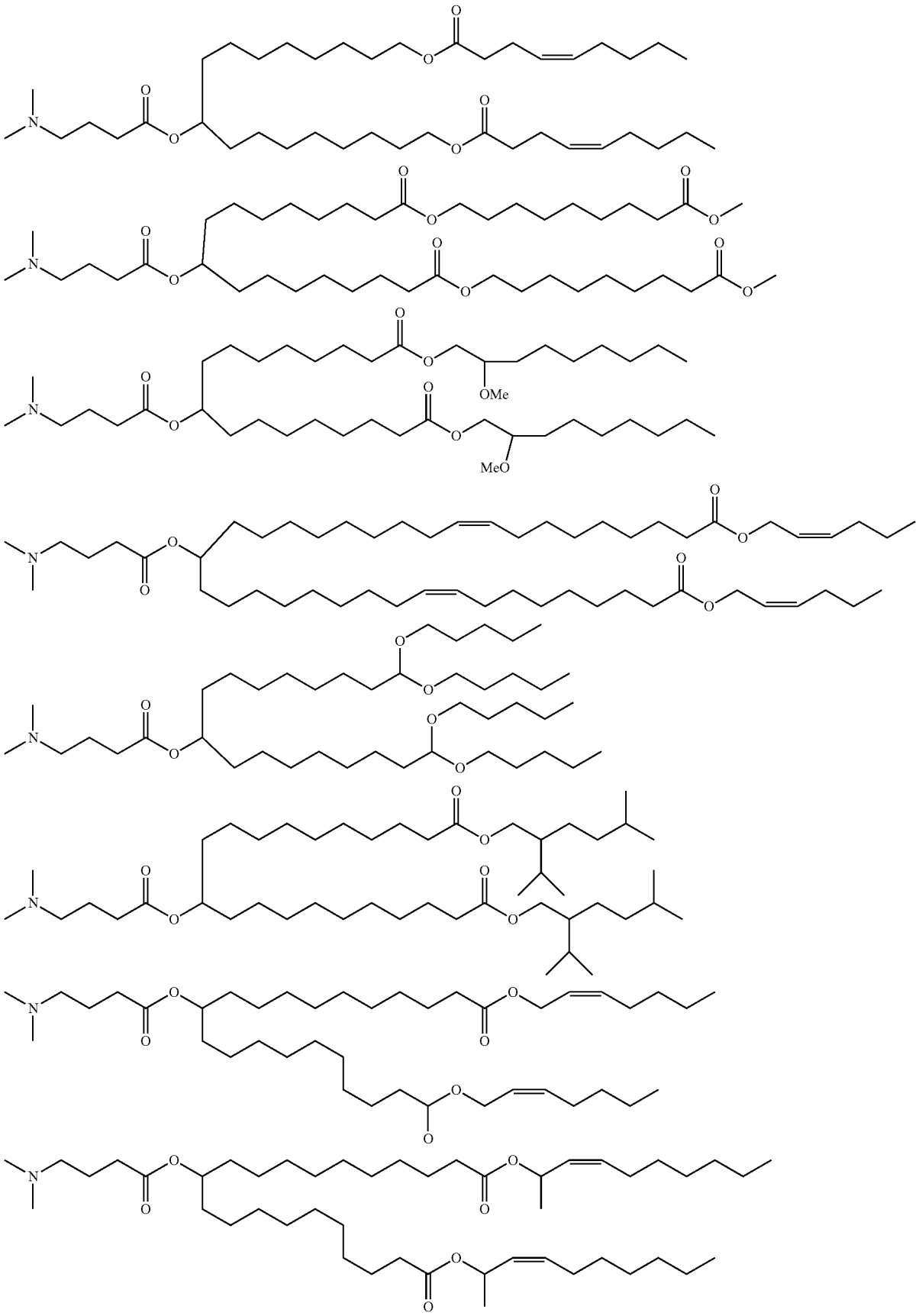

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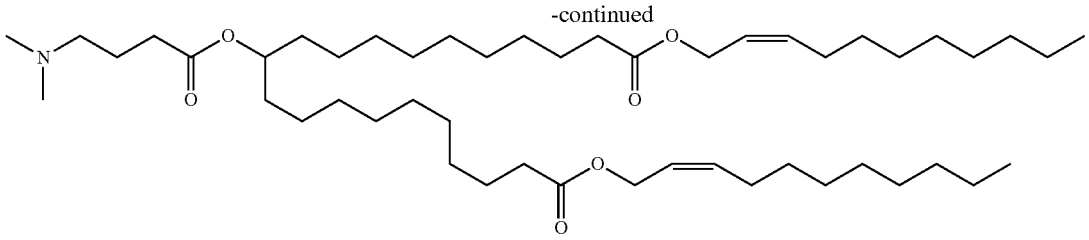

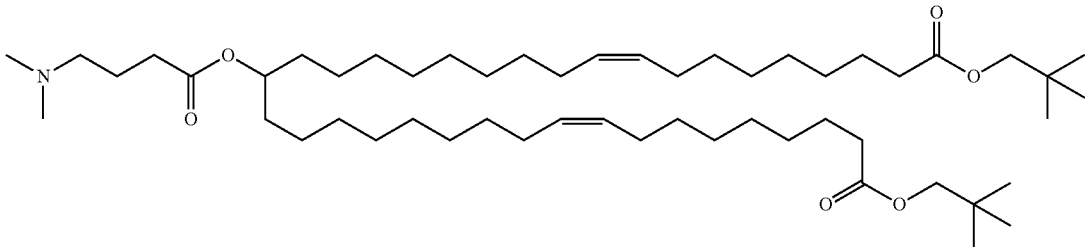

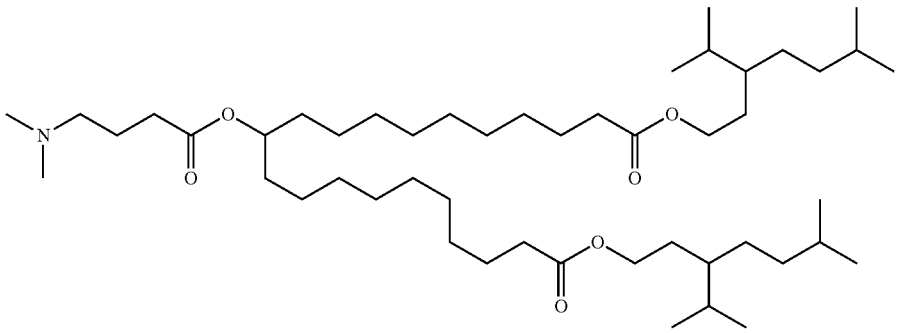

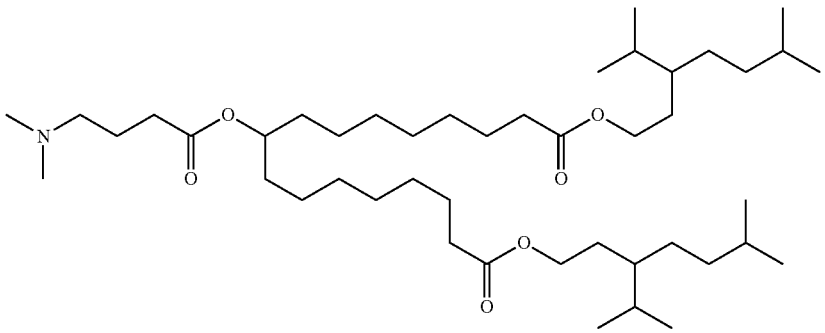

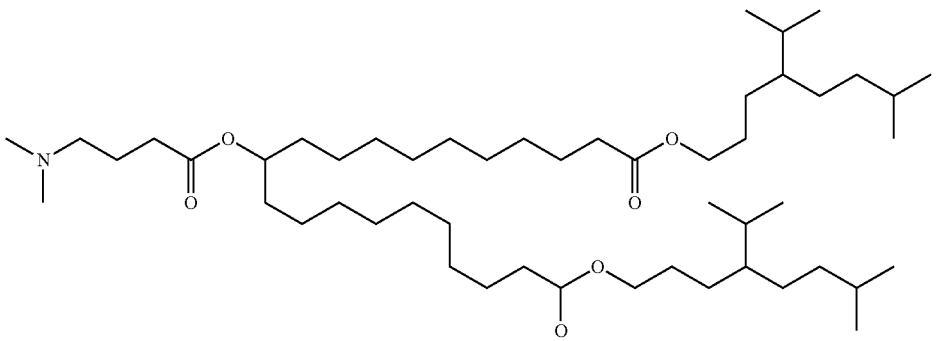

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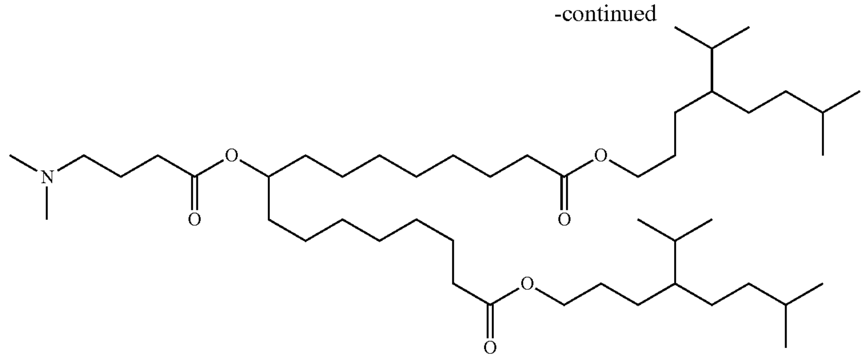
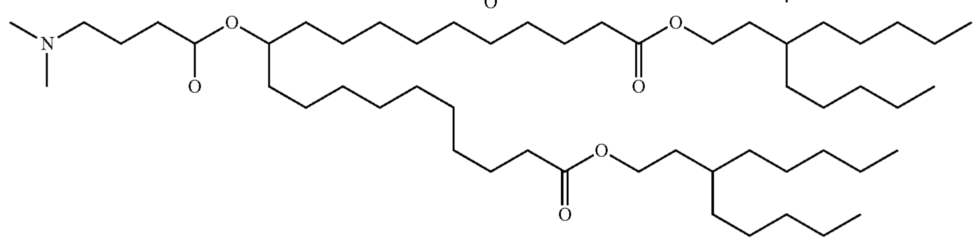
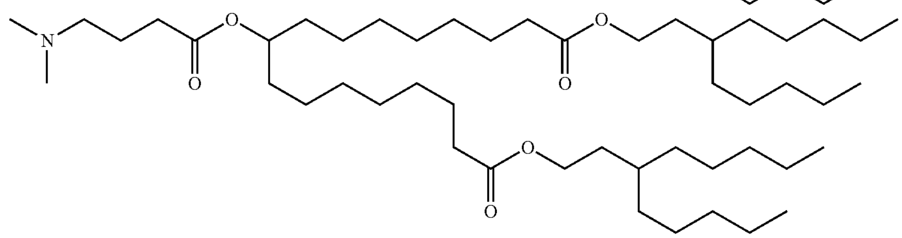
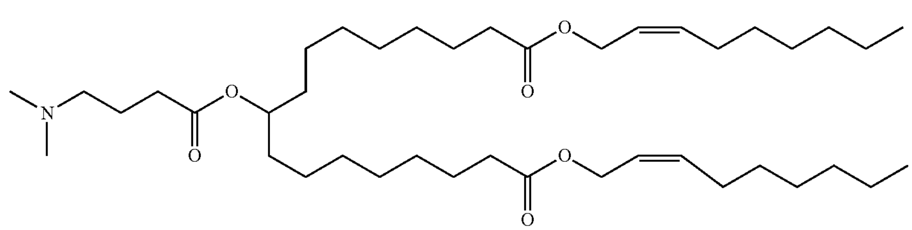
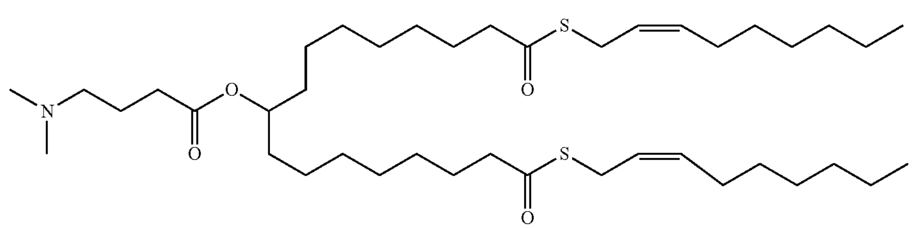


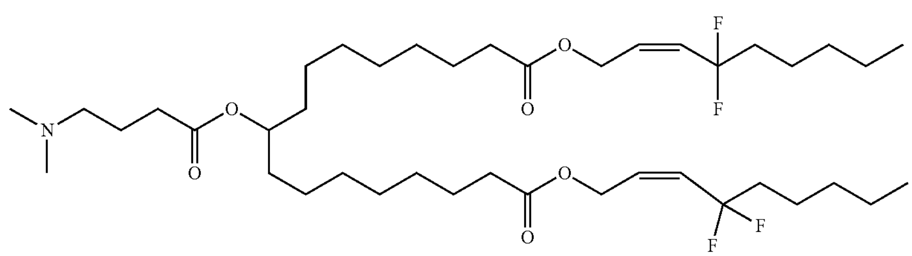




In another embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof):







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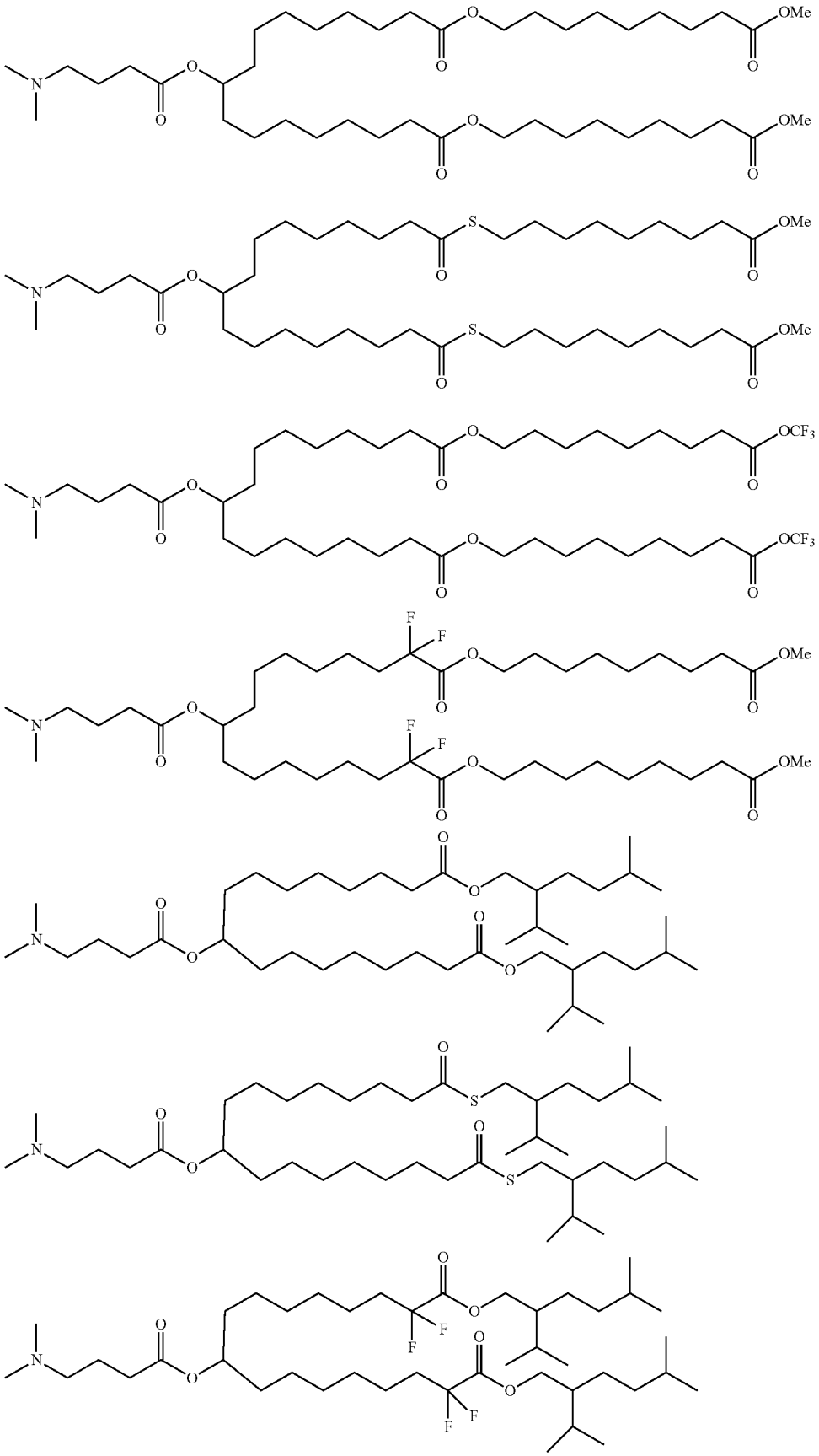

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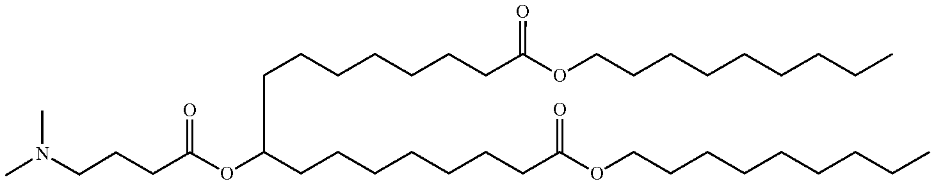

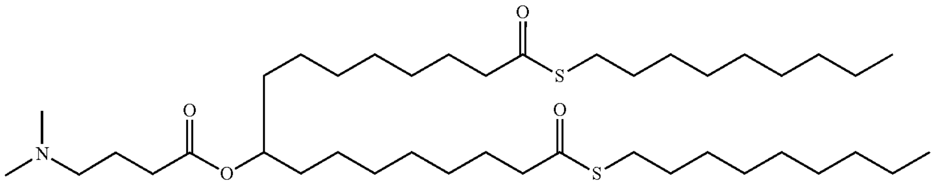

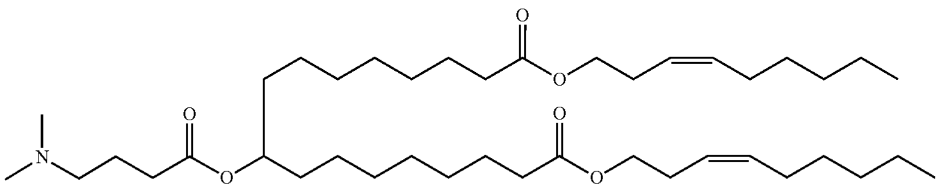

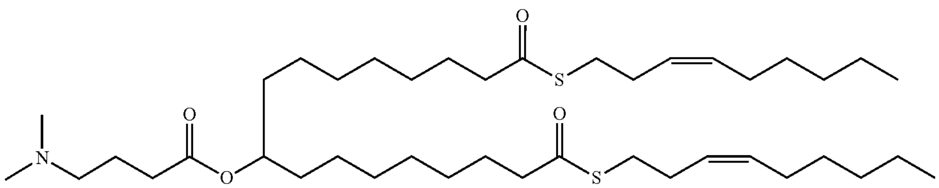

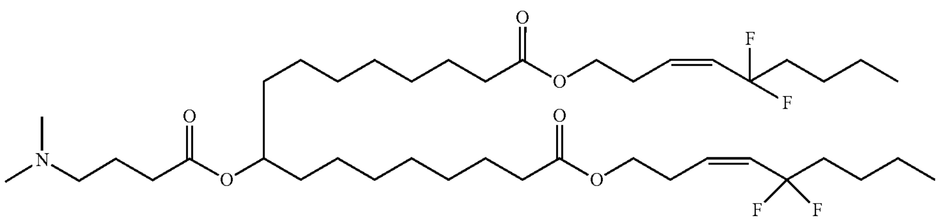

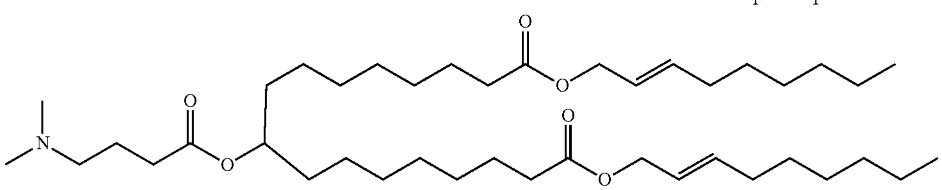

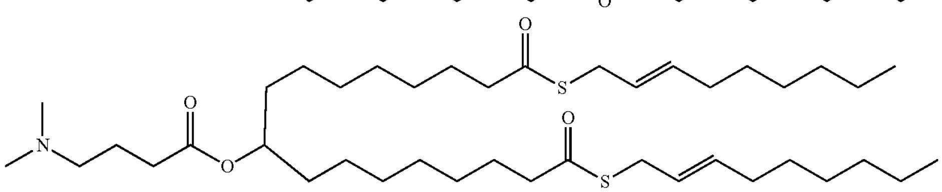

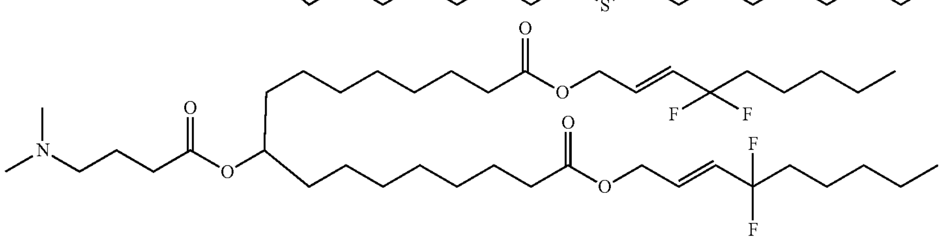

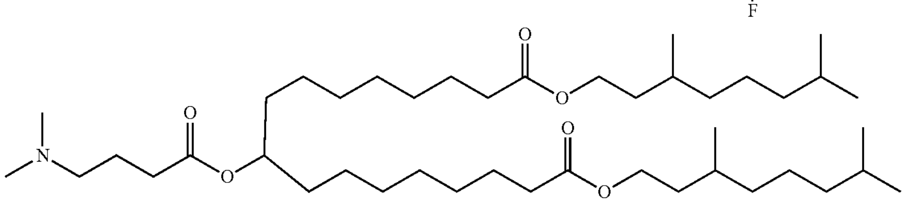

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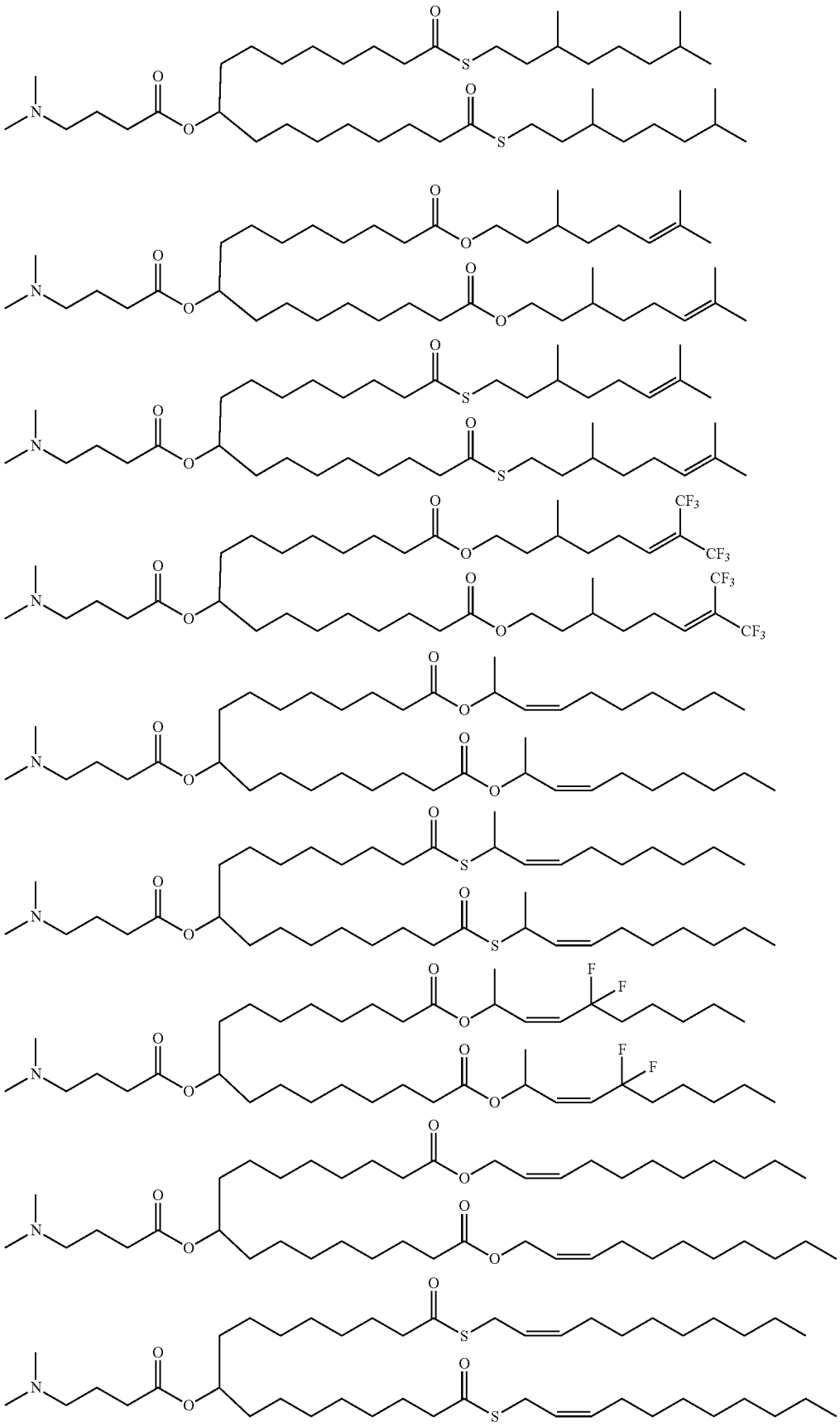

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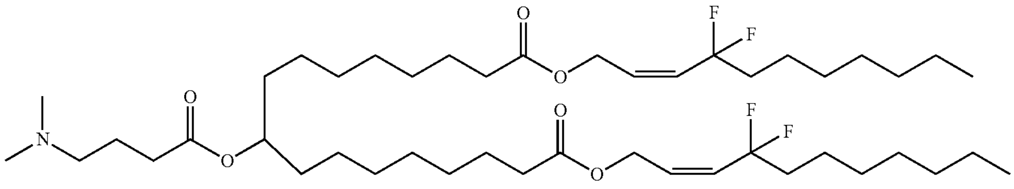
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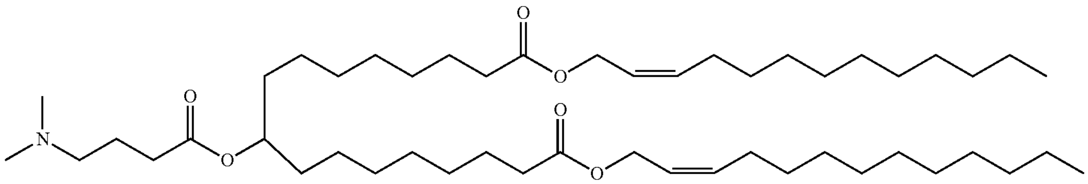
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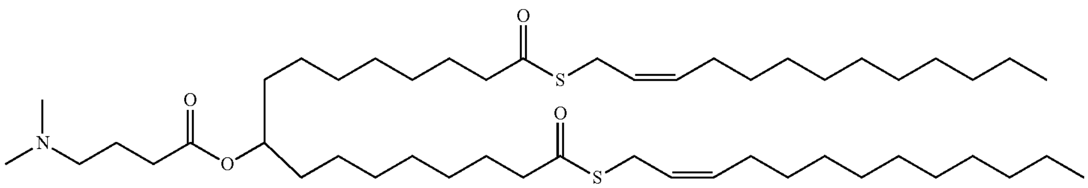
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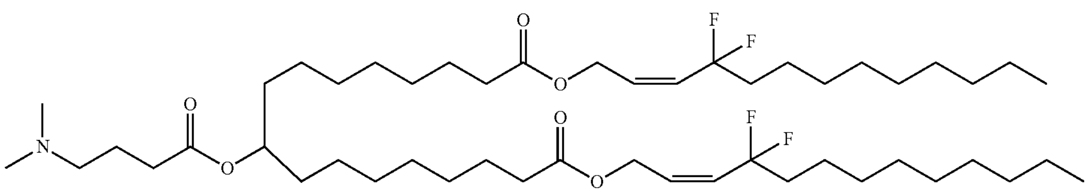
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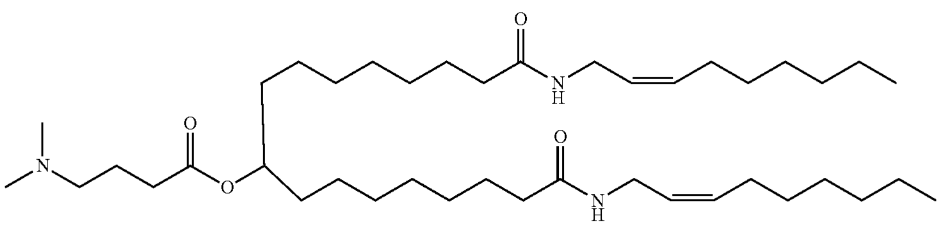
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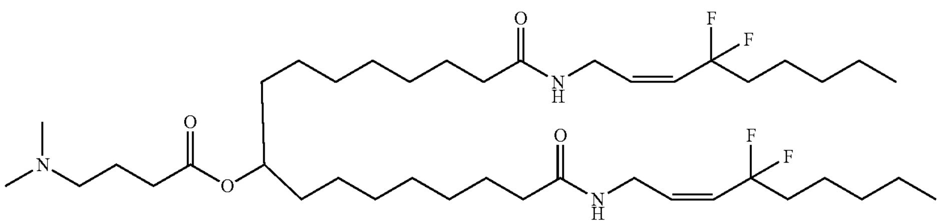
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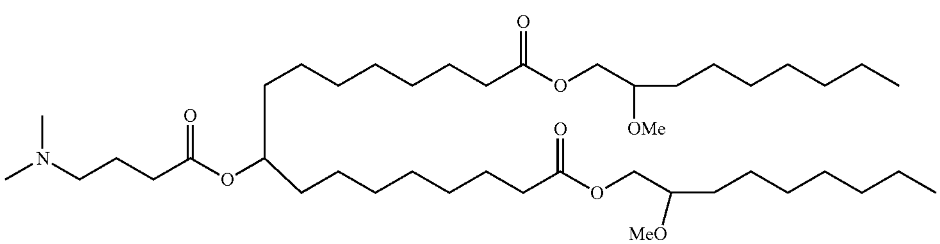
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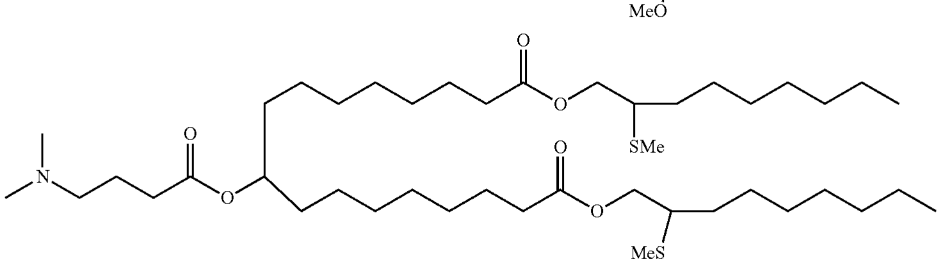
O
N
O
O
MeO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O
MeS

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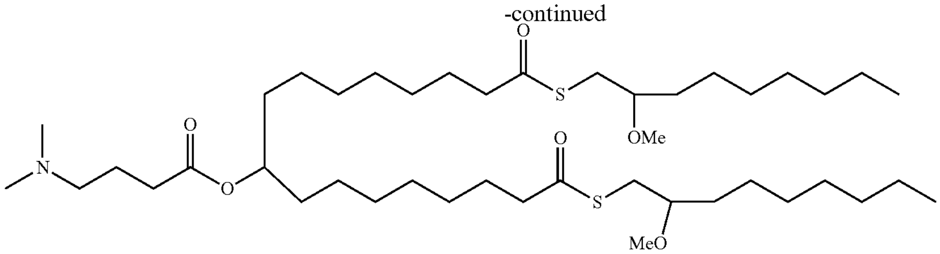
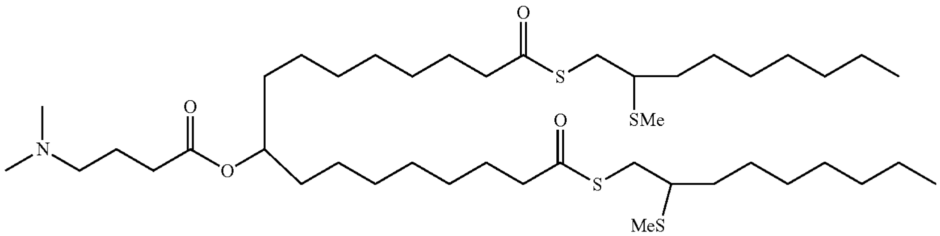
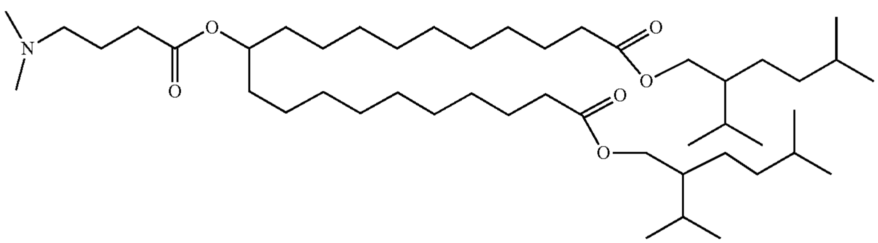
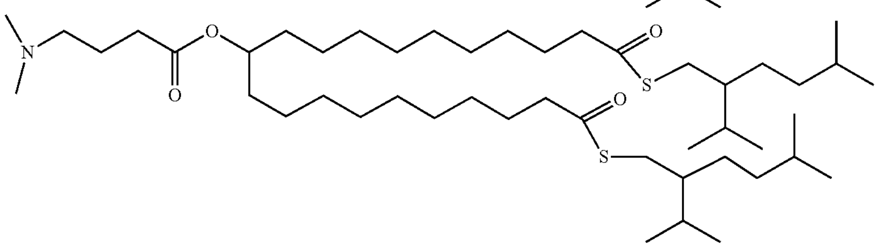
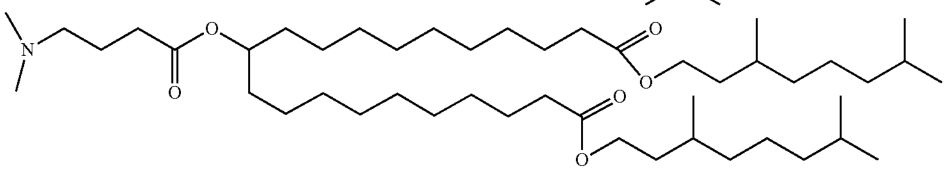
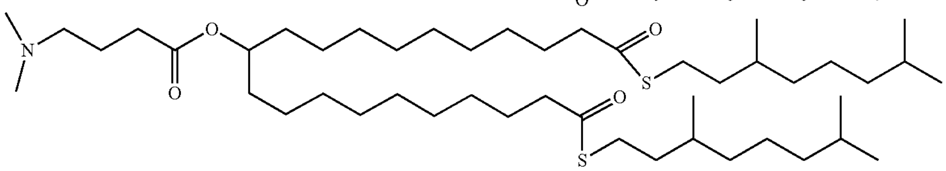


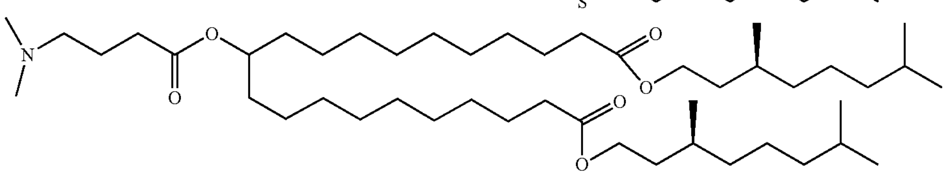


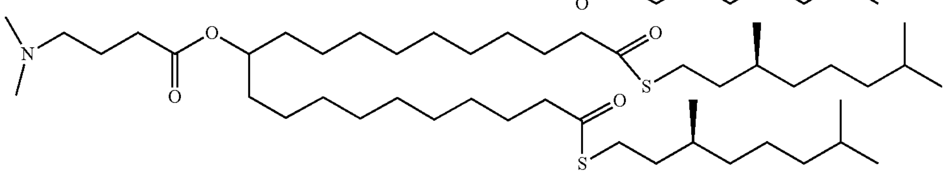




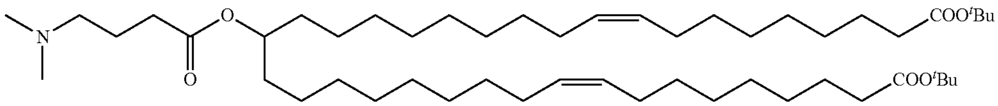


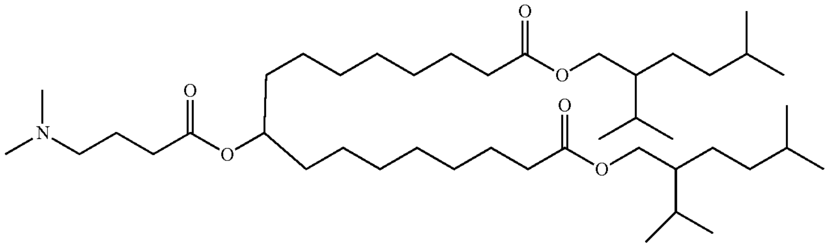


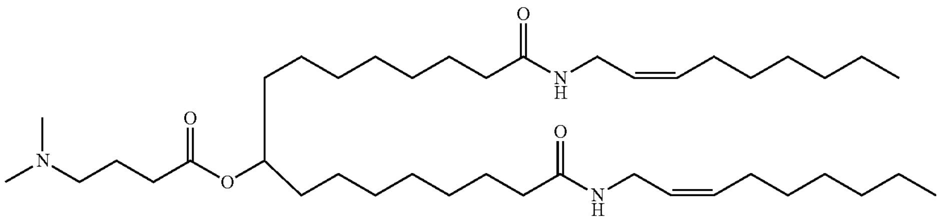


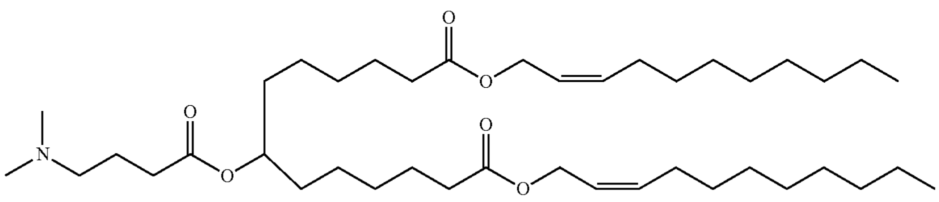


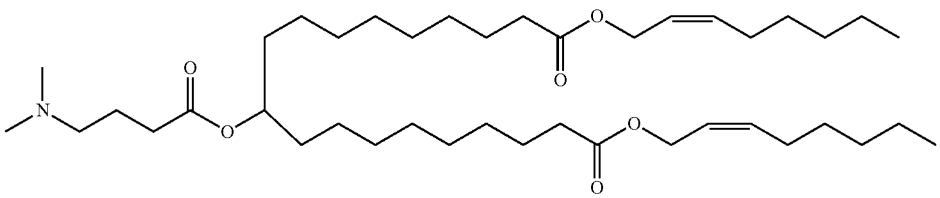
In another embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof):

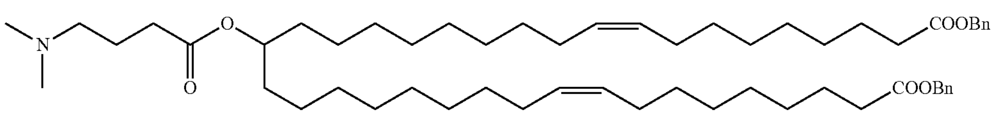


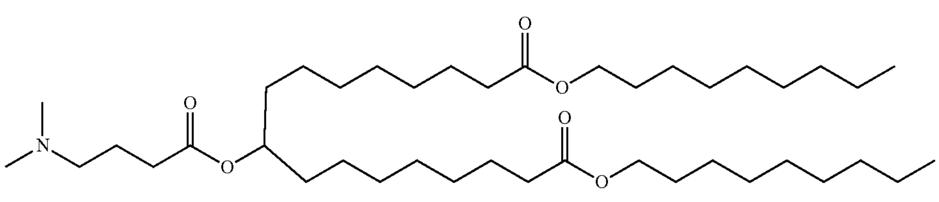


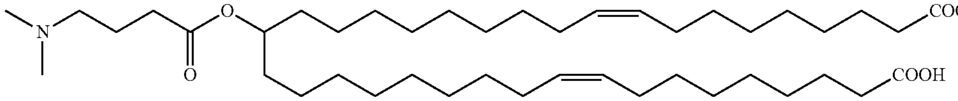


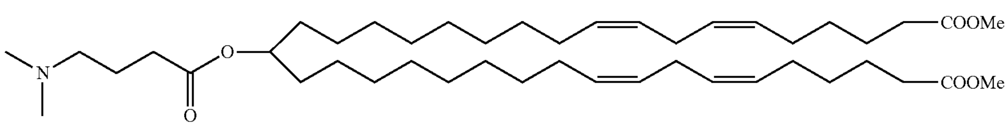


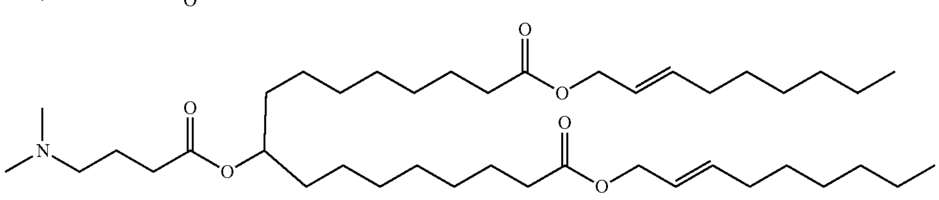




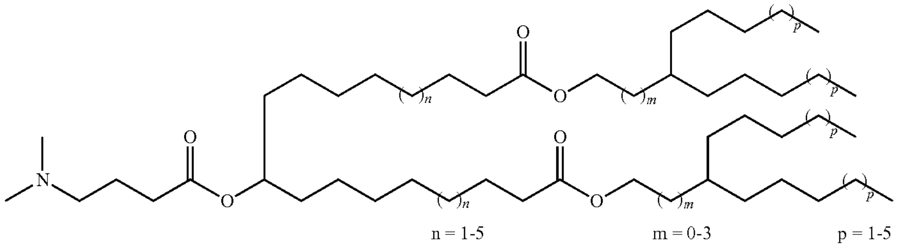


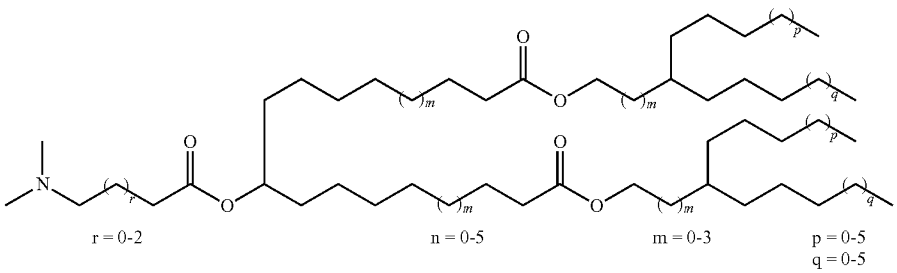


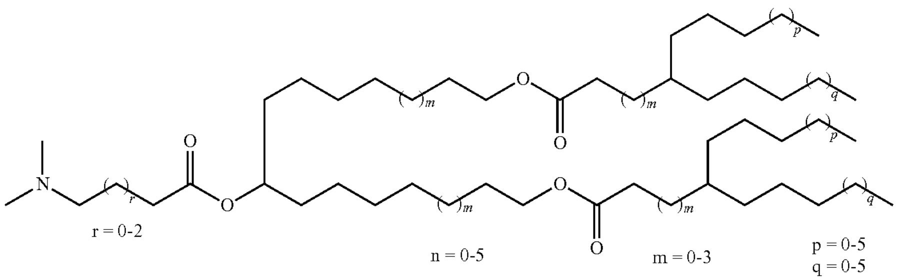


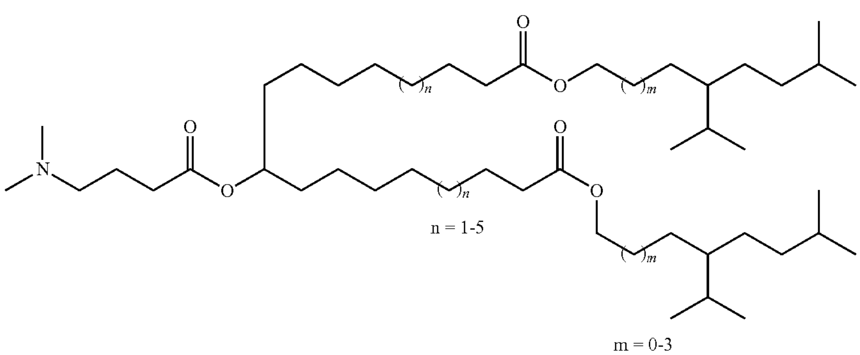


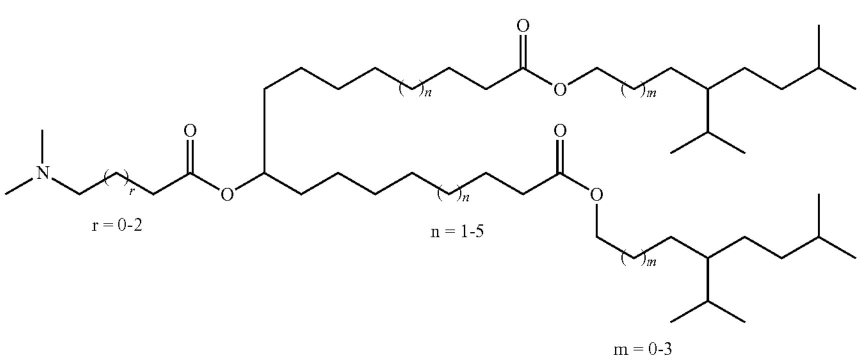
Additional representative cationic lipids include, but are not limited to:











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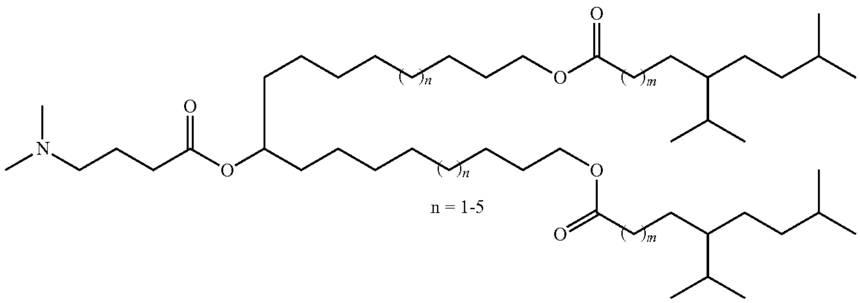
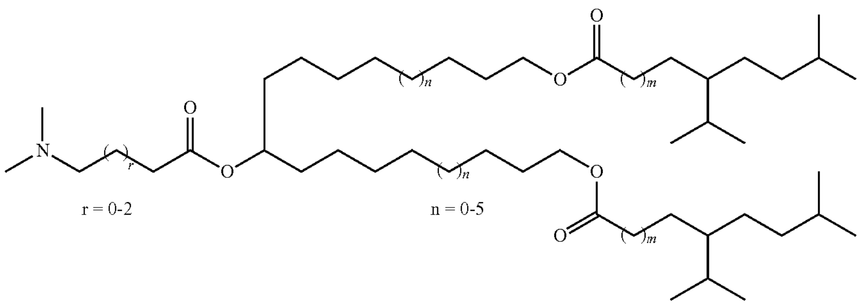


n = 1-5

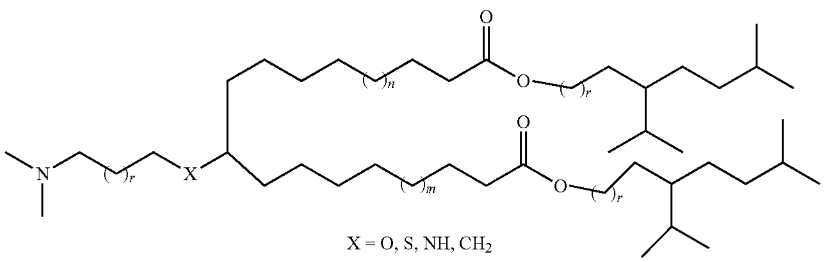
m = 0-3



r = 0-2

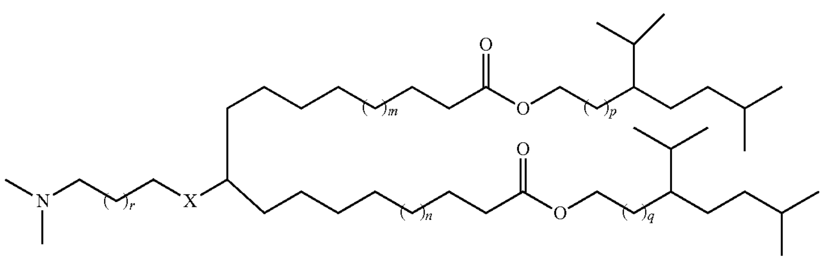
n = 0-5

m = 0-3

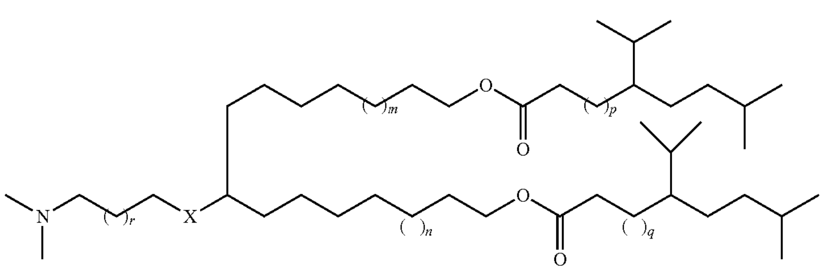


X = O, S, NH, CH_2

r = 0-2, n = 1-5, and m = 1-5



r = 0-2 X = O, S, NH, CH_2 m = 0-5 n = 0-5 p = 0-3 q = 0-3



r = 0-2 X = O, S, NH, CH_2 m = 0-5 n = 0-5 p = 0-3 q = 0-3

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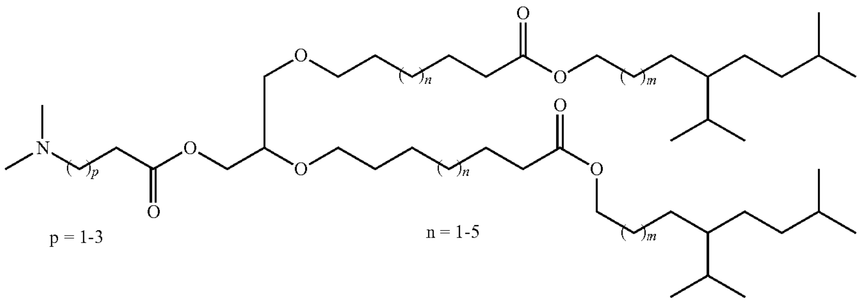
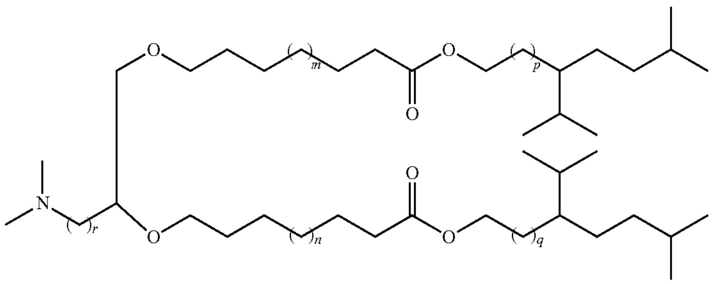
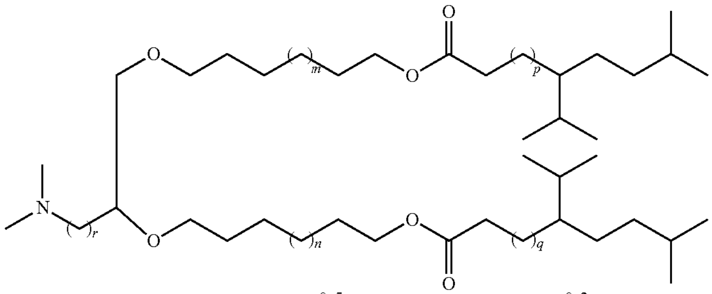


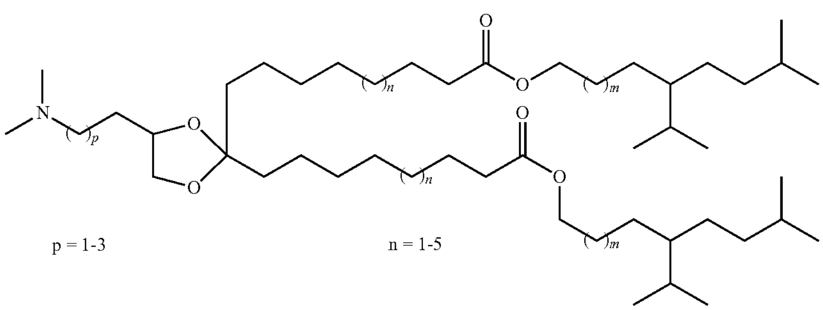
p = 1-3 n = 1-5 m = 0-3



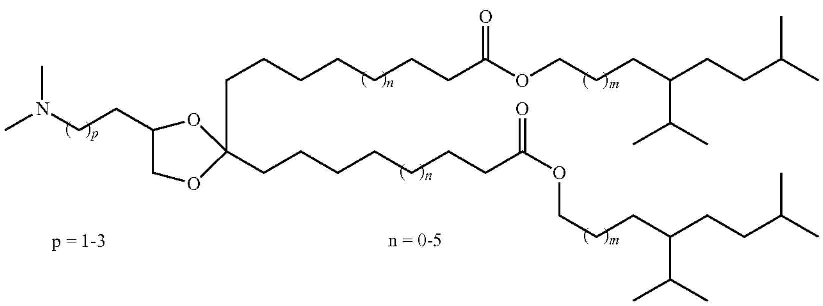
r = 0-2 m = 0-5 n = 0-5 p = 0-3 q = 0-3



r = 0-2 m = 0-5 n = 0-5 p = 0-3 q = 0-3



p = 1-3 n = 1-5 m = 0-3



p = 1-3 n = 0-5 m = 0-3

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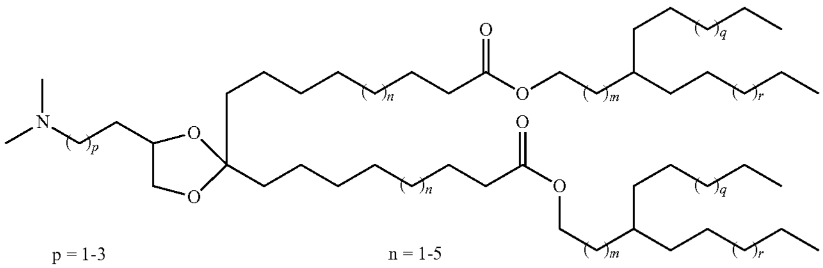
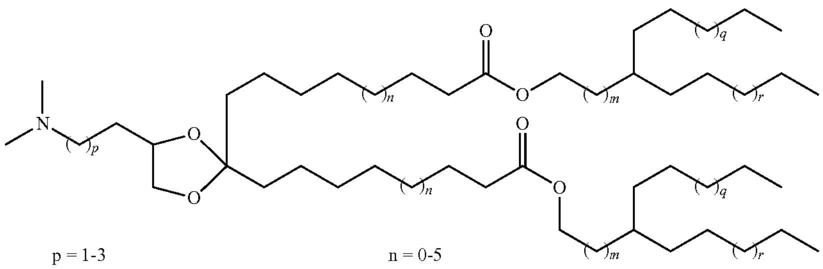
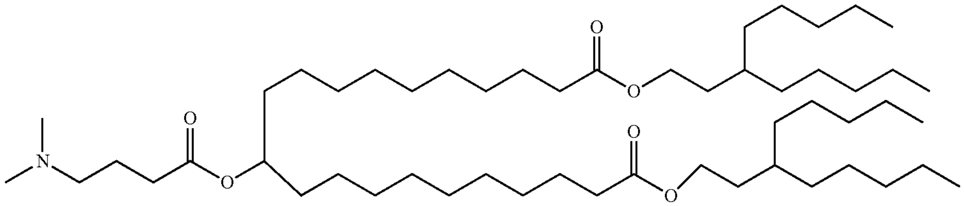
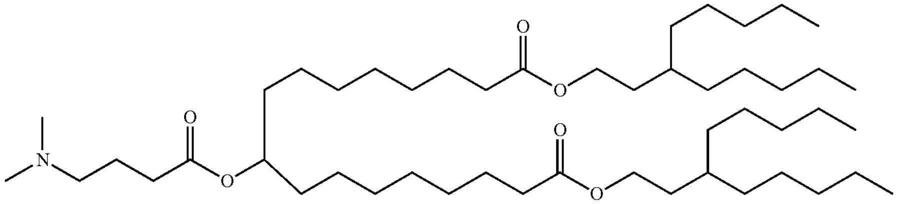


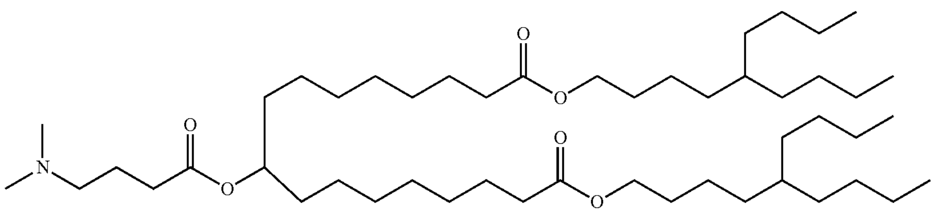
p = 1-3 n = 1-5 m = 0-3; q = 0-4; r = 0-4

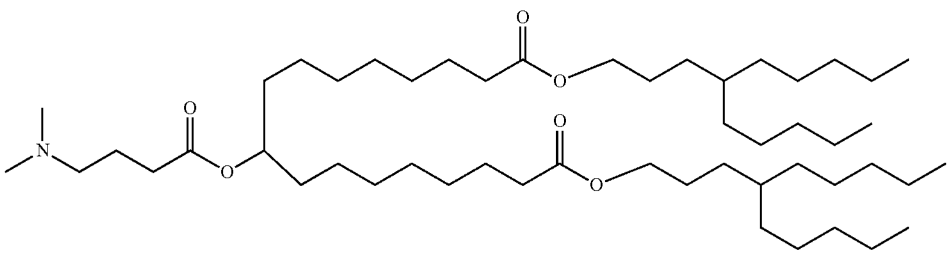


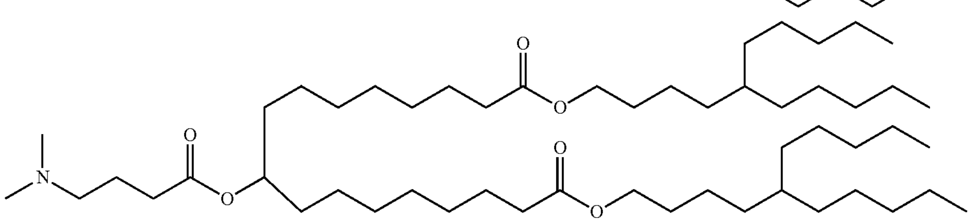
p = 1-3 n = 0-5 m = 0-3; q = 0-4; r = 0-4











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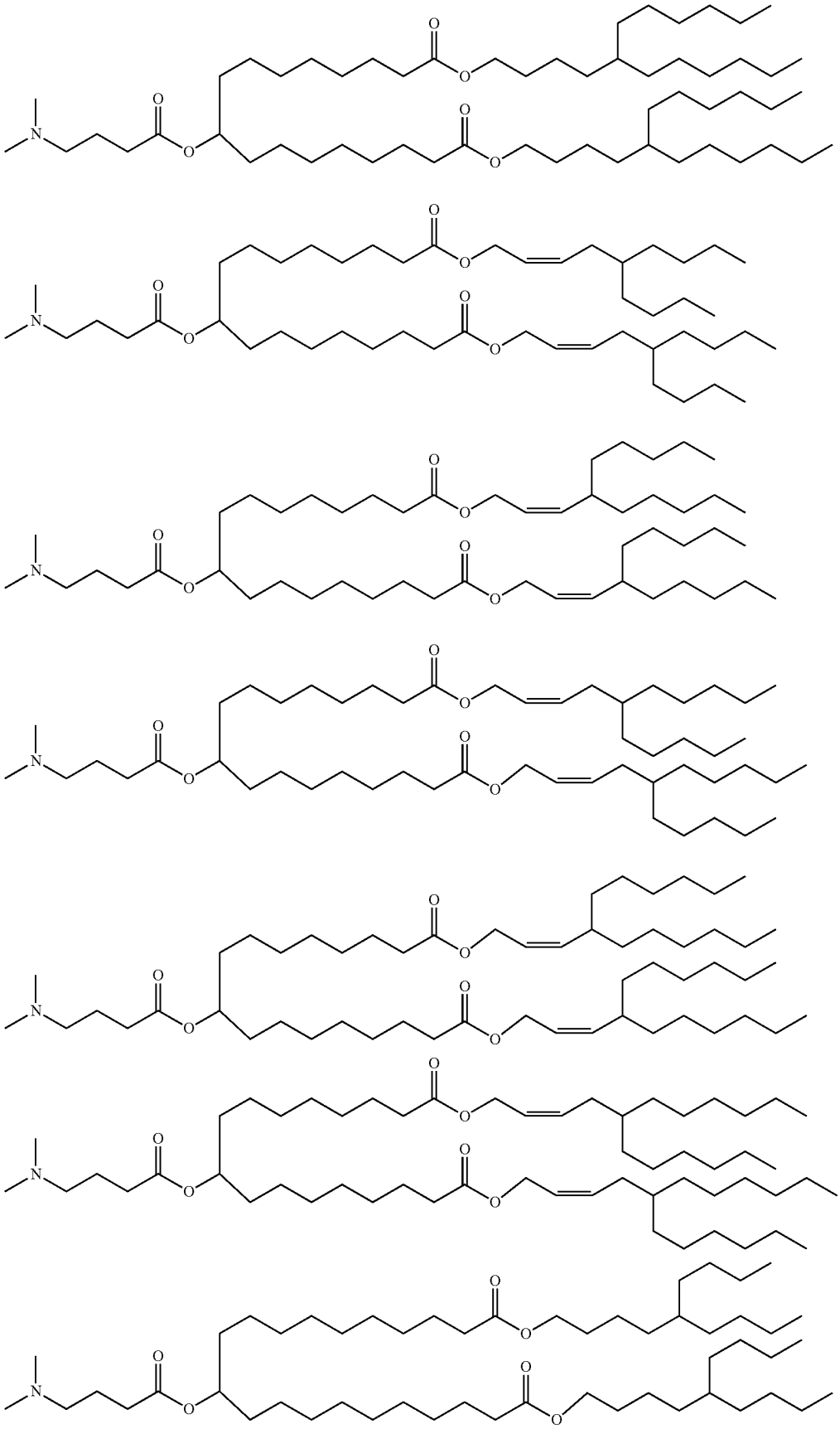

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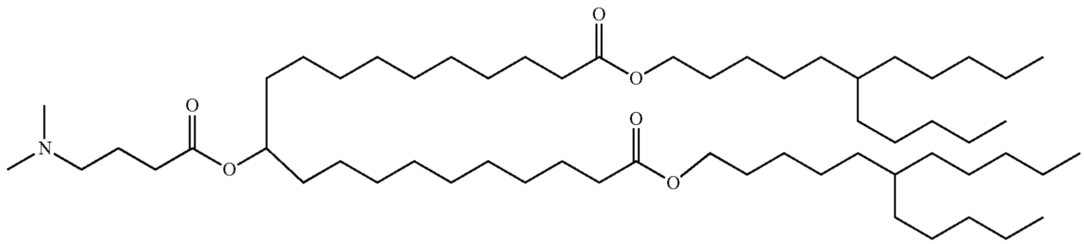
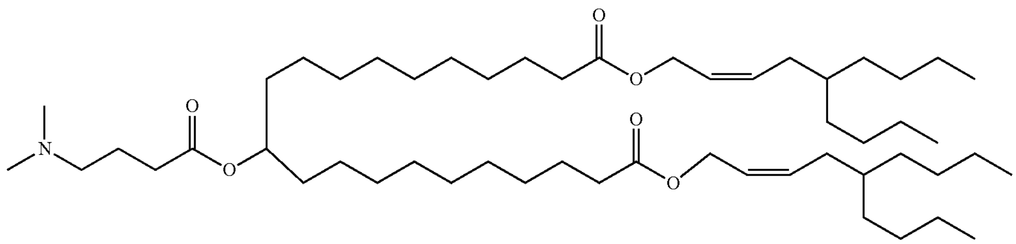
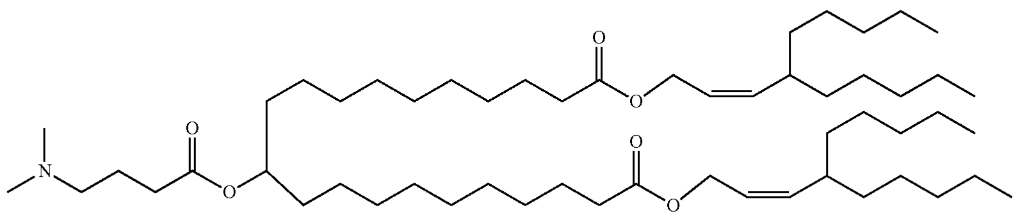
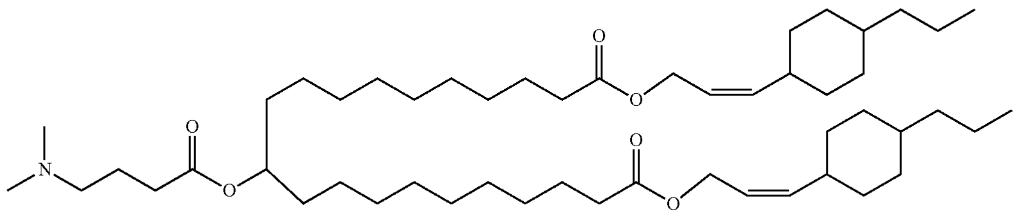
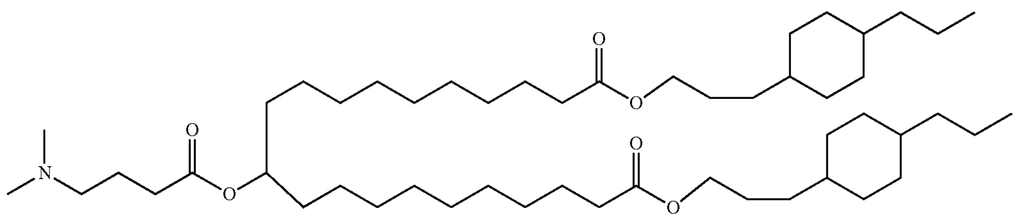
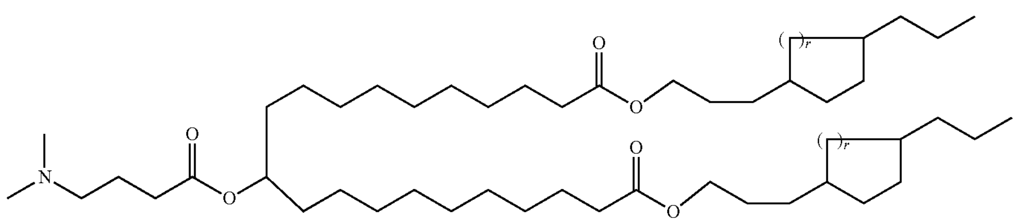




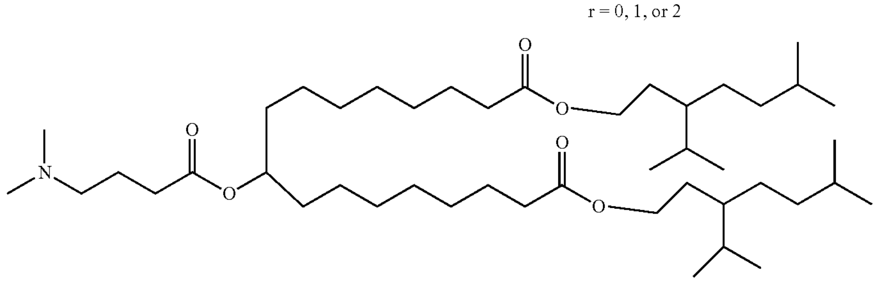








r = 0, 1, or 2



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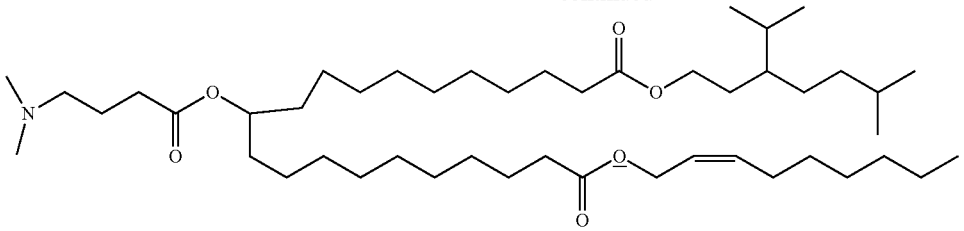
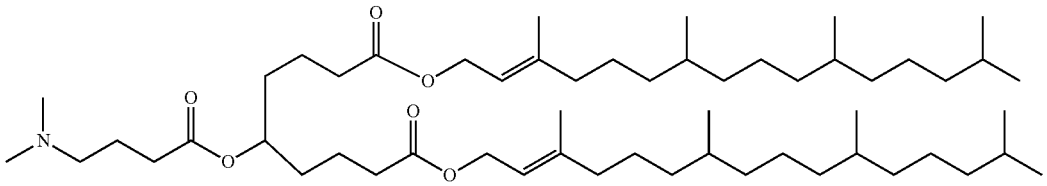
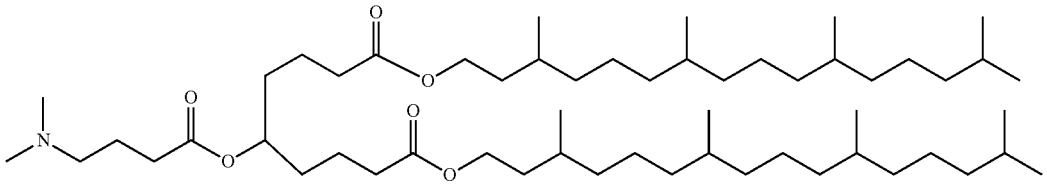
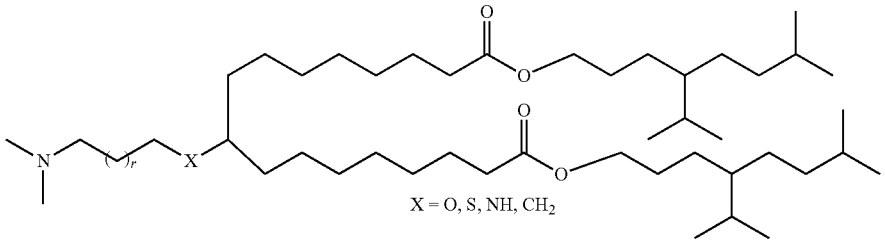


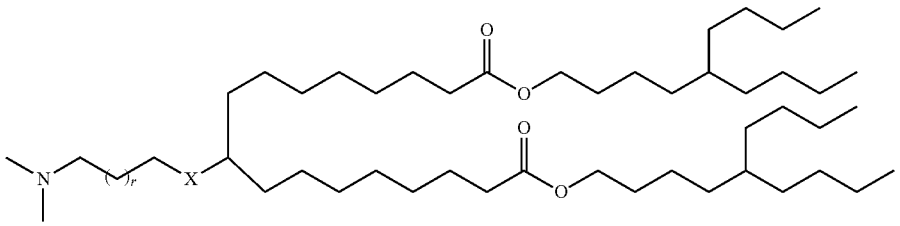






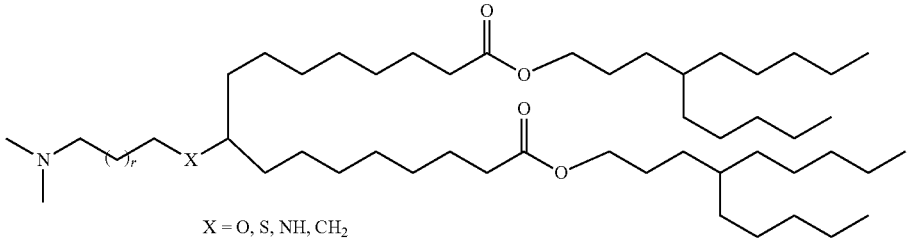
X = O, S, NH, CH_2

r = 0, 1, or 2



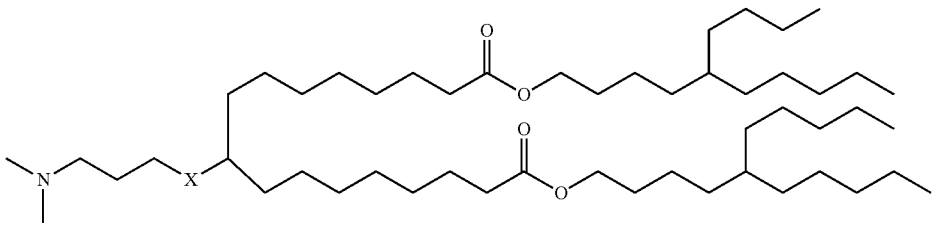
X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

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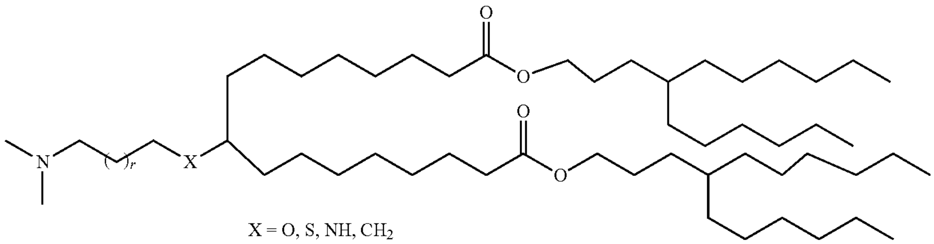
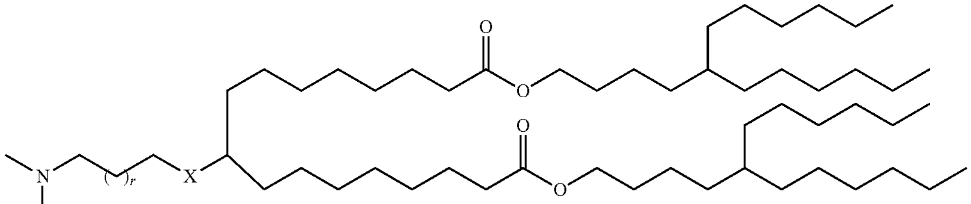


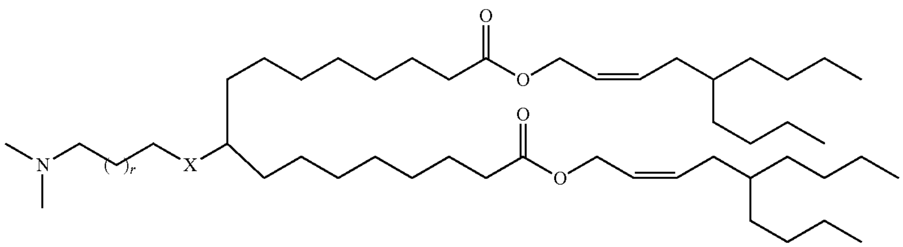
X = O, S, NH, CH_2

r = 0, 1, or 2



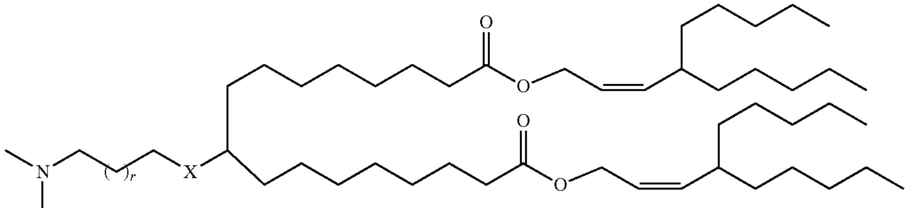
X = O, S, NH, CH_2

r = 0, 1, or 2



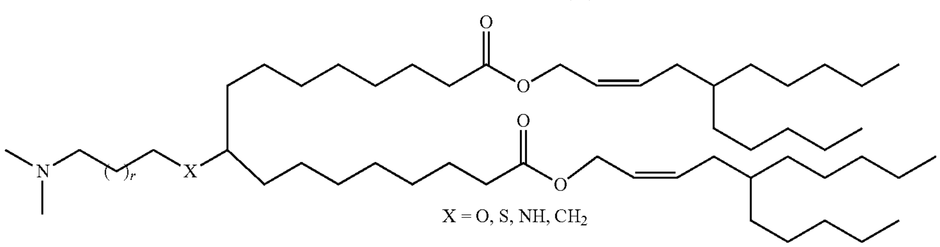
X = O, S, NH, CH_2

r = 0, 1, or 2



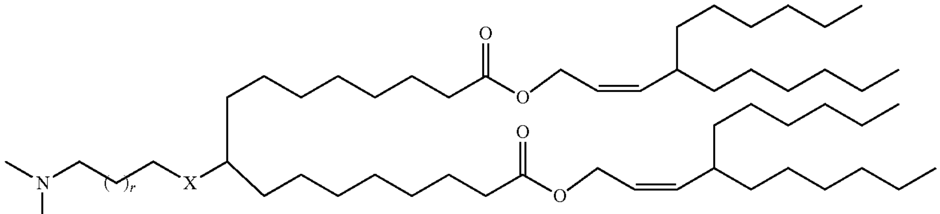
X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

r = 0, 1, or 2

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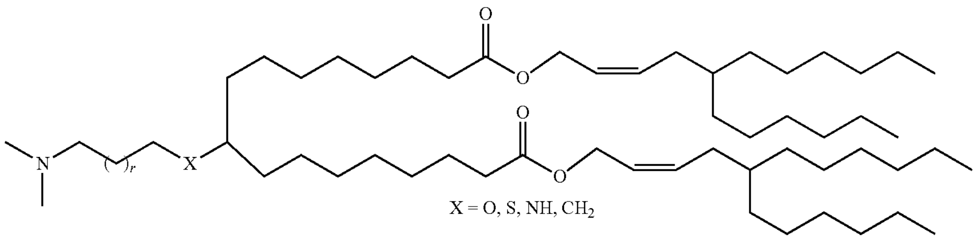
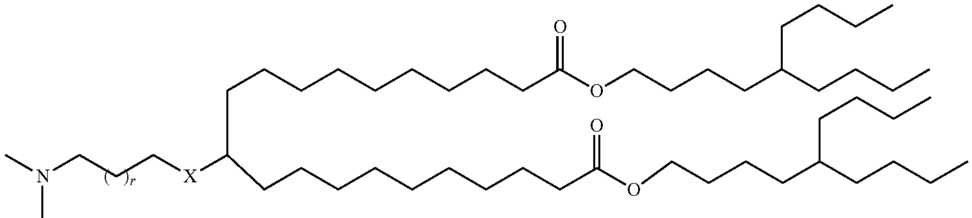


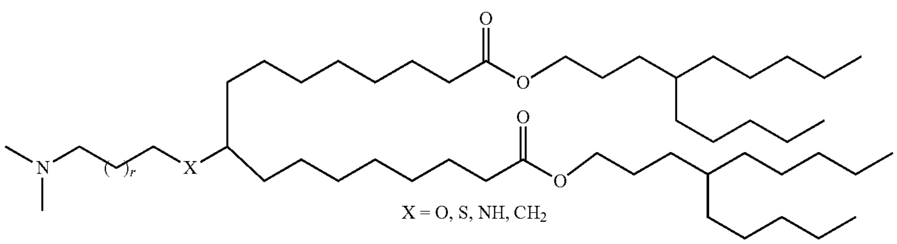
X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

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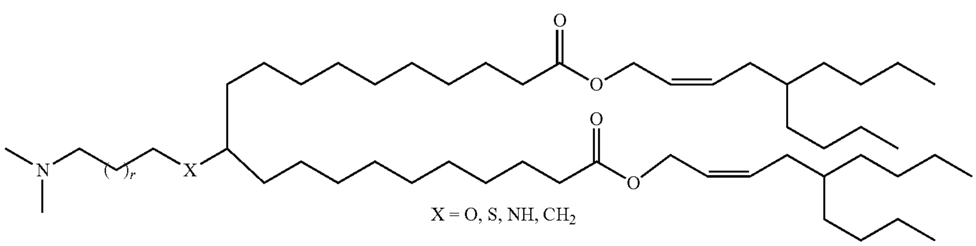

X = O, S, NH, CH_2

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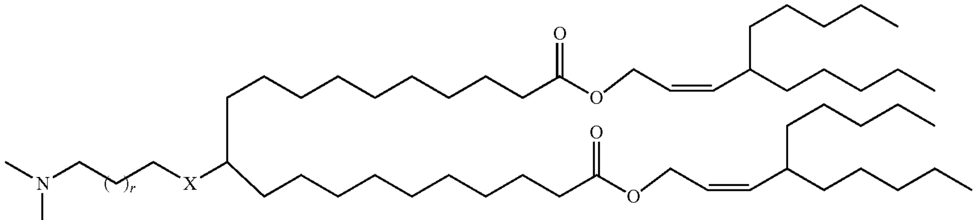
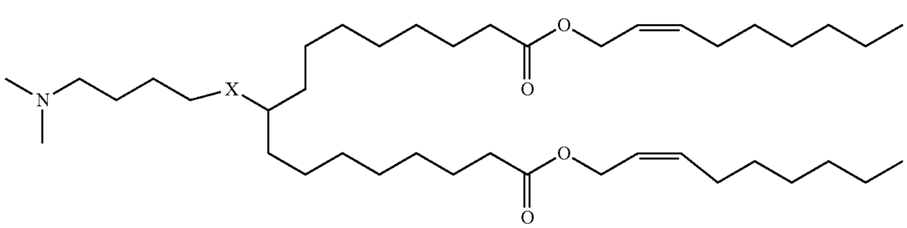


X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

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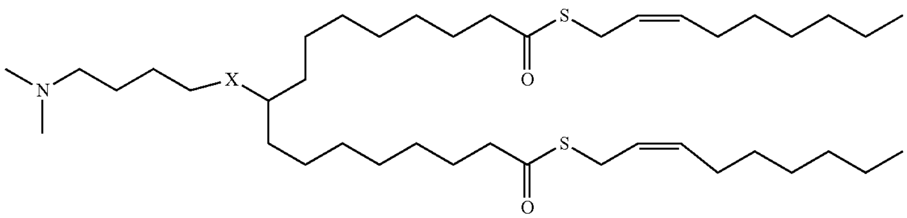

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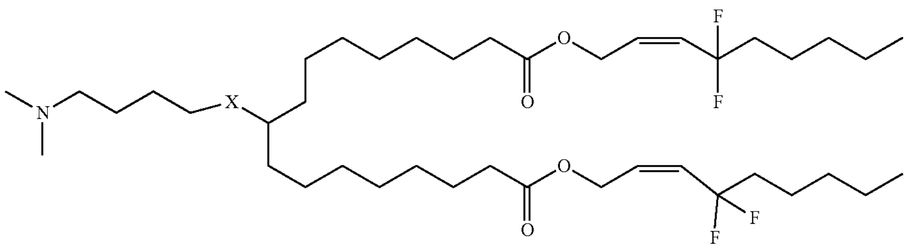

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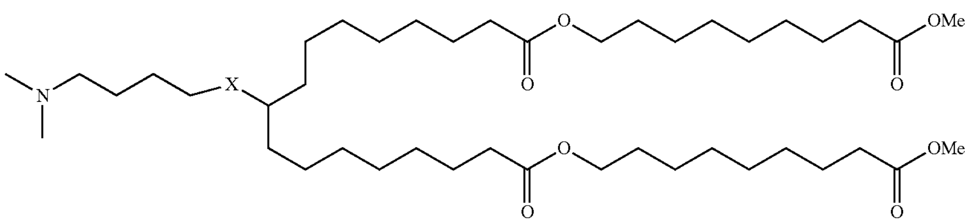

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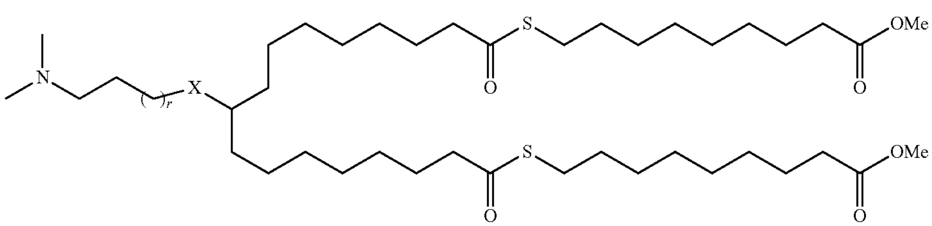
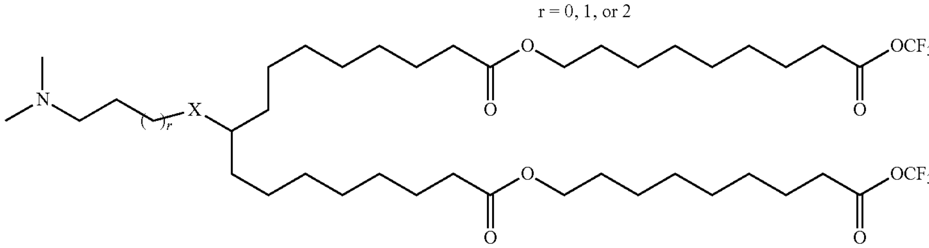


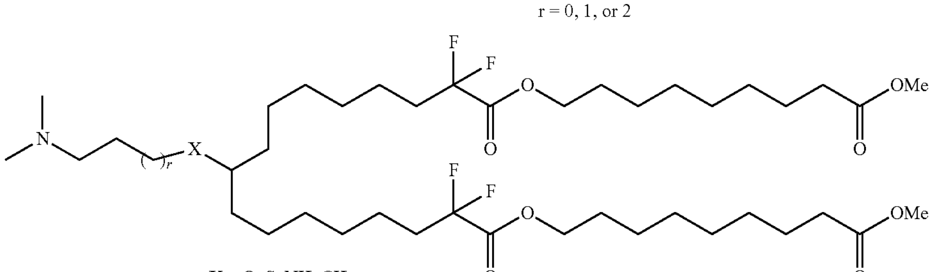
X = O, S, NH, CH_2

r = 0, 1, or 2



X = O, S, NR, CH_2

r = 0, 1, or 2



X = O, S, NH, CH_2

r = 0, 1, or 2

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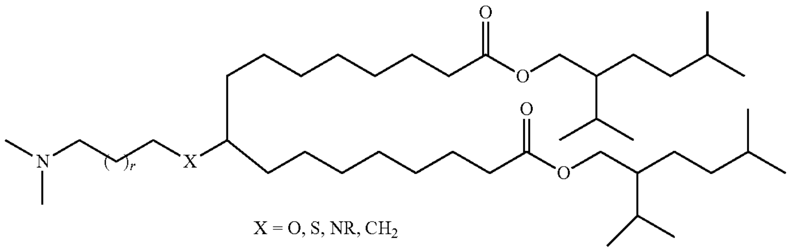
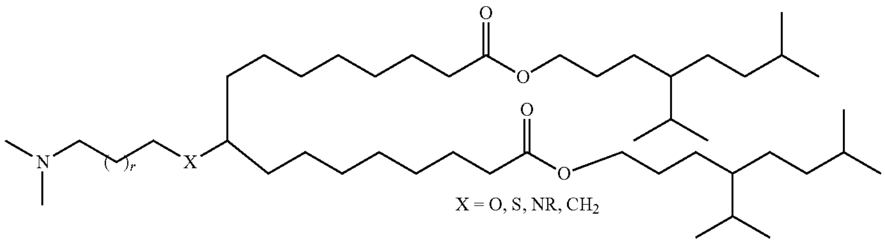


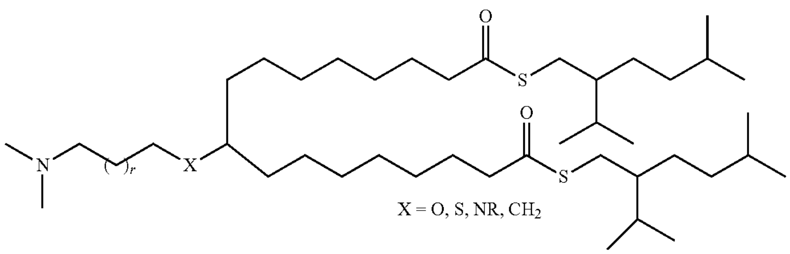
X = O, S, NR, CH_2

r = 0, 1, or 2



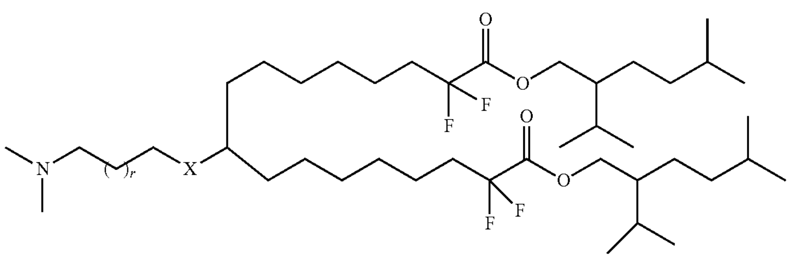
X = O, S, NR, CH_2

r = 0, 1, or 2

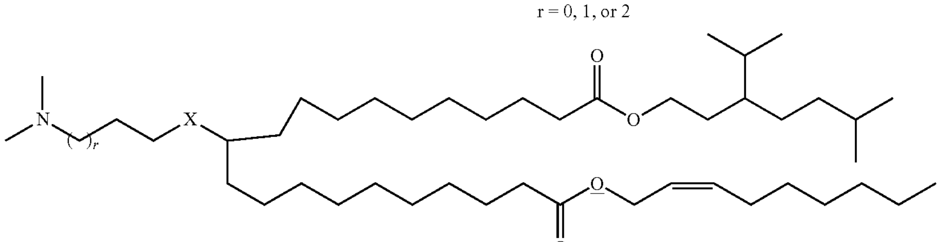


X = O, S, NR, CH_2

r = 0, 1, or 2

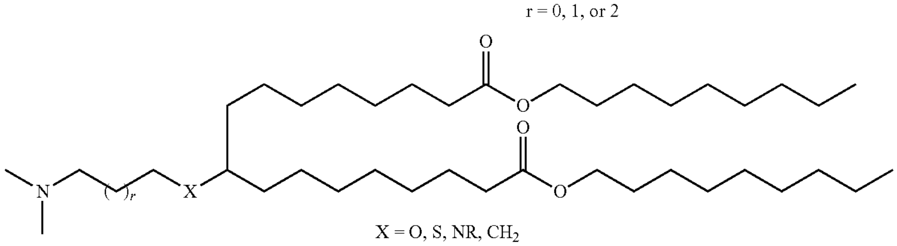


r = 0, 1, or 2



X = O, S, NR, CH_2

r = 0, 1, or 2



X = O, S, NR, CH_2

r = 0, 1, or 2

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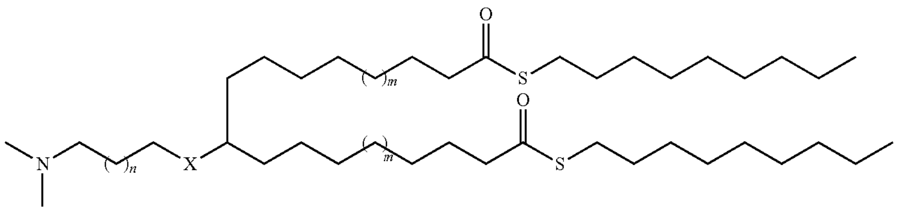
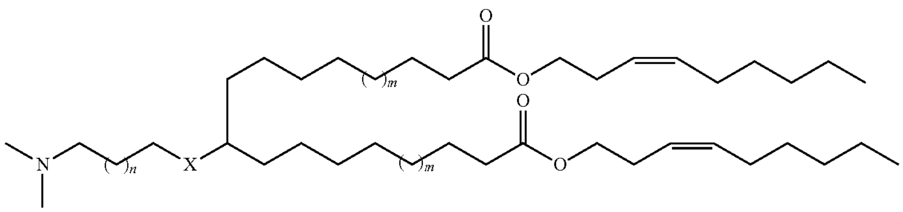
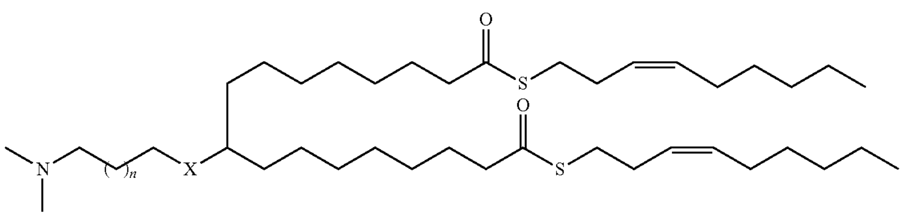


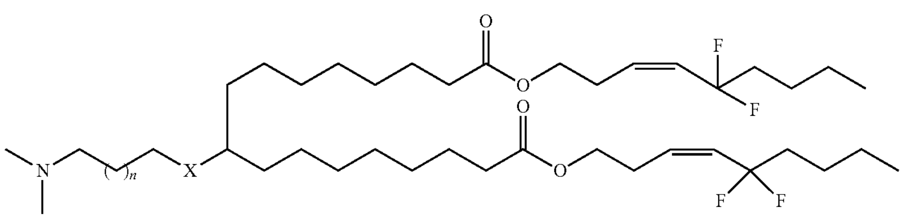
m = 0-5, n = 0, 1, or 2



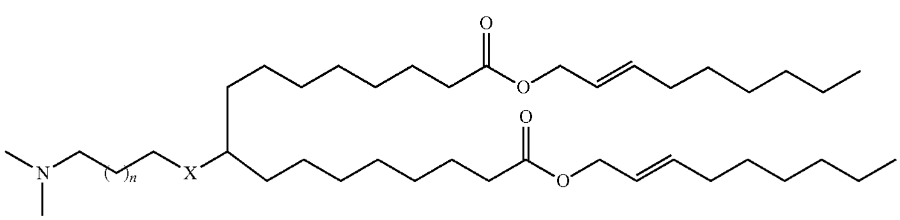
X = O, S, NR, CH_2
m = 0-5, n = 0, 1, or 2



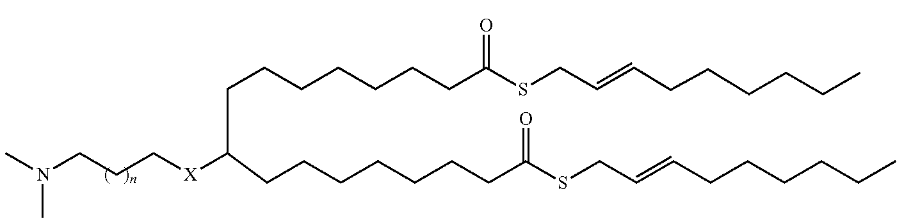
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2

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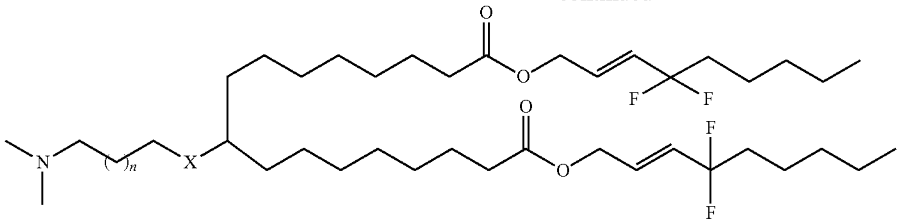
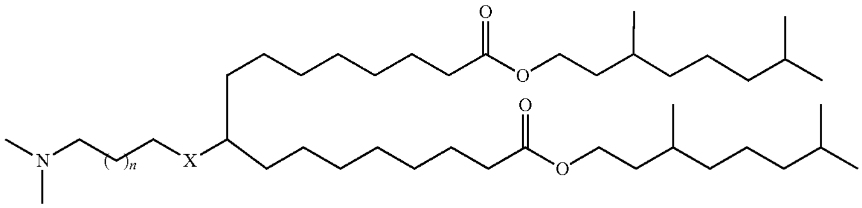


n = 0, 1, or 2



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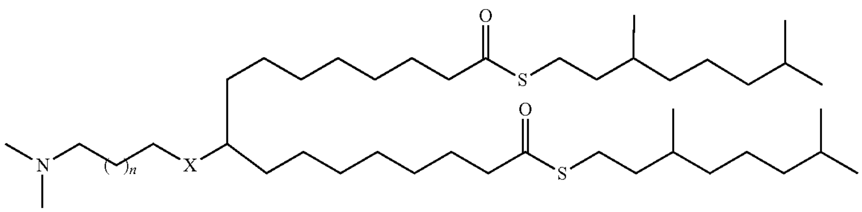
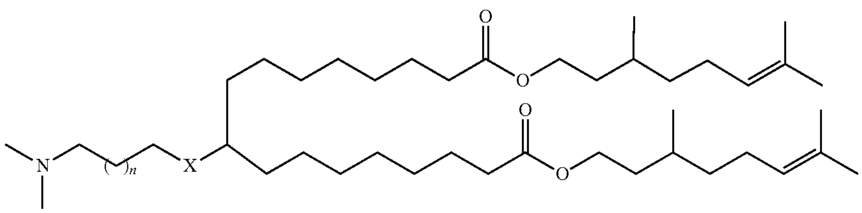
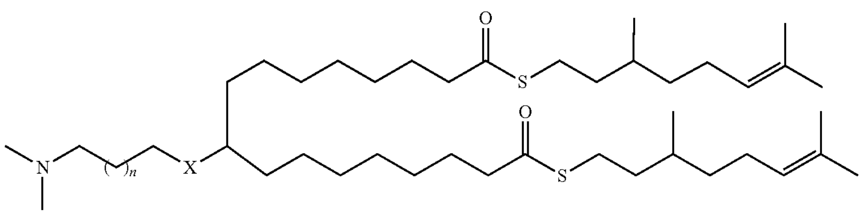


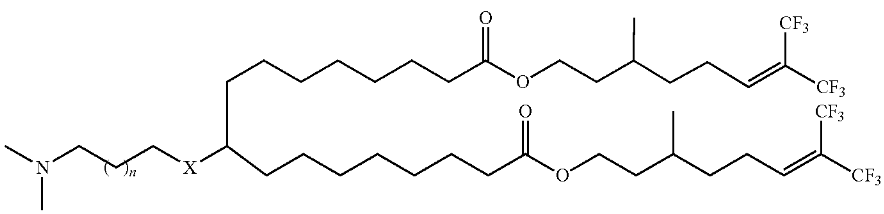
n = 0, 1, or 2



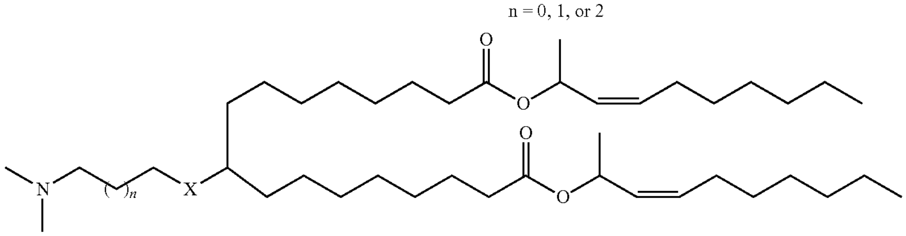
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2

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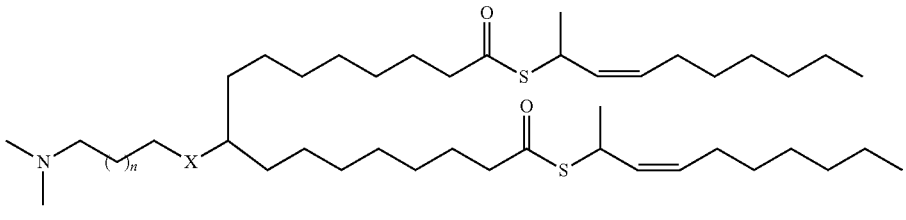
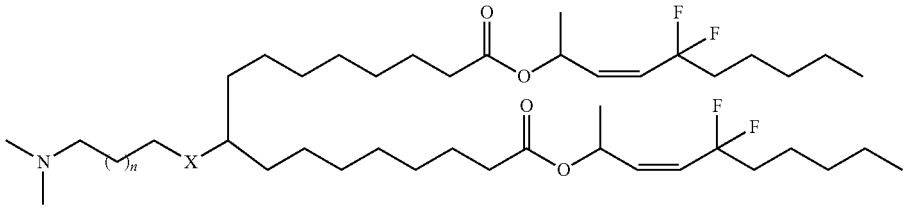
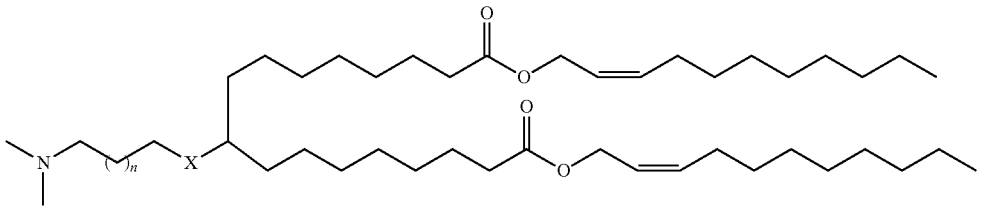


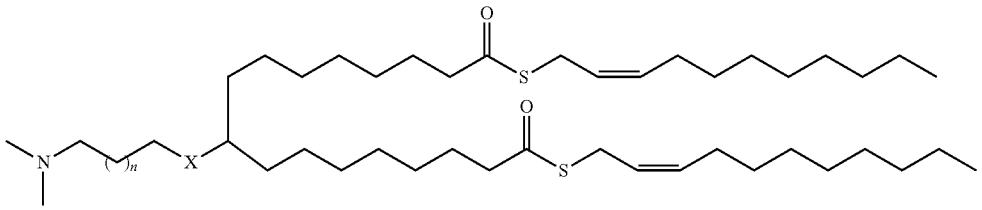
n = 0, 1, or 2



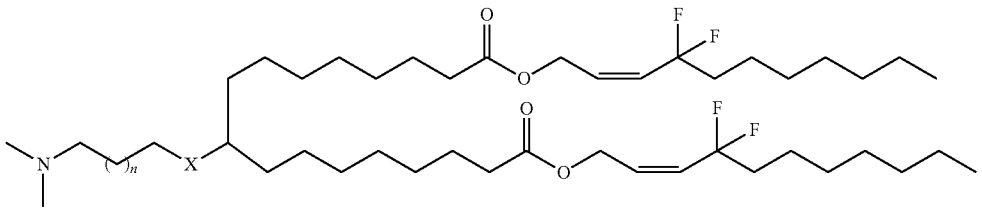
n = 0, 1, or 2



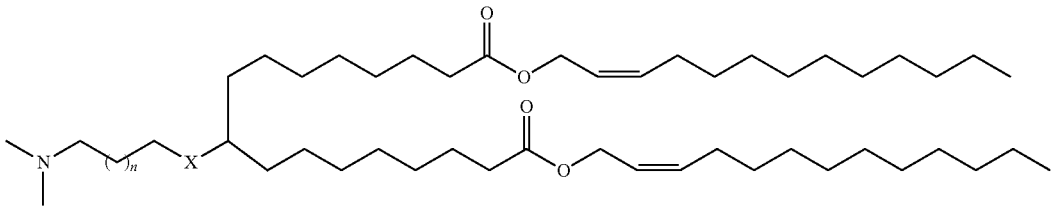
n = 0, 1, or 2



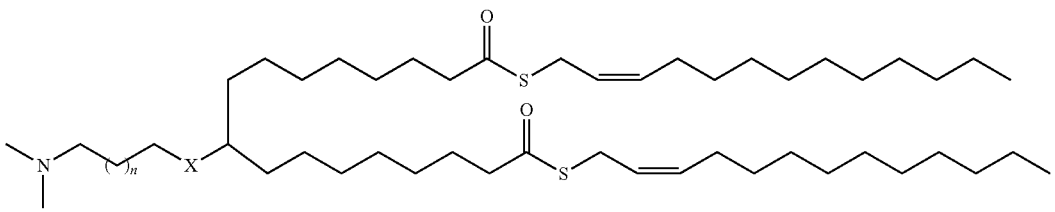
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2

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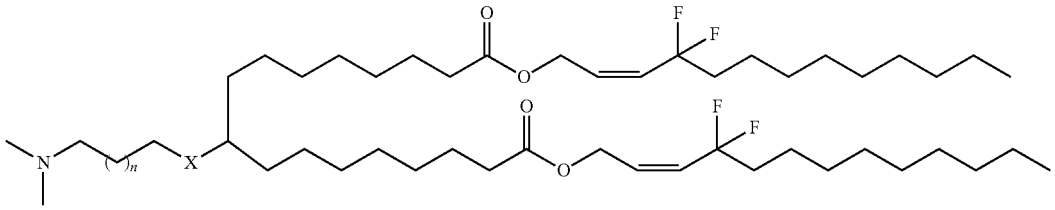
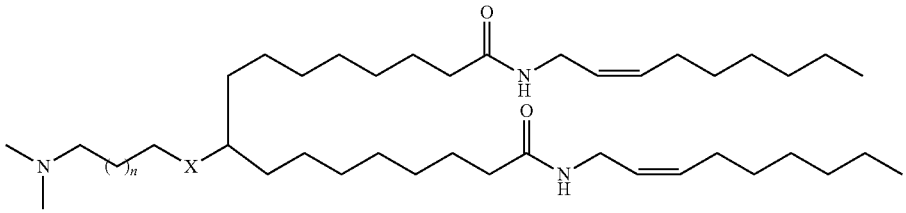
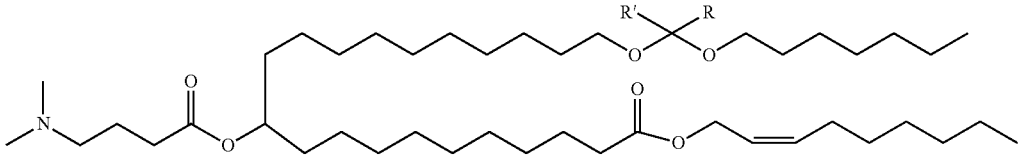


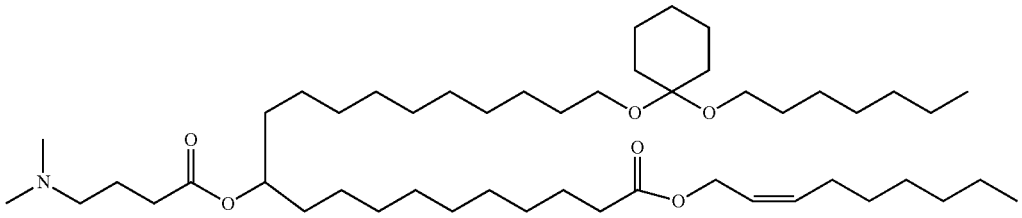
n = 0, 1, or 2

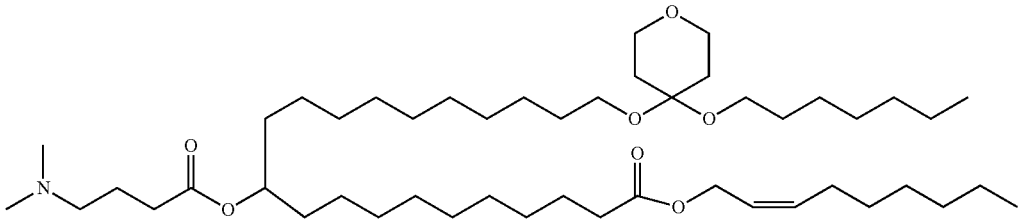


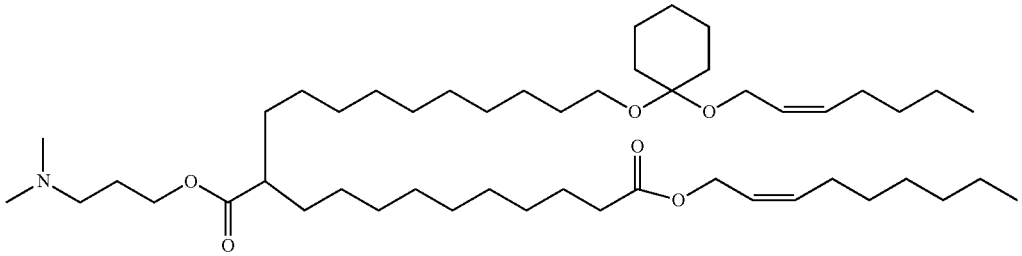
n = 0, 1, or 2

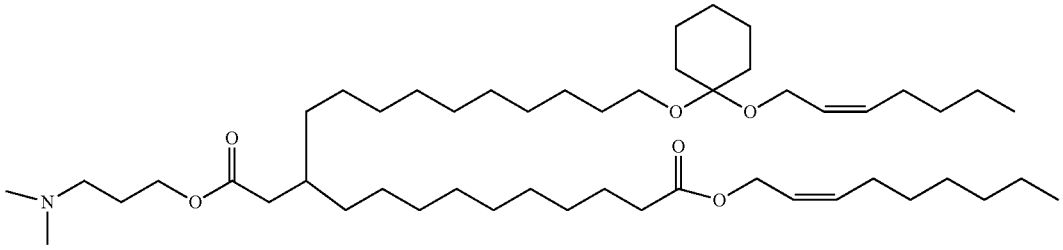


R, R′ = CH_3, Cylcopentyl etc









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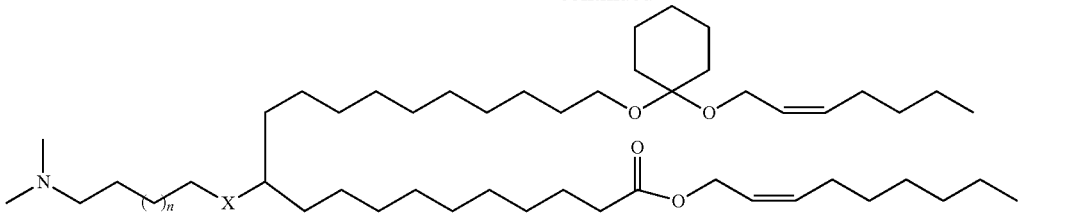
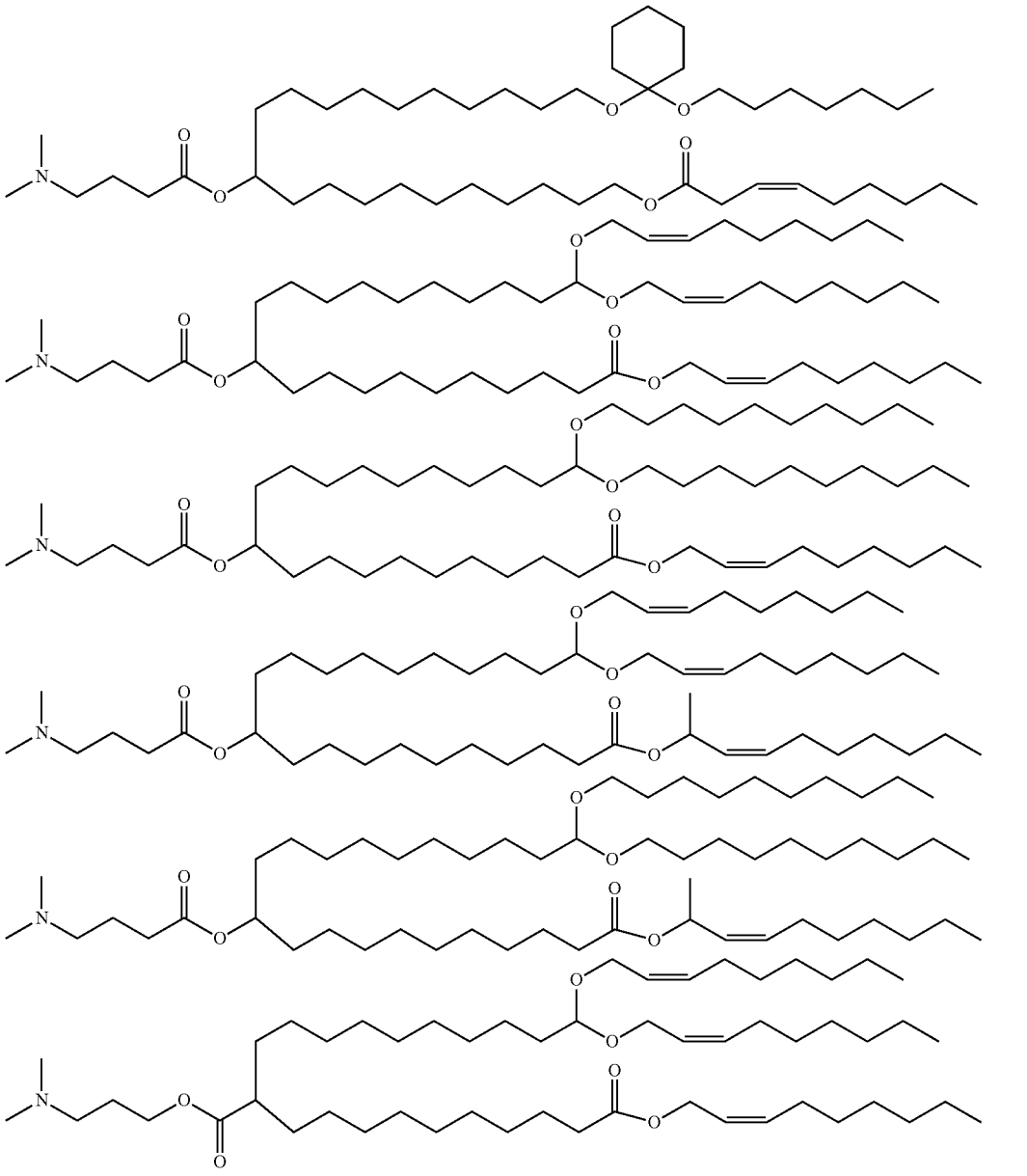


X = O, S, NR, CH_2, COO, NHCOO, OCONH

n = 0, 1, or 2



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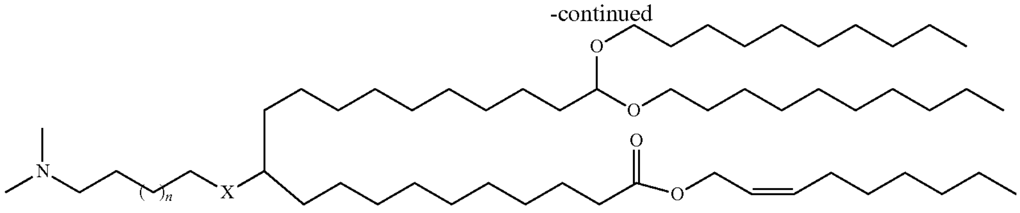
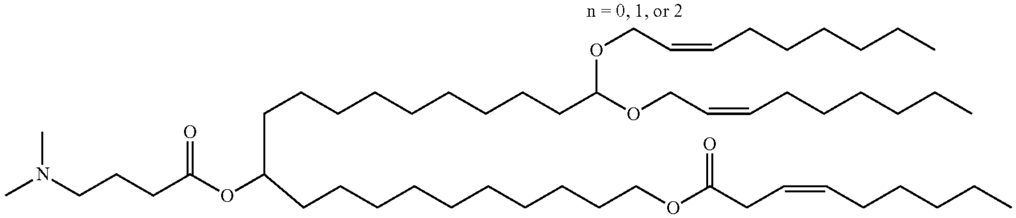
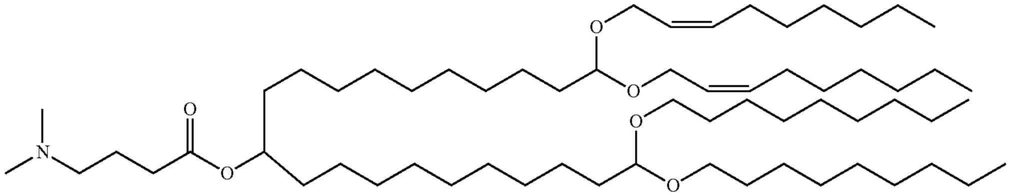
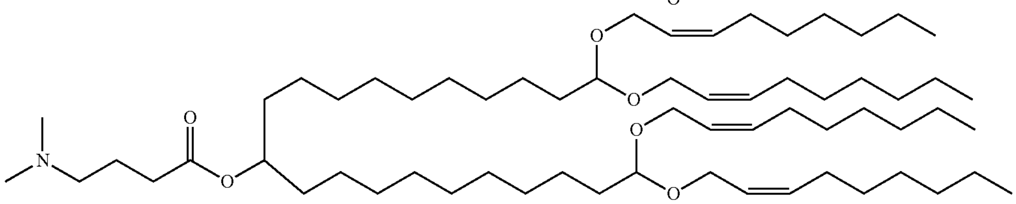


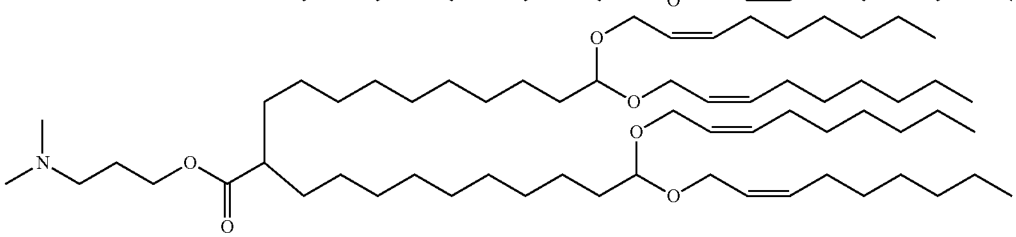
X = O, S, NR, CH_2, COO, NHCOO, OCONH

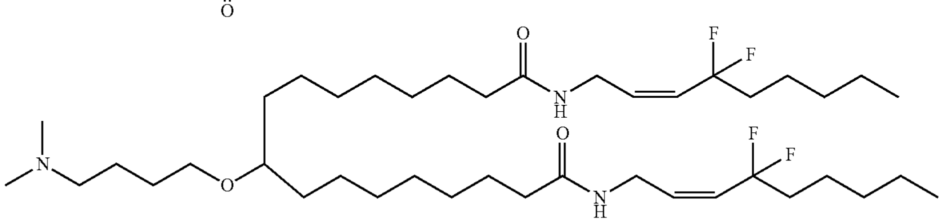
n = 0, 1, or 2

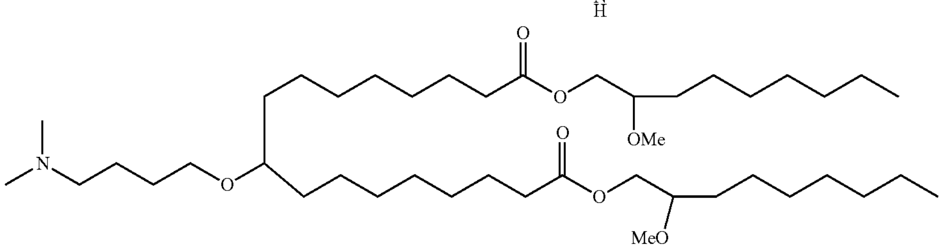


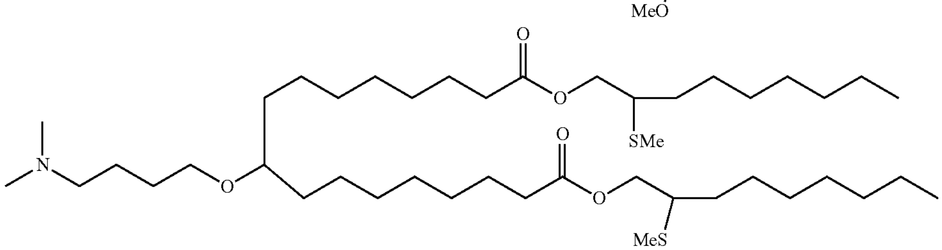












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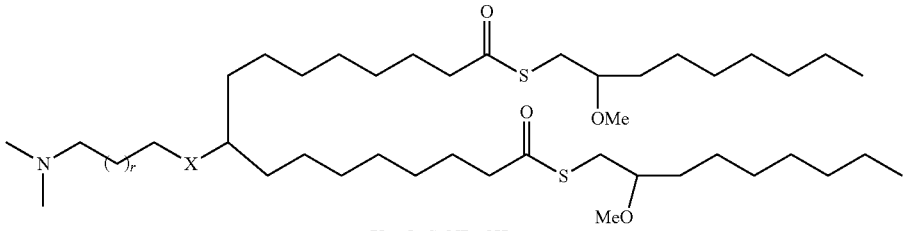
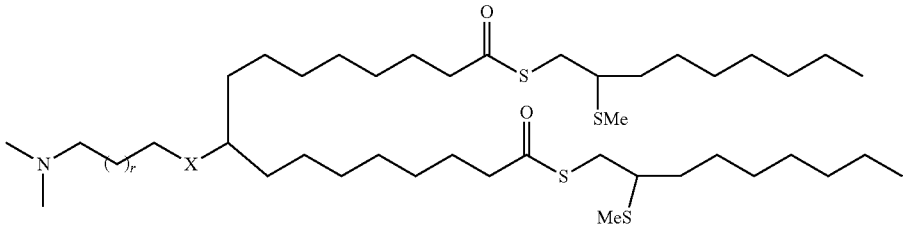


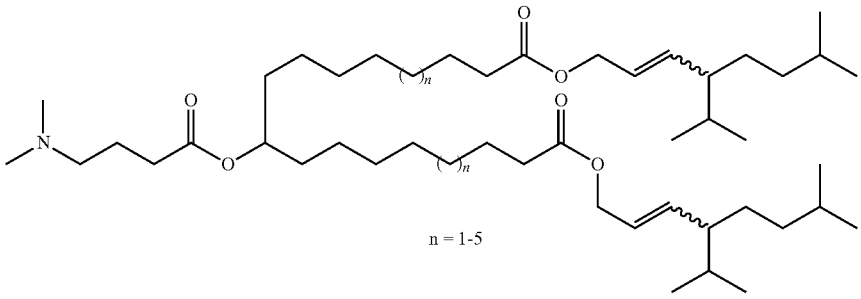
X = O, S, NR, CH_2

r = 0, 1, or 2

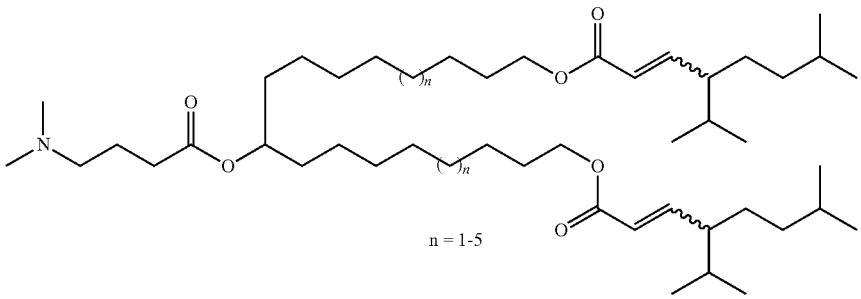


X = O, S, NR, CH_2

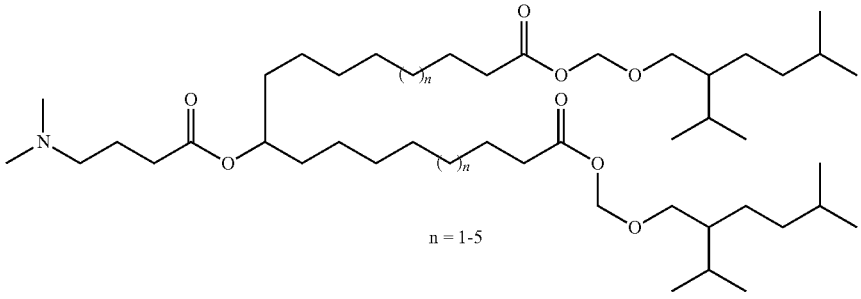
r = 0, 1, or 2



n = 1-5



n = 1-5



n = 1-5

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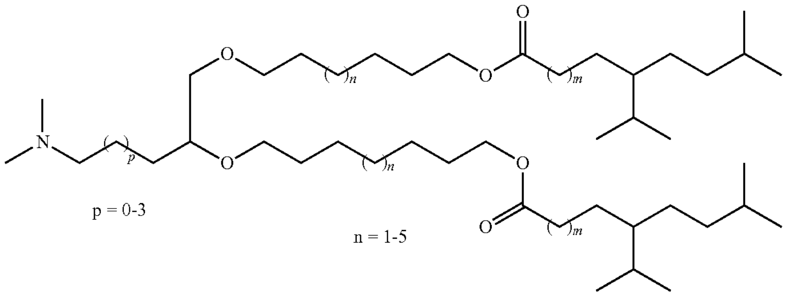
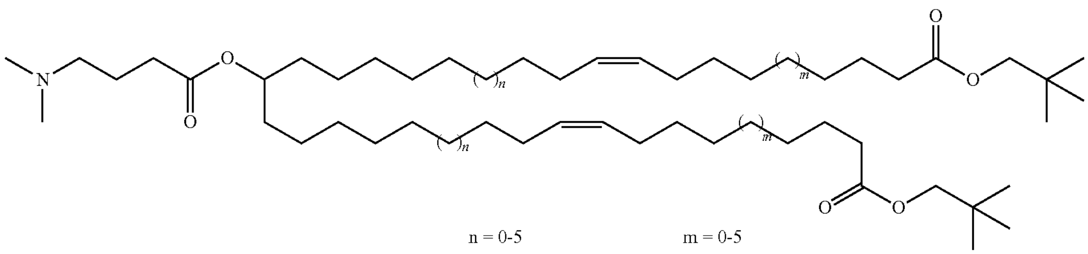


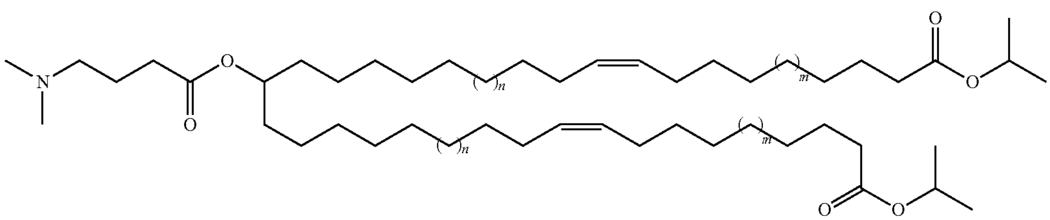
p = 0-3

n = 1-5

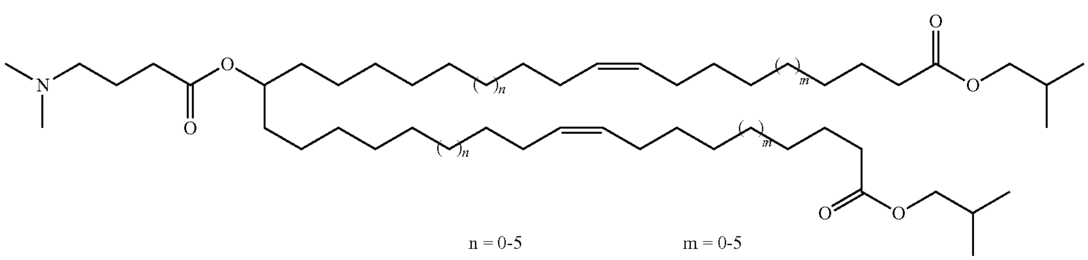
m = 0-3



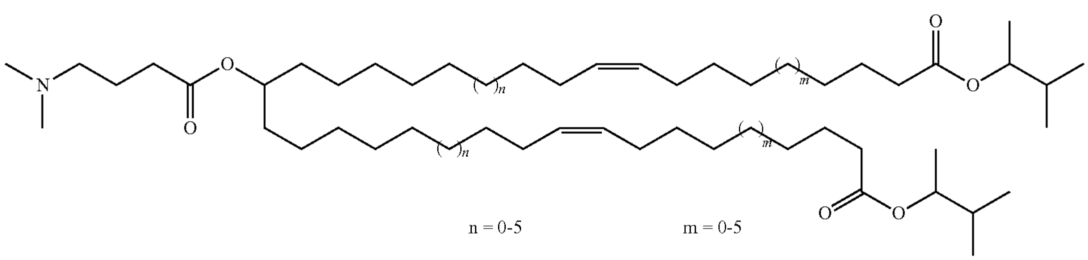
n = 0-5 m = 0-5



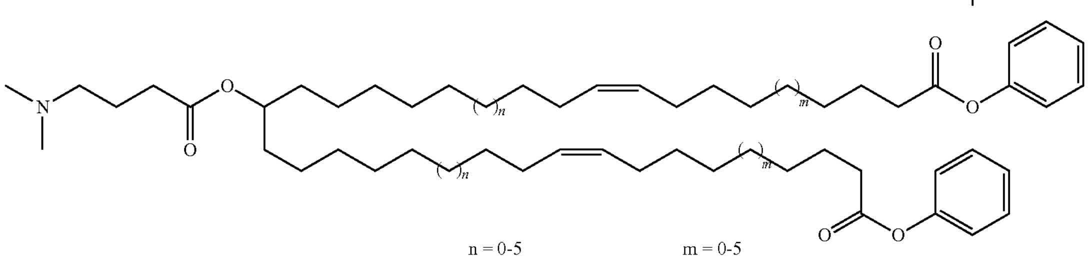
n = 0-5 m = 0-5



n = 0-5 m = 0-5



n = 0-5 m = 0-5



n = 0-5 m = 0-5

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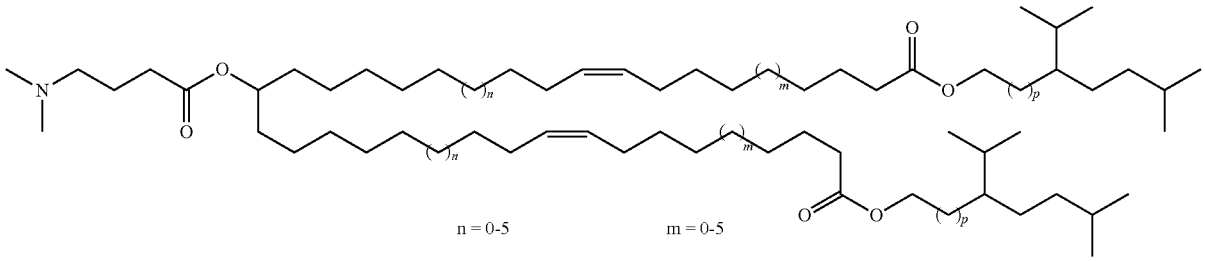
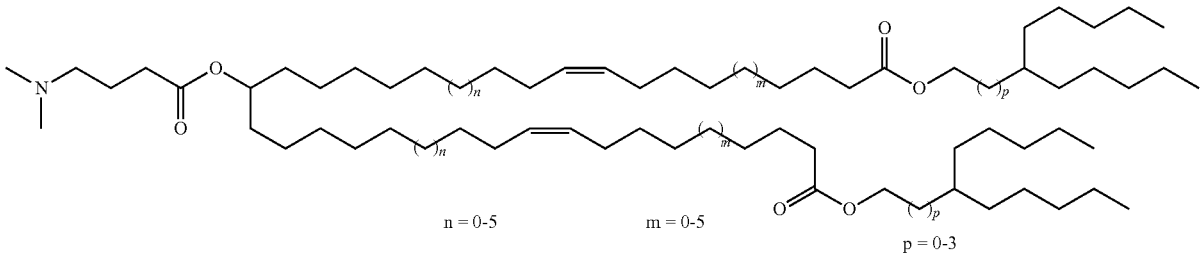
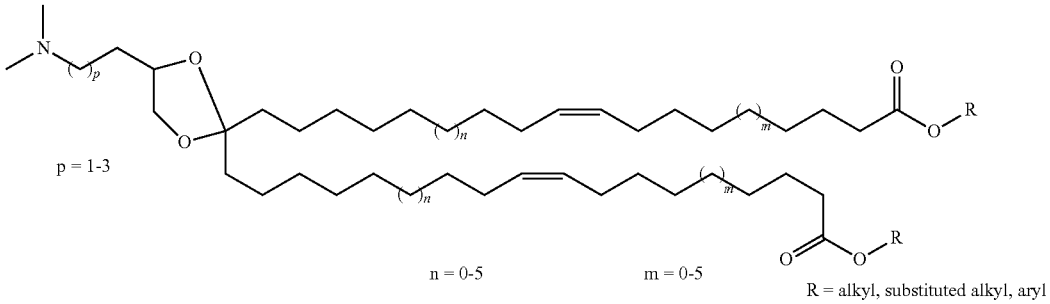
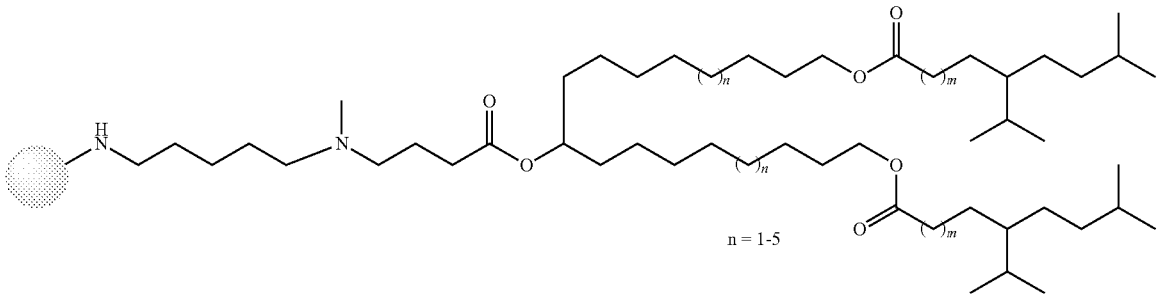
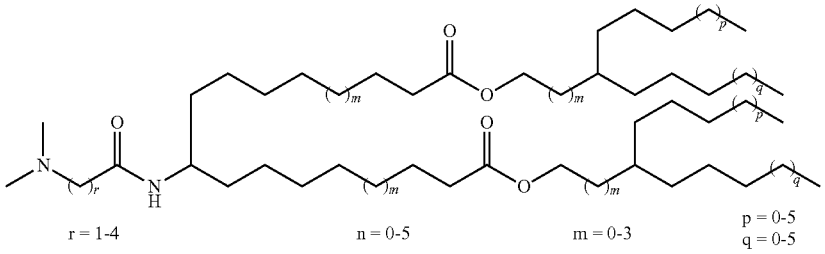

n = 0-5
m = 0-5
p = 0-3

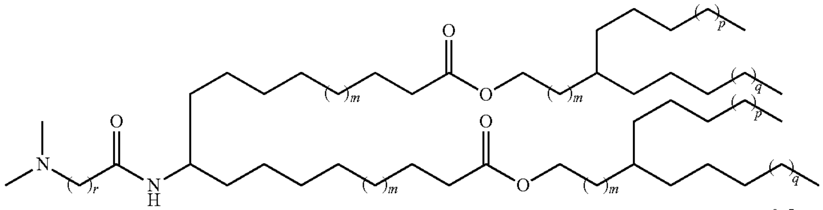

n = 0-5
m = 0-5
p = 0-3


p = 1-3
n = 0-5
m = 0-5
R = alkyl, substituted alkyl, aryl

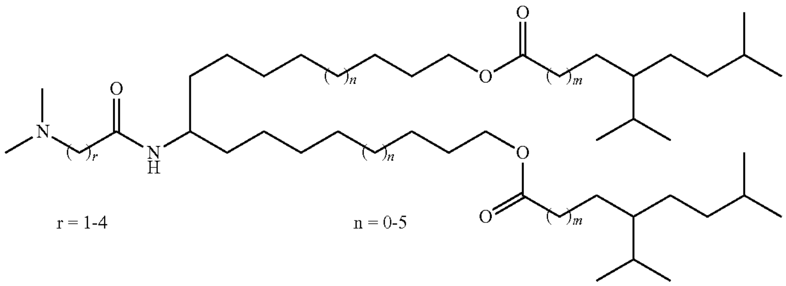

n = 1-5
m = 0-3
= Bodipy, Alexa-647 or other label (e.g., other fluorescent label)


r = 1-4
n = 0-5
m = 0-3
p = 0-5
q = 0-5

-continued



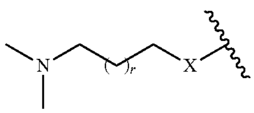
r = 1-4 n = 0-5 m = 0-3 p = 0-5 q = 0-5



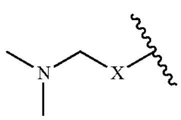
r = 1-4 n = 0-5

m = 0-3

Alternatively, for the compounds above having a head of the formula



(where X can be, for example, —C(O)O—), the head can have one methylene unit between the X group (or other functional group) and nitrogen atom. For example, the head can be:



Cationic lipids include those having alternative fatty acid groups and other dialkylamino groups than those shown, including those in which the alkyl substituents are different (e.g., N-ethyl-N-methylamino-, and N-propyl-N-ethyl-amino-).

In certain embodiments, the cationic lipids have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g. pH 7.4), and neutral at a second pH, preferably at or above physiological pH. Such lipids are also referred to as cationic lipids. It will, of course, be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. The lipids can have more than one protonatable or deprotonatable group, or can be zwiterrionic.

In certain embodiments, protonatable lipids (i.e., cationic lipids) have a pK_a of the protonatable group in the range of about 4 to about 11. For example, the lipids can have a pK_a of about 4 to about 7, e.g., from about 5 to about 7, such as from about 5.5 to about 6.8, when incorporated into lipid particles. Such lipids may be cationic at a lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4.

In particular embodiments, the lipids are charged lipids. As used herein, the term “charged lipid” includes, but is not limited to, those lipids having one or two fatty acyl or fatty alkyl chains and a quaternary amino head group. The quaternary amine carries a permanent positive charge. The head group can optionally include an ionizable group, such as a primary, secondary, or tertiary amine that may be protonated at physiological pH. The presence of the quaternary amine can alter the pKa of the ionizable group relative to the pKa of the group in a structurally similar compound that lacks the quaternary amine (e.g., the quaternary amine is replaced by a tertiary amine).

Included in the instant invention is the free form of the cationic lipids described herein, as well as pharmaceutically acceptable salts and stereoisomers thereof. The cationic lipid can be a protonated salt of the amine cationic lipid. The term “free form” refers to the amine cationic lipids in non-salt form. The free form may be regenerated by treating the salt with a suitable dilute aqueous base solution such as dilute aqueous NaOH, potassium carbonate, ammonia and sodium bicarbonate.

The pharmaceutically acceptable salts of the instant cationic lipids can be synthesized from the cationic lipids of this invention which contain a basic or acidic moiety by conventional chemical methods. Generally, the salts of the basic cationic lipids are prepared either by ion exchange chromatography or by reacting the free base with stoichiometric amounts or with an excess of the desired salt-forming inorganic or organic acid in a suitable solvent or various combinations of solvents. Similarly, the salts of the acidic compounds are formed by reactions with the appropriate inorganic or organic base.

Thus, pharmaceutically acceptable salts of the cationic lipids of this invention include non-toxic salts of the cationic lipids of this invention as formed by reacting a basic instant

cationic lipids with an inorganic or organic acid. For example, non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxy-benzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and trifluoroacetic (TFA).

When the cationic lipids of the present invention are acidic, suitable "pharmaceutically acceptable salts" refers to salts prepared form pharmaceutically acceptable non-toxic bases including inorganic bases and organic bases. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous, potassium, sodium, and zinc. In one embodiment, the base is selected from ammonium, calcium, magnesium, potassium and sodium. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as arginine, betaine caffeine, choline, N,N^1-dibenzylethylenediamine, diethylamin, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethylmorpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine tripropylamine, and tromethamine.

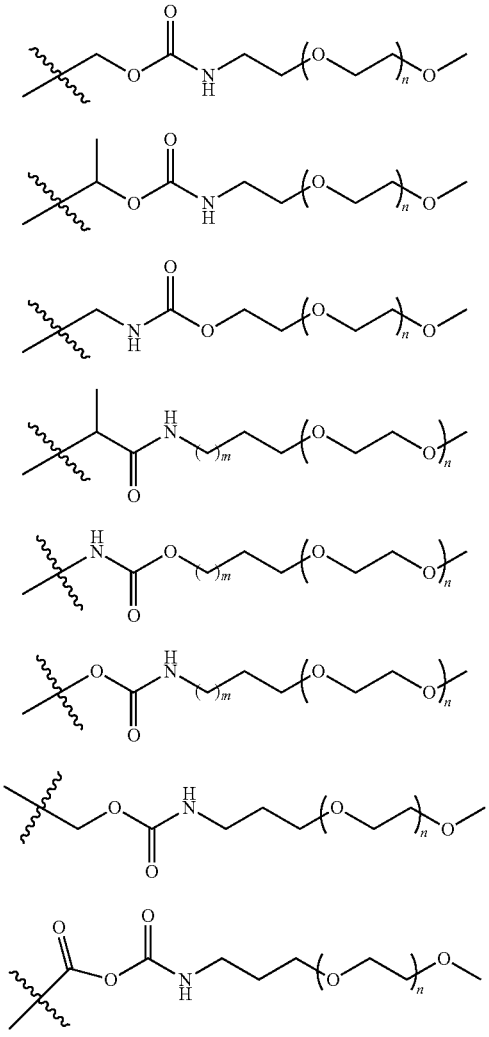
It will also be noted that the cationic lipids of the present invention may potentially be internal salts or zwitterions, since under physiological conditions a deprotonated acidic moiety in the compound, such as a carboxyl group, may be anionic, and this electronic charge might then be balanced off internally against the cationic charge of a protonated or alkylated basic moiety, such as a quaternary nitrogen atom.

One or more additional cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in the lipid particles and compositions described herein. Such cationic lipids include, but are not limited to N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl-N,N—N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N—(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleyloxy)propyl)-N-2-(spermine-carboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dileoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N, N-dimethyl-2,3-dioleyloxy)propylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids can be used, such as, e.g., LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL).

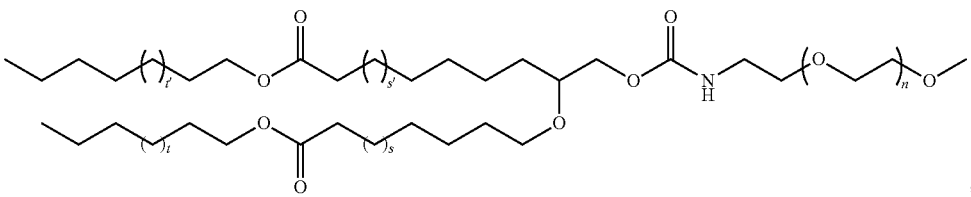
PEG Lipids

Suitable head groups for the PEG lipids include, but are not limited to those shown in Table 3 below.

TABLE 3



Representative PEG lipids include, but are not limited to:



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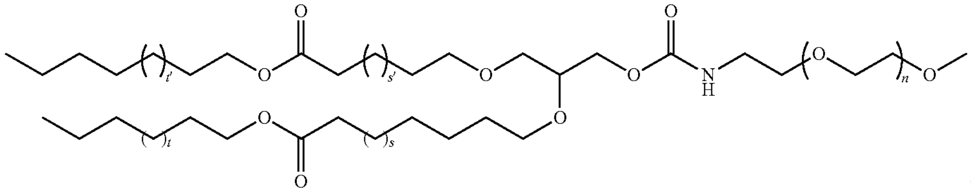
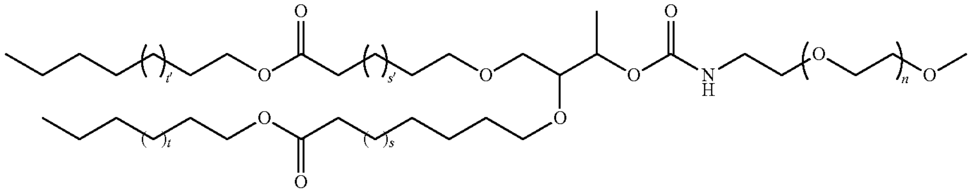
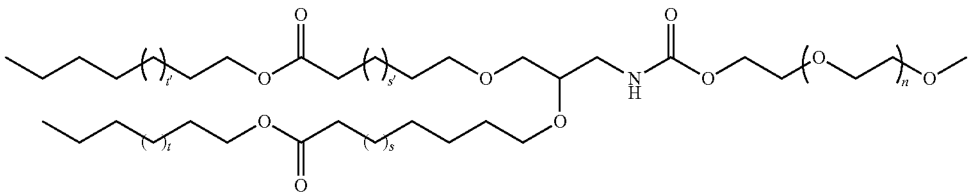
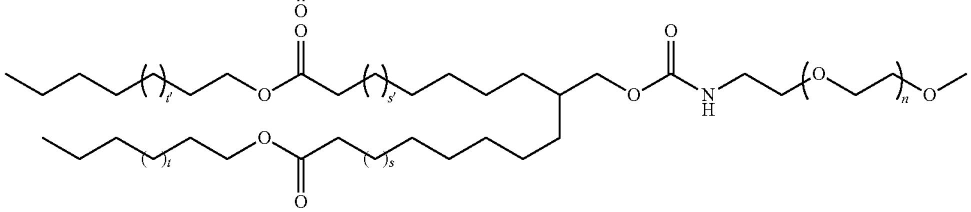


,







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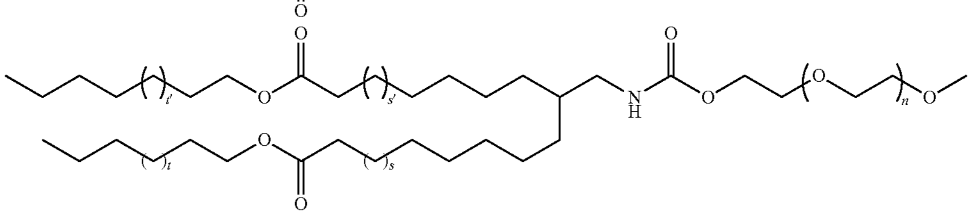

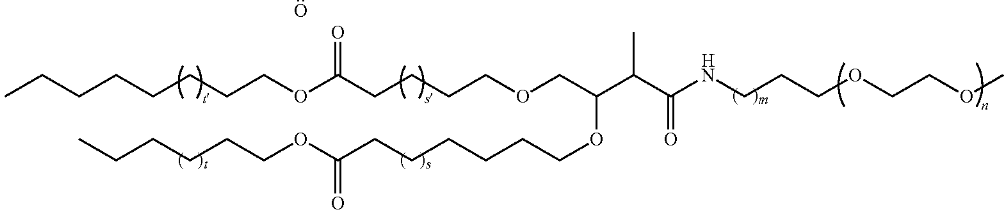

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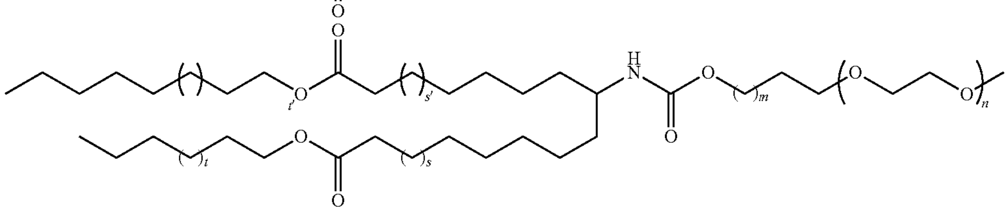

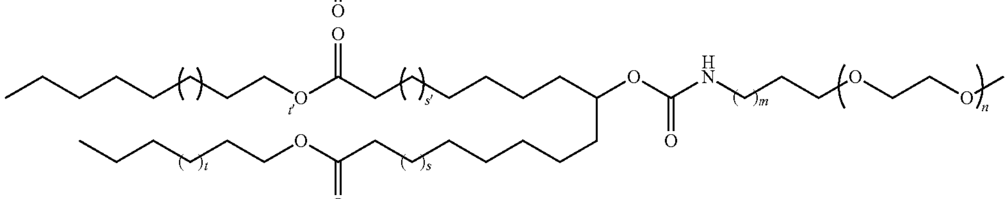

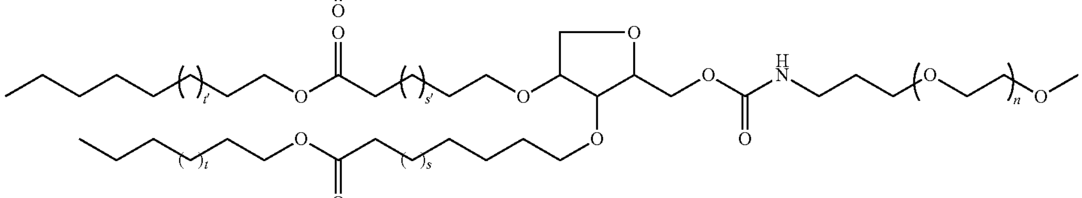

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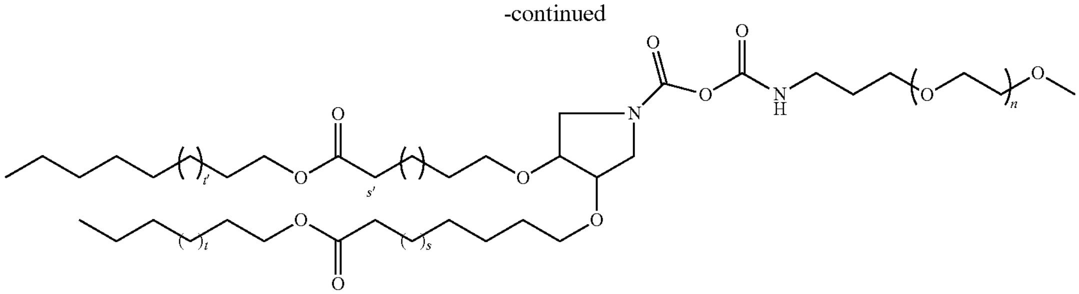
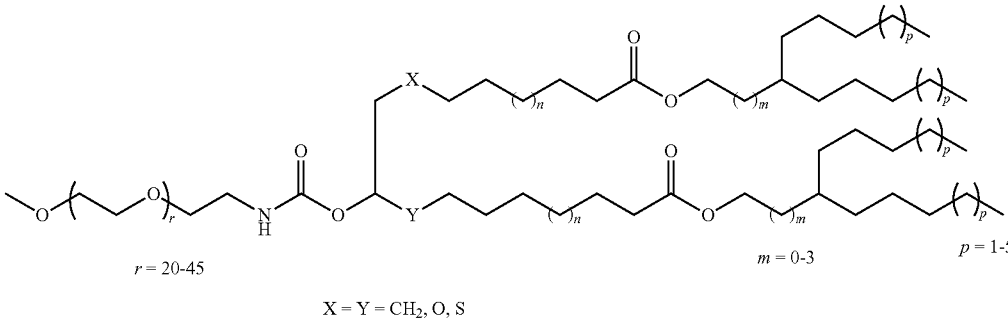


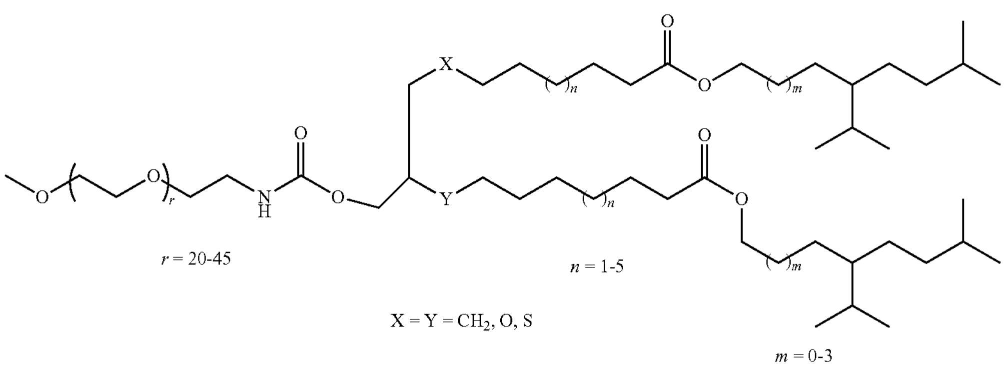
wherein

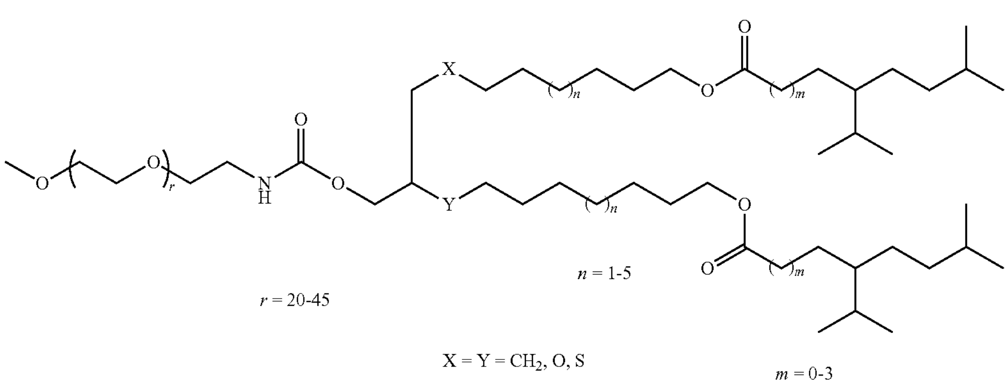
n is an integer from 10 to 100 (e.g. 20-50 or 40-50);

s, s', t and t' are independently 0, 1, 2, 3, 4, 5, 6 or 7; and m is 1, 2, 3, 4, 5, or 6.

Other representative PEG lipids include, but are not limited to:







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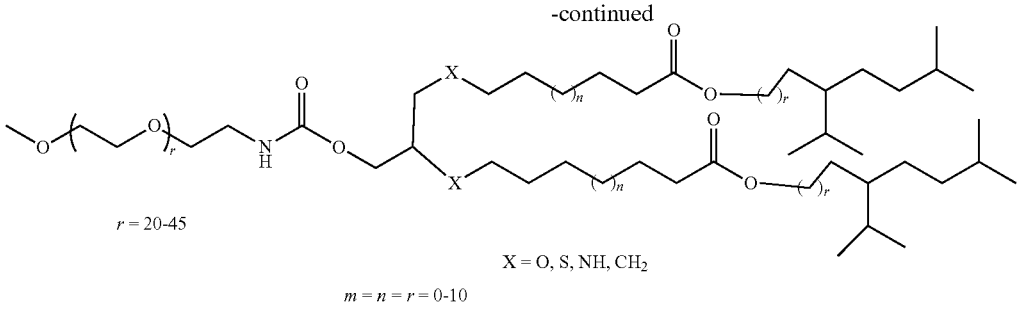
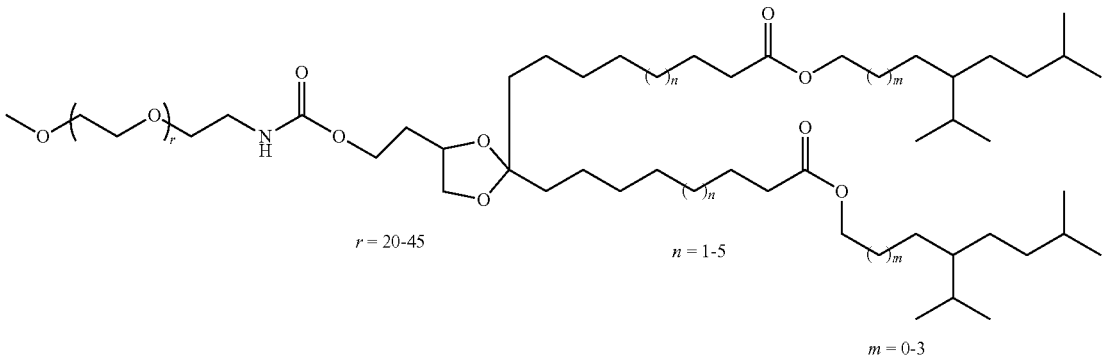
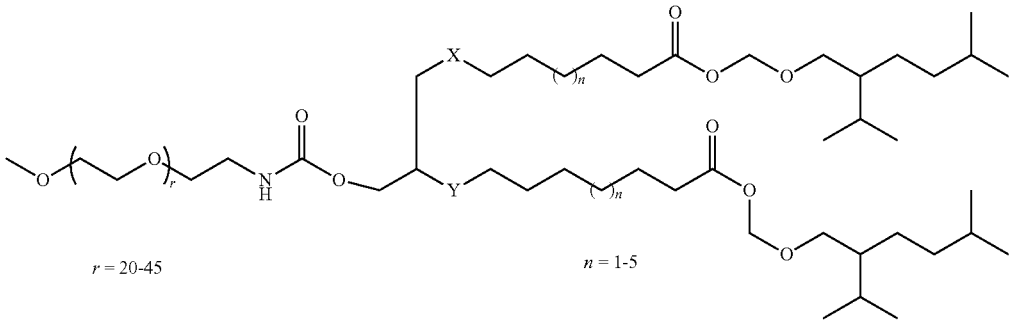
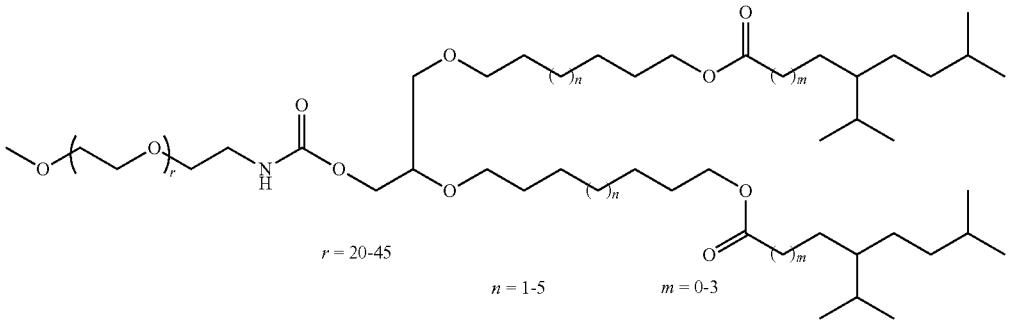
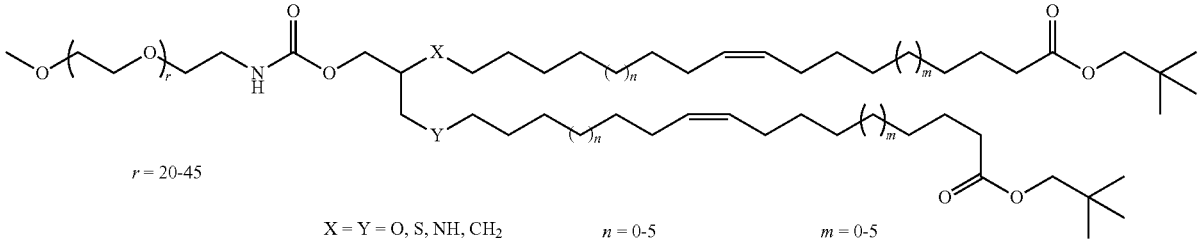

r = 20-45
X = O, S, NH, CH2
m = n = r = 0-10

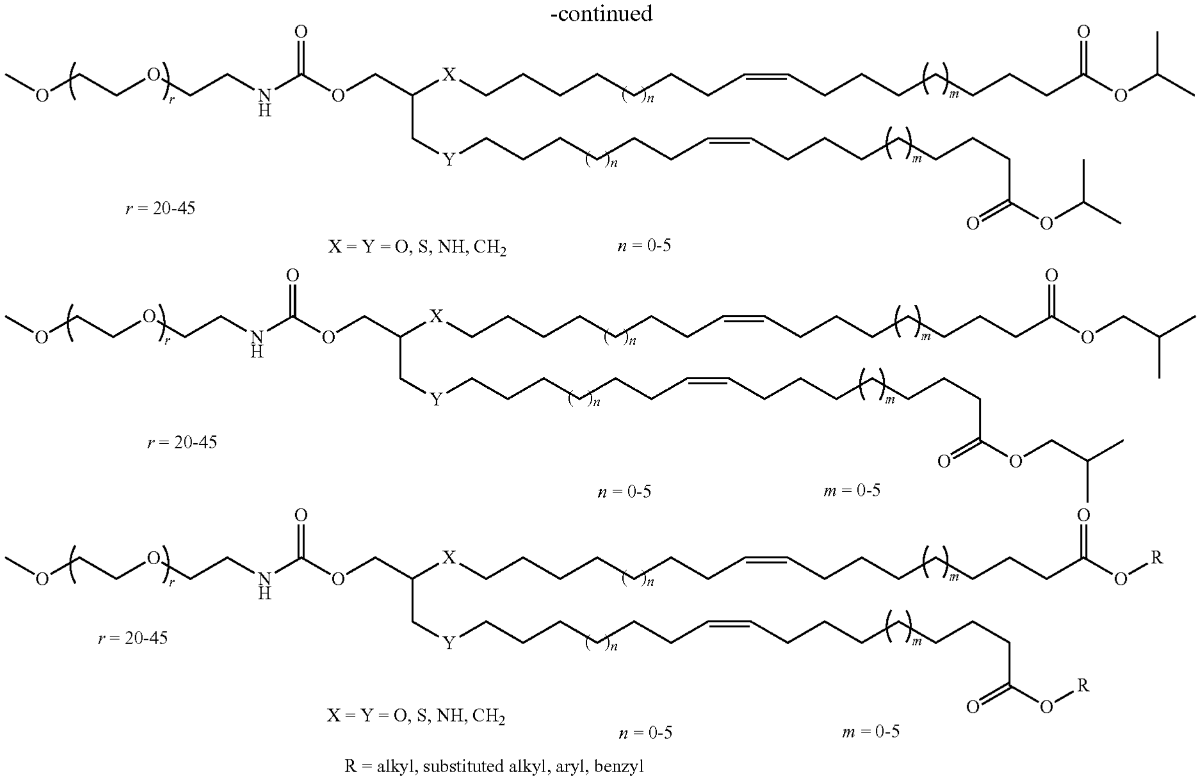

r = 20-45
n = 1-5
m = 0-3


r = 20-45
n = 1-5


r = 20-45
n = 1-5
m = 0-3


r = 20-45
X = Y = O, S, NH, CH2
n = 0-5
m = 0-5

-continued



The Other Lipid Components

The lipid particles and compositions described herein may also include one or more neutral lipids. Neutral lipids, when present, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. In one embodiment, the neutral lipid component is a lipid having two acyl groups (e.g., diacylphosphatidylcholine and diacylphosphatidylethanolamine). In one embodiment, the neutral lipid contains saturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20}. In another embodiment, the neutral lipid includes mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20}. Suitable neutral lipids include, but are not limited to, DSPC, DPPC, POPC, DOPE, DSPC, and SM.

The lipid particles and compositions described herein may also include one or more lipids capable of reducing aggregation. Examples of lipids that reduce aggregation of particles during formation include polyethylene glycol (PEG)-modified lipids (PEG lipids, such as PEG-DMG and PEG-DMA), monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as (described in U.S. Pat. No. 6,320,017, which is incorporated by reference in its entirety). Suitable PEG lipids include, but are not limited to, PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) (such as those described in U.S. Pat. No. 5,820,873, incorporated herein by reference), PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines, PEG-modified diacylglycerols and dialkylglycerols, mPEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE).

The lipid particles and compositions may include a sterol, such as cholesterol.

Lipid Particles

In a further aspect, the present invent relates to lipid particles that include one or more of the cationic lipids described herein. In one embodiment, the lipid particle includes one or more compounds of formula I-VII.

Lipid particles include, but are not limited to, liposomes. As used herein, a liposome is a structure having lipid-containing membranes enclosing an aqueous interior.

Another embodiment is a nucleic acid-lipid particle (e.g., a SNALP) comprising a cationic lipid of the present invention, a non-cationic lipid (such as a neutral lipid), optionally a PEG-lipid conjugate (such as the lipids for reducing aggregation of lipid particles discussed herein), optionally a sterol (e.g., cholesterol), and a nucleic acid. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids, wherein the nucleic acid (e.g., an interfering RNA) is encapsulated within the lipids. In certain instances, SNALPs are useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (e.g., sites physically separated from the administration site), and they can mediate silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a SNALP as set forth in International Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety.

For example, the lipid particle may include a cationic lipid, a fusion-promoting lipid (e.g., DPPC), a neutral lipid, cholesterol, and a PEG-modified lipid. In one embodiment, the lipid particle includes the above lipid mixture in molar ratios of about 20-70% cationic lipid: 0.1-50% fusion pro-

moting lipid: 5-45% neutral lipid: 20-55% cholesterol: 0.5-15% PEG-modified lipid (based upon 100% total moles of lipid in the lipid particle).

In another embodiment of the lipid particle, the cationic lipid is present in a mole percentage of about 20% and about 60%; the neutral lipid is present in a mole percentage of about 5% to about 25%; the sterol is present in a mole percentage of about 25% to about 55%; and the PEG lipid is PEG-DMA, PEG-DMG, or a combination thereof, and is present in a mole percentage of about 0.5% to about 15% (based upon 100% total moles of lipid in the lipid particle).

In particular embodiments, the molar lipid ratio, with regard to mol % cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) is approximately 40/10/40/10, 35/15/40/10 or 52/13/30/5. This mixture may be further combined with a fusion-promoting lipid in a molar ratio of 0.1-50%, 0.1-50%, 0.5-50%, 1-50%, 5%-45%, 10%-40%, or 15%-35%. In other words, when a 40/10/40/10 mixture of lipid/DSPC/Chol/PEG-DMG or PEG-DMA is combined with a fusion-promoting peptide in a molar ratio of 50%, the resulting lipid particles can have a total molar ratio of (mol % cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA/fusion-promoting peptide) 20/5/20/5/50. In another embodiment, the neutral lipid, DSPC, in these compositions is replaced with POPC, DPPC, DOPE or SM.

In one embodiment, the lipid particles comprise a cationic lipid of the present invention, a neutral lipid, a sterol and a PEG-modified lipid. In one embodiment, the lipid particles include from about 25% to about 75% on a molar basis of cationic lipid, e.g., from about 35 to about 65%, from about 45 to about 65%, about 60%, about 57.5%, about 57.1%, about 50% or about 40% on a molar basis. In one embodiment, the lipid particles include from about 0% to about 15% on a molar basis of the neutral lipid, e.g., from about 3 to about 12%, from about 5 to about 10%, about 15%, about 10%, about 7.5%, about 7.1% or about 0% on a molar basis. In one embodiment, the neutral lipid is DPPC. In one embodiment, the neutral lipid is DSPC. In one embodiment, the formulation includes from about 5% to about 50% on a molar basis of the sterol, e.g., about 15 to about 45%, about 20 to about 40%, about 48%, about 40%, about 38.5%, about 35%, about 34.4%, about 31.5% or about 31% on a molar basis. In one embodiment, the sterol is cholesterol.

The lipid particles described herein may further include one or more therapeutic agents. In a preferred embodiment, the lipid particles include a nucleic acid (e.g., an oligonucleotide), such as siRNA or miRNA.

In one embodiment, the lipid particles include from about 0.1% to about 20% on a molar basis of the PEG-modified lipid, e.g., about 0.5 to about 10%, about 0.5 to about 5%, about 10%, about 5%, about 3.5%, about 1.5%, about 0.5%, or about 0.3% on a molar basis. In one embodiment, the PEG-modified lipid is PEG-DMG. In one embodiment, the PEG-modified lipid is PEG-c-DMA. In one embodiment, the lipid particles include 25-75% of cationic lipid, 0.5-15% of the neutral lipid, 5-50% of the sterol, and 0.5-20% of the PEG-modified lipid on a molar basis.

In one embodiment, the lipid particles include 35-65% of cationic lipid, 3-12% of the neutral lipid, 15-45% of the sterol, and 0.5-10% of the PEG-modified lipid on a molar basis. In one embodiment, the lipid particles include 45-65% of cationic lipid, 5-10% of the neutral lipid, 25-40% of the sterol, and 0.5-5% of the PEG-modified lipid on a molar basis. In one embodiment, the PEG modified lipid comprises a PEG molecule of an average molecular weight of 2,000 Da. In one embodiment, the PEG modified lipid is PEG-distyryl glycerol (PEG-DSG).

In one embodiment, the ratio of lipid:siRNA is at least about 0.5:1, at least about 1:1, at least about 2:1, at least about 3:1, at least about 4:1, at least about 5:1, at least about 6:1, at least about 7:1, at least about 11:1 or at least about 33:1. In one embodiment, the ratio of lipid:siRNA ratio is between about 1:1 to about 35:1, about 3:1 to about 15:1, about 4:1 to about 15:1, or about 5:1 to about 13:1. In one embodiment, the ratio of lipid:siRNA ratio is between about 0.5:1 to about 12:1.

In one embodiment, the lipid particles are nanoparticles. In additional embodiments, the lipid particles have a mean diameter size of from about 50 nm to about 300 nm, such as from about 50 nm to about 250 nm, for example, from about 50 nm to about 200 nm.

In one embodiment, a lipid particle containing a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours.

In another embodiment, a lipid particle containing a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 10% (e.g., less than about 7.5%, less than about 5%, less than about 2.5%) of that for the same cationic lipid without the biodegrable group or groups.

Additional Components

The lipid particles and compositions described herein can further include one or more antioxidants. The antioxidant stabilizes the lipid particle and prevents, decreases, and/or inhibits degradation of the cationic lipid and/or active agent present in the lipid particles. The antioxidant can be a hydrophilic antioxidant, a lipophilic antioxidant, a metal chelator, a primary antioxidant, a secondary antioxidant, salts thereof, and mixtures thereof. In certain embodiments, the antioxidant comprises a metal chelator such as EDTA or salts thereof, alone or in combination with one, two, three, four, five, six, seven, eight, or more additional antioxidants such as primary antioxidants, secondary antioxidants, or other metal chelators. In one preferred embodiment, the antioxidant comprises a metal chelator such as EDTA or salts thereof in a mixture with one or more primary antioxidants and/or secondary antioxidants. For example, the antioxidant may comprise a mixture of EDTA or a salt thereof, a primary antioxidant such as a-tocopherol or a salt thereof, and a secondary antioxidant such as ascorbyl palmitate or a salt thereof. In one embodiment, the antioxidant comprises at least about 100 mM citrate or a salt thereof. Examples of antioxidants include, but are not limited to, hydrophilic antioxidants, lipophilic antioxidants, and mixtures thereof. Non-limiting examples of hydrophilic antioxidants include chelating agents (e.g., metal chelators) such as ethylenediaminetetraacetic acid (EDTA), citrate, ethylene glycol tetraacetic acid (EGTA), 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), diethylene triamine pentaacetic acid (DTPA), 2,3-dimercapto-1-propanesulfonic acid (DMPS), dimercaptosuccinic acid (DMSA), cc-lipoic acid, salicylaldehyde isonicotinoyl hydrazone (SIH), hexyl thioethylamine hydrochloride (HTA), desferrioxamine, salts thereof, and mixtures thereof. Additional hydrophilic antioxidants include ascorbic acid, cysteine, glutathione, dihydrolipoic acid, 2-mercaptoethane sulfonic acid, 2-mercaptobenzimidazole sulfonic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, sodium metabisulfite, salts thereof, and mixtures thereof. Non-limiting examples of lipophilic antioxidants include vitamin E isomers such as α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and

δ-tocotrienols; polyphenols such as 2-tert-butyl-4-methyl phenol, 2-fert-butyl-5-methyl phenol, and 2-tert-butyl-6-methyl phenol; butylated hydroxyanisole (BHA) (e.g., 2-teri-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole); butylhydroxytoluene (BHT); tert-butylhydroquinone (TBHQ); ascorbyl palmitate; rc-propyl gallate; salts thereof; and mixtures thereof. Suitable antioxidants and formulations containing such antioxidants are described in International Publication No. WO 2011/066651, which is hereby incorporated by reference.

In another embodiment, the lipid particles or compositions contain the antioxidant EDTA (or a salt thereof), the antioxidant citrate (or a salt thereof), or EDTA (or a salt thereof) in combination with one or more (e.g., a mixture of) primary and/or secondary antioxidants such as α-tocopherol (or a salt thereof) and/or ascorbyl palmitate (or a salt thereof).

In one embodiment, the antioxidant is present in an amount sufficient to prevent, inhibit, or reduce the degradation of the cationic lipid present in the lipid particle. For example, the antioxidant may be present at a concentration of at least about or about 0.1 mM, 0.5 mM, 1 mM, 10 mM, 100 mM, 500 mM, 1 M, 2 M, or 5M, or from about 0.1 mM to about 1 M, from about 0.1 mM to about 500 mM, from about 0.1 mM to about 250 mM, or from about 0.1 mM to about 100 mM.

The lipid particles and compositions described herein can further include an apolipoprotein. As used herein, the term "apolipoprotein" or "lipoprotein" refers to apolipoproteins known to those of skill in the art and variants and fragments thereof and to apolipoprotein agonists, analogues or fragments thereof described below.

In a preferred embodiment, the active agent is a nucleic acid, such as a siRNA. For example, the active agent can be a nucleic acid encoded with a product of interest, including but not limited to, RNA, antisense oligonucleotide, an antagomir, a DNA, a plasmid, a ribosomal RNA (rRNA), a micro RNA (miRNA) (e.g., a miRNA which is single stranded and 17-25 nucleotides in length), transfer RNA (tRNA), a small interfering RNA (siRNA), small nuclear RNA (snRNA), antigens, fragments thereof, proteins, peptides, vaccines and small molecules or mixtures thereof. In one more preferred embodiment, the nucleic acid is an oligonucleotide (e.g., 15-50 nucleotides in length (or 15-30 or 20-30 nucleotides in length)). An siRNA can have, for instance, a duplex region that is 16-30 nucleotides long. In another embodiment, the nucleic acid is an immunostimulatory oligonucleotide, decoy oligonucleotide, supermir, miRNA mimic, or miRNA inhibitor. A supermir refers to a single stranded, double stranded or partially double stranded oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or both or modifications thereof, which has a nucleotide sequence that is substantially identical to an miRNA and that is antisense with respect to its target. miRNA mimics represent a class of molecules that can be used to imitate the gene silencing ability of one or more miRNAs. Thus, the term "microRNA mimic" refers to synthetic non-coding RNAs (i.e. the miRNA is not obtained by purification from a source of the endogenous miRNA) that are capable of entering the RNAi pathway and regulating gene expression.

The nucleic acid that is present in a lipid-nucleic acid particle can be in any form. The nucleic acid can, for example, be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Non-limiting examples of double-stranded RNA include siRNA. Single-stranded nucleic acids include, e.g., antisense oligonucleotides, ribozymes, microRNA, and triplex-forming oligonucleotides. The lipid particles of the present invention can also deliver nucleic acids which are conjugated to one or more ligands.

Pharmaceutical Compositions

The lipid particles, particularly when associated with a therapeutic agent, may be formulated as a pharmaceutical composition, e.g., which further comprises a pharmaceutically acceptable diluent, excipient, or carrier, such as physiological saline or phosphate buffer.

The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, and tonicity adjusting agents, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of lipid particle or lipid-nucleic acid particle in the pharmaceutical formulations can vary, for example, from less than about 0.01%, to at or at least about 0.05-5% to as much as 10 to 30% by weight.

Methods of Manufacture

Methods of making cationic lipids, lipid particles containing them, and pharmaceutical compositions containing the cationic lipids and/or lipid particles are described in, for example, International Publication Nos. WO 2010/054406, WO 2010/054401, WO 2010/054405, WO 2010/054384, WO 2010/042877, WO 2010/129709, WO 2009/086558, and WO 2008/042973, and U.S. Patent Publication Nos. 2004/0142025, 2006/0051405 and 2007/0042031, each of which is incorporated by reference in its entirety.

For example, in one embodiment, a solution of one or more lipids (including a cationic lipid of any of the embodiments described herein) in an organic solution (e.g., ethanol) is prepared. Similarly, a solution of one or more active (therapeutic) agents (such as, for example an siRNA molecule or a 1:1 molar mixture of two siRNA molecules) in an aqueous buffered (e.g., citrate buffer) solution is prepared. The two solutions are mixed and diluted to form a colloidal suspension of siRNA lipid particles. In one embodiment, the siRNA lipid particles have an average particle size of about 80-90 nm. In further embodiments, the dispersion may be filtered through 0.45/2 micron filters, concentrated and diafiltered by tangential flow filtration.

Definitions

As used herein, the term "cationic lipid" includes those lipids having one or two fatty acid or fatty aliphatic chains and an amino acid containing head group that may be protonated to form a cationic lipid at physiological pH. In some embodiments, a cationic lipid is referred to as an "amino acid conjugate cationic lipid."

A subject or patient in whom administration of the complex is an effective therapeutic regimen for a disease or disorder is preferably a human, but can be any animal, including a laboratory animal in the context of a clinical trial or screening or activity experiment. Thus, as can be readily

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appreciated by one of ordinary skill in the art, the methods, compounds and compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, humans, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, and cats, avian species, such as chickens, turkeys, and songbirds, i.e., for veterinary medical use.

Many of the chemical groups recited in the generic formulas above are written in a particular order (for example, —OC(O)—). It is intended that the chemical group is to be incorporated into the generic formula in the order presented unless indicated otherwise. For example, a generic formula of the form —$(R)_i$-$(M^1)_k$-$(R)_m$— where M^1 is —C(O)O— and k is 1 refers to —$(R)_i$—C(O)O—$(R)_m$— unless specified otherwise. It is to be understood that when a chemical group is written in a particular order, the reverse order is also contemplated unless otherwise specified. For example, in a generic formula —$(R)_i$-$(M^1)_k$-$(R)_m$— where M^1 is defined as —C(O)NH— (i.e., —$(R)_i$—C(O)—NH—$(R)_m$—), the compound where M^1 is —NHC(O)— (i.e., —$(R)_i$—NHC(O)—$(R)_m$—) is also contemplated unless otherwise specified.

The term "biodegradable cationic lipid" refers to a cationic lipid having one or more biodegradable groups located in the mid- or distal section of a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid. The incorporation of the biodegradable group(s) into the cationic lipid results in faster metabolism and removal of the cationic lipid from the body following delivery of the active pharmaceutical ingredient to a target area.

As used herein, the term "biodegradable group" refers to a group that include one or more bonds that may undergo bond breaking reactions in a biological environment, e.g., in an organism, organ, tissue, cell, or organelle. For example, the biodegradable group may be metabolizable by the body of a mammal, such as a human (e.g., by hydrolysis). Some groups that contain a biodegradable bond include, for example, but are not limited to esters, dithiols, and oximes. Non-limiting examples of biodegradable groups are —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —$C(R^5)$=N—, —N=$C(R^5)$—, —$C(R^5)$=N—O—, —O—N=$C(R^5)$—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi$(R^5)_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

As used herein, an "aliphatic" group is a non-aromatic group in which carbon atoms are linked into chains, and is either saturated or unsaturated.

The terms "alkyl" and "alkylene" refer to a straight or branched chain saturated hydrocarbon moiety. In one embodiment, the alkyl group is a straight chain saturated hydrocarbon. Unless otherwise specified, the "alkyl" or "alkylene" group contains from 1 to 24 carbon atoms. Representative saturated straight chain alkyl groups include methyl, ethyl, n-propyl, n-butyl, n-pentyl, and n-hexyl. Representative saturated branched alkyl groups include isopropyl, sec-butyl, isobutyl, tert-butyl, and isopentyl.

The term "alkenyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon double bonds. In one embodiment, the alkenyl group contains 1, 2, or 3 double bonds and is otherwise saturated. Unless otherwise specified, the "alkenyl" group contains from 2 to 24 carbon atoms. Alkenyl groups include both cis and trans isomers. Representative straight chain and branched alkenyl groups include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, and 2,3-dimethyl-2-butenyl.

The term "alkynyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon triple bonds. Unless otherwise specified, the "alkynyl" group contains from 2 to 24 carbon atoms. Representative straight chain and branched alkynyl groups include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, and 3-methyl-1-butynyl.

Unless otherwise specified, the terms "branched alkyl", "branched alkenyl", and "branched alkynyl" refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group. For example, a spirocyclic group in an alkyl, alkenyl, or alkynyl group is not considered a point of branching.

Unless otherwise specified, the term "acyl" refers to a carbonyl group substituted with hydrogen, alkyl, partially saturated or fully saturated cycloalkyl, partially saturated or fully saturated heterocycle, aryl, or heteroaryl. For example, acyl groups include groups such as (C_1-C_{20})alkanoyl (e.g., formyl, acetyl, propionyl, butyryl, valeryl, caproyl, and t-butylacetyl), (C_3-C_{20})cycloalkylcarbonyl (e.g., cyclopropylcarbonyl, cyclobutylcarbonyl, cyclopentylcarbonyl, and cyclohexylcarbonyl), heterocyclic carbonyl (e.g., pyrrolidinylcarbonyl, pyrrolid-2-one-5-carbonyl, piperidinylcarbonyl, piperazinylcarbonyl, and tetrahydrofuranylcarbonyl), aroyl (e.g., benzoyl) and heteroaroyl (e.g., thiophenyl-2-carbonyl, thiophenyl-3-carbonyl, furanyl-2-carbonyl, furanyl-3-carbonyl, 1H-pyrroyl-2-carbonyl, 1H-pyrroyl-3-carbonyl, and benzo[b] thiophenyl-2-carbonyl).

The term "aryl" refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system. Unless otherwise specified, the "aryl" group contains from 6 to 14 carbon atoms. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, anthracenyl, and pyrenyl.

The terms "cycloalkyl" and "cycloalkylene" refer to a saturated monocyclic or bicyclic hydrocarbon moiety such as cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl. Unless otherwise specified, the "cycloalkyl" or "cycloalkylene" group contains from 3 to 10 carbon atoms.

The term "cycloalkylalkyl" refers to a cycloalkyl group bound to an alkyl group, where the alkyl group is bound to the rest of the molecule.

The term "heterocycle" (or "heterocyclyl") refers to a non-aromatic 5- to 8-membered monocyclic, or 7- to 12-membered bicyclic, or 11- to 14-membered tricyclic ring system which is either saturated or unsaturated, and which contains from 1 to 3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized. For instance, the heterocycle may be a cycloalkoxy group. The heterocycle may be attached to the rest of the molecule via any heteroatom or carbon atom in the heterocycle. Heterocycles include, but are not limited to, morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, and tetrahydrothiopyranyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 7-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, where the heteroatoms are selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively). The heteroaryl groups herein described may also contain fused rings that share a common carbon-carbon bond.

The term "substituted", unless otherwise indicated, refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, oxo, thioxy, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy, aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aryloxycarbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxycarbonylalkyl, aminocarbonylalkyl, acyl, aralkoxycarbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and an aliphatic group. It is understood that the substituent may be further substituted. Exemplary substituents include amino, alkylamino, dialkylamino, and cyclic amino compounds.

The term "halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

The following abbreviations may be used in this application:

DSPC: distearoylphosphatidylcholine; DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; POPC: 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine; DOPE: 1,2-dileoyl-sn-3-phosphoethanolamine; PEG-DMG generally refers to 1,2-dimyristoyl-sn-glycerol-methoxy polyethylene glycol (e.g., PEG 2000); TBDPSCl: tert-Butylchlorodiphenylsilane; DMAP: dimethylaminopyridine; HMPA: hexamethylphosphoramide; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DIPEA: diisopropylethylamine; DCM: dichloromethane; TEA: triethylamine; TBAF: tetrabutylammonium fluoride

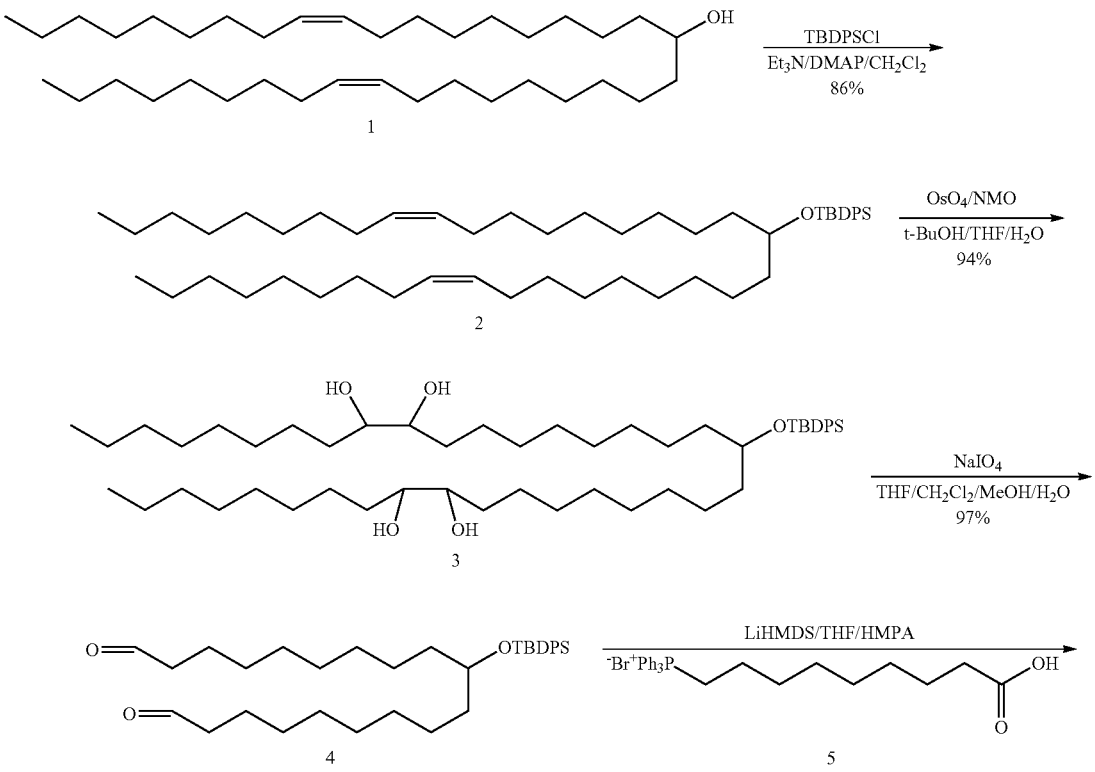
Methods to prepare various organic groups and protective groups are known in the art and their use and modification is generally within the ability of one of skill in the art (see, for example, Green, T. W. et. al., *Protective Groups in Organic Synthesis* (1999); Stanley R. Sandler and Wolf Karo, *Organic Functional Group Preparations* (1989); Greg T. Hermanson, *Bioconjugate Techniques* (1996); and Leroy G. Wade, *Compendium Of Organic Synthetic Methods* (1980)). Briefly, protecting groups are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

The compounds may be prepared by at least one of the techniques described herein or known organic synthesis techniques.

EXAMPLES

Example 1

Scheme 1. ALNY-322/quaternary salts



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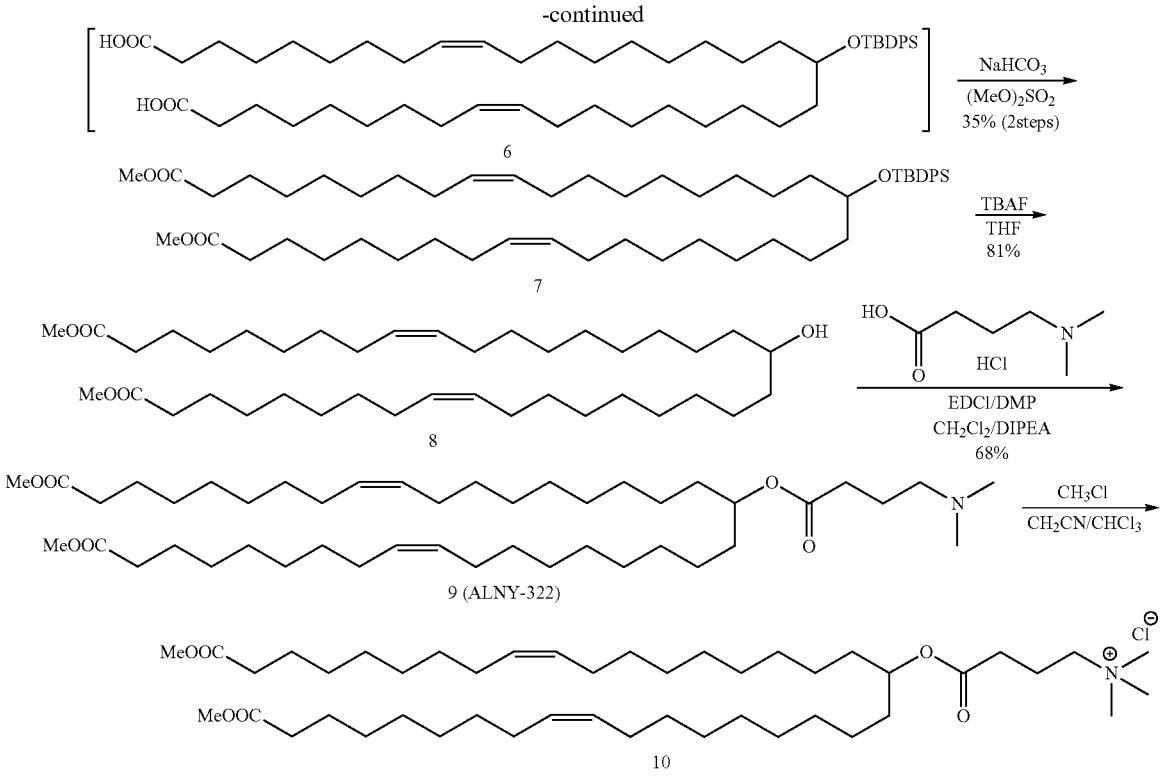


Compound 2: To a solution of compound 1 (10.0 g, 18.8 mmol, see International Publication No. WO 2010/054406) in CH_2Cl_2 (80 mL) were added triethylamine (7.86 mL, 56.4 mmol), DMAP (459 mg, 3.76 mmol) and tert-butyl(chloro) diphenylsilane (9.62 mL, 37.6 mmol). The reaction mixture was stirred for 24 hours. The mixture was then diluted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$ solution. The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (0-5% EtOAc in hexane) to afford 2 (12.4 g, 16.1 mmol, 86%, R_f=0.24 with hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 7.66-7.68 (m, 4H), 7.33-7.42 (m, 6H), 5.30-5.39 (m, 4H), 3.67-3.72 (m, 1H), 1.97-2.04 (m, 8H), 1.07-1.42 (m, 52H), 1.05 (s, 9H), 0.88 (t, J=6.8 Hz, 6H).

Compound 3: To a solution of 2 (12.4 g, 16.1 mmol) in tert-butanol (100 mL), THF (30 mL) and H_2O (10 mL) were added 4-methylmorpholine N-oxide (4.15 g, 35.4 mmol) and osmium tetroxide (41 mg, 0.161 mg). The reaction mixture was stirred for 16 hours, then quenched by adding sodium bisulfite. After removing the solvents by evaporation, the residue was extracted with Et_2O (500 mL) and H_2O (300 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude was purified by silica gel column chromatography (hexane: EtOAc=1:1, R_f=0.49) to afford 3 (12.7 g, 15.1 mmol, 94%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.66-7.68 (m, 4H), 7.33-7.43 (m, 6H), 3.67-3.73 (m, 1H), 3.57-3.62 (m, 4H), 1.82 (t, J=5.0 Hz, 4H), 1.10-1.51 (m, 60H), 1.04 (s, 9H), 0.88 (t, J=6.8 Hz, 6H).

Compound 4: To a solution of 3 (12.6 g, 15.0 mmol) in 1,4-dioxane (220 mL), CH_2Cl_2 (70 mL), MeOH (55 mL), and H_2O (55 mL) was added $NaIO_4$ (7.70 g, 36.0 mmol). The reaction mixture was stirred for 16 hours at room temperature. The mixture was extracted with Et_2O (500 mL) and H_2O (300 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (Hexane:EtOAc=9:1, R_f=0.30) to afford 4 (7.98 g, 14.5 mmol, 97%). Molecular weight for $C_{35}H_{54}NaO_3Si$ $(M+Na)^+$ Calc. 573.3740, Found 573.3.

Compound 7: To a solution of 5 (see, Tetrahedron, 63, 1140-1145, 2006; 1.09 g, 2.18 mmol) in THF (20 mL) and HMPA (4 mL), LiHMDS (1 M THF solution, 4.36 mL, 4.36 mmol) was added at −20° C. The resulting mixture was stirred for 20 minutes at the same temperature, then cooled to −78° C. A solution of 4 (500 mg, 0.908 mmol) in THF (4 mL) was added. The mixture was stirred and allowed to warm to room temperature overnight. MS analysis showed the formation of the di-acid (6; $C_{53}H_{85}O_5Si$ $(M-H)^-$ calc. 829.6166, observed 829.5). To the mixture, $NaHCO_3$ (1.10 g, 13.1 mmol) and dimethyl sulfate (1.24 mL, 13.1 mmol) were added and stirred for 2 hours at room temperature. The reaction was quenched by adding saturated NH_4Cl aqueous solution (50 mL) then extracted with Et_2O (2×100 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (Hexane: EtOAc=9:1, R_f=0.35) to afford 7 (270 mg, 0.314 mmol, 35%). Molecular weight for $C_{55}H_{90}NaO_5Si$ $(M+Na)^+$ Calc. 881.6455, Found 881.6484.

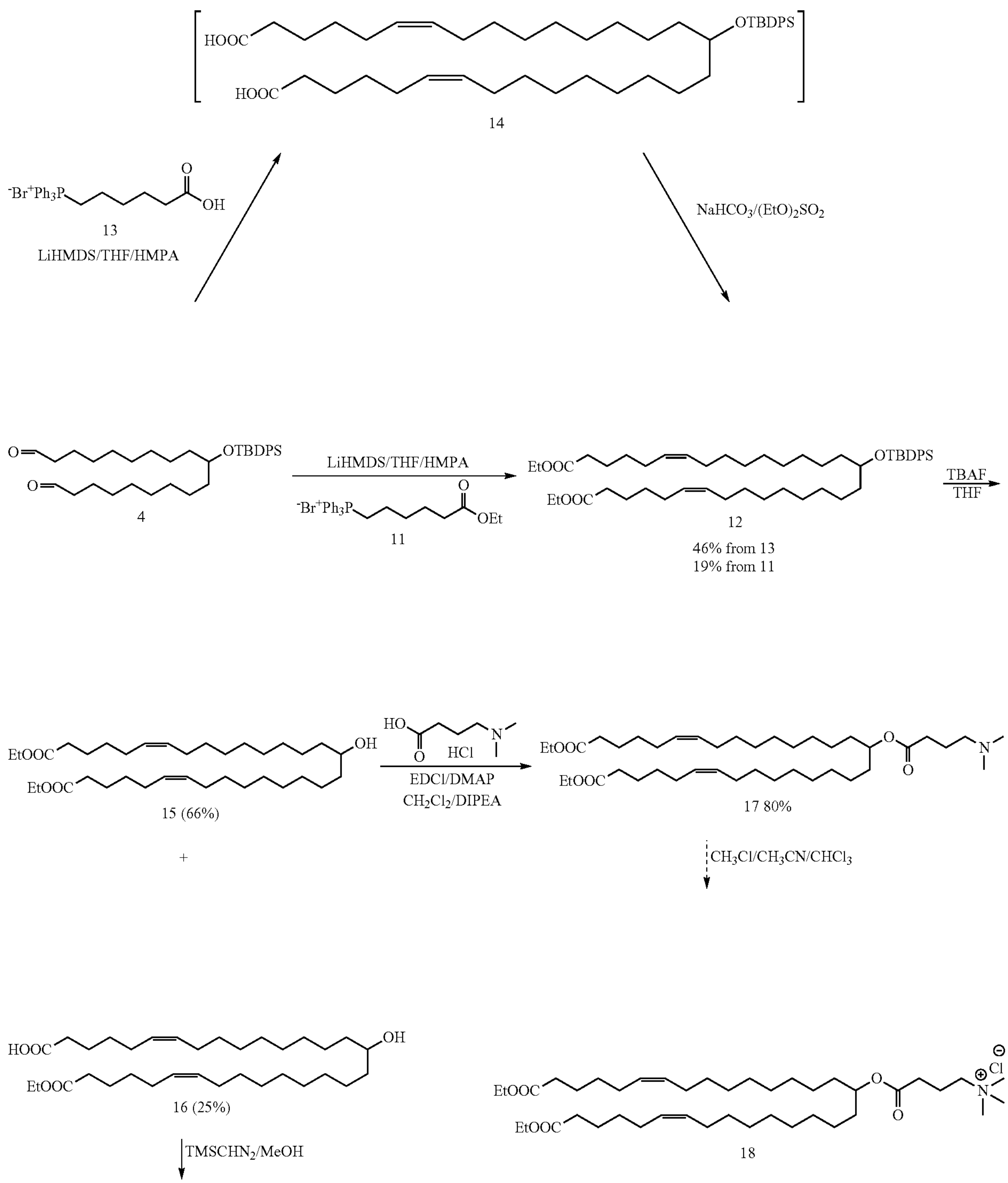
Compound 8: To a solution of 7 (265 mg, 0.308 mmol) in THF (2.5 mL), n-TBAF (1 M THF solution, 0.555 mL, 0.555 mmol) was added. The reaction mixture was stirred for 14 hours at 45° C. After concentration, the mixture was purified by silica gel column chromatography (Hexane: EtOAc=3:1, R_f=0.52) to afford 8 (155 mg, 0.250 mmol, 81%). Molecular weight for $C_{39}H_{72}NaO_5$ $(M+Na)^+$ Calc. 643.5277, Found 643.5273.

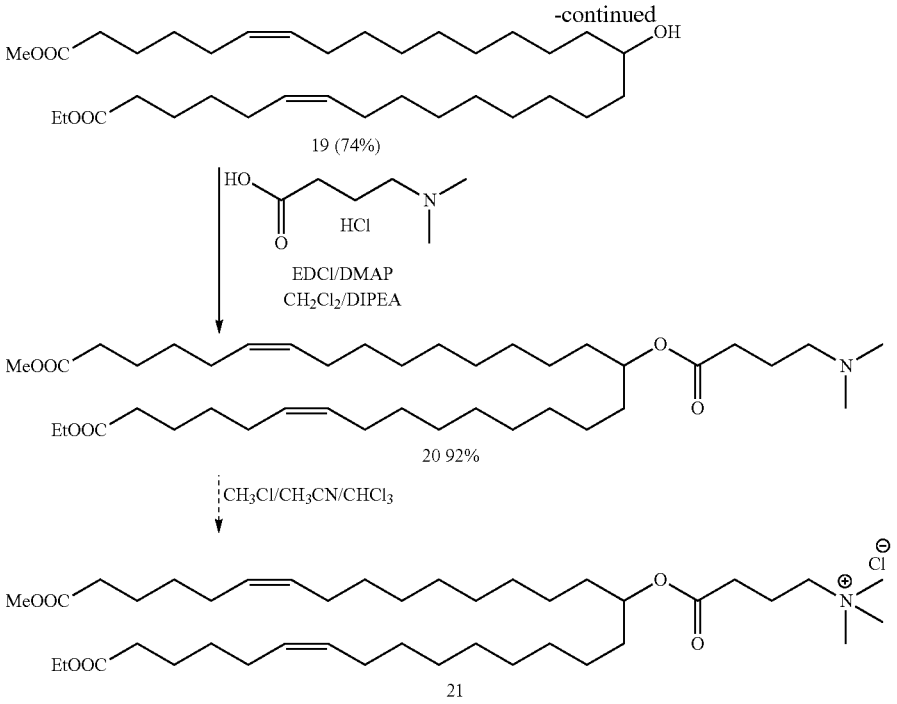
Compound 9: To a solution of compound 8 (150 mg, 0.242 mmol) and 4-(dimethylamino)butyric acid hydrochloride (49 mg, 0.290 mmol) in CH_2Cl_2 (5 mL) were added diisopropylethylamine (0.126 mL, 0.726 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (56 mg, 0.290 mmol) and DMAP (6 mg, 0.0484 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was then diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude product was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to afford compound 9 (121 mg, 0.165 mmol, 68%, R_f=0.25 developed with 5% MeOH in CH_2Cl_2). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.5.

Compound 10: Treatment of compound 9 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 10.

Example 2

Scheme 2





Compound 12: To a solution of 11 (Journal of Medicinal Chemistry (1995), 38, 636-46; 1.25 g, 2.58 mmol) in THF (20 mL) and HMPA (4 mL), LiHMDS (1 M THF solution, 2.58 mL, 2.58 mmol) was added at −20° C. The mixture was stirred for 20 min at the same temperature, then cooled to −78° C. A solution of 4 (500 mg, 0.908 mmol) in THF (9 mL) and HMPA (0.9 mL) was added. The mixture was stirred from −78° C. to room temperature overnight. The reaction was quenched by adding H_2O (40 mL) then extracted with Et_2O (150 mL×3). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude was purified by silica gel column chromatography (Hexane:EtOAc=9:1, R_f=0.35) to give 12 (136 mg, 0.169 mmol, 19%). Molecular weight for $C_{51}H_{82}NaO_5Si$ $(M+Na)^+$ Calc. 825.5829, Found 825.5.

Using 13 in place of 5, a procedure analogous to that described for compound 7 was followed to afford compound 12 (135 mg, 0.168 mmol, 46%).

Compound 15/Compound 16: To a solution of 12 (800 mg, 0.996 mmol) in THF (5 mL), n-TBAF (1 M THF solution, 5 mL, 5.00 mmol) was added. The reaction mixture was stirred for 16 h at 45° C. After concentration, the mixture was purified by silica gel column chromatography to give 15 (Hexane:EtOAc=3:1, R_f=0.46, 372 mg, 0.659 mmol, 66%) and 16 (CH_2Cl_2:MeOH=95:5, R_f=0.36, 135 mg, 0.251 mmol, 25%). Molecular weight for 15; $C_{35}H_{64}NaO_5$ $(M+Na)^+$ Calc. 587.4651, Found 587.4652. Molecular weight for 16; $C_{33}H_{61}O_5$ $(M+H)^+$ Calc. 537.4519, Found 537.5.

Compound 17: To a solution of compound 15 (164 mg, 0.290 mmol) and 4-(dimethylamino)butyric acid hydrochloride (58 mg, 0.348 mmol) in CH_2Cl_2 (5 mL) were added diisopropylethylamine (0.152 mL, 0.870 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (67 mg, 0.348 mmol) and DMAP (7 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to give compound 17 (158 mg, 0.233 mmol, 80%, R_f=0.24 developed with 5% MeOH in CH_2Cl_2). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.5.

Compound 18: Treatment of compound 17 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 18.

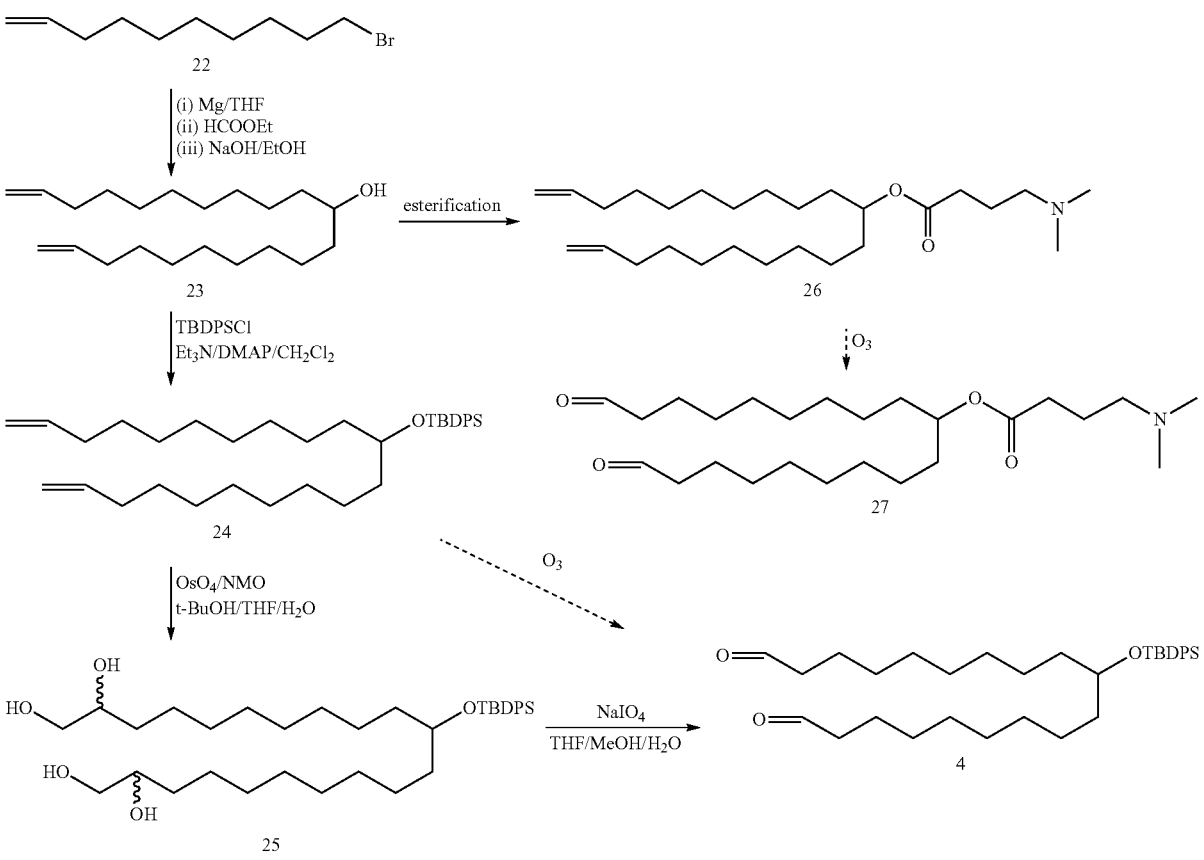
Compound 19: To a solution of 16 (130 mg, 0.242 mmol) in THF (2 mL) and MeOH (2 mL), trimethylsilyldiazomethane (2 M solution in Et_2O, 0.158 mL, 0.315 mmol) was added. The reaction mixture was stirred for 14 h. After evaporation, the residue was purified by silica gel column chromatography (Hexane:EtOAc=3:1, R_f=0.50) to give 19 (99 mg, 0.180 mmol, 74%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.29-5.40 (m, 4H), 4.12 (q, J=7.1 Hz, 2H), 3.66 (s, 3H), 3.55-3.59 (m, 1H), 2.30 (dd, J=14.7, 7.2 Hz, 4H), 1.98-2.07 (m, 8H), 1.60-1.68 (m, 4H), 1.23-1.43 (m, 37H).

Compound 20: To a solution of compound 19 (95 mg, 0.168 mmol) and 4-(dimethylamino)butyric acid hydrochloride (42 mg, 0.252 mmol) in CH_2Cl_2 (3 mL) were added diisopropylethylamine (0.088 mL, 0.504 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (48 mg, 0.504 mmol) and DMAP (4 mg, 0.034 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to give compound 20 (103 mg, 0.155 mmol, 92%, R_f=0.19 developed with 5% MeOH in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$) δ 5.29-5.40 (m, 4H), 4.83-4.89 (m, 1H), 4.12 (q, J=7.1 Hz, 2H), 3.67 (s, 3H), 2.28-2.34 (m, 8H), 2.23 (s, 6H), 1.98-2.07 (m, 8H), 1.76-1.83 (m, 2H), 1.60-1.68 (m, 4H), 1.23-1.51 (m, 35H).

Compound 21: Treatment of compound 20 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 21.

Example 3: Alternate Synthesis for Di-Aldehyde Intermediate 4

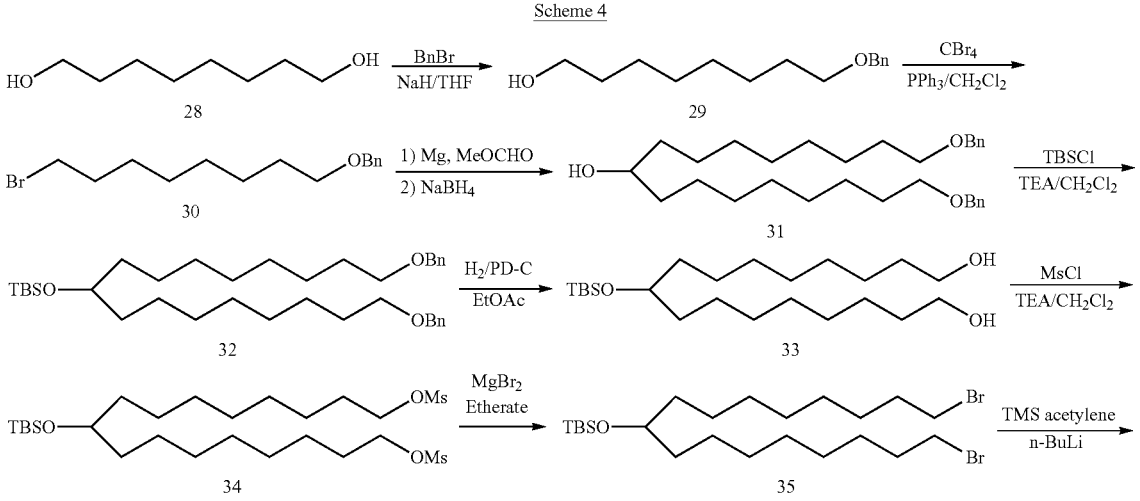
Scheme 3



The di-aldehyde 4 can be synthesized as shown in Scheme 3, using 1-bromo-9-decene. Di-aldehyde containing a head group 27 can be useful for the synthesis of terminal ester-substituted lipids using, e.g., a Wittig reaction. Ozonolysis can afford di-aldehyde 4 and 27.

Example 4: Alternate Synthesis for Compound 8

Scheme 4



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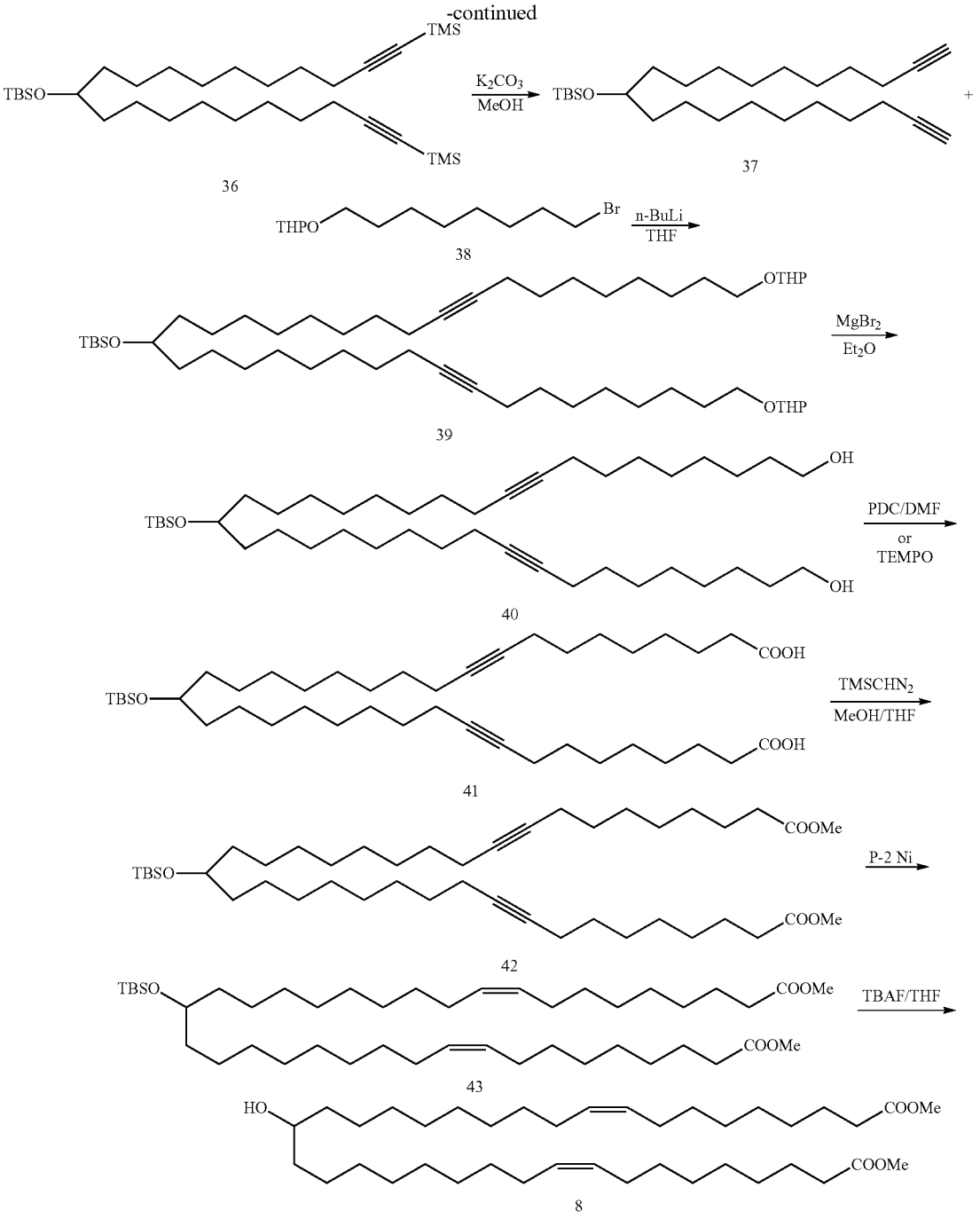

Compound 8 can be synthesized as shown in Scheme 4.

Compound 29: To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 28 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly using a dropping funnel at 0° C. The reaction mixture was stirred for 30 minutes, then benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under an atmosphere of nitrogen. The reaction was then warmed to ambient temperature and stirred for 10 hours. The mixture was then quenched with crushed ice (~2 kg) and extracted with ethyl acetate (2×1 L). The organic layer was washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness in vacuo. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 29 (220 g, 54%) as a pale yellow liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

Compound 30: Compound 29 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM, CBr_4 (280.35 g, 0.8456 mol) was added into this stirring solution and the reaction mixture was cooled to 0° C. under an inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions keeping the temperature below 20° C. After complete addition, the reaction mixture was stirred for 3 hours at room temperature. After completion of the reaction, the solid (PPh_3O) that precipi-

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tated from the reaction mixture was removed by filtration, and the filtrate was diluted with crushed ice (~1.5 kg) and extracted with DCM (3×750 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and distilled under vacuum. The resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to afford compound 30 (150 g, 89%) as pale yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

Compound 31: To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF, followed by the addition of pinch of iodine into the mixture under an inert atmosphere. A solution of Compound 30 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly, controlling the exothermic reaction. The reaction was then heated to reflux for 1 hour, then cooled to room temperature. Methyl formate (60.24 g, 1.0033 mol) was then added slowly and the reaction was continued for 2 hours. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with ethyl acetate (3×1 L). The organic layer was taken in 5 litre beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution, an excess of $NaBH_4$ (~5 eq) was added in portions to ensure hydrolysis of the formate ester which was not cleaved by addition of HCl. The resulting solution was stirred for an hour and then volatilites were removed under vacuum. The residue was taken in water (1 L) and acidified by 10% HCl solution (pH 4). The product was then extracted with ethyl acetate (3×1 L). the organic phase was then dried and concentrated on rotary evaporator to afford the desired compound 31 (57 g, 24%) as solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

Compound 32: Compound 31 (56 g, 0.1196 mol) was dissolved in 700 mL dry THF and cooled to 0° C. TBSCl (36.06 g, 0.2396 mol) was added slowly followed by the addition of imidazole (32.55 g, 0.4786 mol) under an inert atmosphere. The reaction was then stirred at room temperature for 18 hours. Upon completion, the reaction was quenched with ice (~1 kg) and extracted with ethyl acetate (3×500 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution to remove acidic impurities, dried over Na_2SO_4 and evaporated under reduce pressure to afford a crude compound that was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to afford (60 g, 82%) of compound 32 as yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s, 4H), 3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 33: Compound 32 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 minutes. (10 wt %) Pd on carbon (12 g) was added and the reaction was stirred under an atmosphere of hydrogen for 18 hours. After completion, the mixture was filtered through a bed of celite and washed with ethyl acetate. The filtrate was evaporated under vacuum to afford compound 33 (19 g, 46%) that was pure enough to use in the next synthetic sequence. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 34: Compound 33 (8.2 g, 0.0199 mol) was dissolved in 100 mL dry DCM and cooled to 0° C. TEA (22.14 mL, 0.1592 mol) was added under an inert atmosphere. After stirring the mixture for 5 minutes, mesyl chloride (4.6 mL, 0.059 mol) was added drop wise and the reaction was stirred further for 3 hours. After completion of the reaction, the mixture was quenched with ice (~200 g) and extracted with DCM (3×75 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to afford a crude compound which was purified on a 60-120 mesh silica gel column using 0-30% ethyl acetate in hexane as eluting system to afford compound 34 (8.2 g, 73%) as a pale yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=4.22-4.19 (m, 4H), 3.60-3.58 (m, 1H), 2.99 (s, 6H), 1.75-1.69 (m, 4H), 1.38-1.28 (m, 28H), 0.86 (s, 9H), 0.02 (s, 6H).

Compound 35: To a solution of compound 34 (8.2 g, 0.0146 mol) in 400 mL dry ether was added $MgBr_2Et_2O$ (22.74 g, 0.08817 mol) in portions at 0° C. under a nitrogen atmosphere. After complete addition, the reaction mixture was heated to reflux for 28 hours. After completion of reaction, inorganic material formed in the reaction was removed by filtration. The filtrate was evaporated and the resulting crude compound was purified on 60-120 mesh silica gel column using 0-3% ethyl acetate in hexanes as eluting system to afford compound 35 (6.6 g, 85%) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.61-3.58 (m, 1H), 3.41-3.37 (t, 4H, J=6.8 Hz), 1.87-1.80 (m, 4H), 1.42-1.25 (m, 24H), 0.87 (s, 9H), 0.012 (s, 6H).

Compound 36: A solution of ethynyl trimethyl silane (5.3 mL, 0.0378 mol) in 60 mL dry THF was cooled to −78° C. and 1.4 M n-BuLi (23 mL, 0.03405 mol) in hexane was added slowly under an inert atmosphere. The reaction was stirred for 10 minutes, then HMPA (2.3 g, 0.01324 mol) was added and the resulting mixture was then stirred for 2 hours at 0° C., then cooled to −78° C. To this a solution of compound 35 (5 g, 0.0094 mol) in 60 mL dry THF was added slowly and after complete addition, the reaction was warmed to room temperature and maintained for 18 hours. The reaction progress was monitored by ^{1}H NMR. After completion, the reaction mixture was cooled to 0° C. and quenched by careful addition of saturated NH_4Cl solution (50 mL) followed by water (200 mL). The aqueous phase was extracted with hexane (3×250 mL). The organic layer was dried and solvent removed under vacuum to afford compound 36 (5 g, 94%), which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.62-3.56 (m, 1H), 2.21-2.17 (m, 4H), 1.49-1.47 (m, 4H), 1.37-1.26 (m, 24H), 0.87 (s, 9H), 0.13 (s, 18H), 0.021 (s, 6H).

Compound 37: To a stirred solution of compound 36 (5 g, 0.0088 mol) in 50 mL methanol, was added K_2CO_3 (6.1 g, 0.044 mol) in one portion, and the resulting mixture was stirred for 18 hours at ambient temperature. Volatilities were then removed on a rotary evaporator and the crude mixture was diluted with 100 mL water and extracted with hexane (3×100 mL). The organic layer was dried over Na_2SO_4 and evaporated under vacuum to afford compound 37 (3.5 g, 97%) which was used which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.60-3.58 (m, 1H), 2.19-2.14 (m, 4H), 1.93-1.92 (m, 2H), 1.54-1.49 (m, 4H), 1.37-1.27 (m, 24H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 39: Compound 37 (2.5 g, 0.00598 mol) was dissolved in 25 mL dry THF and cooled to −40° C. n-BuLi (1.4 M in hexane 12.9 mL, 0.01794 mol) was added slowly, followed, after a 10 minute interval, by slow addition of HMPA (25 mL). The resulting mixture was maintained for 30 minutes −40° C. under a nitrogen atmosphere. A solution of compound 38 (3.5 g, 1.01196 mol) in 25 mL dry THF was then added drop wise to the cooled reaction mixture. The resulting mixture was warmed to room temperature over 2

hours, then stirred at room temperature for 18 hours. The mixture was then quenched by adding saturated NH_4Cl solution (~50 mL) and the product was extracted with ethyl acetate (3×50 mL). The solvent was removed on a rotary evaporator and the resulting crude product was purified by (100-200 mesh) silica gel column using 0-3% ethyl acetate in dichloromethane as eluting system to afford compound 39 (0.9 g, 18%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ=4.56-4.55 (m, 2H), 3.87-3.83 (m, 2H), 3.74-3.68 (m, 2H), 3.59-3.57 (m, 1H), 3.49-3.46 (m, 2H), 3.39-3.33 (m, 2H), 2.13-2.10 (m, 8H), 1.87-1.75 (m, 2H), 1.74-1.66 (m, 2H), 1.57-1.42 (m, 20H), 1.40-1.19 (m, 40H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 40: To a solution of compound 39 (504 mg, 0.598 mmol) in 10 mL dry ether was added $MgBr_2Et_2O$ (926 mg, 3.59 mmol). The reaction mixture was stirred for 14 hours, then quenched by adding saturated $NaHCO_3$ aqueous solution. The product was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography to afford compound 40 (307 mg, 0.455 mmol, 76%, R_f=0.36 developed with hexane:EtOAc=2:1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.59-3.66 (m, 5H), 2.14 (t, J=6.6 Hz, 8H), 1.21-1.59 (m, 52H), 0.88 (s, 9H), 0.03 (s, 6H).

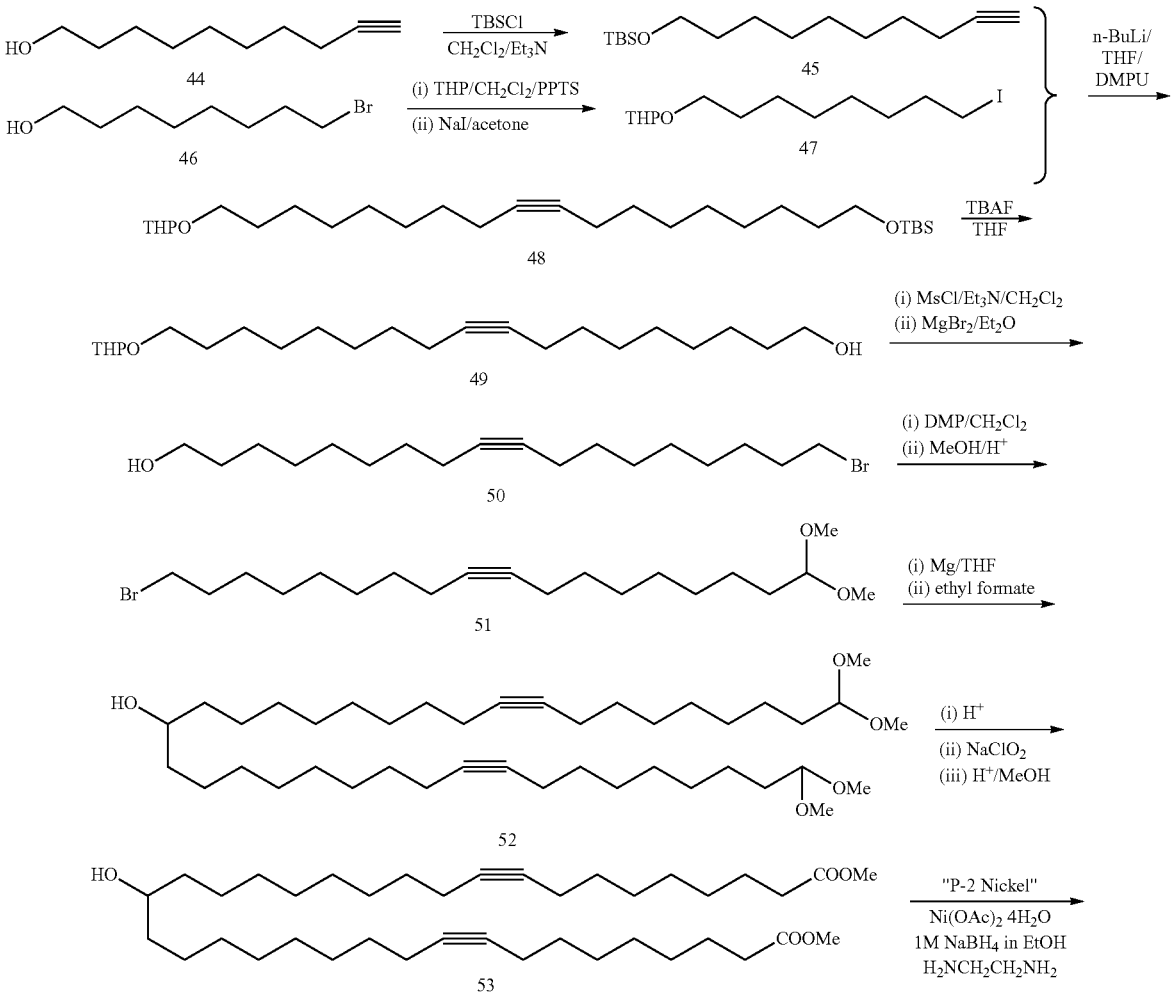
Compound 41: To a stirred solution of 40 (180 mg, 0.267 mmol) in anhydrous DMF (5 mL) was added pyridinium dichromate (603 mg, 1.60 mmol). The reaction mixture was stirred for 48 hours. After dilution with water (20 mL), the mixture was extracted with Et_2O (3×40 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography to afford compound 41 (53 mg, 0.075 mmol, 28%, R_f=0.25 developed with CH_2Cl_2:MeOH:AcOH=95:4.5:0.5). Molecular weight for $C_{43}H_{77}O_5Si$ $(M-H)^-$ Calc. 701.5540, Found 701.5. This compound can be synthesized by TEMPO oxidation.

Compound 42: A procedure analogous to that described for compound 19 afforded compound 42 (23 mg 0.032 mmol, 21% from compound 40). ^1H NMR (400 MHz, $CDCl_3$) δ 3.67 (s, 6H), 3.59-3.62 (m, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.13 (t, J=6.8 Hz, 8H), 1.27-1.64 (m, 48H), 0.88 (s, 9H), 0.03 (s, 6H).

Reduction using P-2 nickel conditions can give compound 43 and subsequent deprotection by TBAF can afford compound 8.

Example 5: Alternate Synthesis for Compound 8

Scheme 5



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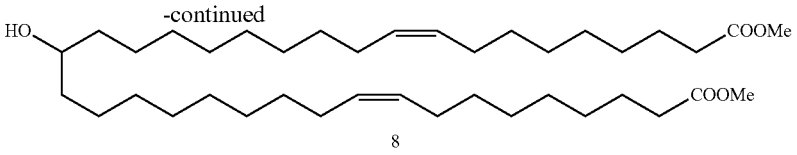

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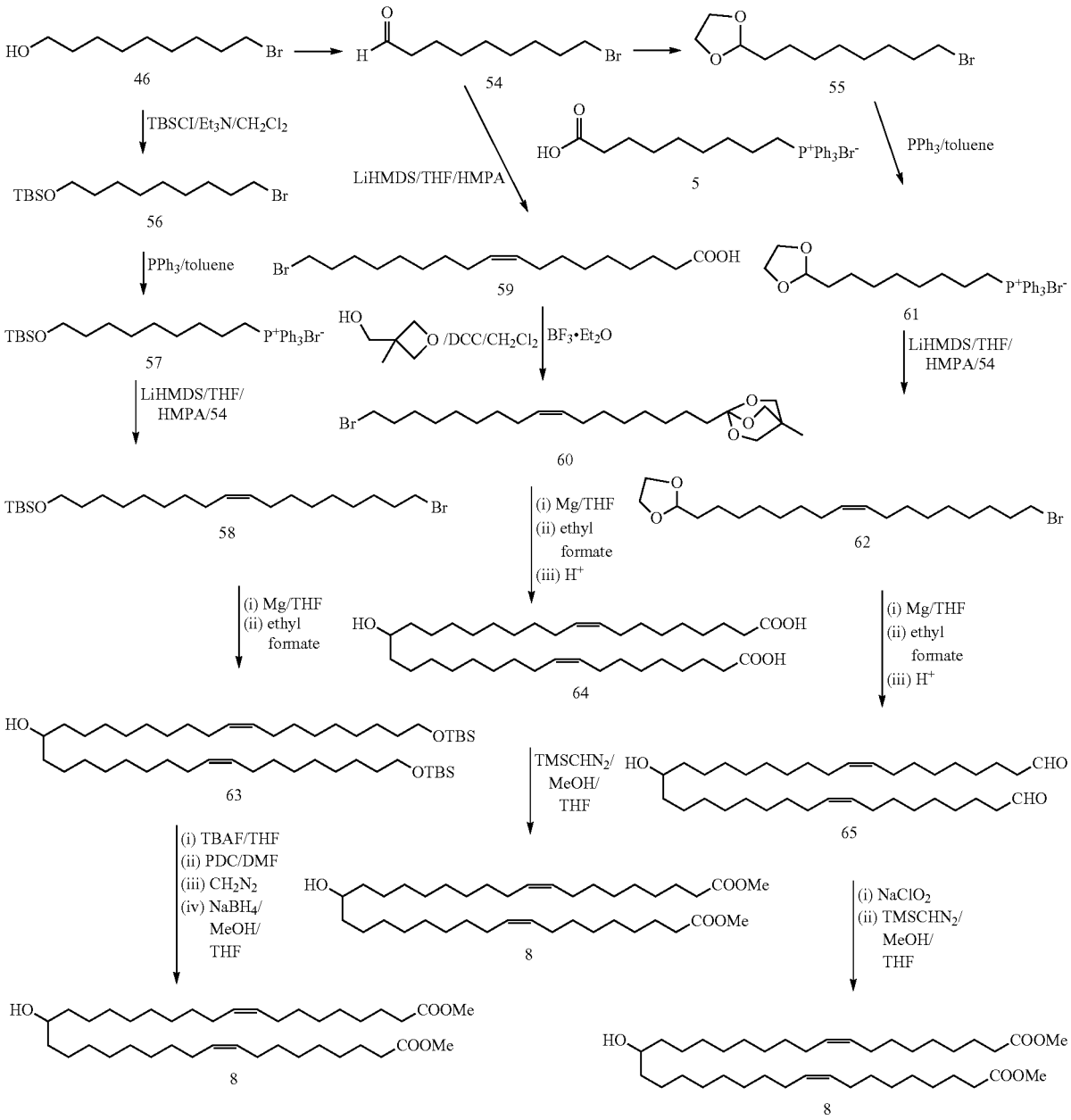
Compound 8 can be synthesized as shown in Scheme 5. The bromide 51 can be converted to its Grignard reagent then coupled with ethyl formate to afford compound 52. Subsequent acid treatment, oxidation, and reduction can give compound 8.

Example 6: Alternate Synthesis for Compound 8

Scheme 6



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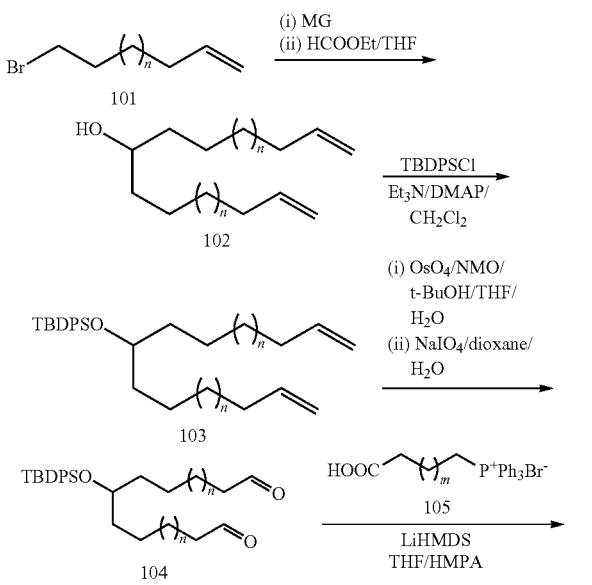
Compound 8 can be synthesized as shown in Scheme 6. Either bromides of compound 58, 60, or 62 can be reacted with ethyl formate to generate terminal-functionalized di-olefin chain. Compound 8 can then be prepared from the diolefin chain compounds using standard chemical reactions.

Example 7: General Synthetic Scheme for Terminal Ester Lipids

Scheme 7



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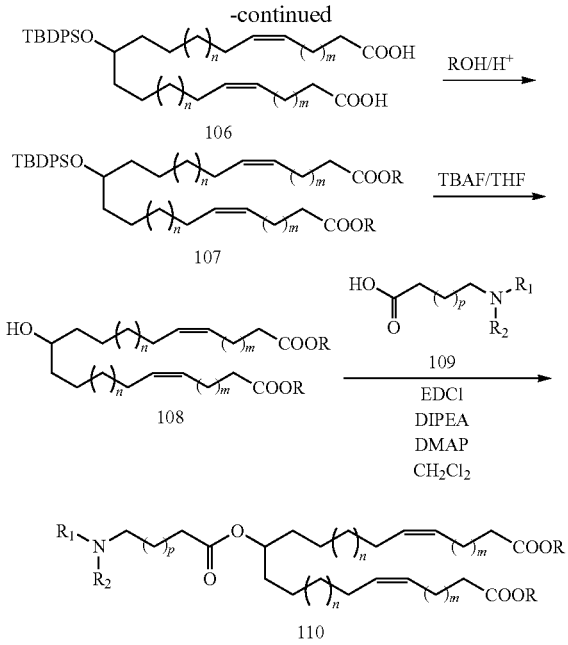
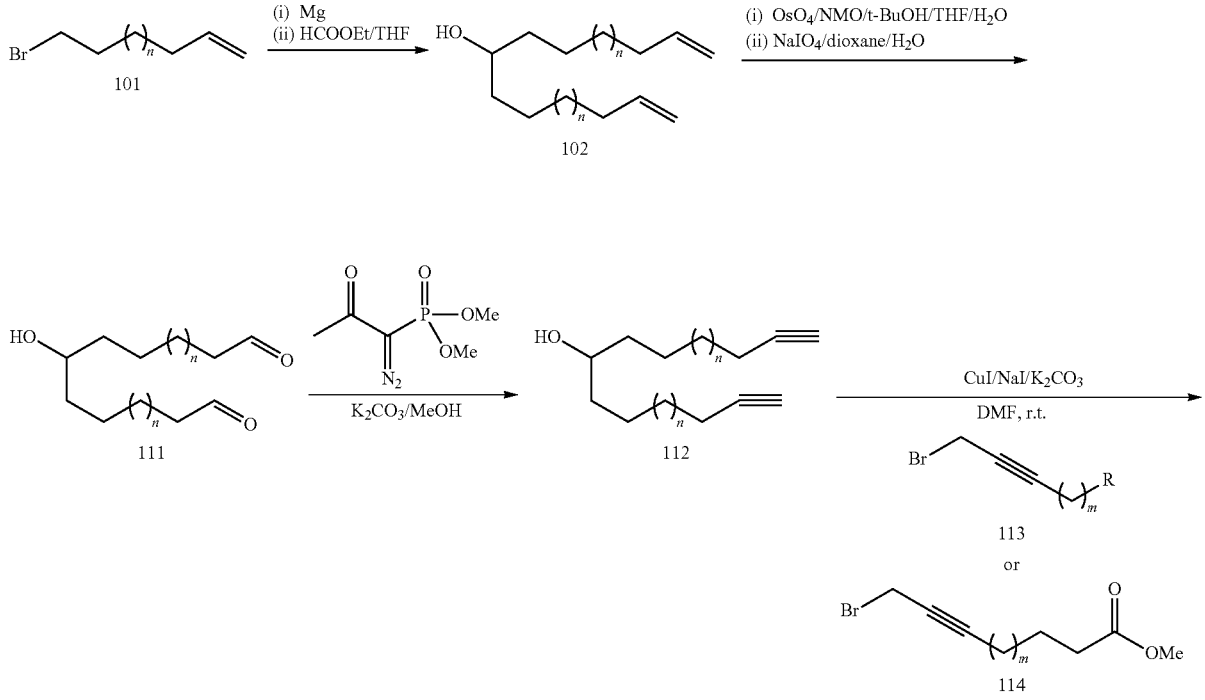


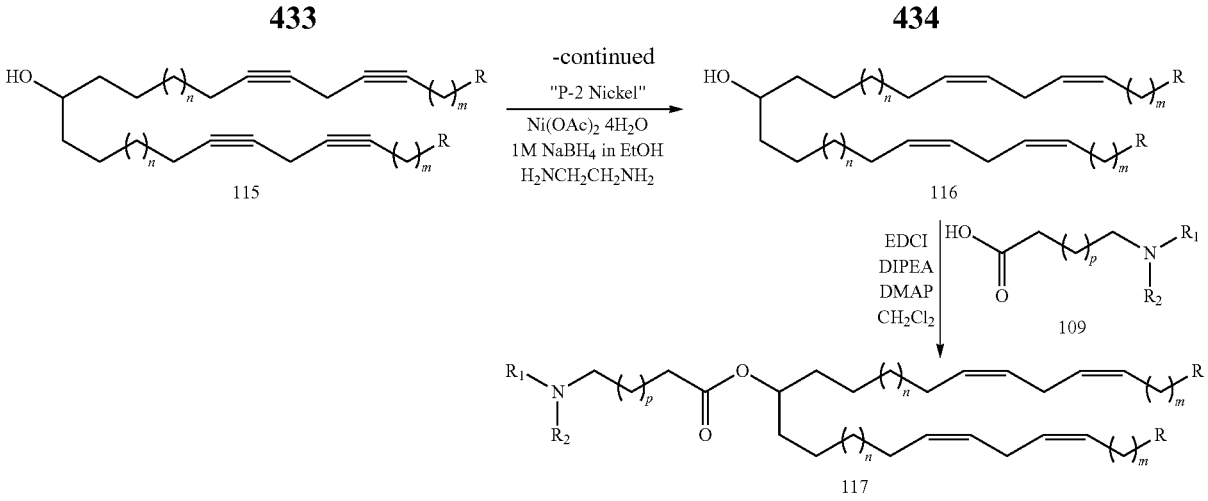
n = 0-8
m = 0-8
p = 0-3
R = R1 = R2 = Me, Et, Pr, BN, t-Bu, pH, alkyl, aryl, cycloalkyl, etc.

As shown in Scheme 7, chain length and linker length as well as alkyl groups in ester functionality and substituents on nitrogen atom can be derivatized.

Example 8: General Synthetic Scheme 2 for Terminal Ester Lipids

Scheme 8





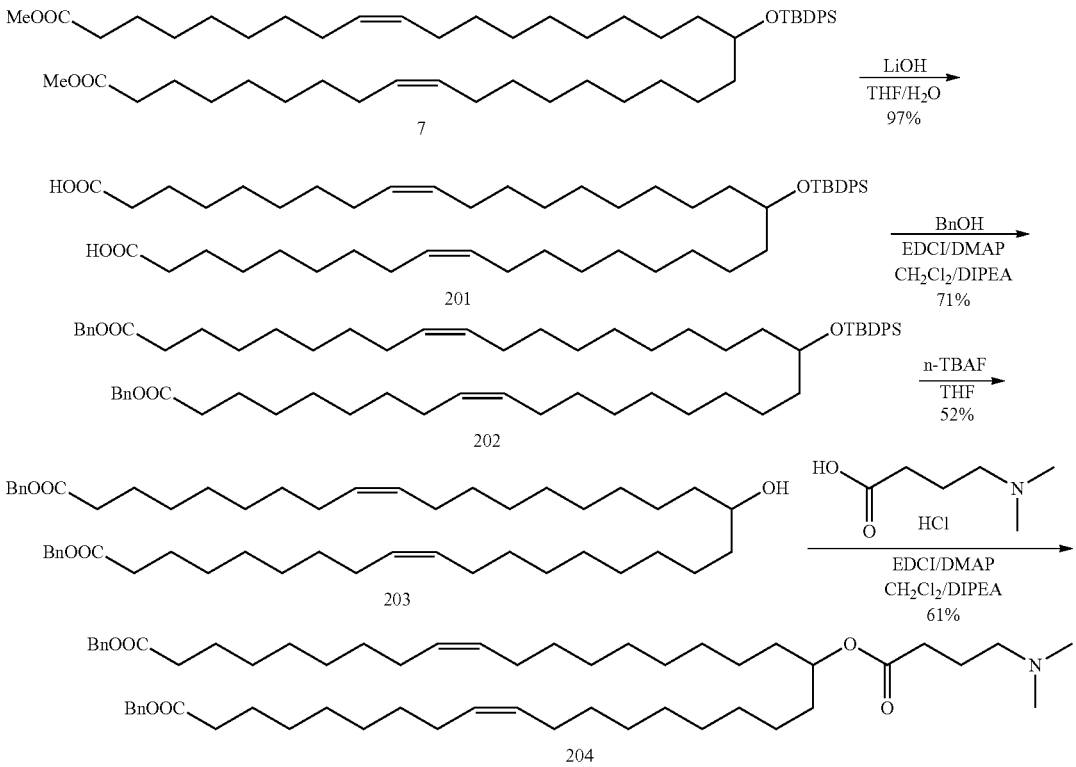
n = 0-8
m = 0-8
p = 0-3
R = Me, Et, Pr, Bn, t-Bu, Ph, alkyl, aryl, cycloalkyl, and alkyl esters, etc.
$R_1 = R_2$ = Me, Et, Pr, Bn, t-Bu, Ph, alkyl, aryl, cycloalkyl

As shown in Scheme 8, copper-mediated coupling affords di-yne containing lipid chain with terminal functional groups, which can be easily reduced to generate di-ene containing lipid chains. The length of linker and lipid chain as well as functional substituent groups (R, R_1, R_2) can be derivatized.

Example 9: Synthesis of Terminal Benzyl Ester Lipid

Compound 201: Compound 7 (1.30 g, 1.51 mmol) was treated with lithium hydroxide monohydrate (317 mg, 7.55 mmol) in THF (25 mL) and H_2O (5 mL) for 12 h. Amberlite IR-120 (plus) ion exchange resin was added then stirred for 10 minutes. The resulting clear solution was filtered, washed with THF/H_2O and evaporated. Co-evaporation with toluene gave the compound 201 (1.22 g, 1.47 mmol, 97%). Molecular weight for $C_{53}H_{85}O_5Si$ $(M-H)^-$ Calc. 829.6166, Found 829.5.

Scheme 9



Compound 202: A procedure analogous to that described for compound 9 was followed with benzylalcohol and 201 (101 mg, 0.121 mmol) to afford compound 202 (87 mg, 0.0860 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.30 (m, 16H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 3.71-3.68 (m, 1H), 2.35 (t, J=7.6 Hz, 4H), 2.04-1.97 (m, 8H), 1.66-1.62 (m, 4H), 1.40-1.07 (m, 44H), 1.04 (s, 9H).

Compound 203: A procedure analogous to that described for compound 8 was followed with 202 (342 mg, 0.338 mmol) to afford compound 202 (136 mg, 0.176 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.30 (m, 10H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 3.57 (brs, 1H), 2.35 (t, J=7.6 Hz, 4H), 2.01-1.98 (m, 8H), 1.66-1.60 (m, 4H), 1.45-1.25 (m, 44H).

Compound 204: A procedure analogous to that described for compound 9 was followed with 203 (133 mg, 0.172 mmol) to afford compound 204 (93 mg, 0.105 mmol, 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.26 (m, 10H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 4.88-4.83 (m, 1H), 2.37-2.27 (m, 8H), 2.22 (s, 6H), 2.03-1.97 (m, 8H), 1.81-1.26 (m, 50H).

Example 10: Synthesis of Terminal t-Butyl Ester Lipid and the Derivatives

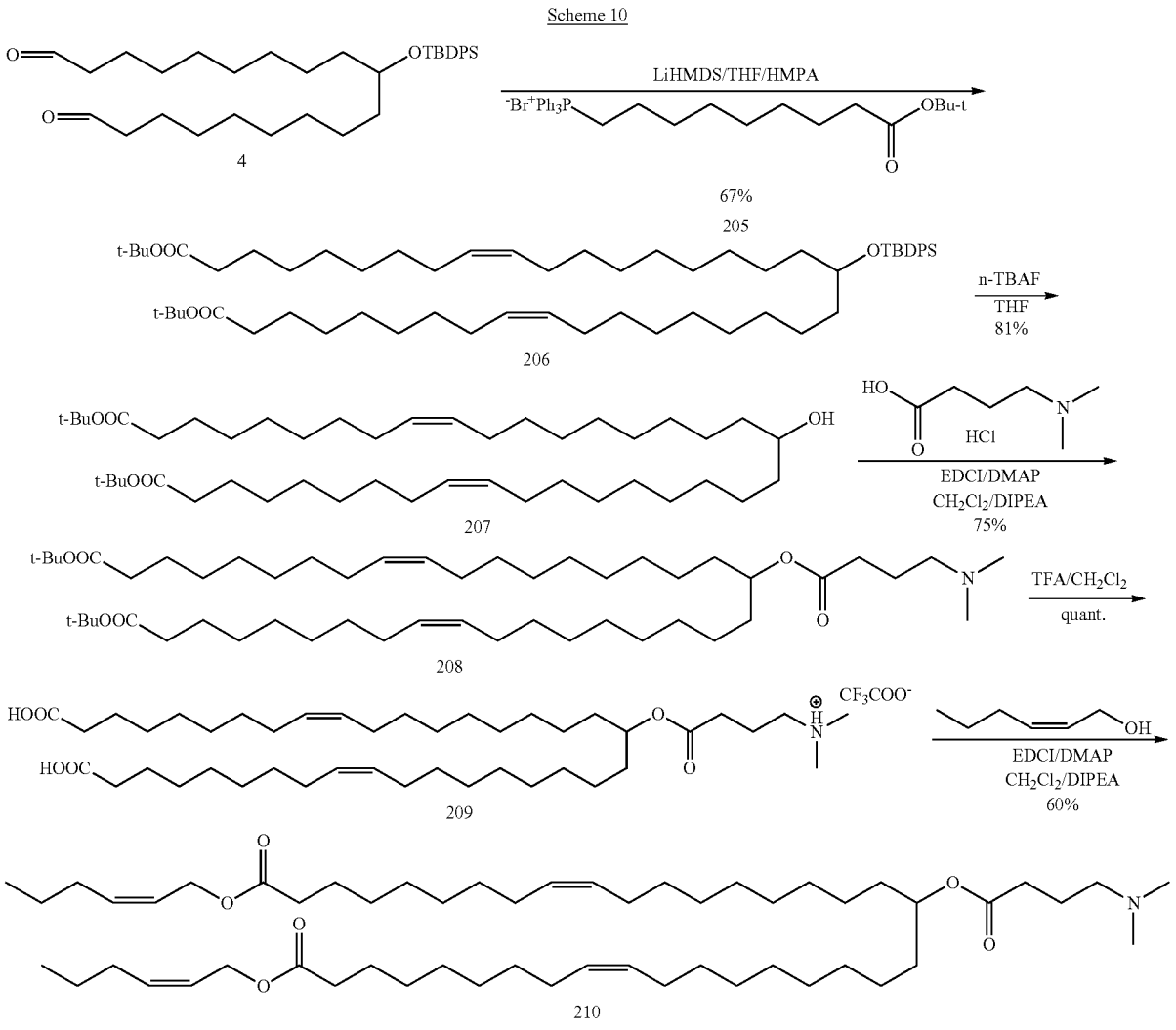
Compound 206: A procedure analogous to that described for compound 12 was followed with 205 (3.80 g, 0.7.61 mmol) and 4 (1.75 g, 3.17 mmol) to afford compound 206 (2.00 g, 2.12 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.33 (m, 6H), 5.39-5.31 (m, 4H), 3.71-3.68 (m, 1H), 2.20 (t, J=7.6 Hz, 4H), 2.01-1.98 (m, 8H), 1.59-1.55 (m, 4H), 1.44 (s, 18H), 1.41-1.11 (m, 44H), 1.04 (s, 9H).

Compound 207: A procedure analogous to that described for compound 8 was followed with 206 (265 mg, 0.281 mmol) to afford compound 207 (161 mg, 0.228 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38-5.30 (m, 4H), 3.58 (brs, 1H), 2.20 (t, J=7.4 Hz, 4H), 2.01-1.98 (m, 8H), 1.59-1.55 (m, 4H), 1.44 (s, 18H), 1.35-1.26 (m, 44H).

Compound 208: A procedure analogous to that described for compound 9 was followed with 207 (158 mg, 0.224 mmol) to afford compound 208 (138 mg, 0.169 mmol, 75%). Molecular weight for $C_{51}H_{96}NO_6$ $(M+H)^+$ Calc. 818.7238, Found 818.7.

Compound 209: Compound 208 (148 mg, 0.181 mmol) was treated with TFA (1.5 mL) in CH_2Cl_2 (6 mL) for 2.5 h. After evaporation and co-evaporation with toluene gave the compound 209 (154 mg, quant.). Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5980, Found 706.5.

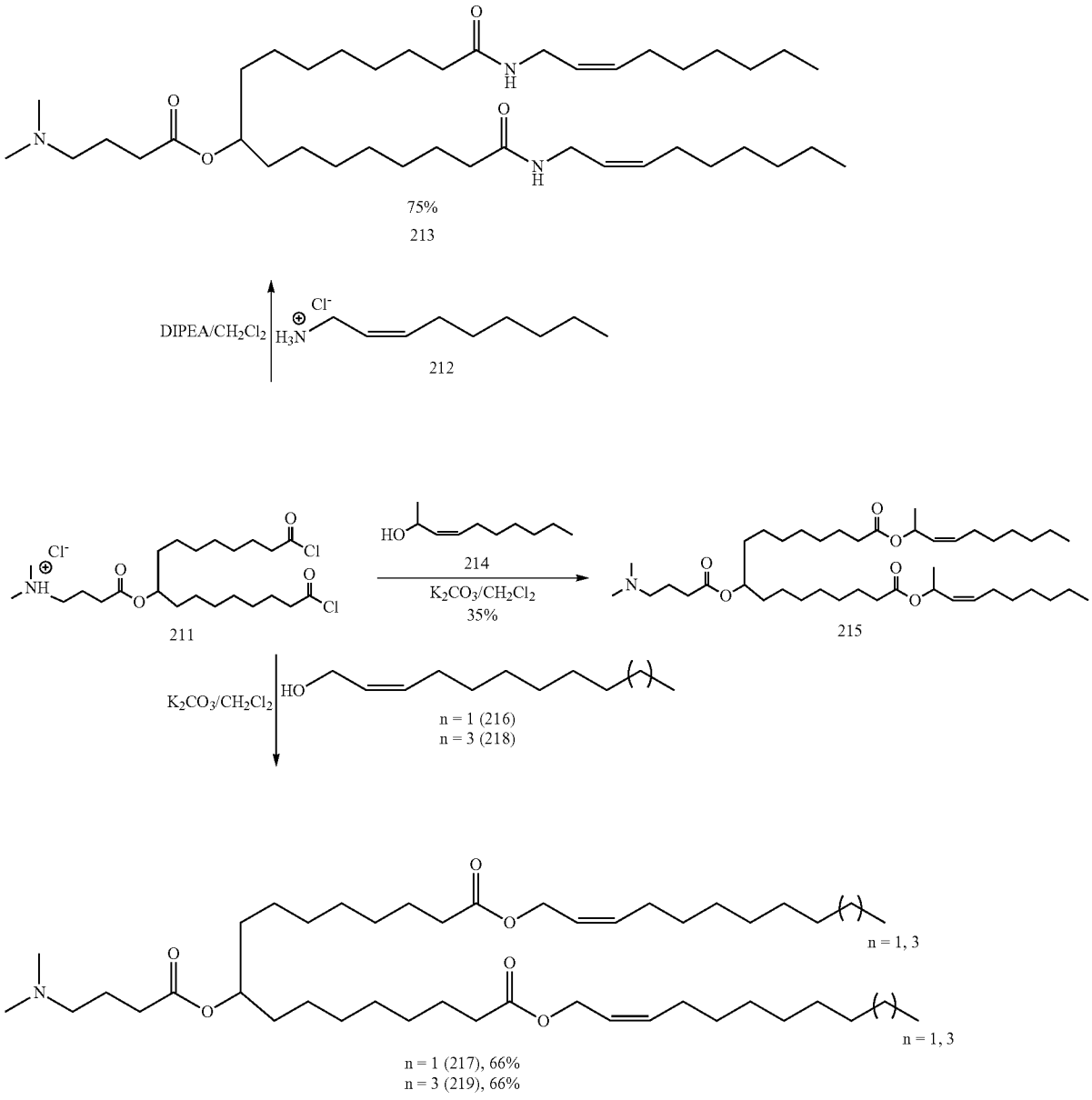
Scheme 10



Compound 210: A procedure analogous to that described for compound 9 was followed with 209 (0.061 mmol) and cis-2-Hexen-1-ol (18.3 mg, 0.183 mmol) to afford compound 210 (32 mg, 0.0368 mmol, 60%). Molecular weight for $C_{55}H_{100}NO_6$ $(M+H)^+$ Calc. 870.7551, Found 870.5.

Example 11: Synthesis of Internal Ester/Amide Lipids-1

Scheme 11



Compound 213: Compound 211 (503 mg, 1.0 mmol) was treated with 212 (533 mg, 3.0 mmol) in CH_2Cl_2 (35 mL) and DIPEA (1.74 mL, 10 mmol) for 14 h. Aqueous work-up then column chromatography gave compound 213 (506 mg, 0.748 mmol, 75%). Molecular weight for $C_{41}H_{78}N_3O_4$ $(M+H)^+$ Calc. 676.5992, Found 676.4.

Compound 215: Compound 211 (503 mg, 1.0 mmol) was treated with 214 (469 mg, 3.0 mmol) and K_2CO_3 (1.38 g, 10 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 215 (244 mg, 0.346 mmol, 35%). Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5986, Found 706.4.

Compound 217: Compound 211 (425 mg, 0.845 mmol) was treated with 216 (525 mg, 3.08 mmol) and K_2CO_3 (1.17 g, 8.45 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 217 (407 mg, 0.554 mmol, 66%). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.4.

Compound 219: Compound 211 (503 mg, 1.0 mmol) was treated with 218 (595 mg, 3.0 mmol) and K_2CO_3 (1.38 g, 10 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 219 (519 mg, 0.657 mmol, 66%). Molecular weight for $C_{49}H_{92}NO_6$ $(M+H)^+$ Calc. 790.6925, Found 790.7.

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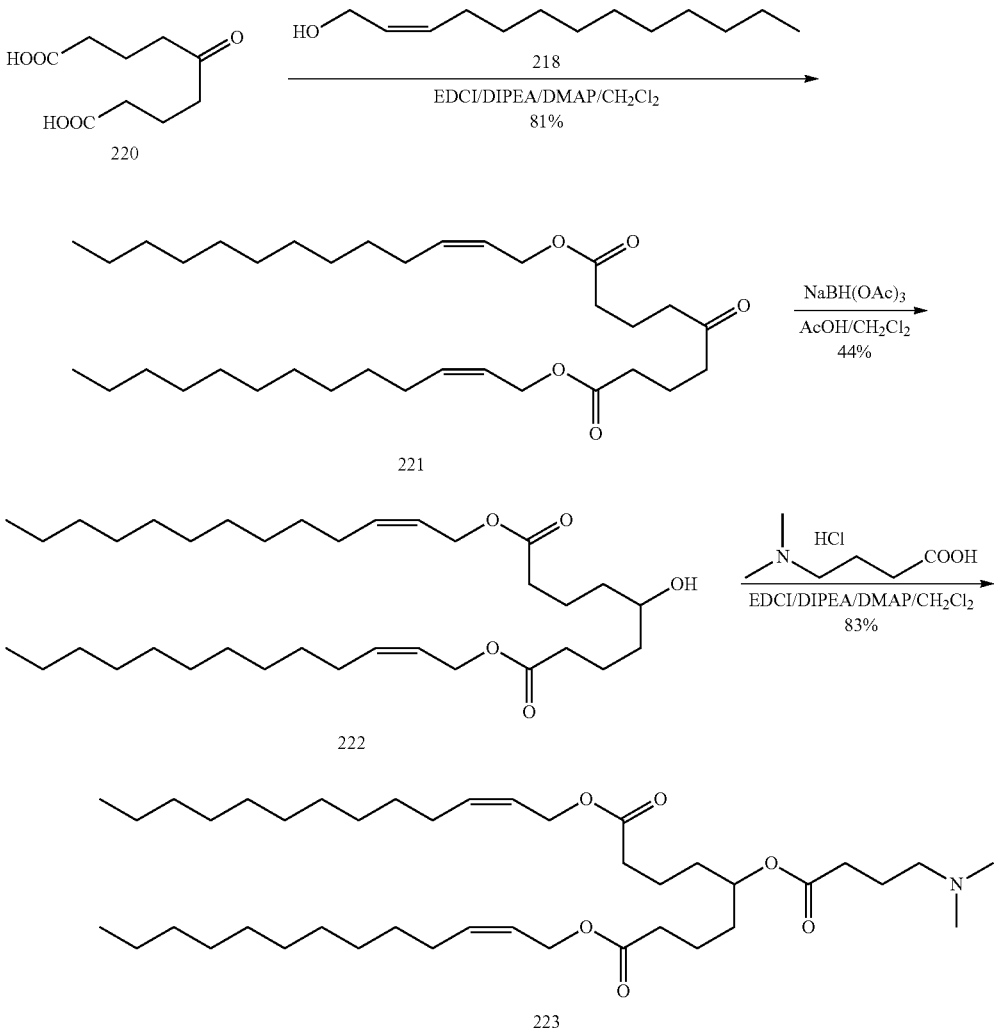
Example 12: Synthesis of Internal Ester Lipid-223

Scheme 12



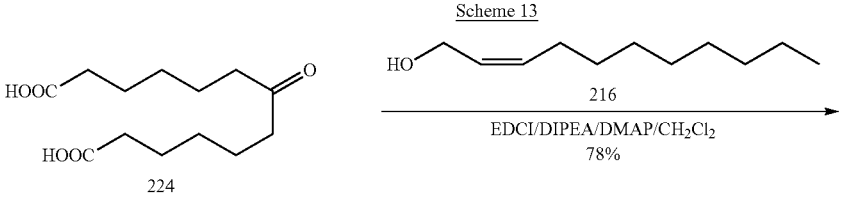
Compound 221: A procedure analogous to that described for compound 9 was followed with 220 (390 mg, 1.93 mmol) and 218 (765 mg, 3.86 mmol) to afford compound 221 (878 mg, 1.56 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.54-5.48 (m, 2H), 4.62 (d, J=6.8 Hz, 4H), 2.47 (t, J=7.2 Hz, 4H), 2.33 (t, J=7.2 Hz, 4H), 2.12-2.06 (m, 4H), 1.93-1.86 (m, 4H), 1.38-1.26 (m, 32H), 0.88 (t, J=6.8 Hz, 6H).

Compound 222: Compound 221 (318 mg, 0.565 mmol) was treated with $NaBH(OAc)_3$ (360 mg, 1.70 mmol) in CH_2Cl_2 (5 mL) and AcOH (0.2 mL) for 16 h. After evaporation, column chromatography gave compound 222 (141 mg, 0.250 mmol, 44%). Molecular weight for $C_{35}H_{65}O_5$ $(M+H)^+$ Calc. 565.4832, Found 565.4.

Compound 223: A procedure analogous to that described for compound 9 was followed with 222 (137 mg, 0.243 mmol) to afford compound 223 (137 mg, 0.202 mmol, 83%). Molecular weight for $C_{41}H_{76}NO_6$ $(M+H)^+$ Calc. 678.5673, Found 678.5.

Example 13: Synthesis of Internal Ester Lipid-227

Scheme 13



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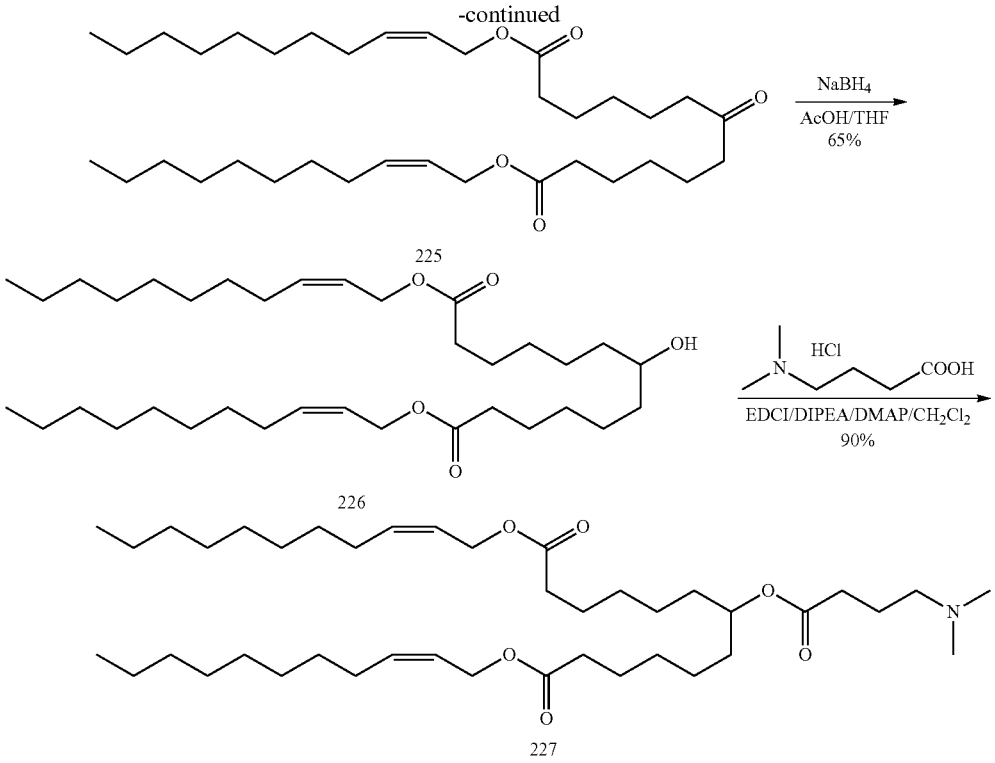


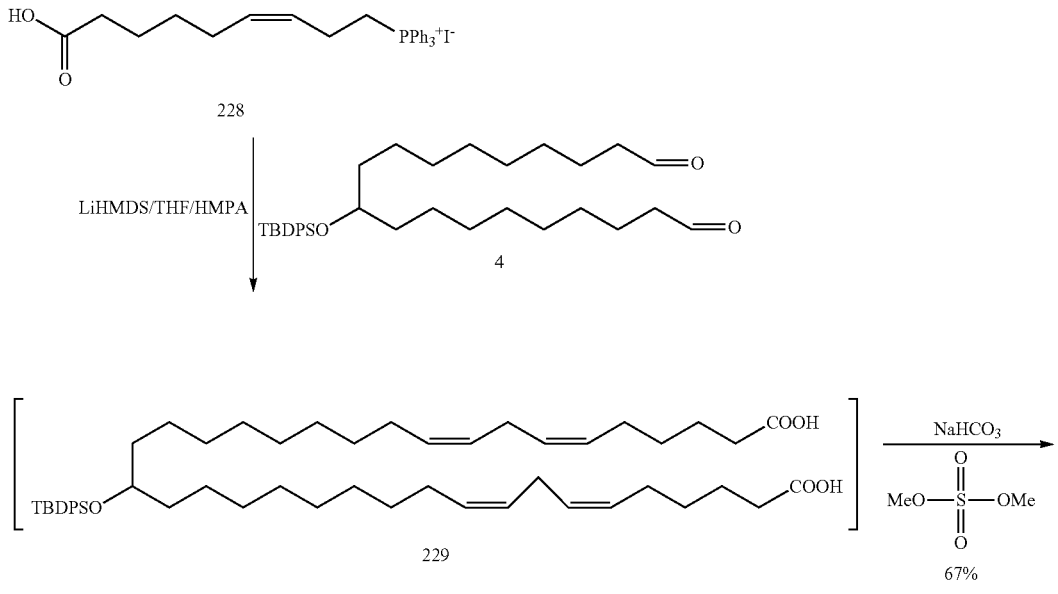
Compound 225: A procedure analogous to that described for compound 9 was followed with 224 (200 mg, 0.774 mmol) and 216 (264 mg, 1.55 mmol) to afford compound 225 (341 mg, 0.606 mmol, 78%). Molecular weight for $C_{35}H_{62}NaO_5$ $(M+Na)^+$ Calc. 585.4495, Found 585.5.

Compound 226: Compound 225 (283 mg, 0.503 mmol) was treated with $NaBH_4$ (57 mg, 1.51 mmol) in THF (5 mL) and AcOH (0.2 mL) for 8 h. After evaporation, column chromatography gave compound 226 (185 mg, 0.328 mmol, 65%). Molecular weight for $C_{35}H_{64}NaO_5$ $(M+Na)^+$ Calc. 587.4651, Found 587.3.

Compound 227: A procedure analogous to that described for compound 9 was followed with 226 (230 mg, 0.407 mmol) to afford compound 227 (248 mg, 0.366 mmol, 90%). Molecular weight for $C_{41}H_{76}NO_6$ $(M+H)^+$ Calc. 678.5673, Found 678.5.

Example 14: Synthesis of Terminal Ester Lipid with Linoleyl Chain-232

Scheme 14



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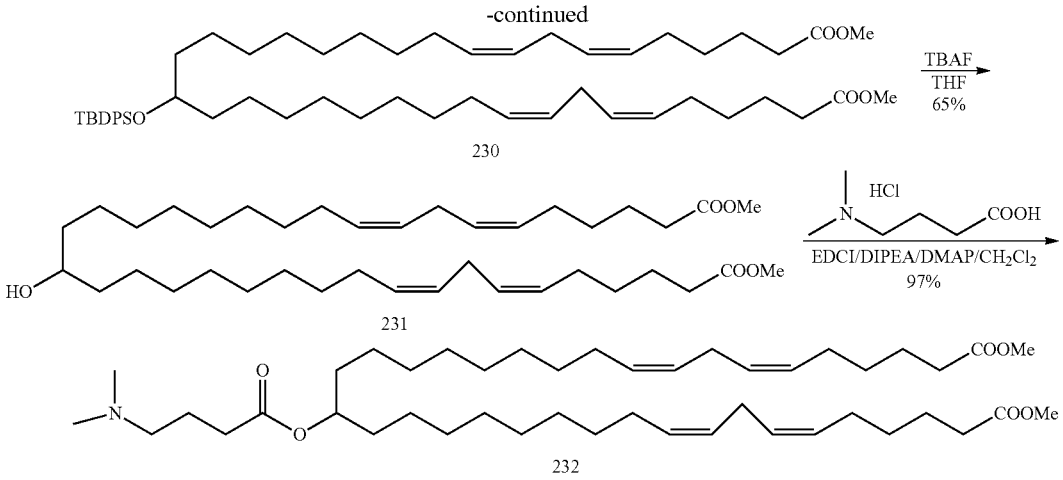


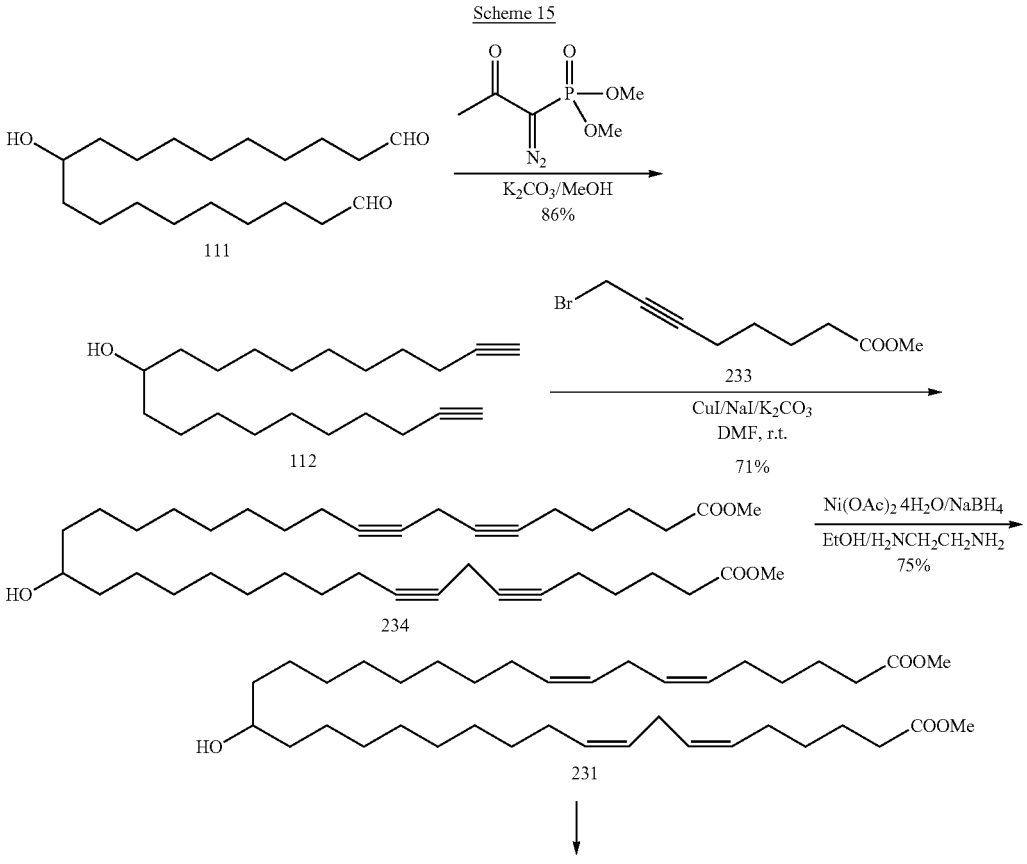
Compound 230: A procedure analogous to that described for compound 7 was followed with 228 (3.27 g, 6.0 mmol) and 4 (1.27 g, 2.30 mmol) to afford compound 230 (1.31 g, 1.53 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.33 (m, 6H), 5.42-5.29 (m, 8H), 3.71-3.68 (m, 1H), 3.66 (s, 6H), 2.77 (t, J=5.8 Hz, 4H), 2.33-2.28 (m, 4H), 2.11-2.01 (m, 8H), 1.69-1.60 (m, 4H), 1.43-1.10 (m, 32H), 1.04 (s, 9H).

Compound 231: A procedure analogous to that described for compound 8 was followed with 230 (1.30 g, 1.52 mmol) to afford compound 231 (611 mg, 0.990 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41-5.29 (m, 8H), 3.67 (s, 6H), 3.58 (brs, 1H), 2.77 (t, J=5.8 Hz, 4H), 2.32 (t, J=7.4 Hz, 4H), 2.10-2.00 (m, 8H), 1.69-1.60 (m, 4H), 1.43-1.29 (m, 32H).

Compound 232: A procedure analogous to that described for compound 9 was followed with 231 (520 mg, 0.843 mmol) to afford compound 232 (600 mg, 0.822 mmol, 97%). Molecular weight for $C_{45}H_{80}NO_6$ $(M+H)^+$ Calc. 730.5986, Found 730.5.

Example 15: Synthesis of Terminal Ester Lipid with Linoleyl Chain-232

Scheme 15



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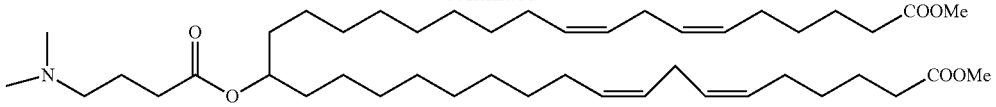


232

Compound 231 was also synthesized as shown Scheme 15.

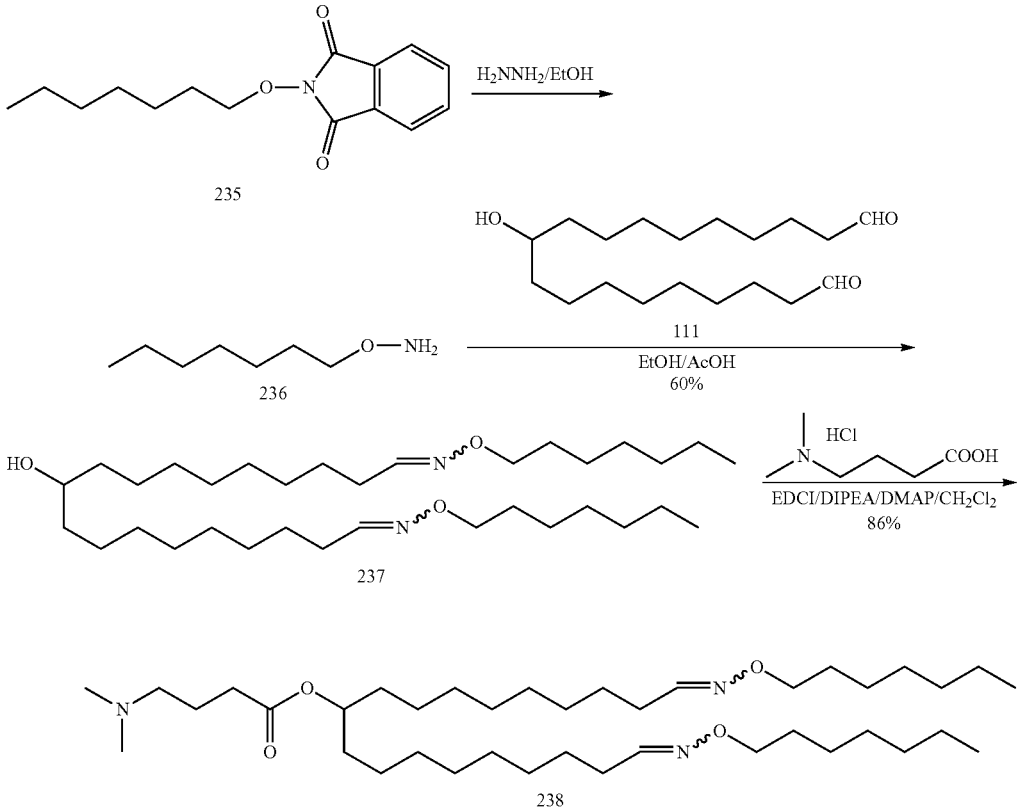
Compound 112: Compound 111 (840 mg, 2.69 mmol) was treated with dimethyl (1-diazo-2-oxopropyl)phosphonate (0.970 mL, 6.46 mmol) and K_2CO_3 (1.49 g, 10.8 mmol) in MeOH (40 mL) for 6 h. Aqueous work-up then column chromatography gave compound 112 (700 mg, 2.30 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.58 (brs, 1H), 2.18 (td, J=7.1, 2.6 Hz, 4H), 1.94 (t, J=2.6 Hz, 2H), 1.56-1.25 (m, 28H).

Compound 234: Compound 112 (207 mg, 0.680 mmol) was treated with 233 (316 mg, 1.36 mmol), K_2CO_3 (282 mg, 2.04 mmol), NaI (408 mg, 2.72 mmol) and CuI (518 mg, 2.72 mmol) in DMF (3.5 mL) for 18 h. Aqueous work-up then column chromatography gave compound 234 (292 mg, 0.480 mmol, 71%). Molecular weight for $C_{39}H_{61}O_5$ $(M+H)^+$ Calc. 609.4519, Found 609.5.

Compound 231: To a stirred solution of nickel(II) acetate tetrahydrate (533 mg, 2.14 mmol) in EtOH (28.5 mL), 1 M solution of $NaBH_4$ in EtOH (2.14 mL) was added at room temperature. After 30 min, ethylenediamine (0.574 mL, 8.57 mmol) and a solution of 234 (290 mg, 0.476 mmol) in EtOH (3 mL) was added then stirred for 1 h. The reaction mixture was filtered through Celite and evaporated. Aqueous work-up then column chromatography gave compound 231 (219 mg, 0.355 mmol, 75%). Molecular weight for $C_{39}H_{69}O_5$ $(M+H)^+$ Calc. 617.5145, Found 617.3.

Example 16: Synthesis of Internal Oxime Lipid-238

Scheme 16



235

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237

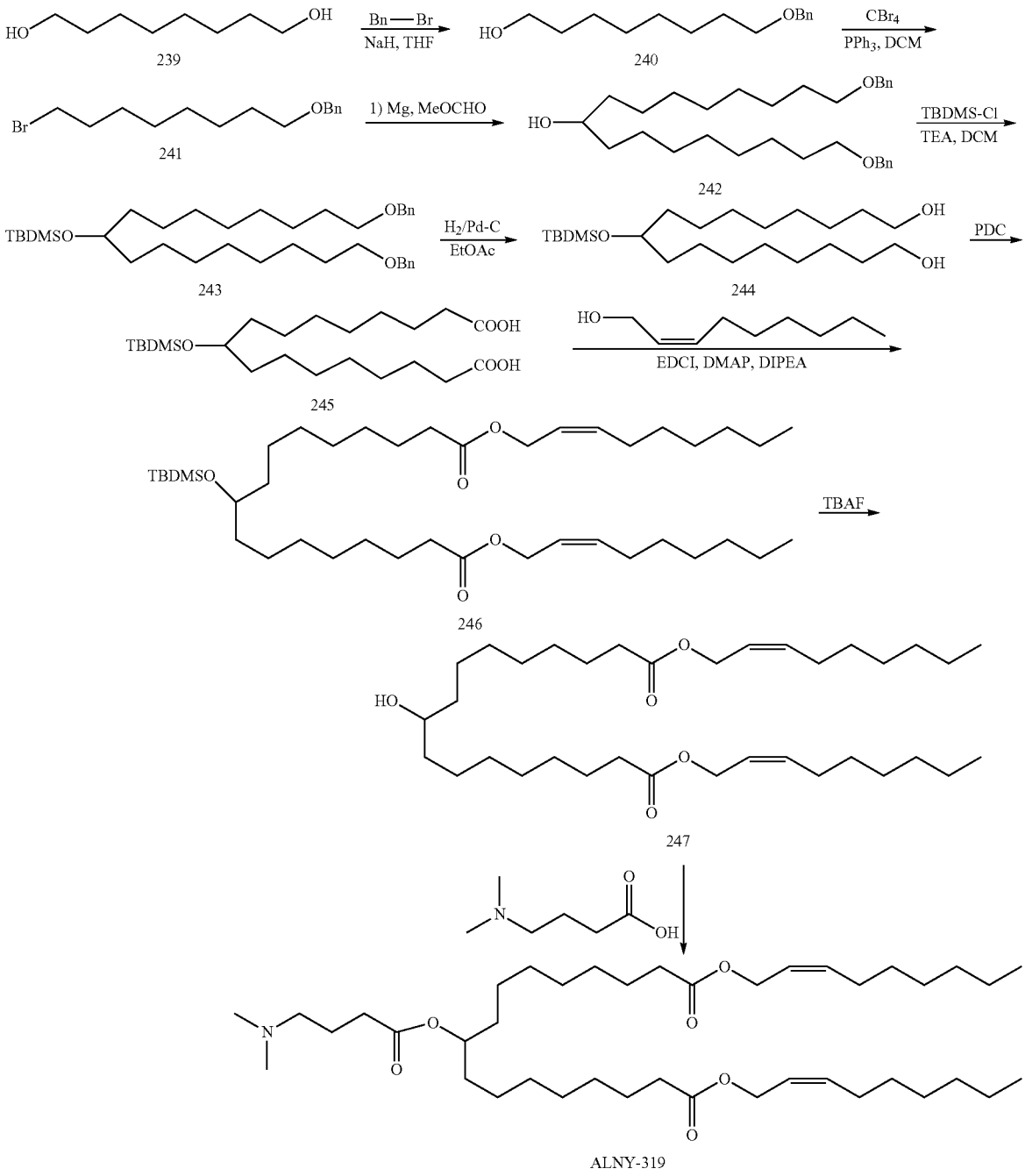
238

Compound 237: Compound 235 (465 mg, 1.78 mmol) was treated with hydrazine monohydrate (64-65%, 0.135 mL, 1.78 mmol) in EtOH (15 mL) for 4 h. After filtration then evaporation, the crude was re-suspended in EtOH (5 mL). To this solution was added compound 111 (160 mg, 0.512 mmol) and AcOH (a few drops). Aqueous work-up then column chromatography gave compound 237 (165 mg, 0.306 mmol, 60%). Molecular weight for $C_{33}H_{67}N_2O_3$ $(M+H)^+$ Calc. 539.5152, Found 539.3.

Compound 238: A procedure analogous to that described for compound 9 was followed with 237 (162 mg, 0.301 mmol) to afford compound 238 (168 mg, 0.258 mmol, 86%). Molecular weight for $C_{39}H_{78}N_3O_4$ $(M+H)^+$ Calc. 652.5992, Found 652.4.

Example 17

Scheme 17



8-benzyloxy-octan-1-ol (240): To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 239 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly using a dropping funnel at 0° C. The reaction mixture was stirred for 30 minutes, then benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under a nitrogen atmosphere. The reaction was then warmed to ambient temperature and stirred for 10 hours. After completion of reaction, the mixture was quenched with crushed ice (~2 kg) and extracted with ethyl acetate (2×1 L). The organic layer washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness under vacuum. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 240 (220 g, 54%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

(8-bromo-octyloxymethyl)-benzene (241): Compound 240 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM,

CBr_4 (280.35 g, 0.8456 mol) was added to this stirring solution and the reaction mixture was cooled to 0° C. under an inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions maintaining the temperature below 20° C. and after complete addition, the reaction mixture was stirred for 3 hours at room temperature. After completion of reaction, solid (PPh_3O) precipitated out from the reaction mixture was isolated by filtration and the filtrate was diluted with crushed ice (~1.5 kg) and extracted with DCM (3×750 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and distilled under vacuum. The resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to afford compound 241 (150 g, 89%) as pale yellow liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

1, 17-bis-benzyloxy-heptadecan-9-ol (242): To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF, followed by the addition of pinch of iodine into the mixture under inert atmosphere. After initiation of the Grignard formation a solution of Compound 241 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly controlling the exothermic reaction. After complete addition, the reaction was heated to reflux for 1 hour, then cooled to room temperature. Methyl formate (60.24 g, 1.0033 mol) was then added slowly and reaction was continued for 2 hours. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with ethyl acetate (3×1 L). The organic layer was taken in 5 litre beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution excess of $NaBH_4$ (~5 eq) was added in portions to ensure the hydrolysis of formate ester which was not cleaved by addition of HCl. The resulting solution was stirred for an hour and then volatilites were removed under vacuum. The residue was taken in water (1 L) and acidified by 10% HCl solution (P^H 4). The product was then extracted with ethyl acetate (3×1 L). The organic phase was then dried and concentrated on rotary evaporator to afford compound 242 (57 g, 24%) as solid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). C^{13} NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

[9-benzyloxy-1-(8-benzylozy-octyl)-nonyloxy]-tert-butyl-dimethyl-silane (243): Compound 242 (56 g, 0.1196 mol) was dissolved in 700 mL of anhydrous THF and cooled to 0° C. TBMS-Cl (36.06 g, 0.2396 mol) was added slowly followed by addition of imidazole (32.55 g, 0.4786 mol) under an inert atmosphere. The reaction was then stirred at room temperature for 18 hours, then quenched with ice (~1 kg). The product was extracted with ethyl acetate (3×500 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution to remove the acidic impurity, dried over Na_2SO_4 and evaporated under reduce pressure to obtain crude compound which was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to afford (60 g, 82%) of compound 243 as yellowish oil. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s, 4H), 3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H) 9-(tert-butyl-dimethyl-silanyloxy)-heptadecane-1, 17-diol (244): Compound 243 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 min. (10 wt %) Pd on carbon (12 g) was added and reaction was stirred under an atmosphere of hydrogen for 18 hours. After completion, the mixture was filtered through a bed of celite and washed with ethyl acetate. The filtrate was evaporated under vacuum. Compound 244 (19 g, 46%) thus obtained was pure enough to carry out the next reaction. ^1H NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

9-(tert-butyl-dimethyl-silanyloxy)-heptadecanedioic acid (245): To a stirred solution of 244 (2 g, 0.0049 mol) in anhydrous DMF (40 mL) was added pyridinium dirchromate (2.7 g, 0.0074 mol) at 0° C. under an inert atmosphere. The reaction mixture was then allowed to warm to room temperature over a period of 10-15 minutes and continued for 24 hours. Then, the reaction was diluted with water (100 mL). The aqueous phase was extracted using DCM (3×40 mL). The organic phase was washed with brine (1×25 mL) and concentrated under vacuum to afford crude acid which was then purified by (100-200 mesh) silica gel column using 0-30% ethyl acetate in hexanes system. Pure product (245) was obtained (0.7 g, 33%) as a pale yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ=3.61-3.56 (m, 1H), 2.35-2.32 (m, 4H), 1.64-1.59 (m, 4H), 1.40-1.19 (m, 24H), 0.86 (s, 9H), 0.017 (s, 6H); LC-MS [M+H]−431.00; HPLC (ELSD) purity −96.94%

Di((Z)-non-2-en-1-yl) 9-((tert-butyldimethylsilyl)oxy) heptadecanedioate (246): The diacid 245 (0.42 g, 0.97 mmol) was dissolved in 20 mL of dichloromethane and to it cis-2-nonen-1-ol (0.35 g, 2.44 mmol) was added followed by Hunig's base (0.68 g, 4.9 mmol) and DMAP (12 mg). To this mixture EDCI (0.47 g, 2.44 mmol) was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash Rf purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to afford the pure product 246 (0.35 g, 53%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.58-5.43 (m, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.71-3.48 (m, 1H), 2.30 (t, J=7.6 Hz, 4H), 2.20-1.98 (m, 4H), 1.71-1.53 (m, 4H), 1.31 (ddd, J=8.3, 7.0, 3.7 Hz, 34H), 1.07-0.68 (m, 14H), 0.02 (s, 5H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.18, 139.81, 127.78, 81.73, 81.42, 81.10, 76.72, 64.59, 41.52, 41.32, 38.76, 36.09, 34.10, 33.93, 33.80, 33.70, 33.59, 33.55, 33.26, 31.95, 30.34, 29.69, 29.58, 29.39, 27.01, 22.56, 18.48, 0.01.

Di((Z)-non-2-en-1-yl) 9-hydroxyheptadecanedioate (247): The silyl protected diester 246 (0.3 g, 0.44 mmol) was dissolved in 1 M solution of TBAF in THF (6 mL) and the solution was kept at 40° C. for two days. The reaction mixture was diluted with water (60 mL) and extracted with ether (2×50 mL). The combined organic layers were concentrated and the thus obtained crude product was purified by column to isolate the pure product (0.097 g, 39%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.52 (dt, J=11.0, 6.8 Hz, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.57 (s, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.09 (q, J=7.1 Hz, 4H), 1.75-1.53 (m, 4H), 1.53-1.06 (m, 36H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.98, 135.64, 123.57, 77.54, 77.22, 76.91, 72.14, 60.41, 37.69, 34.54, 31.89, 29.70, 29.60, 29.44, 29.29, 29.07, 27.76, 25.80, 25.15, 22.82, 14.29.

Di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl) oxy)heptadecanedioate: The alcohol 247 (0.083 g, 0.147 mmol) was dissolved in 20 mL of dichloromethane and to it dimethylaminobutyric acid hydrochloride (0.030 g, 0.176

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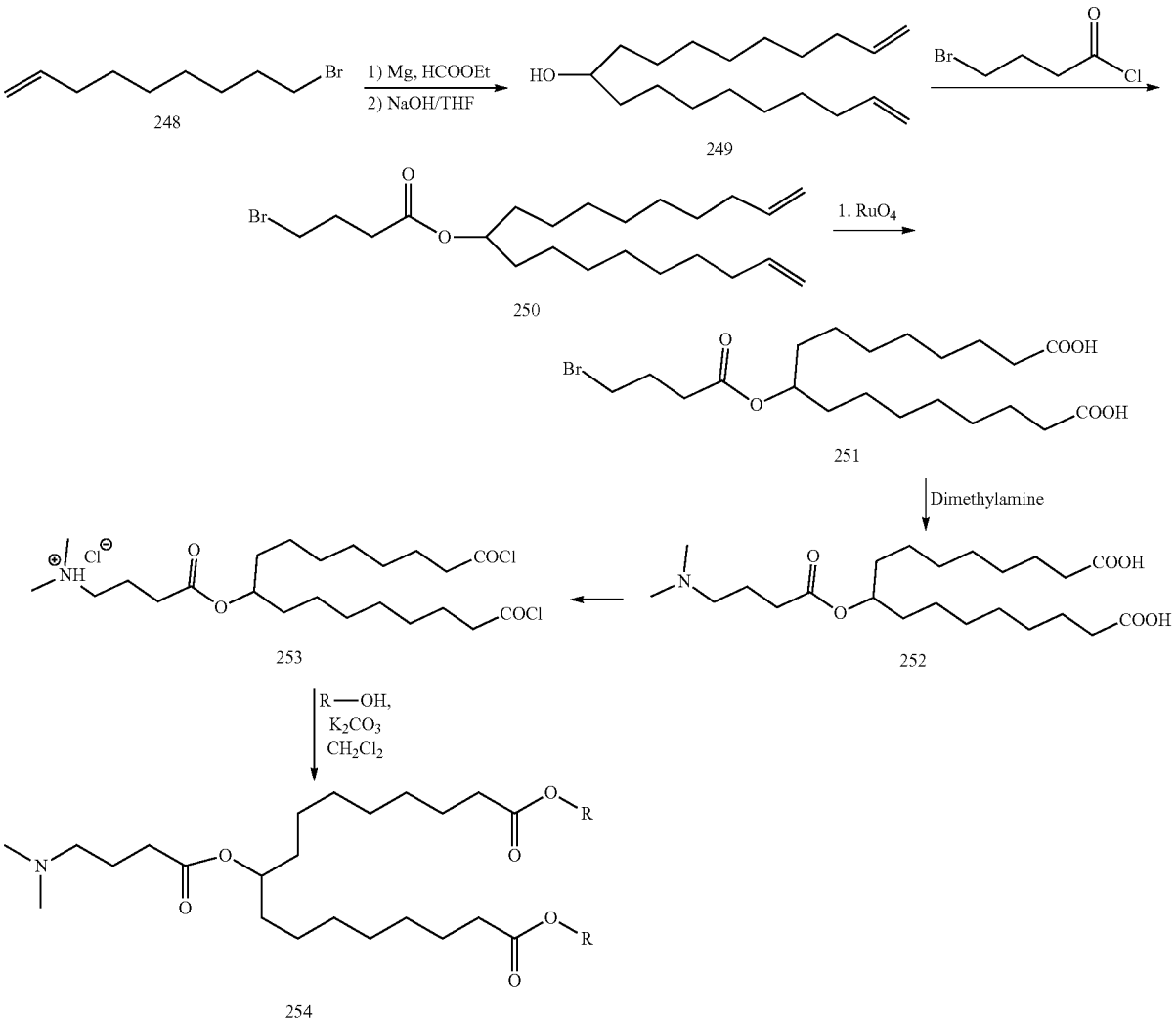
mmol) was added followed by Hunig's base (0.045 g, 0.44 mmol) and DMAP (2 mg). To this mixture EDCI (0.034 g, 0.176 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 10% MeOH in CH_2Cl_2) showed complete disappearance of the starting alcohol. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash Rf purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product (0.062 g, 62%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 5.74-5.58 (m, 2H), 5.51 (dtt, J=9.7, 6.8, 1.3 Hz, 2H), 4.95-4.75 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 2.35-2.24 (m, 8H), 2.22 (d, J=7.9 Hz, 6H), 2.09 (q, J=6.9 Hz, 4H), 1.83-1.72 (m, 2H), 1.60 (dd, J=14.4, 7.2 Hz, 4H), 1.49 (d, J=5.7 Hz, 4H), 1.41-1.13 (m, 30H), 0.88 (t, J=6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.72, 173.36, 135.40, 123.35, 74.12, 60.18, 58.95, 45.46, 34.30, 34.11, 32.45, 31.67, 29.38, 29.35, 29.17, 29.07, 28.84, 27.53, 25.28, 24.93, 23.16, 22.59, 14.06. MW calc. for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

Example 18

The following shorter route was used for the synthesis of analogs of Compound 1 of the present invention The commercial 9-bromonon-1-ene 248 was treated with magnesium to form the corresponding Grignard reagent which was reacted with ethylformate to give the corresponding adduct 249 which on treatment with bromobutyryl chloride to provide the bromoester 250. The bromoester 250 on treatment with RuO_4 provided the diacid 251. The bromodiacid 251 on treatment with dimethylamine provided the amino diacid 252. The diacid 252 on treatment with oxalyl chloride in the presence of DMF provided the diacid chlorides 253. The lipids 254a-n were synthesized by treating the acid Chloride 253 with respective alcohols.

Scheme 18



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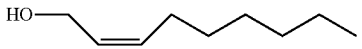
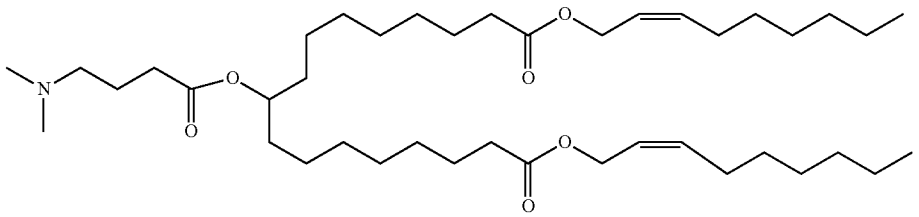
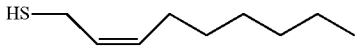
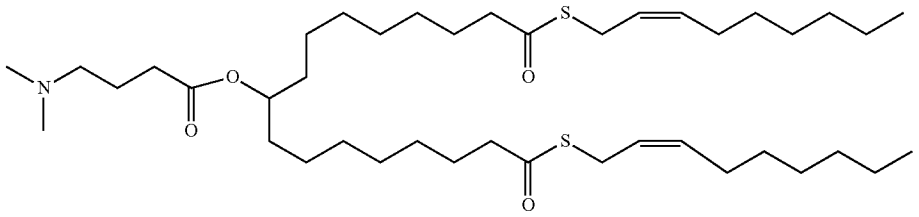
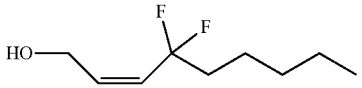
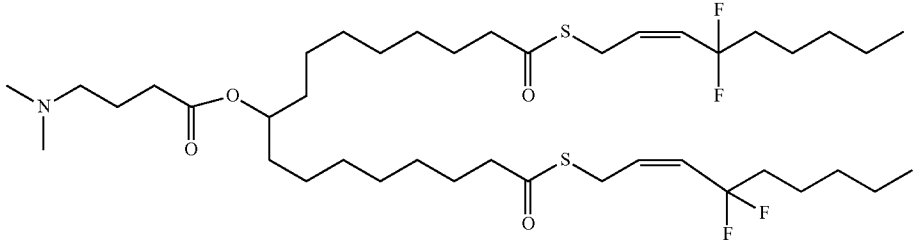
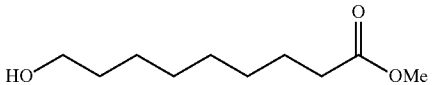
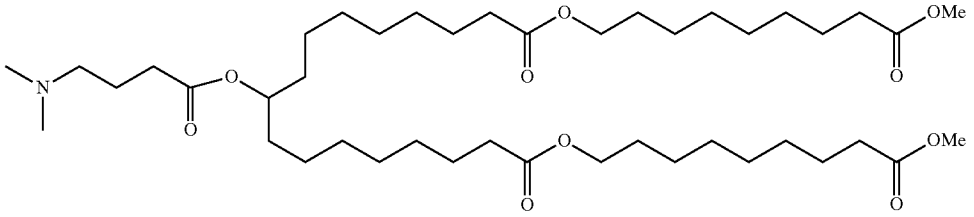
254a		
254aS		
254aF		
254b		

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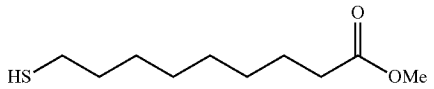
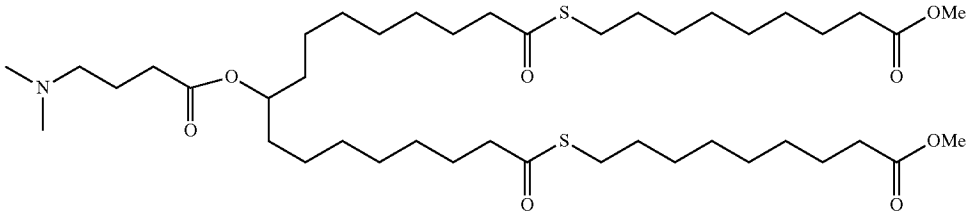
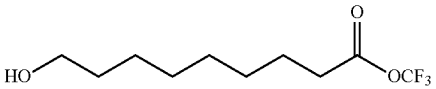
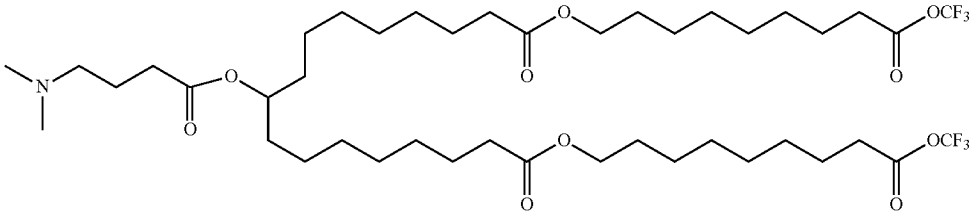
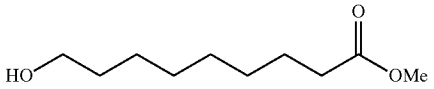
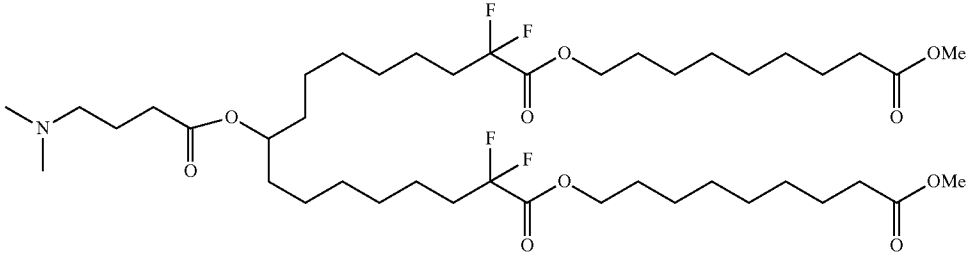
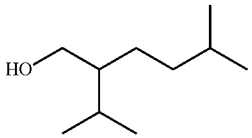
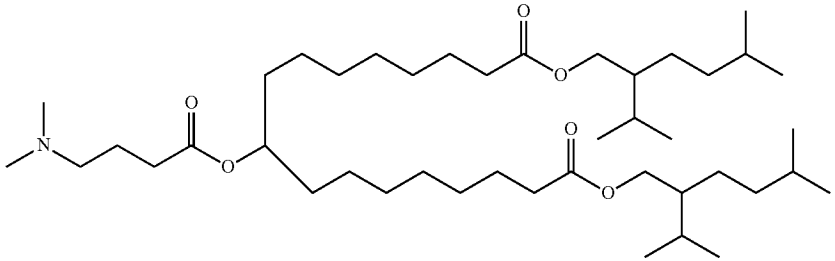
254bS		
254bF		
254bF2		
254c		

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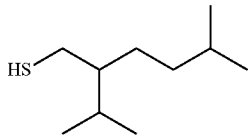
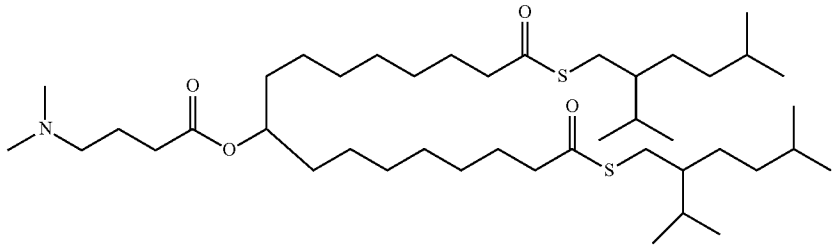
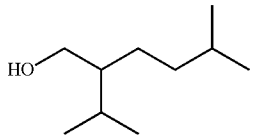
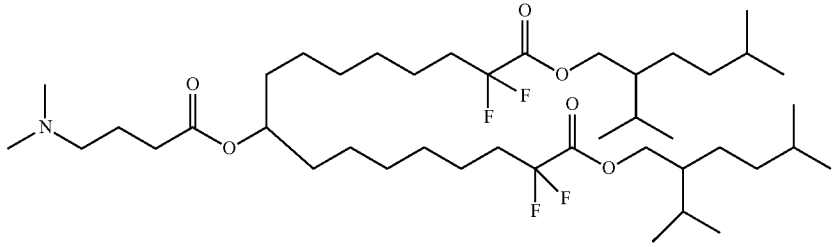
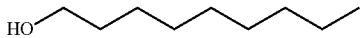
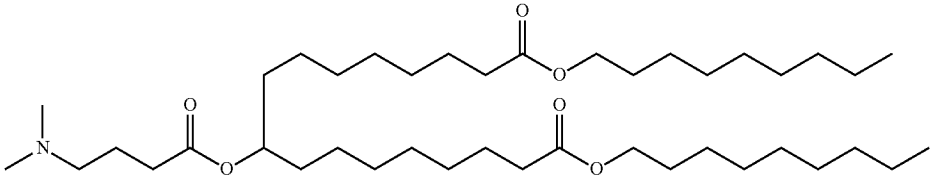
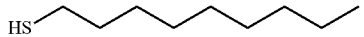
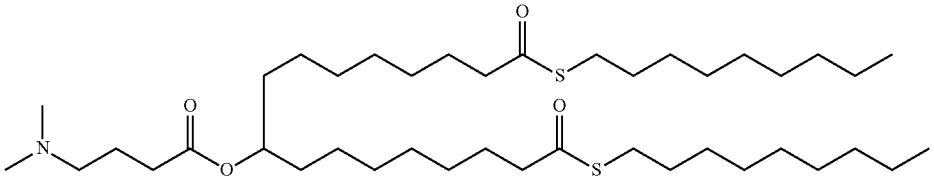
254cS		
254cF		
254d		
254ds		

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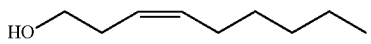
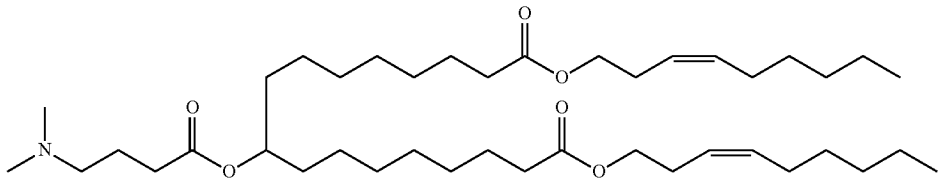
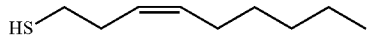
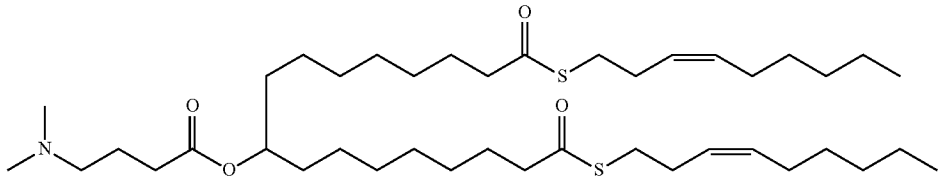
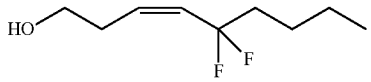
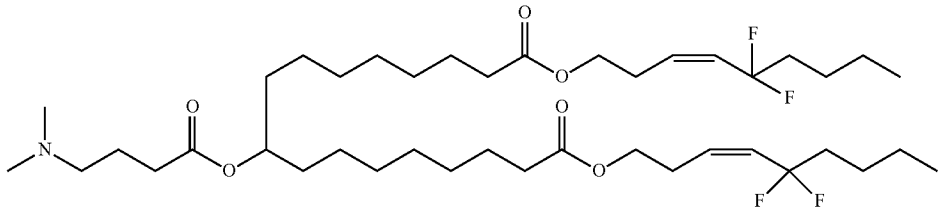
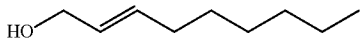
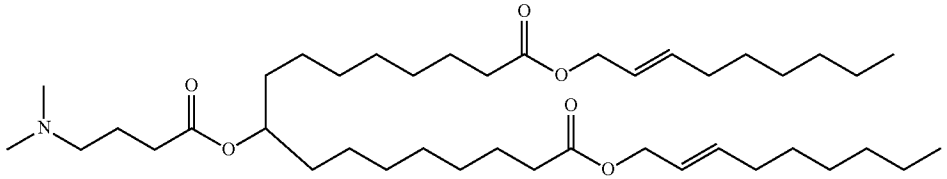
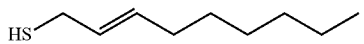
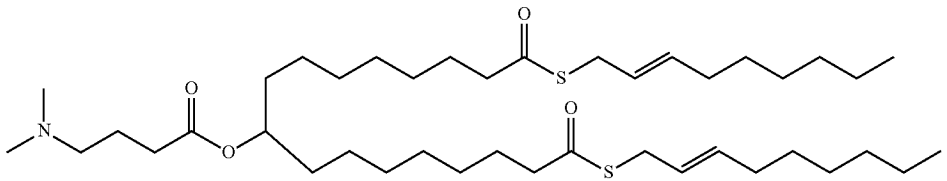
254e		
254es		
254eF		
254f		
254fs		

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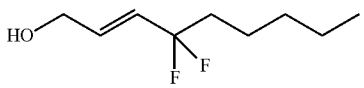
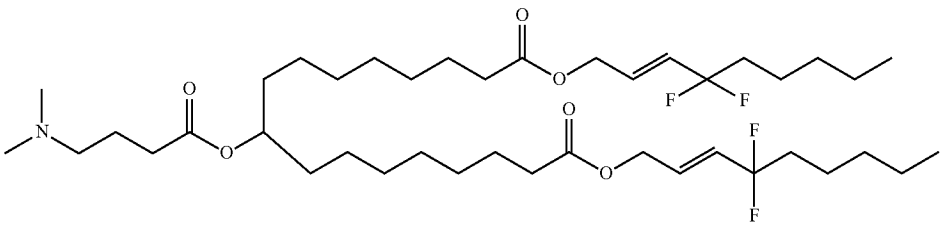
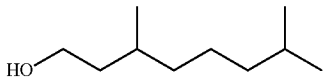
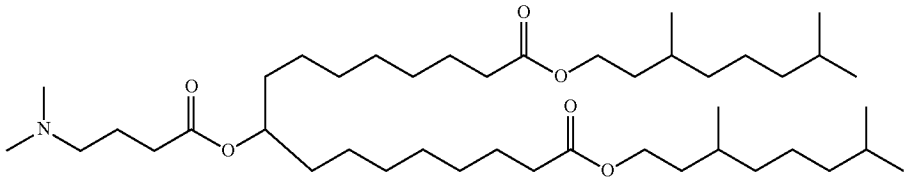
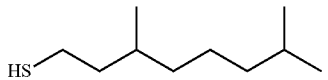
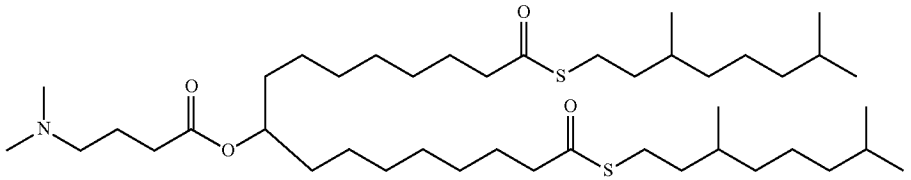
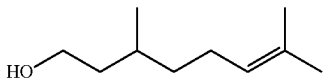
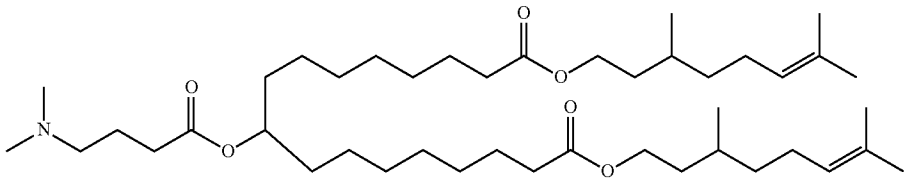
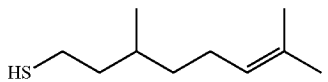
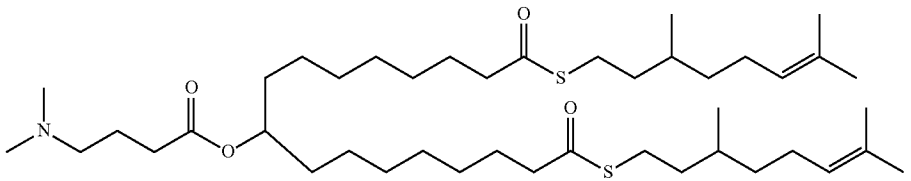
No	Starting Alcohol (ROH)	Product
254fF		
254g		
254gs		
254h		
254hs		

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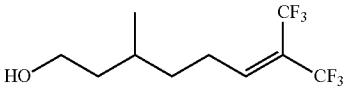
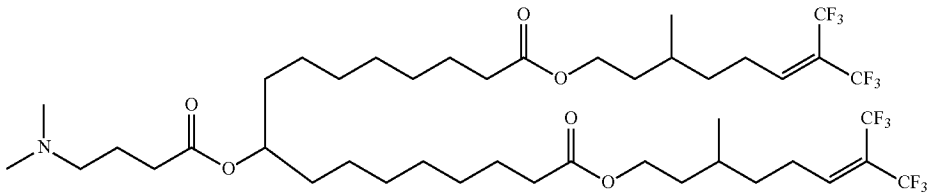
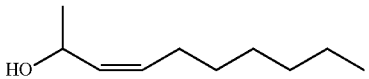
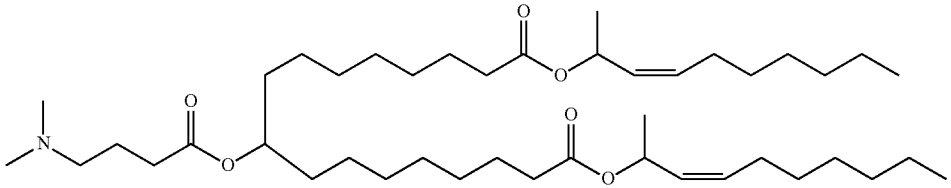
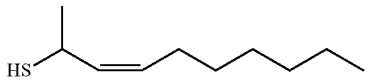
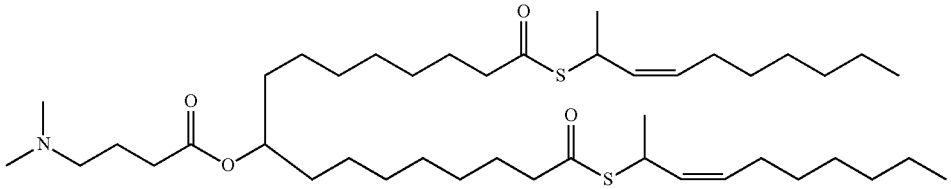
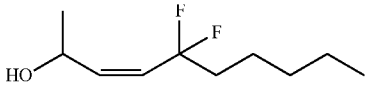
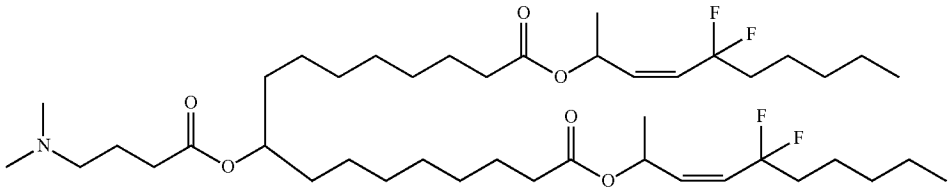
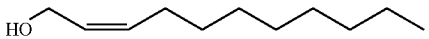
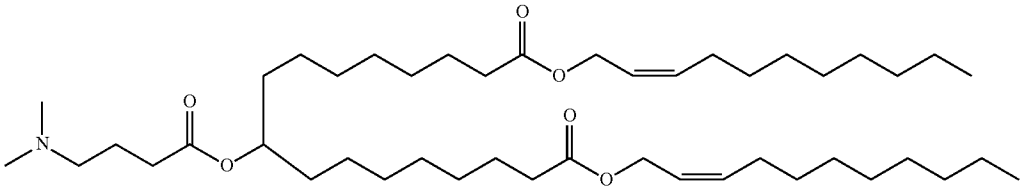
No	Starting Alcohol (ROH)	Product
254hF		
254i		
254is		
254iF		
254j		

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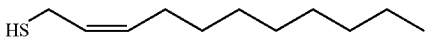
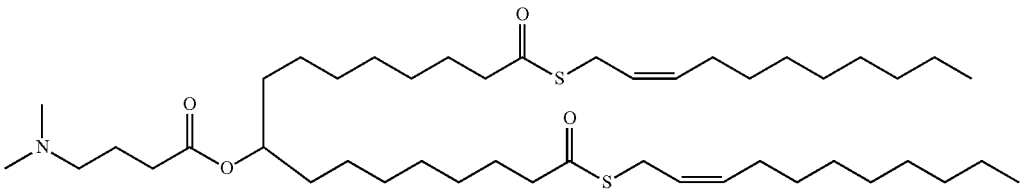
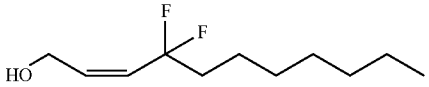
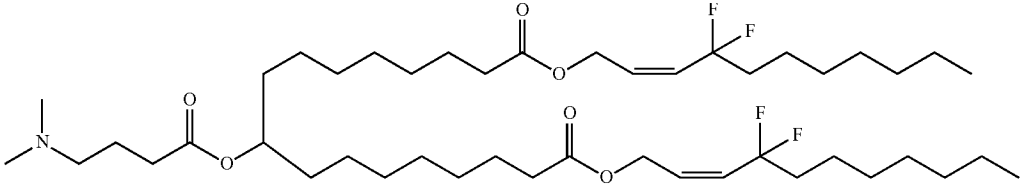
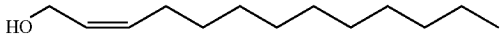
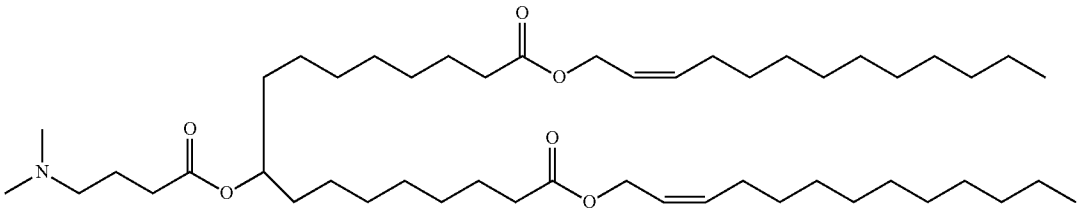
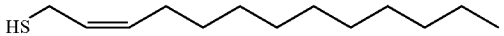
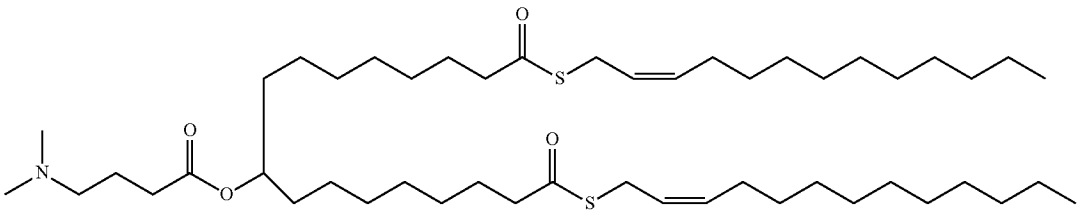
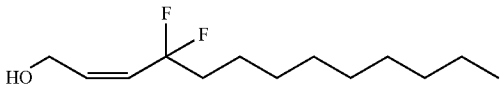
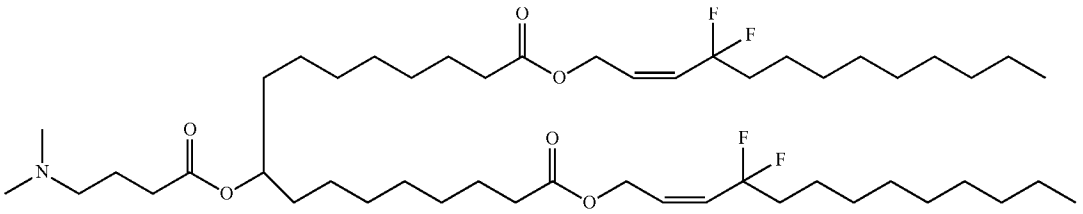
No	Starting Alcohol (ROH)	Product
254js		
254jF		
254k		
254ks		
254kS		

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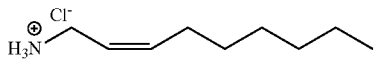
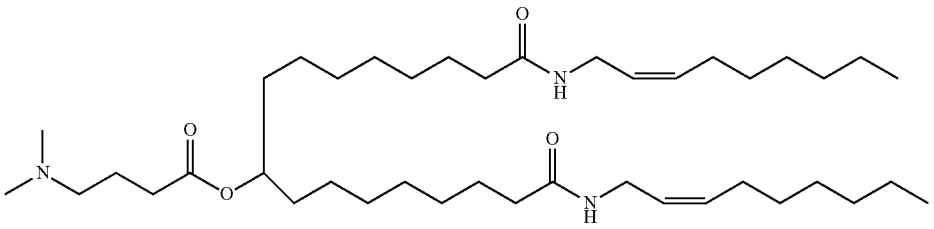
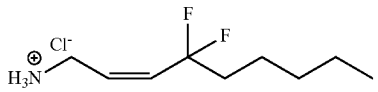
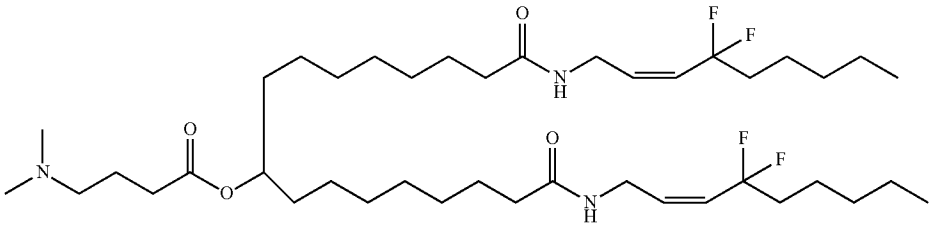
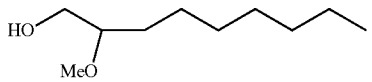
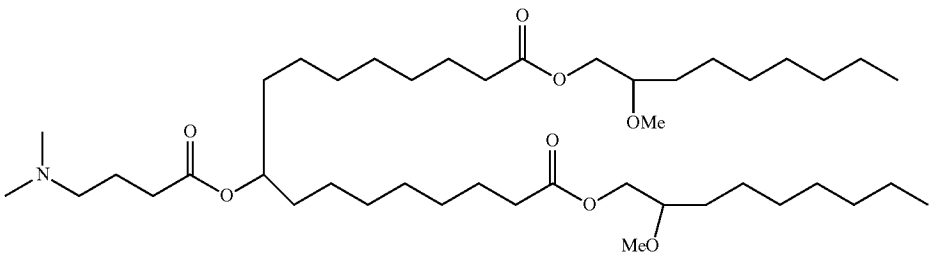
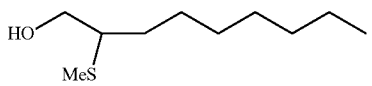
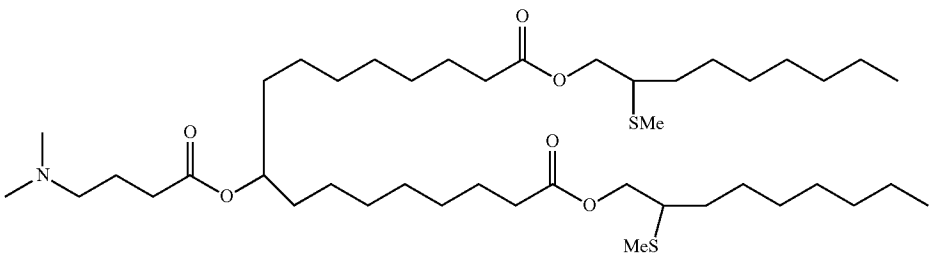
No	Starting Alcohol (ROH)	Product
254l		
254lF		
254m		
254ms		

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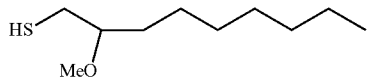
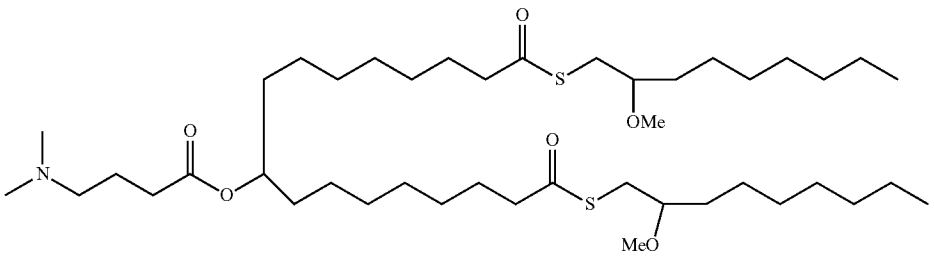
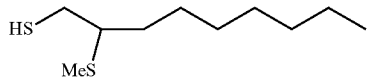
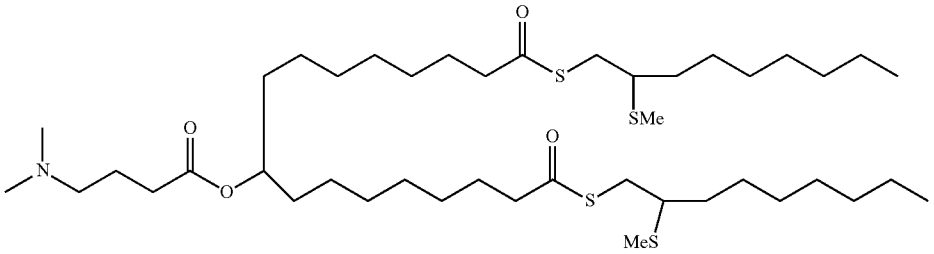
No	Starting Alcohol (ROH)	Product
254ns		
254os		

Synthesis of nonadeca-1,18-dien-10-ol (249)

To a flame dried 500 mL RB flask, freshly activated Mg turnings (9 g) were added and the flask was equipped with a magnetic stir bar, an addition funnel and a reflux condenser. This set-up was degassed and flushed with argon and 100 mL of anhydrous ether was added to the flask via syringe. The bromide 3 (51.3 g, 250 mmol) was dissolved in anhydrous ether (100 mL) and added to the addition funnel. About 5 mL of this ether solution was added to the Mg turnings while stirring vigorously. An exothermic reaction was noticed (to confirm/accelerate the Grignard reagent formation, 5 mg of iodine was added and immediate decolorization was observed confirming the formation of the Grignard reagent) and the ether started refluxing. The rest of the solution of the bromide was added dropwise while keeping the reaction under gentle reflux by cooling the flask in water. After the completion of the addition the reaction mixture was kept at 35° C. for 1 hour and then cooled in ice bath. Ethyl formate (9 g, 121 mmol) was dissolved in anhydrous ether (100 mL) and transferred to the addition funnel and added dropwise to the reaction mixture with stirring. An exothermic reaction was observed and the reaction mixture started refluxing. After the initiation of the reaction the rest of the ethereal solution of formate was quickly added as a stream and the reaction mixture was stirred for a further period of 1 h at ambient temperature. The reaction was quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture was treated with aq. H_2SO_4 (10% by volume, 300 mL) until the solution became homogeneous and the layers were separated. The aq. phase was extracted with ether (2×200 mL). The combined ether layers were dried (Na_2SO_4) and concentrated to afford the crude product which was purified by column (silica gel, 0-10% ether in hexanes) chromatography. The product fractions were evaporated to provide the pure product 249 as a white solid (30.6 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 1H), 5.81 (ddt, J=16.9, 10.2, 6.7 Hz, 8H), 5.04-4.88 (m, 16H), 3.57 (dd, J=7.6, 3.3 Hz, 4H), 2.04 (q, J=6.9 Hz, 16H), 1.59 (s, 1H), 1.45 (d, J=7.5 Hz, 8H), 1.43-1.12 (m, 94H), 0.88 (t, J=6.8 Hz, 2H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 139.40, 114.33, 77.54, 77.22, 76.90, 72.21, 37.70, 34.00, 29.86, 29.67, 29.29, 29.12, 25.85.

Synthesis of nonadeca-1,18-dien-10-yl 4-bromobutanoate (250)

To a solution of the alcohol 249 (5.6 g, 20 mol) in anhydrous DCM (300 mL) was added slowly and carefully Bromobutryl chloride (20 mmol) at 0° C. under inert atmosphere. The reaction mixture was warmed to room temperature, stirred for 20 h and monitored by TLC (silica gel, 10% ethyl acetate in hexanes). Upon completion of the reaction, mixture was diluted with water (400 mL) and organic layer was separated out. Organic phase was then washed with sat. solution of $NaHCO_3$ (1×400 mL) followed by brine (1×100 mL) and concentrated under vacuum. Crude product was then purified by silica gel (100-200 mesh) column, eluted with 2-3% ethyl acetate in hexane solution to give 6 g (90%) of desired product 250 as colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (ddt, J=16.9, 10.2, 6.7 Hz, 2H), 5.05-4.81 (m, 5H), 3.46 (t, J=6.5 Hz, 2H), 2.48 (t, J=7.2 Hz, 2H), 2.17 (p, J=6.8 Hz, 2H), 2.11-1.93 (m, 4H), 1.65-1.44 (m, 4H), 1.43-1.17 (m, 19H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 172.51, 139.37, 114.35, 77.54, 77.23, 76.91, 74.86, 34.31, 33.99, 33.01, 32.96, 29.65, 29.56, 29.24, 29.09, 28.11, 25.52.

Synthesis of 9-((4-bromobutanoyl)oxy)heptadecanedioic acid (251)

To a solution of the bromoester 250 (12.1 g, 28.2 mmol) in dichloromethane (300 mL) and acetonitrile (300 mL), $RuCl_3$ (1.16 g, 5 mol %) was added and the mixture was cooled to 10° C. and sodium metaperiodate (60 g) in water (400 mL) was added dropwise. It was stirred at 10° C. for 20 hr. The reaction mixture was diluted with water, The layers were separated and to the organic layer, was added saturated brine solution with stirring followed by 3% sodium sulfide solution drop wise for the decolourisation (dark green to pale yellow). The layers were separated, the organic layer was dried over sodium sulfate and evaporated at reduced pressure to afford pure product. MW calcd for $C_{20}H_{35}BrO_7$ 467.39; Found 465.4 (M−2H). ^{1}H NMR (400 MHz, DMSO) δ 11.94 (s, 2H), 4.88-4.69 (m, 1H), 3.53 (t, J=6.6 Hz, 2H), 2.43 (t, J=7.2 Hz, 2H), 2.17 (t, J=7.4 Hz, 4H), 2.09-1.95 (m, 2H), 1.90 (s, 3H), 1.46 (s, 7H), 1.23 (s, 15H).

Synthesis of 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioic acid (252)

The Bromoacid 251 (2 mmol) is dissolved in 2M solution of dimethylamine in THF (20 mL) and to it 1 g of anhudrous K_2CO_3 was added and the mixture was heated in a pressure bottle at 50° C. overnight. The TLC showed the completion of the reaction. The reaction mixture was acidified with acetic acid and diluted with water (100 mL) and extracted with dichloromethane (2×60 mL). The combined organic layers were concentrated dried and used as such in the next reaction. MW calcd for $C_{23}H_{43}NO_6$ 429.59; Found 430.6 $(MH)^+$. 1H NMR (400 MHz, DMSO) δ 11.87-11.82 (m, 7H), 5.75 (d, J=0.7 Hz, 15H), 4.85-4.69 (m, 38H), 3.64-3.55 (m, 12H), 3.35-2.83 (m, 106H), 3.01-2.90 (m, 59H), 2.94 (ddd, J=30.6, 7.7, 4.0 Hz, 63H), 2.90-2.73 (m, 9H), 2.70 (s, 221H), 2.57-2.46 (m, 91H), 2.44-2.30 (m, 76H), 2.17 (t, J=7.3 Hz, 147H), 1.89 (tq, J=15.5, 7.6 Hz, 88H), 1.79-1.69 (m, 13H), 1.65-1.32 (m, 311H), 1.28 (d, J=46.0 Hz, 598H).

Synthesis of 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioyl chloride (253)

The diacid 252 is converted to the corresponding diacid chloride 253 by treating it with oxalyl chloride in dichloromethane in the presence of catalytic DMF and the crude acid chloride obtained after the concentration of the reaction mixture was used as such for the coupling with different alcohols.

General Procedure for the Synthesis of Cationic Lipids 254a-n

To a solution of the acid chloride 253 (500 mg, 1 mmol) in dichloromethane (30 mL) the corresponding alcohol (5 equivalent) was added at room temperature followed by solid K_2CO_3 (1 g) and the solution was stirred for 16 h at room temperature. The reaction mixture was diluted with dichloromethane (100 mL) and washed with satd. $NaHCO_3$ (100 mL) and the organic layer was dried (Anhyd. Na_2SO_4)

and concentrated to obtain the crude product which was purified by Combiflash Rf purification system.

Compound 254b: By using the above procedure the lipid 254b was isolated in 72% yield (554 mg). 1H NMR (400 MHz, CDCl3) δ 4.91-4.78 (m, 1H), 4.05 (t, J=6.7 Hz, 4H), 3.81 (s, 6H), 3.63 (t, J=6.4 Hz, 1H), 2.29 (dt, J=15.2, 7.5 Hz, 8H), 2.21 (s, 6H), 1.84-1.69 (m, 2H), 1.57 (dt, J=13.4, 5.2 Hz, 9H), 1.53-1.40 (m, 4H), 1.27 (s, 43H). 13C NMR (101 MHz, cdcl$_3$) δ 174.45, 174.13, 173.59, 77.54, 77.22, 76.91, 74.34, 64.54, 59.17, 51.65, 45.67, 34.56, 34.35, 34.27, 32.67, 29.59, 29.40, 29.33, 29.31, 29.25, 28.83, 26.06, 25.51, 25.18, 25.11, 23.38. MW calcd for $C_{43}H_{79}NO_{10}$ 770.09; Found 770.68.

Compound 254c: By using the above procedure the lipid 254c was isolated in 69% (490 mg). 1H NMR (400 MHz, CDCl3) δ 5.71-5.36 (m, 4H), 4.89-4.72 (m, 1H), 4.59 (d, J=6.8 Hz, 4H), 2.26 (ddd, J=22.3, 13.0, 8.6 Hz, 9H), 2.19 (s, 6H), 2.12-1.95 (m, 4H), 1.82-1.68 (m, 2H), 1.63-1.37 (m, 8H), 1.37-1.00 (m, 32H), 0.85 (t, J=6.8 Hz, 6H). 13C NMR (101 MHz, cdcl3) δ 173.94, 173.57, 135.61, 123.57, 77.54, 77.22, 76.91, 74.34, 60.40, 59.16, 45.65, 34.52, 34.33, 32.66, 31.88, 29.59, 29.57, 29.38, 29.28, 29.06, 27.75, 25.49, 25.14, 23.35, 22.81, 14.28. MW calcd for $C_{43}H_{83}NO_6$: 710.12; Found 710.81.

Compound 254d: By using the above procedure the lipid 254d was isolated in 67% yield (456 mg). 1H NMR (400 MHz, CDCl3) δ 4.92-4.78 (m, 1H), 4.05 (t, J=6.7 Hz, 4H), 3.63 (t, J=6.4 Hz, 1H), 2.39-2.24 (m, 8H), 2.21 (s, 6H), 1.89-1.70 (m, 2H), 1.69-1.54 (m, 8H), 1.51 (dd, J=17.2, 6.3 Hz, 4H), 1.27 (s, 42H), 0.88 (t, J=6.8 Hz, 6H). MW calcd for: $C_{41}H_{79}NO_6$: 682.07; Found 682.96.

Compound 254e: By using the above procedure the lipid 254e was isolated in 70% (474 mg). 1H NMR (400 MHz, CDCl3) δ 5.49 (ddd, J=12.9, 9.8, 7.3 Hz, 2H), 5.40-5.23 (m, 2H), 4.92-4.77 (m, 1H), 4.05 (t, J=6.9 Hz, 4H), 2.32 (ddd, J=23.4, 14.5, 7.1 Hz, 12H), 2.21 (s, 6H), 2.07-1.91 (m, 4H), 1.84-1.70 (m, 2H), 1.66-1.39 (m, 8H), 1.40-1.15 (m, 26H), 0.88 (t, J=6.8 Hz, 5H). MW calc. for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

Compound 254f: By using the above procedure the lipid 254f was isolated in 73% (559 mg). 1H NMR (400 MHz, CDCl3) δ 5.87-5.62 (m, 2H), 5.55 (dtt, J=9.1, 6.4, 1.3 Hz, 2H), 4.93-4.75 (m, 1H), 4.50 (dd, J=6.5, 0.6 Hz, 4H), 2.40-2.17 (m, 13H), 2.12-1.95 (m, 4H), 1.89-1.67 (m, 2H), 1.69-1.44 (m, 7H), 1.41-1.12 (m, 25H), 0.88 (t, J=6.9 Hz, 5H). MW calc. for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

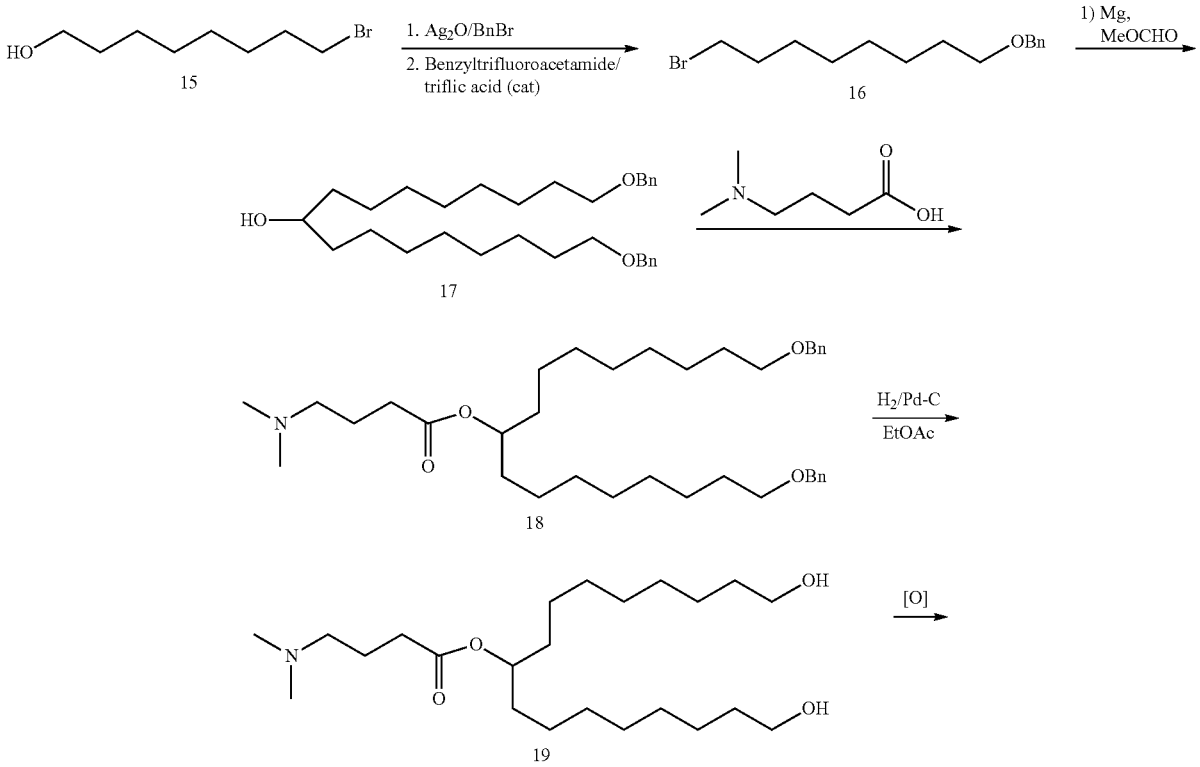
Compound 254g: By using the above procedure the lipid 254g was isolated in 63% (432 mg). 1H NMR (400 MHz, CDCl3) δ 4.93-4.77 (m, 1H), 4.20-3.95 (m, 4H), 2.44-2.23 (m, 8H), 2.21 (s, 6H), 1.84-1.66 (m, 3H), 1.68-1.34 (m, 15H), 1.35-1.17 (m, 20H), 1.17-1.04 (m, 5H), 0.88 (ddd, J=12.4, 6.6 Hz, 16H). MW calcd for $C_{43}H_{83}NO_6$: 710.12; Found 710.81.

Compound 254h: By using the above procedure the lipid 254h was isolated in 66% (466 mg). 1H NMR (400 MHz, CDCl3) δ 5.08 (ddd, J=7.1, 5.9, 1.3 Hz, 2H), 4.91-4.75 (m, 1H), 4.22-3.97 (m, 4H), 2.39-2.22 (m, 8H), 2.23 (d, J=16.7 Hz, 7H), 2.09-1.84 (m, 4H), 1.86-1.71 (m, 3H), 1.71-1.02 (m, 44H), 0.91 (t, J=4.9 Hz, 6H). MW calcd for $C_{43}H_{79}NO_6$: 706.12; Found 706.81.

Example 19

In another approach the following synthetic approach is used for the synthesis of Compound 1 of the present invention.

Scheme 19



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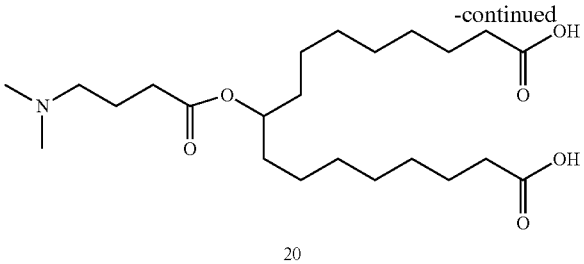

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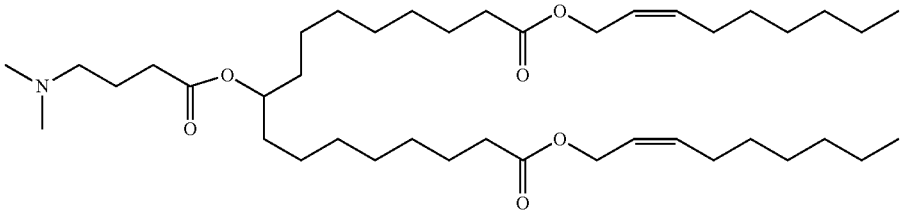
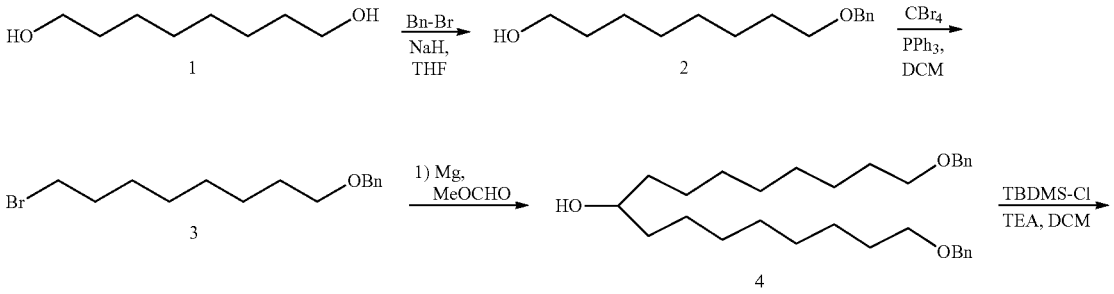


Example 20

Scheme 20



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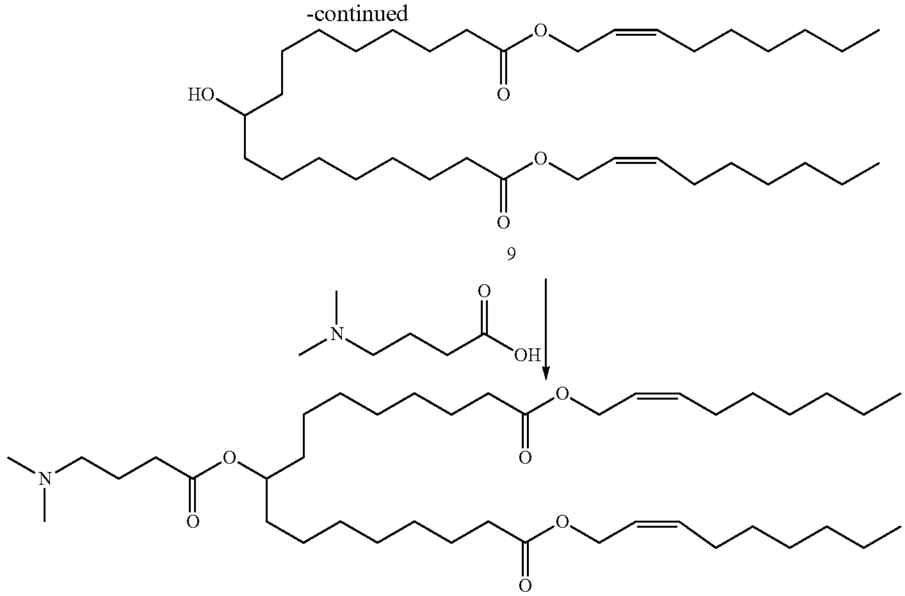


9

ALNY-319

8-benzyloxy-octan-1-ol (2): To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 1 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly with dropping funnel at 0° C. Reaction mixture was stirred for 30 min and to it Benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under nitrogen atmosphere. Reaction was then warmed to ambient temperature and stirred for 10 h. After completion of reaction, mixture was quenched with crushed ice (~2 kg) and extracted with Ethyl acetate (2×1 L). Organic layer washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness under vacuum. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 2 (220 g, 54%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

(8-bromo-octyloxymethyl)-benzene (3): Compound 2 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM, CBr_4 (280.35 g, 0.8456 mol) was added into this stirring solution and reaction mixture was cooled to 0° C. under inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions keeping the temperature below 20° C. and after complete addition reaction was stirred for 3 h at room temperature and monitored by TLC. After completion of reaction, solid (PPh_3O) precipitated out from the reaction mixture was filtered off and filtrate was diluted with crushed ice (~1.5 kg) and extracted with DCM (3×750 mL). Organic layer was separated, dried over an. Na_2SO_4 and distilled under vacuum. Resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to give compound (150 g, 89%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

1, 17-bis-benzyloxy-heptadecan-9-ol (4): To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF was added followed by the addition of pinch of iodine into the mixture under inert atmosphere. After initiation of the Grignard formation a solution of Compound 3 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly controlling the exothermic reaction. After complete addition reaction was refluxed for 1 h and then cooled to room temperature. (60.24 g, 1.0033 mol) methyl formate was then added slowly and reaction was continued for 2 h. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with Ethyl Acetate (3×1 L). Organic layer was taken in 5 lit beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution excess of $NaBH_4$ (~5 eq) was added in portions to ensure the hydrolysis of formate ester which was not cleaved by addition of HCl. Resulting solution was stirred for an hour and then volatilites were stripped off under vacuum. Residue was taken in water (1 L) and acidified by 10% HCl solution (P^H 4).

Product was then extracted out with ethyl acetate (3×1 L). Organic phase was then dried and concentrated on rotary evaporator to get the desired compound 4 (57 g, 24%) as solid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). C^{13} NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

[9-benzyloxy-1-(8-benzylozy-octyl)-nonyloxy]-tert-butyl-dimethyl-silane (5): Compound 4 (56 g, 0.1196 mol) was dissolved in 700 mL of anhydrous THF and cooled to 0° C. TBMS-Cl (36.06 g, 0.2396 mol) was added slowly followed by addition of Imidazole (32.55 g, 0.4786 mol) under inert atmosphere. Reaction was then stirred at room temperature for 18 h. Reaction was judged complete by TLC and then quenched with ice (~1 kg) and extracted with Ethyl acetate (3×500 mL). Organic layer was separated, washed with Sat $NaHCO_3$ solution to remove the acidic impurity, dried over Na_2SO_4 and evaporated under reduce pressure to obtain crude compound which was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to yield (60 g, 82%) of compound 5 as yellowish oil. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s,

4H), 3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H)

9-(tert-butyl-dimethyl-silanyloxy)-heptadecane-1, 17-diol (6): Compound 5 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 min. (10 wt %) Pd on carbon (12 g) was added and reaction was stirred under H_2 atmosphere for 18 h. After completion of reaction (by TLC) mixture was filtered through celite bed and washed with ethyl acetate. Filtrate was evaporated under vacuum. The compound 6 (19 g, 46%) thus obtained was pure enough to carry out the next reaction. H^1 NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

9-(tert-butyl-dimethyl-silanyloxy)-heptadecanedioic acid (7): To a stirred solution of 6 (2 g, 0.0049 mol) in anhydrous DMF (40 mL) was added pyridinium dirchromate (2.7 g, 0.0074 mol) at 0° C. under inert atmosphere. Reaction mixture was then allowed to warm to room temperature over a period of 10-15 minutes and continued for 24 h. Progress of the reaction was monitored by TLC. After complete oxidation reaction was diluted with water (100 mL). Aqueous phase was extracted with DCM (3×40 mL). Organic phase was washed with brine (lx 25 mL) and concentrated under vacuum to afford crude acid which was then purified by (100-200 mesh) silica gel column using 0-30% ethyl acetate in hexanes system. Pure product 26-003 was obtained (0.7 g, 33%) as pale yellow oil. H^1 NMR (400 MHz, $CDCl_3$): δ=3.61-3.56 (m, 1H), 2.35-2.32 (m, 4H), 1.64-1.59 (m, 4H), 1.40-1.19 (m, 24H), 0.86 (s, 9H), 0.017 (s, 6H); LC-MS [M+H]−431.00; HPLC (ELSD) purity −96.94%

Di((Z)-non-2-en-1-yl) 9-((tert-butyldimethylsilyl)oxy) heptadecanedioate (8): The diacid 7 (0.42 g, 0.97 mmol) was dissolved in 20 mL of dichloromethane and to it cis-2-nonen-1-ol (0.35 g, 2.44 mmol) was added followed by Hunig's base (0.68 g, 4.9 mmol) and DMAP (12 mg). To this mixture EDCI (0.47 g, 2.44 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 5% MeOH in CH_2Cl_2) showed complete disappearance of the starting acid. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash Rf purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product 8 (0.35 g, 53%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.58-5.43 (m, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.71-3.48 (m, 1H), 2.30 (t, J=7.6 Hz, 4H), 2.20-1.98 (m, 4H), 1.71-1.53 (m, 4H), 1.31 (ddd, J=8.3, 7.0, 3.7 Hz, 34H), 1.07-0.68 (m, 14H), 0.02 (s, 5H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.18, 139.81, 127.78, 81.73, 81.42, 81.10, 76.72, 64.59, 41.52, 41.32, 38.76, 36.09, 34.10, 33.93, 33.80, 33.70, 33.59, 33.55, 33.26, 31.95, 30.34, 29.69, 29.58, 29.39, 27.01, 22.56, 18.48, 0.01.

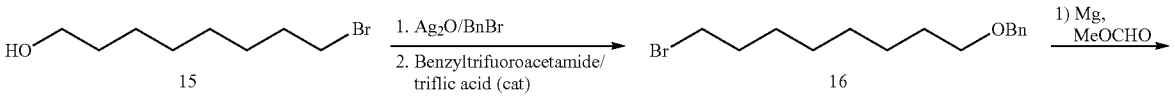
Di((Z)-non-2-en-1-yl) 9-hydroxyheptadecanedioate (9): The silyl protected diester 8 (0.3 g, 0.44 mmol) was dissolved in 1 M solution of TBAF in THF (6 mL) and the solution was kept at 40° C. for two days after which the TLC showed the completion of the reaction. The reaction mixture was diluted with water (60 mL) and extracted with ether (2×50 mL). The combined organic layers were concentrated and the thus obtained crude product was purified by column to isolate the pure product (0.097 g, 39%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.52 (dt, J=11.0, 6.8 Hz, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.57 (s, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.09 (q, J=7.1 Hz, 4H), 1.75-1.53 (m, 4H), 1.53-1.06 (m, 36H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.98, 135.64, 123.57, 77.54, 77.22, 76.91, 72.14, 60.41, 37.69, 34.54, 31.89, 29.70, 29.60, 29.44, 29.29, 29.07, 27.76, 25.80, 25.15, 22.82, 14.29.

Di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl) oxy)heptadecanedioate: The alcohol 9 (0.083 g, 0.147 mmol) was dissolved in 20 mL of dichloromethane and to it dimethylaminobutyric acid hydrochloride (0.030 g, 0.176 mmol) was added followed by Hunig's base (0.045 g, 0.44 mmol) and DMAP (2 mg). To this mixture EDCI (0.034 g, 0.176 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 10% MeOH in CH_2Cl_2) showed complete disappearance of the starting alcohol. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash Rf purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product (0.062 g, 62%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 5.74-5.58 (m, 2H), 5.51 (dtt, J=9.7, 6.8, 1.3 Hz, 2H), 4.95-4.75 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 2.35-2.24 (m, 8H), 2.22 (d, J=7.9 Hz, 6H), 2.09 (q, J=6.9 Hz, 4H), 1.83-1.72 (m, 2H), 1.60 (dd, J=14.4, 7.2 Hz, 4H), 1.49 (d, J=5.7 Hz, 4H), 1.41-1.13 (m, 30H), 0.88 (t, J=6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.72, 173.36, 135.40, 123.35, 74.12, 60.18, 58.95, 45.46, 34.30, 34.11, 32.45, 31.67, 29.38, 29.35, 29.17, 29.07, 28.84, 27.53, 25.28, 24.93, 23.16, 22.59, 14.06. MW calc. for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

In another embodiment the following shorter route was used for the synthesis of the di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate. The commercial 9-bromonon-1-ene 10 was treated with magnesium to form the corresponding Grignard reagent which was reacted with ethylformate to give the corresponding adduct 11 which on treatment with bromobutyryl chloride to provide the bromoester 12. The bromoester 12 on treatment with RuO_4 provided the diacid 13. The bromodiacid 13 on treatment with dimethylamine provided the amino diacid 14. The aminodiacid 14 on coupling with the alcohol 15 provided the product in good yields.

Example 21

Scheme 21



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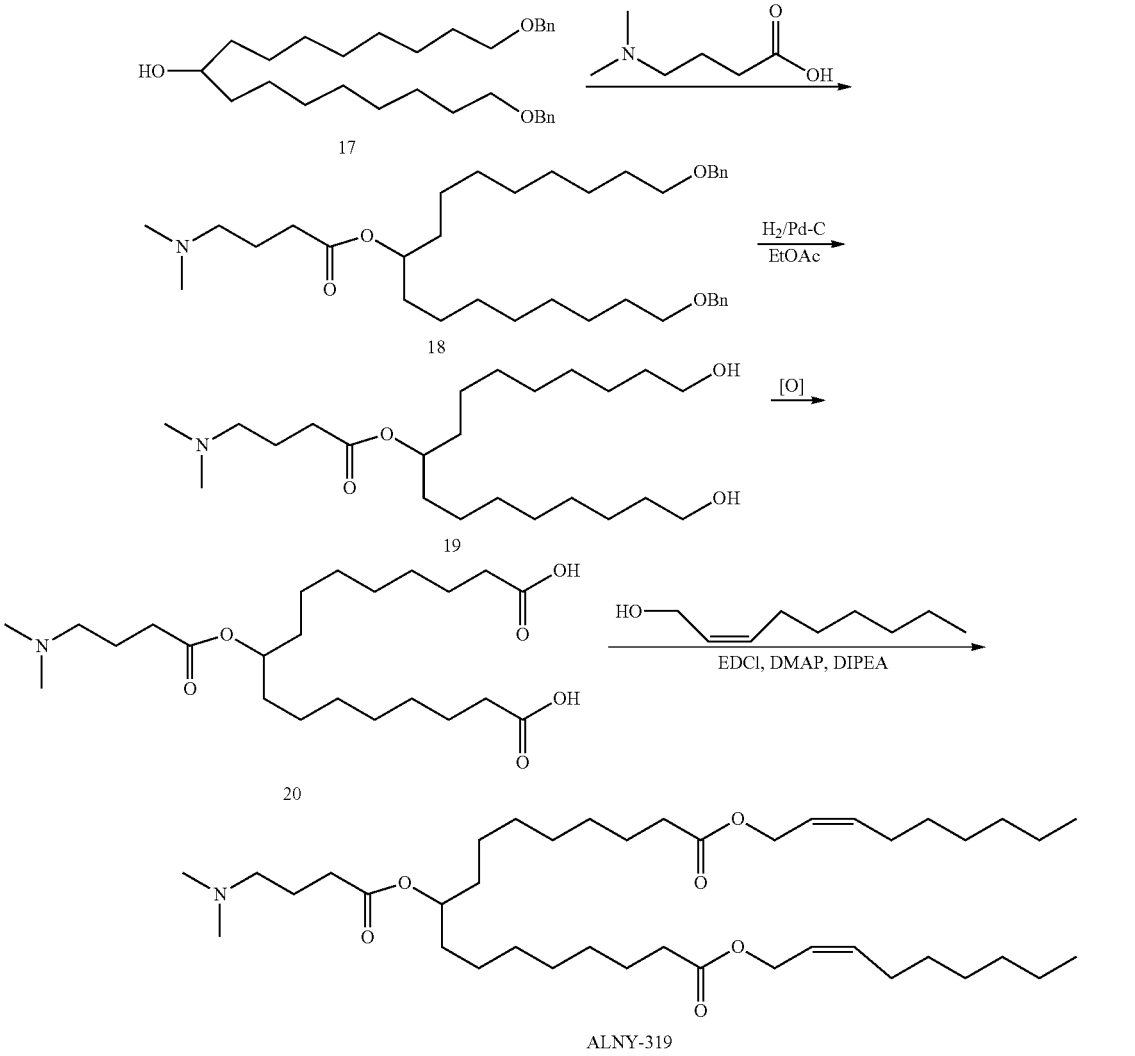
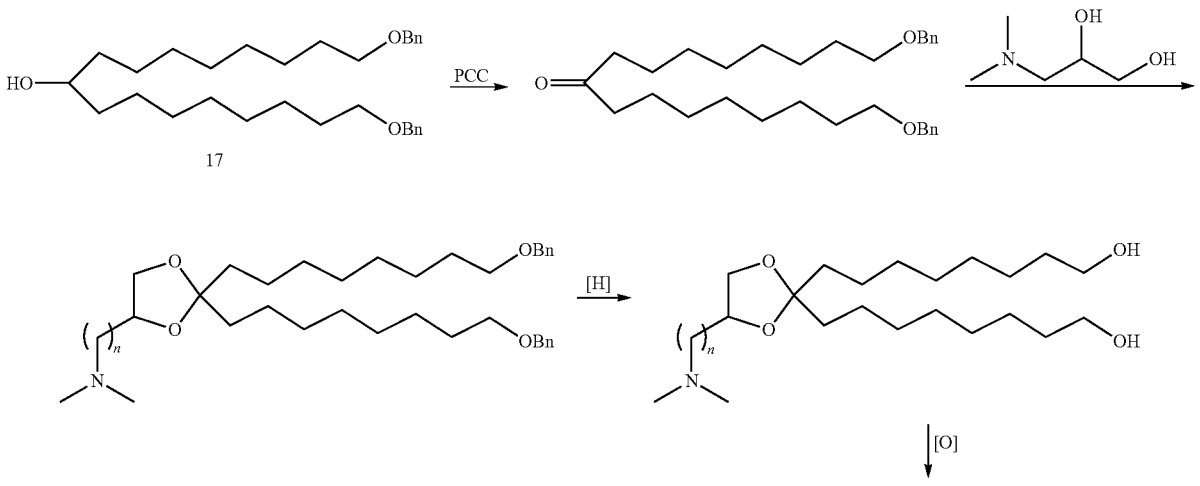


Example 22

Scheme 22



483 **484**

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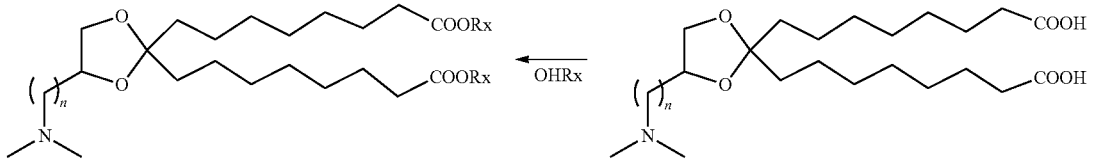
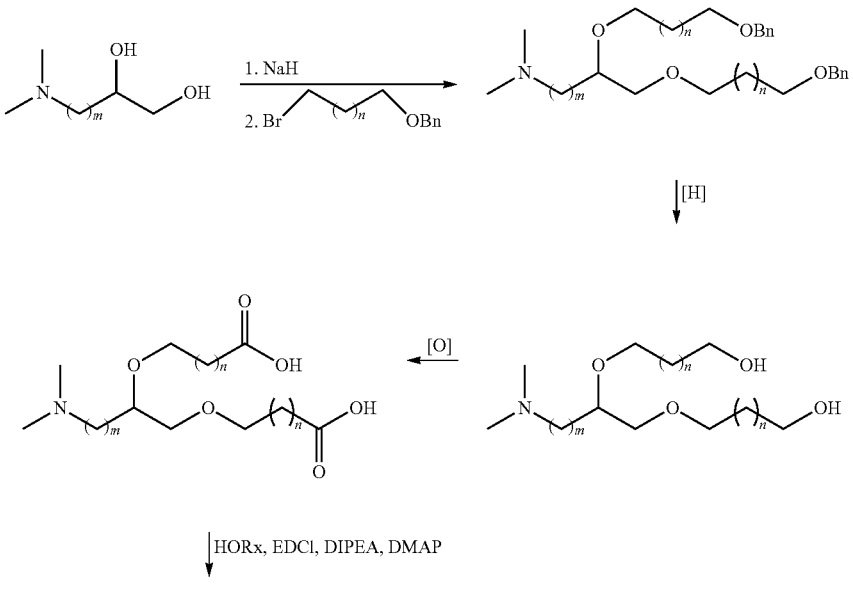


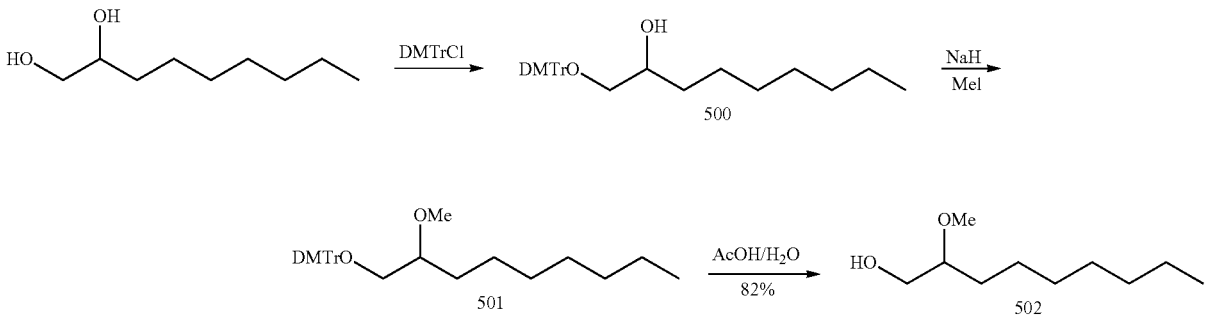
Example 23

Scheme 23



Example 24

Scheme 24



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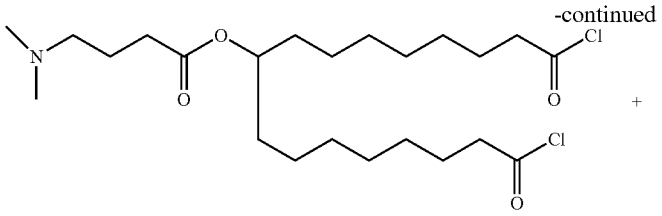

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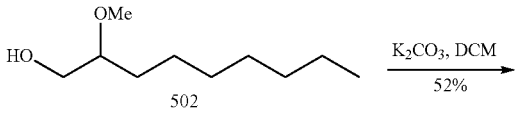
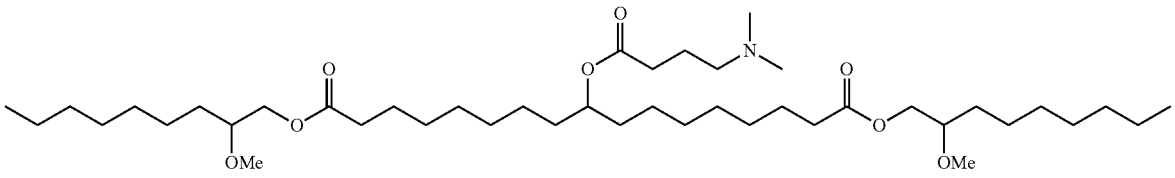


502



503

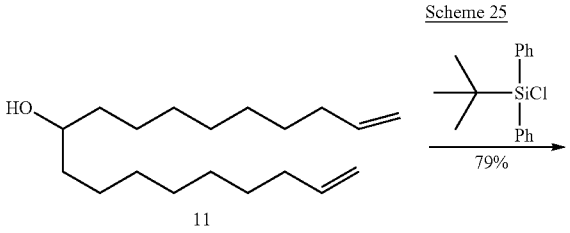
Compound 501: To a stirred solution of 2-hydroxy 1-octanol 5 g (31.25 mmol), DMAP 0.38 g (3.1 mmol) in dry pyridine (100 mL) was added DMTr-Cl and stirred at room temperature for 14 h. 10 mL of water was added and extracted with ethyl acetate, washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentration of the solvent gave 20 g of crude product 500 which was co-evaporated with toluene twice and used for the next step without further purification. To the above crude DMTr ether in dry THF (250 mL) were added NaH and iodo methane at 0° C. and then brought to room temperature over 30 min. and then stirred for two days. 5 mL of water was added and concentrated followed by column chromatography (0-30% ethyl acetate in hexane) gave the corresponding product 501 (10.25 g, R_f: 0.45, 20% ethyl acetate in hexane) and 8.4 g of recovered starting material 500. ^1H NMR (400 MHz, $CDCl_3$) δ 7.47-6.8 (m, 13H), 3.79 (s, 6H), 3.42 (s, 3H), 3.29-3.26 (m, 1H), 3.13-3.04 (m, 2H), 1.55-1.47 (m, 2H), 1.3-1.2 (m, 10H), 0.89 (t, J=6.4 Hz, 3H).

Alcohol 502: The compound 501 (10.25 g, 21.5 mmol) was dissolved in 75 mL of 80% acetic acid and stirred at room temperature for 14 h. 10 mL of methanol was added and concentrated, followed by column chromatography (0-50% ethyl acetate in hexane) yielded the expected product 502 as colorless oil (1.8 g, 82%, R_f: 0.3, 30% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 3.71-3.65 (m, 1H), 3.5-3.45 (m, 1H), 3.41 (s, 3H), 3.28-3.25 (m, 1H), 1.93-1.9 (m, 1H), 1.45-1.41 (m, 2H), 1.39-1.27 (m, 10H), 0.88 (s, J=6.8 Hz, 3H).

Compound 503: Compound 503 was synthesized following general experimental procedure for compound 213. 0.3 g as pale yellow oil (52%, R_f=0.2, 5% methanol in dichloromethane). ^1H NMR (400 MHz, $CDCl_3$) δ 4.87-4.84 (m, 1H), 4.18-4.00 (m, 4H), 3.4 (s, 6H), 3.37-3.19 (m, 2H), 2.34-2.26 (m, 6H), 2.2 (s, 6H), 1.8-1.6 (m, 2H), 1.63-1.2 (m, 50H), 0.88 (s, J=6.8 Hz, 6H).

Example 25

Scheme 25



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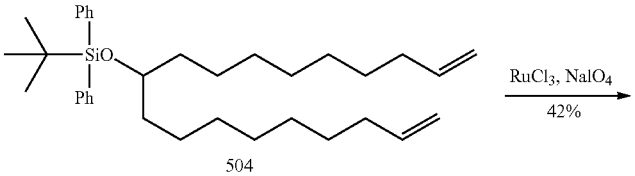

504

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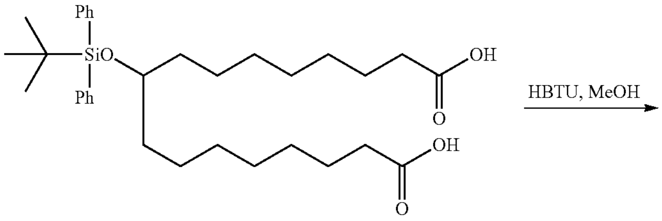

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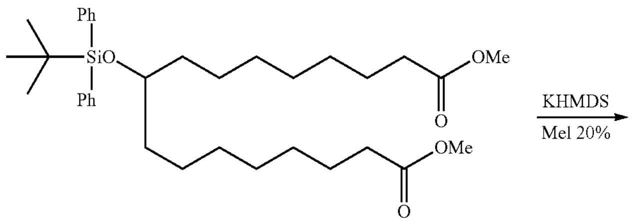

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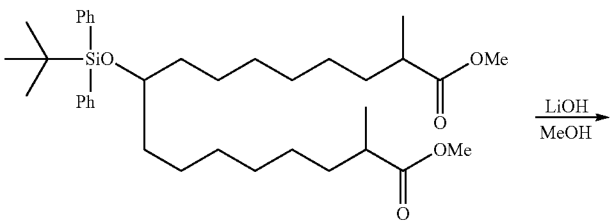

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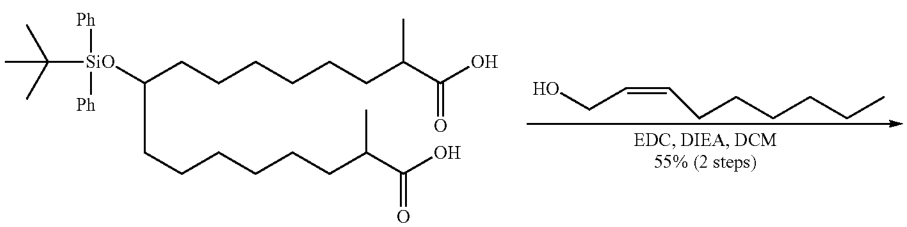

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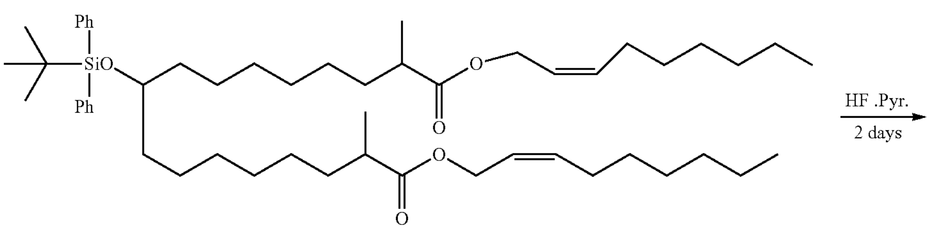

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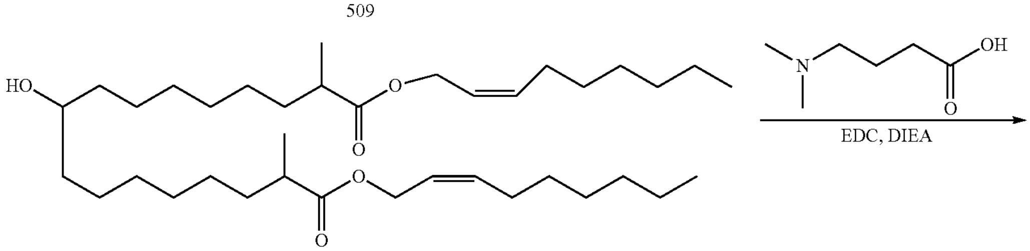

510

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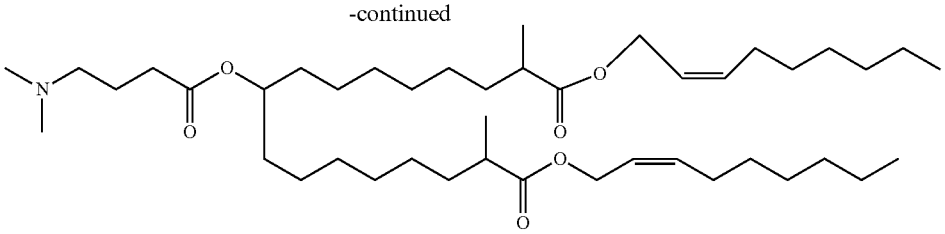


511

167 mg, 66% (2 steps)

Compound 504: To a stirred solution of alcohol 11 (4.01 g, 22.25 mmol), TBDPS-Cl (12.24 g, 44.5 mmol) and DMAP (0.54 g, 4.42 mmol) was added triethyl amine (8.99 g, 90 mmol) and stirred at room temper for 14 h. To the above solution was added imidazole (1.51 g, 22.25 mmol) and continued to stir for 14 h at room temperature. 20 mL of water was added and extracted with DCM followed by washing with 2N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 504 (7.38 g, 79%, R_f: 0.8, 5% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.43-7.33 (m, 6H), 5.86-5.76 (m, 2H), 5.02-4.91 (m, 4H), 3.73-3.67 (m, 1H), 2.04-1.99 (m, 4H), 1.42-1.08 (m, 24H), 1.05 (s, 9H).

Compound 505: To a stirred solution of diene 504 (7.38 g, 17.6 mmol) and $RuCl_3$ (0.18 g, 0.88 mmol) in 400 mL of DCM/CH_3CN(1:1) was added $NaIO_4$ (37.6 g, 176 mmol) dissolved in 400 mL of water drop wise around 5° C. over 30 min. and stirred at room temperature for 3 h. The organic layer was separated followed by washing with 3% Na_2S solution (100 mL), water (250 mL) brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product 505 (4 g, 42%, R_f: 0.3, 40% ethyl acetate in hexane), which was used for the next step without further purification.

Compound 506: To a stirred solution of the acid 505 (4 g, 7.22 mmol), HBTU (6.02 g, 15.88 mmol), HOBt (2.14 g, 15.88 mmol) and DMAP (88 mg, 0.72 mmol) in 75 mL of dry DCM was added 5 mL of methanol and stirred at room temperature for 14 h. 10 mL of water was added followed by extraction with DCM (3×50 mL), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in hexane) to yield compound 506 (2 g, 47.6%, R_f: 0.3, 10% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (m, 4H), 7.41-7.33 (m, 6H), 3.70-3.64 (m, 1H), 3.66 (s, 6H), 2.28 (t, J=7.2 Hz, 4H), 1.63-1.07 (m, 24H), 1.04 (s, 9H).

Compound 507: To a stirred solution of dimethyl ester 506 (1.0 g, 1.79 mmol) in dry THF (20 mL) were added KHMDS (0.752 g, 3.76 mmol) and methyl iodide (0.762 g, 5.37 mmol) at 0° C. and then brought to room temperature over 30 min. and stirred for 24 h. 10 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×50 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was purified by column chromatography (0-5% ethyl acetate in hexane) to obtain the product 507 (0.218 g, 20%, R_f: 0.8, 5% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 7.68-7.65 (m, 4H), 7.41-7.33 (m, 6H), 3.70-3.67 (m, 1H), 3.67 (s, 6H), 2.43-2.38 (m, 2H), 1.59-1.07 (m, 24H), 1.13 (d, J=7.2 Hz, 6H), 1.04 (s, 9H).

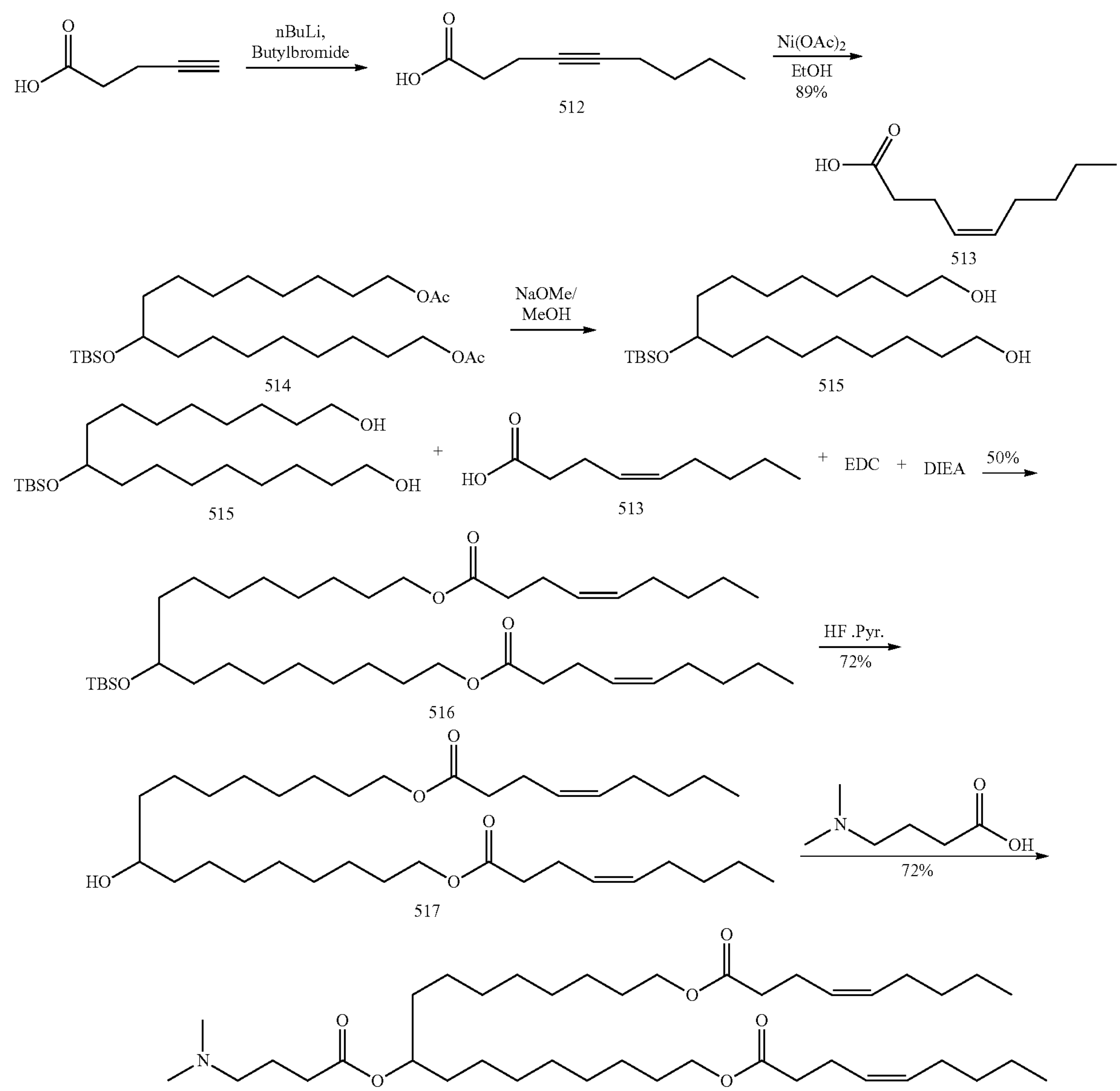
Compound 509: To a stirred solution of methyl ester 507 (0.4 g, 0.66 mmol) in 10 mL of MeOH/THF (1:1) was added LiOH (0.079 g, 3.27 mmol) in 1 mL of water and stirred at room temperature for 24 h. To the above solution was added KOH (0.183 g, 3.27 mmol) in 1 mL of water and stirred for another 2 days. 2 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×25 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product 508 (0.45 g, R_f: 0.2, 10% ethyl acetate in hexane), which was used for the next step without further purification. To a stirred solution of the above di-acid 508 (0.45 g), cis-2-Nonen-1-ol (0.66 g, 4.6 mmol) and EDC.HCl (0.82 g, 4.6 mmol) in dry DCM (15 mL) was added DIEA (1.2 g, 9.24 mmol) and stirred at room temperature for 3 days. 10 mL of water was added followed by extraction with DCM followed by washing with 2N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 509 (0.3 g, 55%, R_f: 0.5, 3% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (m, 4H), 7.42-7.33 (m, 6H), 5.67-5.6 (m, 2H), 5.55-5.49 (m, 2H), 4.615 (d, J=4 Hz, 4H), 3.71-3.65 (m, 1H), 2.44-2.35 (m, 2H), 2.10 (q, J=8.0 Hz, 4H), 1.64-1.07 (m, 40H), 1.13 (d, J=8.0 Hz, 6H), 1.04 (s, 9H), 0.86 (t, J=10 Hz, 6H).

Compound 511: To a stirred solution of silyl ether 509 (0.3 g, 0.36 mmol) in dry THF were added pyridine (1 mL) and HF.Pyr., (1 mL) drop wise and stirred at 45° C. for 48 h. The solvent was evaporated and used for the next step without purification.

To a stirred solution of the above crude alcohol 510, N,N-Dimethyl amino butyric acid (0.34 g, 2.04 mmol), EDC.HCl (0.39 g, 2.04 mmol) and DMAP (0.06 g, 0.51 mmol) in dry DCM (10 mL) was added DIEA (0.5 g, 3.88 mmol) and stirred at room temperature for 2 days. 10 mL of water was added followed by extraction with DCM (3×25 mL), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in 1% TEA containing hexane) to yield compound 511 (0.167 g, 66%, R_f: 0.4, 10% MeOH in DCM). Molecular weight for $C_{43}H_{79}NO_6$ $(M+H)^+$ Calc. 706.59, Found 706.5.

Example 26

Scheme 26



Compound 512: To a stirred solution of 4-Pentynoic acid in 100 mL of THF/HMPA (4:1) at −78° C. was added nBuLi (3.1 g, 49 mmol) drop wise and stirred for 30 min. Then the reaction mixture was brought to 0° C. and stirred for 2 h. Again, the reaction mixture was cooled to −78° C. and n-butyl bromide (3.07 g, 22.44 mmol) was added drop wise and stirred at room temperature for 14 h. 10 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×25 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was purified by column chromatography (0-30% ethyl acetate in hexane) to yield compound 512 (0.4 g, R_f: 0.8, 30% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.59-2.55 (m, 2H), 2.49-2.44 (m, 2H), 2.16-2.11 (m, 2H), 1.49-1.34 (m, 4H), 0.9 (t, J=6.0 Hz, 3H).

Compound 513: To a suspension of $Ni(OAc)_2$ (0.45 g, 2.53 mmol) in EtOH (20 mL) was added $NaBH_4$ (0.096 g, 12.65 mmol) portion wise at room temperature and stirred for 15 min. under H_2 atm. Filtered off the solid followed by concentration of the solvent gave compound 513 (0.35 g, 88.6%, R_f: 0.6, 20% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.88 (br s, 1H), 5.47-5.41 (m, 1H), 5.35-5.31 (m, 1H), 2.43-2.33 (m, 4H), 2.07-2.03 (m, 2H), 1.36-2.27 (m, 4H), 0.9 (t, J=8.0 Hz, 3H).

Compound 515: To a stirred solution of di-acetate 514 (1.5 g, 3.09 mmol) in MeOH (100 mL) was added a piece of sodium metal (0.05 g, 2.17 mmol) and stirred at room temperature for 14 h. Neutralized with dry ice and concentrated followed by extraction with ethyl acetate (3×50 mL), washing with water, dried over anhydrous Na_2SO_4. Con-

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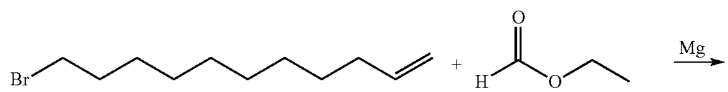
centration of the solvent gave the crude product 515 (1.1 g, 88.7%), which was used for the next step without purification.

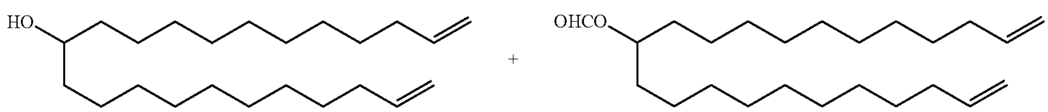
Compound 516: To a stirred solution of the above diol 515 (0.4 g, 1 mmol), 513 (0.341 g, 2.19 mmol), DMAP (0.1 g, 0.82 mmol) and EDC.HCl (0.57 g, 2.98 mmol) in dry DCM (15 mL) was added DIEA (5.97 g, 6 mmol) and stirred at room temperature for 2 days. 10 mL of water was added followed by extraction with ethyl acetate followed by washing with 1N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 516 (0.335 g, 50%, R_f: 0.6, 5% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.45-5.38 (m, 2H), 5.36-5.29 (m, 2H), 4.06 (t, J=8 Hz, 4H), 3.63-3.58 9 m, 1H), 2.39-2.31 (m, 8H), 2.07-2.02 (m, 4H), 1.65-1.57 (m, 4H), 1.4-1.28 (m, 32H), 0.9 (t, J=6.0 Hz, 6H), 0.88 (s, 9H), 0.03 (s, 6H).

Compound 517: To a stirred solution of silyl ether 516 (0.3 g, 0.36 mmol) in dry THF (5 mL) were added pyridine (1 mL) and HF.Pyr. (1 mL) drop wise and stirred at 45° C. for 24 h. The solvent was evaporated followed by purification by column chromatography gave product 517 (0.2 g, 72%, R_f: 0.4, 10% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.43-5.36 (m, 2H), 5.34-5.27 (m, 2H), 4.04 (t, J=8 Hz, 4H), 3.59-3.53 (m, 1H), 2.37-2.3 (m, 8H), 2.05-2.0 (m, 4H), 1.61-1.29 (m, 37H), 0.88 (t, J=8.0 Hz, 6H).

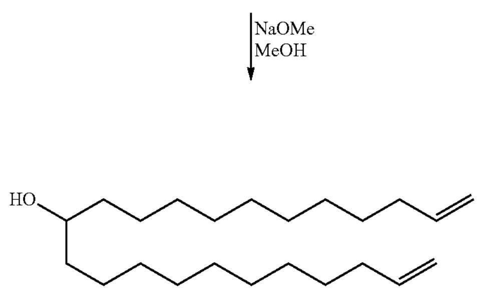
Compound 518: To a stirred solution of the alcohol 517 (0.2 g, 0.355 mmol), N,N-Dimethyl amino butyric acid (0.36 g, 2.14 mmol), EDC.HCl (0.406 g, 2.14 mmol) and DMAP (0.043 g, 0.36 mmol) in dry DCM (10 mL) was added DIEA (0.55 g, 4.26 mmol) and stirred at room temperature for 2 days. 10 mL of water was added followed by extraction with DCM (3×25 mL), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in 1% TEA containing hexane) to yield compound 518 (0.172 g, 72%, R_f: 0.2, 5% MeOH in DCM). ^1H NMR (400 MHz, $CDCl_3$) δ 5.43-5.36 (m, 2H), 5.32-5.27 (m, 2H), 4.87-4.83 (m, 1H), 4.03 (t, J=6 Hz, 4H), 2.36-2.2 (m, 6H), 2.32 (s, 6H), 2.03-1.25 (m, 40H), 0.88 (t, J=6.0 Hz, 6H).

Example 27



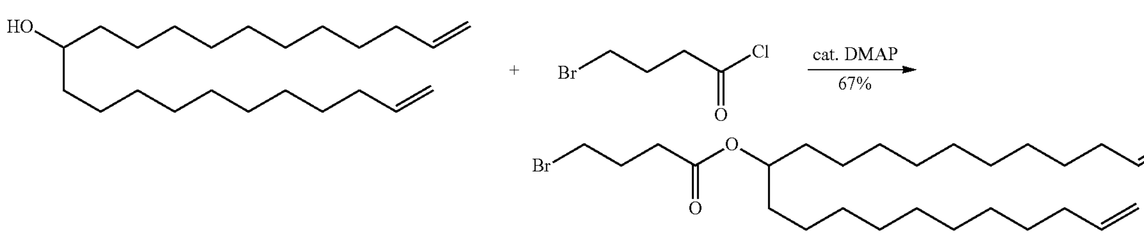


519 520

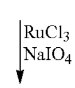


94%

521



522



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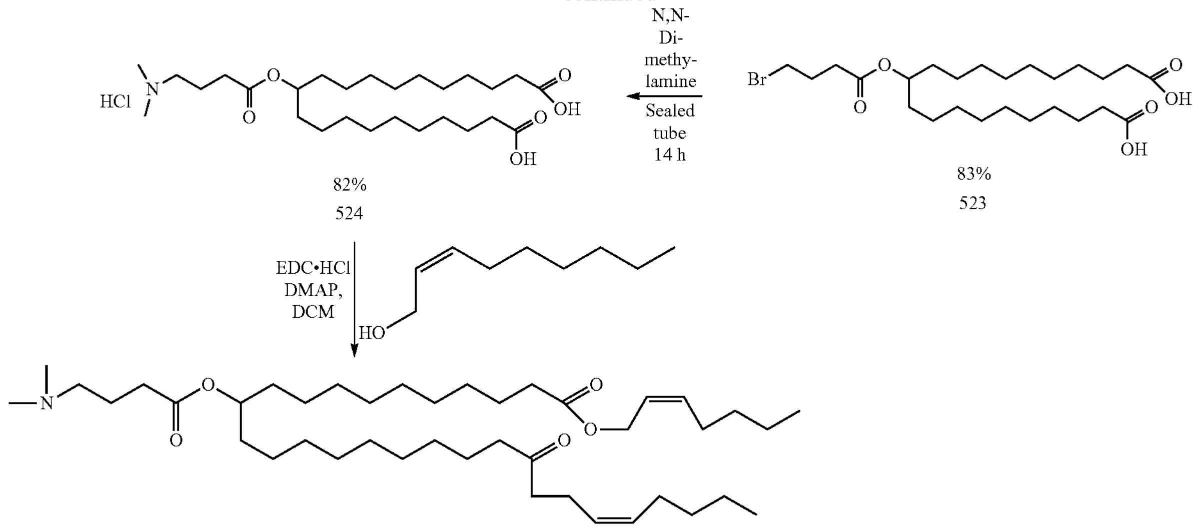


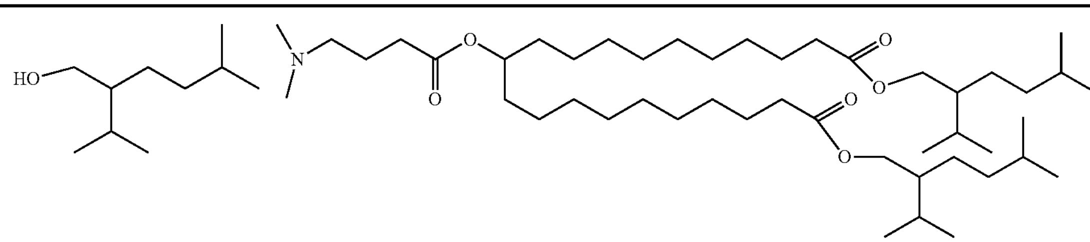
60%
525

Compound 521: To a suspension of Mg in Et_2O was added alkyl bromide (25 g, 107.7 mmol) drop wise at 40° C. over one hour. Ethyl formate was added to the above reaction mixture at 0-5° C. and then the reaction mixture was stirred at room temperature for 14 h. The reaction mixture was poured onto the ice cold sat. NH_4Cl solution followed by extraction with Et_2O (3×250 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was re-dissolved in MeOH (250 mL) and a small piece of sodium (0.1 g) was added and stirred at room temperature for 14 h. The solvent was evaporated and 100 mL of water was added followed by filtration of the solid, washing with water (2×100 mL) gave pale yellow powder 521 (17 g, 94%, %, R_f: 0.8, 10% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.84-5.74 (m, 2H), 5.0-4.89 (m, 4H), 3.64-3.49 (m, 1H), 2.04-1.99 (m, 4H), 1.79 (br s, 1H), 1.44-1.23 (m, 32H).

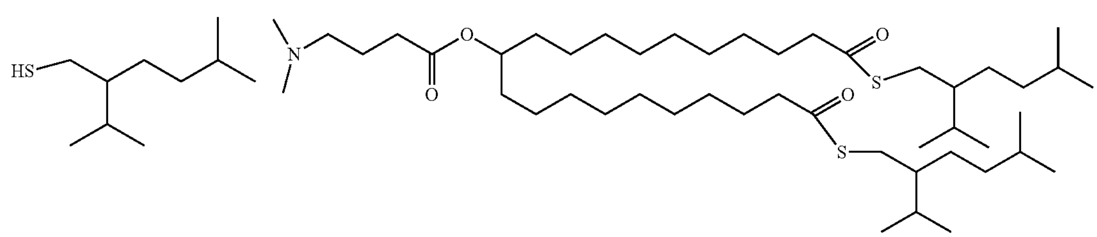
Compound 522: To a stirred solution of 521 (10 g, 29.73 mmol) and DMAP (0.1 g, 0.82 mmol) in dry DCM (50 mL) was added 4-bromo butyryl chloride (6.56 g, 35.68 mmol) and stirred at room temperature for 14 h. 5 mL of saturated $NaHCO_3$ was added and the organic layer was separated and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 522 (9.6 g, 66.7%, R_f: 0.9, 5% ethyl acetate in hexane).

Compound 524: Oxidation was carried out to get compound 523 (8.6 g, 83.5%, R_f: 0.1, 5% MeOH in DCM) following same experimental procedure as for compound 505. This crude material was dissolved in 2N N,N-dimethyl amine in THF (20 mL) and heated to 60° C. in a sealed tube for 14 h. Concentrated the reaction mixture and then pH of the reaction mixture was brought to 3. This mixture was freeze-dried to obtain compound 524 as HCl salt (4 g, 82%). Molecular weight for C27H51NO6 $(M+H)^+$ Calc. 486.37, Found 486.2. ^1H NMR (400 MHz, $CDCl_3$) δ 4.94-4.89 (m, 1H), 3.32-3.3 (m, 2H), 3.2-3.16 (m, 2H), 2.91 (s, 6H), 2.47 (t, J=8 Hz, 2H), 2.28 (t, J=8 Hz, 4H), 2.05-1.97 (m, 2H), 1.61-1.56 (8H), 1.4-1.25 (m, 22H).

526



526s



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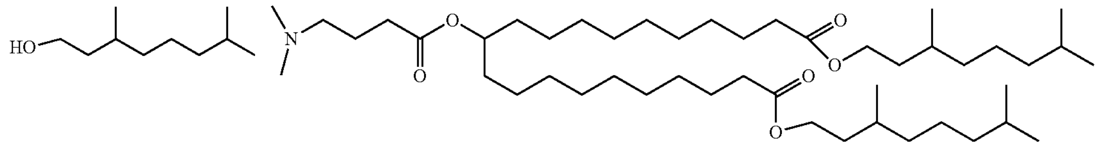
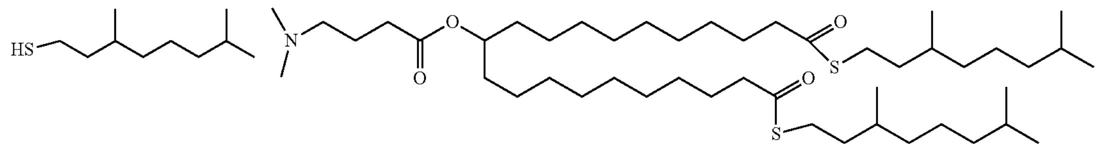
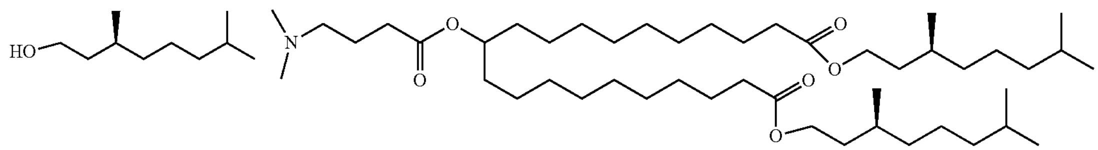
527



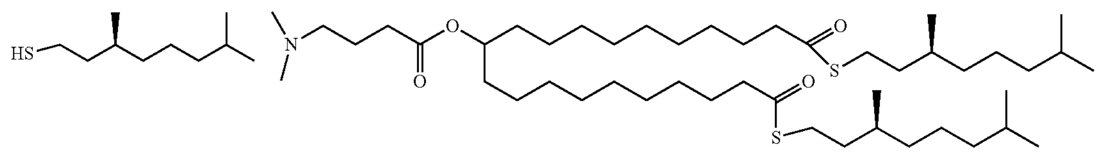
527s



528



528s



Synthesis of Ester 525, 526, 527 and 528

The title compounds were synthesized following the experimental procedure as for compound 516.

Compound 525: (0.75 g, 60%, R_f: 0.3, 5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.65-5.59 (m, 2H), 5.53-5.47 (m, 2H), 4.87-4.81 (m, 1H), 4.595 (d, J=4.0 Hz, 4H), 2.43-2.25 (m, 8H), 2.2 (s, 6H), 2.1-2.03 (m, 4H), 1.81-1.73 (m, 2H), 1.61-1.56 (m, 4H), 1.48-1.47 (m, 4H), 1.36-1.23 (m, 32H), 0.86 (t, J=8.0 Hz, 6H).

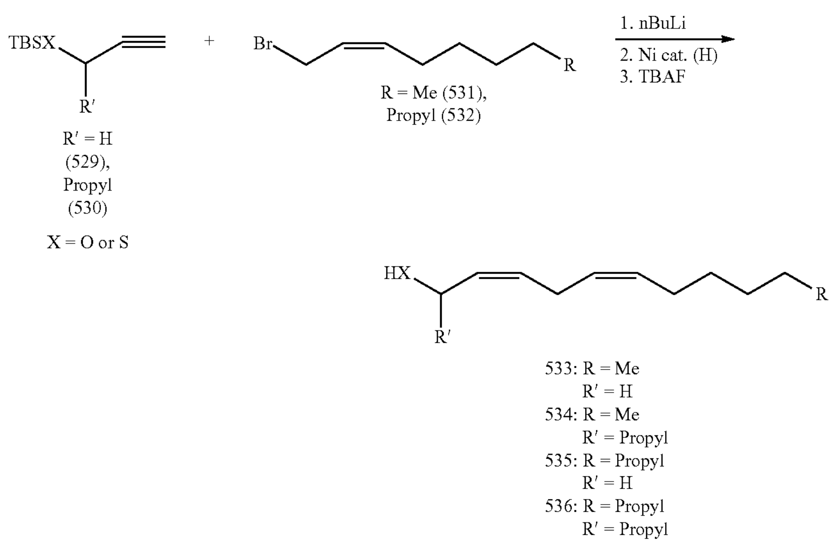
Compound 526: (0.358 g, 60.9%, R_f: 0.5, 5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.87-4.81 (m, 1H), 4.07-3.95 (m, 4H), 2.32-2.24 (m, 6H), 2.2 (s, 6H), 1.80-1.69 (m, 4H), 1.6-1.14 (m, 46H), 0.88-0.84 (m, 24H).

Compound 527: (0.258 g, 56.8%, R_f: 0.5, 5% MeOH in DCM). Molecular weight for C47H91NO6 $(M+H)^+$ Calc. 766.23; Found: 766.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.86-4.80 (m, 1H), 4.12-4.02 (m, 4H), 2.31-2.23 (m, 8H), 2.19 (s, 6H), 1.80-1.72 (m, 2H), 1.66-1.06 (m, 52H), 0.87 (d, J=8.0 Hz, 6H), 0.84 (d, J=8.0 Hz, 12H).

Compound 528: (0.3 g, 68.1%, R_f: 0.5, 5% MeOH in DCM). Molecular weight for C47H91NO6 $(M+H)^+$ Calc. 766.23; Found: 766.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.86-4.80 (m, 1H), 4.12-4.02 (m, 4H), 2.31-2.21 (m, 8H), 2.19 (s, 6H), 1.79-1.72 (m, 2H), 1.66-0.98 (m, 52H), 0.87 (d, J=8.0 Hz, 6H), 0.835 (d, J=4.0 Hz, 12H).

Example 28

Scheme 28:



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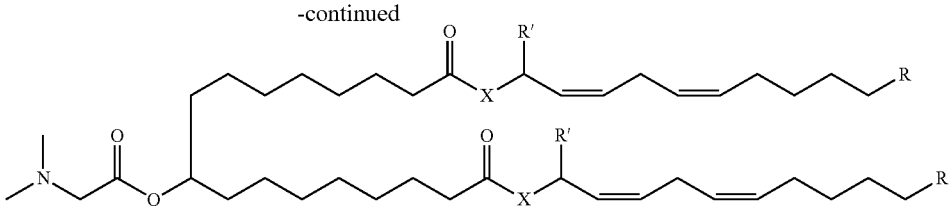


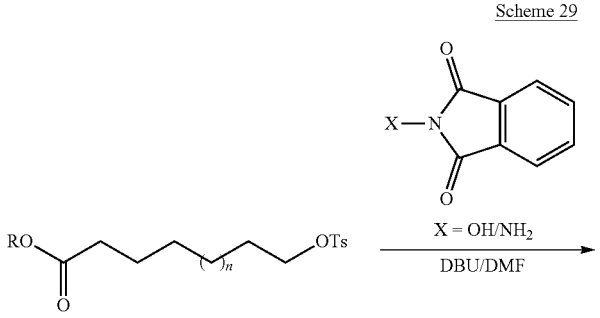
537: R = Me
R′ = H
538: R = Me
R′ = Propyl
539: R = Propyl
R′ = H
540: R = Propyl
R′ = Propyl

Synthesis of compounds 533, 534, 535 and 536: The title compounds (1 mmol) are synthesized following the experimental procedure of compound 513 except de-silylation step and it is done using TBAF in THF at room temperature.

Synthesis of compounds 537, 538, 539 and 540: The title compounds (1 mmol) are synthesized following the experimental procedure of compound 525.

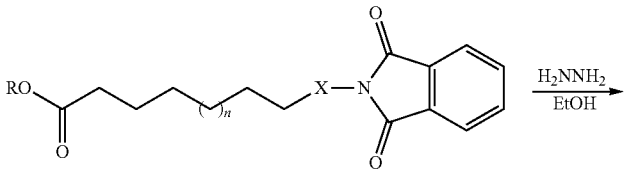
Example 29

Scheme 29



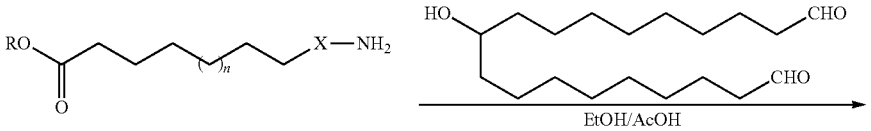
R = alkyl/aryl
N = 0-10

239



X = O/NH
R = alkyl/aryl
n = 0-10

240

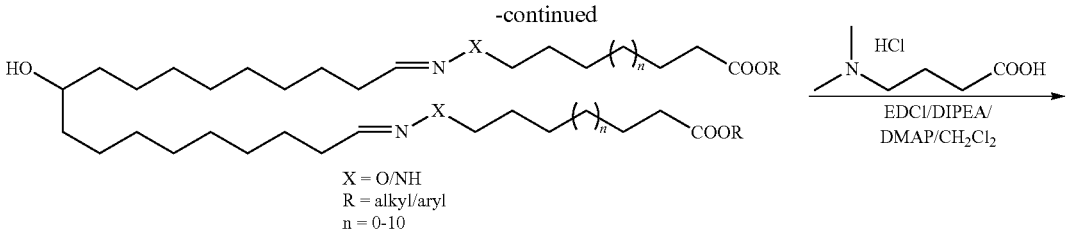


X = O/NH
R = alkyl/aryl
n = 0-10

241

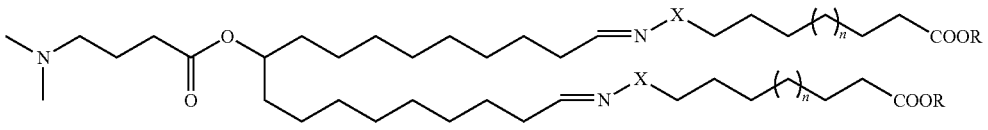
501 502

-continued



X = O/NH
R = alkyl/aryl
n = 0-10

242

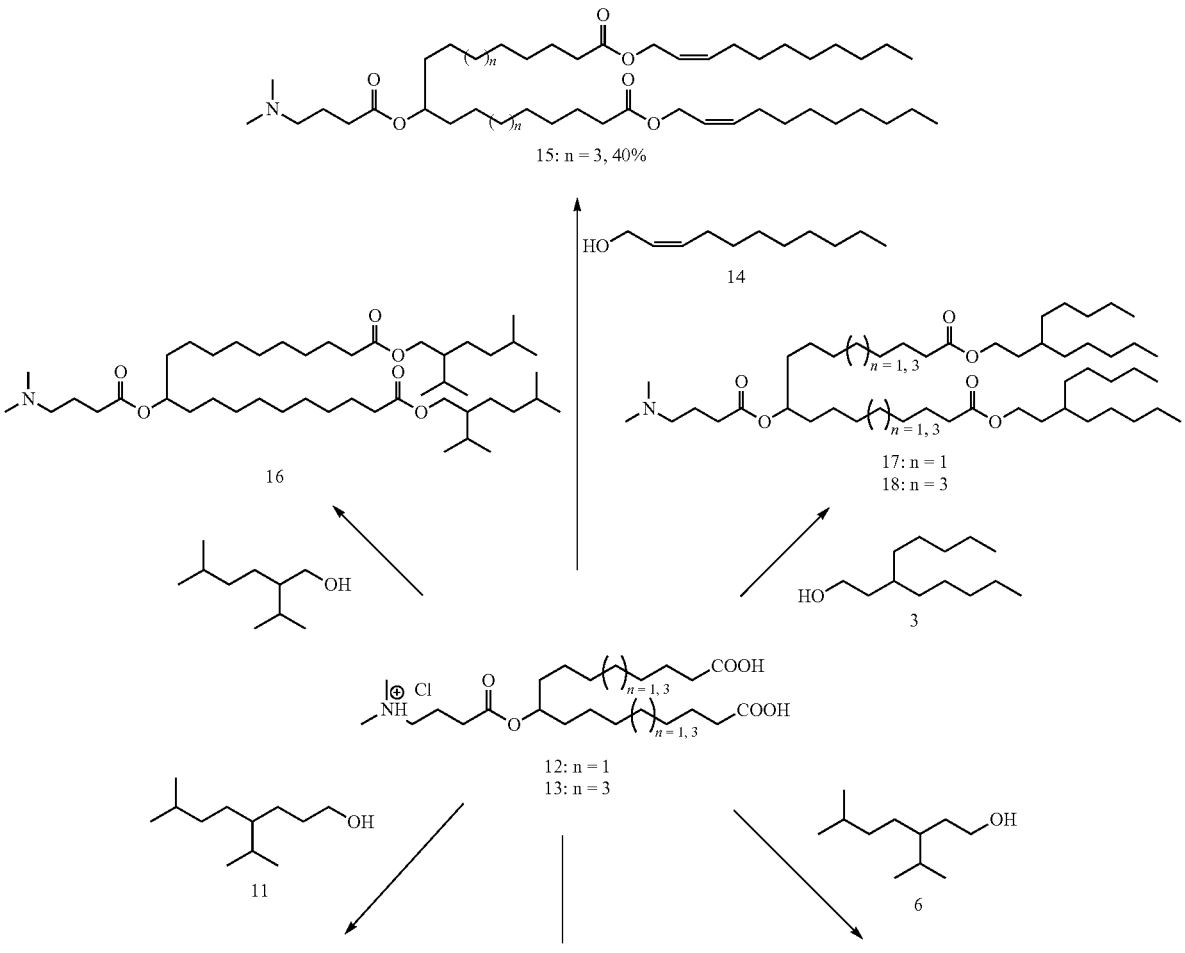


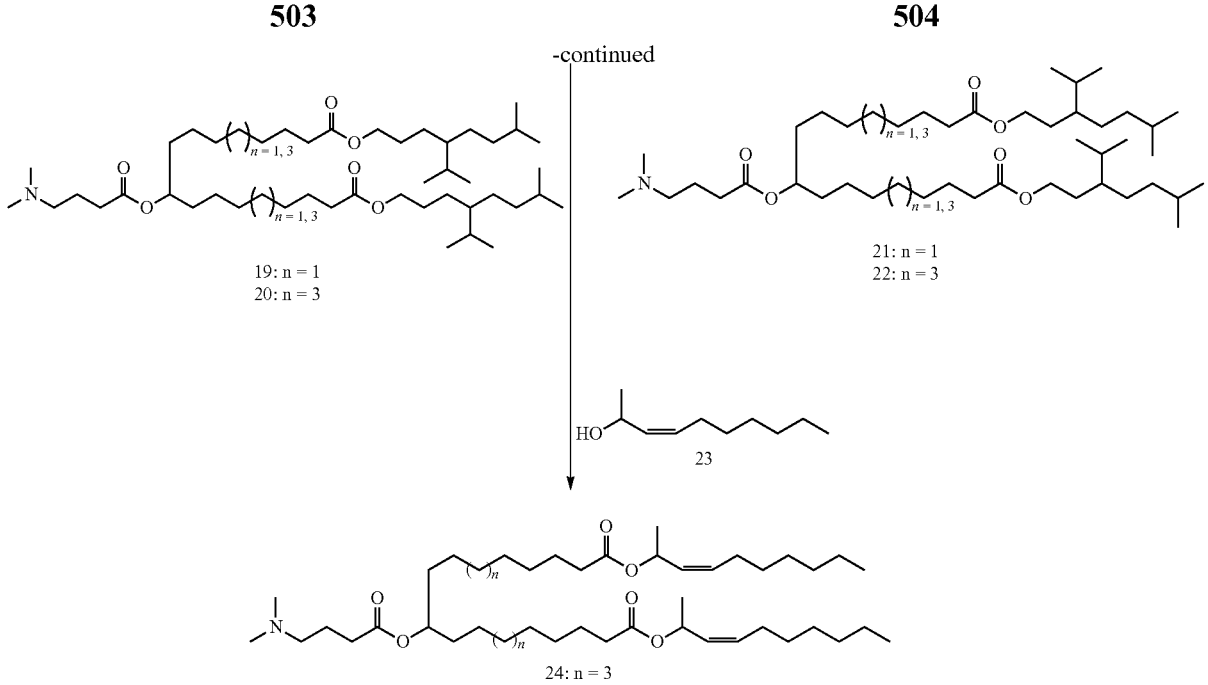
X = O/NH
R = alkyl/aryl
n = 0-10

243

Compound 243 (X═O/NH, R=alkyl/aryl) can be synthesized as shown in Scheme 16-2. Tosyl group of 239 can be replaced with phthalimide group by nucleophilic substitution. After deprotection followed by coupling with 111 under acidic conditions, 242 can be synthesized. Standard esterification gives cationic lipid 243 and its analogs.

Example 30: Synthesis of Ester-Containing Lipids





Compound 15: Compound 13 (503 mg, 1.0 mmol) was treated with 14 (469 mg, 3.0 mmol) in the presence of EDCI (2.30 g, 12.0 mmol), DMAP (235 mg, 1.92 mmol) and DIEA (8.34 mL, 47.9 mmol) in CH_2Cl_2 (50 mL) for 14 h. Aqueous work-up then column chromatography gave compound 15 (1.22 g, 1.54 mmol, 40%).

Molecular weight for $C_{49}H_{92}NO_6$ $(M+H)^+$ Calc. 790.6925, Found 790.7.

Compound 16: This compound was synthesized from 13 and tetrahydrolavandulol using a procedure analogous to that described for compound 15. Yield: 0.358 g, 61%. ^1H NMR (400 MHz, $CDCl_3$) δ 4.87-4.81 (m, 1H), 4.07-3.95 (m, 4H), 2.32-2.24 (m, 6H), 2.2 (s, 6H), 1.80-1.69 (m, 4H), 1.6-1.14 (m, 46H), 0.88-0.84 (m, 24H).

Compound 17: This compound was synthesized from 12 (1.0 g, 2.15 mmol) and 3 (1.03 g, 5.16 mmol) using a procedure analogous to that described for compound 15.

Yield: 856 mg (50%). ^1H NMR (400 MHz, $CDCl_3$) δ 4.91-4.79 (m, 1H), 4.08 (t, J=7.1 Hz, 4H), 2.35-2.25 (m, 14H), 1.89-1.76 (m, 2H), 1.67-1.13 (m, 62H), 0.88 (t, J=7.0 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.08, 74.45, 63.08, 45.27, 34.76, 34.56, 34.28, 33.70, 32.61, 32.39, 29.54, 29.36, 29.28, 26.36, 25.47, 25.13, 22.83, 14.26. Molecular weight for $C_{49}H_{96}NO_6$ $(M+H)^+$ Calc. 794.7238, Found 794.6.

Compound 18: This compound was synthesized from 13 (1.0 g, 2.15 mmol) and 3 (1 g) using a procedure analogous to that described for compound 15.

Yield: 1 g (59%). ^1H NMR (400 MHz, $CDCl_3$) δ 4.94-4.74 (m, 1H), 4.17-3.85 (m, 4H), 2.46-2.19 (m, 12H), 1.93-1.79 (m, 2H), 1.74-1.45 (m, 10H), 1.37 (d, J=20.2 Hz, 2H), 1.35-1.13 (m, 44H), 0.88 (t, J=6.9 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.19, 77.53, 77.21, 76.90, 63.12, 34.81, 34.66, 34.35, 33.76, 32.66, 32.45, 29.76, 29.73, 29.63, 29.48, 29.39, 26.42, 25.57, 25.23, 22.89, 14.32. Molecular weight for $C_{53}H_{103}NO_6$ $(M+H)^+$ Calc. 850.38, Found 850.7.

Compound 19: This compound was synthesized from 12 and 11 using a procedure analogous to that described for compound 15.

Yield: 860 mg (51%). ^1H NMR (400 MHz, $CDCl_3$) δ 4.90-4.81 (m, 1H), 4.04 (t, J=6.8 Hz, 4H), 2.37-2.17 (m, 14H), 1.84-1.06 (m, 48H), 0.93-0.78 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.06, 74.35, 65.51, 64.91, 59.05, 45.51, 43.77, 37.10, 34.55, 34.29, 32.55, 29.54, 29.37, 29.34, 29.28, 28.58, 28.19, 26.99, 26.74, 25.47, 25.15, 22.90, 22.82, 19.60, 19.41, 19.28. Molecular weight for $C_{47}H_{92}NO_6$ $(M+H)^+$ Calc. 766.6925, Found 766.5.

Compound 20: This compound was synthesized from 13 and 11 using a procedure analogous to that described for compound 15.

^1H NMR (400 MHz, $CDCl_3$) δ 4.86 (p, J=6.2 Hz, 1H), 4.04 (t, J=6.7 Hz, 4H), 2.38-2.17 (m, 14H), 1.84-1.07 (m, 56H), 0.93-0.76 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.11, 173.46, 74.44, 64.90, 59.06, 45.51, 43.77, 37.11, 34.59, 34.32, 32.57, 29.71, 29.67, 29.57, 29.43, 29.34, 28.58, 28.20, 27.00, 26.75, 25.51, 25.20, 22.90, 22.82, 19.41, 19.28. Molecular weight for $C_{51}H_{100}NO_6$ $(M+H)^+$ Calc. 822.7551, Found 822.6.

Compound 21: This compound was synthesized from 12 and 6 using a procedure analogous to that described for compound 15.

^1H NMR (400 MHz, $CDCl_3$) δ 4.91-4.78 (m, 1H), 4.15-3.98 (m, 4H), 2.39-2.18 (m, 14H), 1.84-1.11 (m, 44H), 0.92-0.77 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.06, 173.44, 74.36, 63.73, 59.03, 45.48, 41.00, 36.98, 34.56, 34.29, 32.54, 29.60, 29.54, 29.49, 29.36, 29.28, 28.52, 25.47, 25.13, 23.15, 22.85, 22.81, 19.49, 18.89. Molecular weight for $C_{45}H_{88}NO_6$ $(M+H)^+$ Calc. 738.6612, Found 738.6.

Compound 22: This compound was synthesized from 13 and 6 using a procedure analogous to that described for compound 15.

Yield: 900 mg (57%). ^1H NMR (400 MHz, $CDCl_3$) δ 4.92-4.78 (m, 1H), 4.15-3.91 (m, 4H), 3.33-3.08 (m, 1H), 2.36-2.15 (m, 14H), 1.79 (dq, J=14.3, 7.2 Hz, 2H), 1.74-1.55 (m, 8H), 1.55-1.37 (m, 9H), 1.35-0.95 (m, 36H), 0.96-0.61

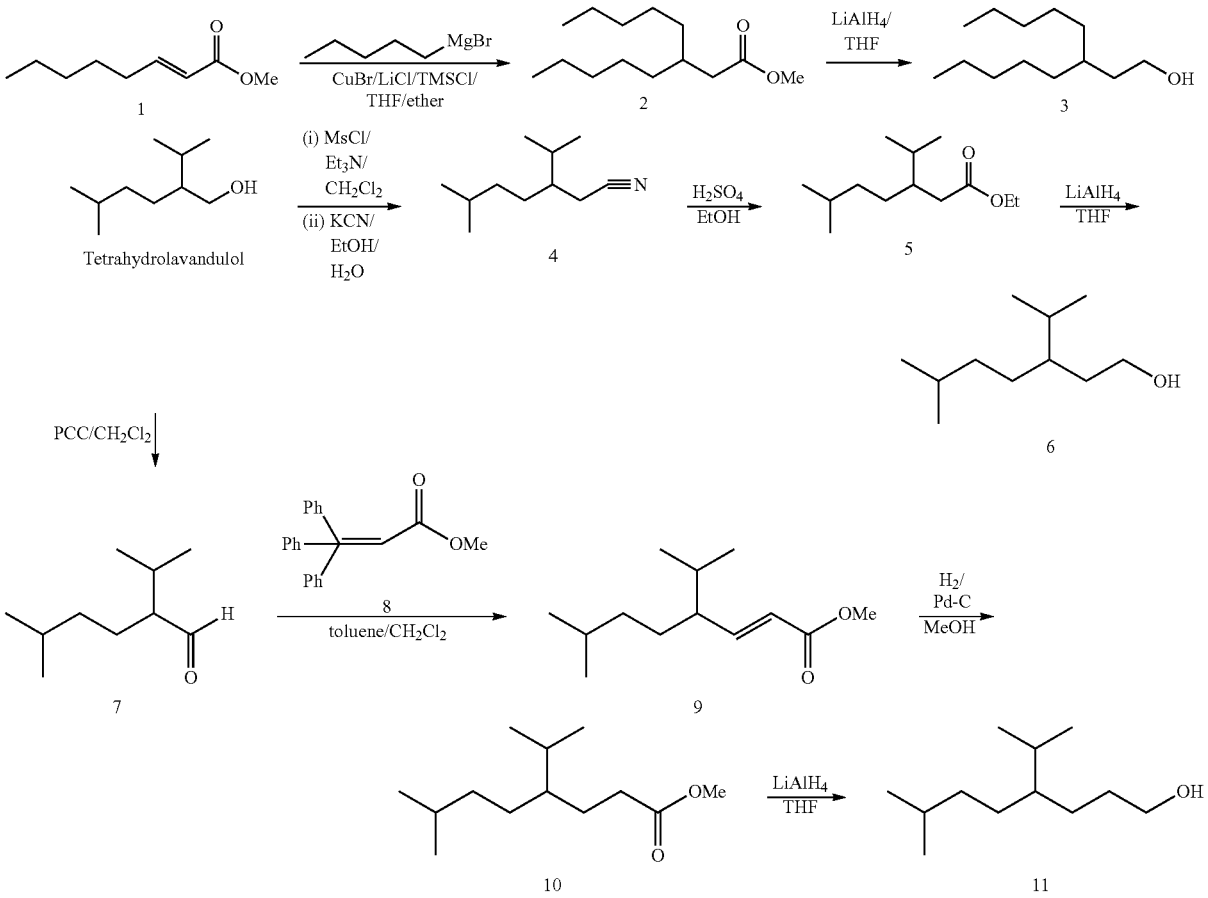
Case 1:22-cv-00333-UNA Document 1-1 Filed 03/17/22 Page 253 of 357 PageID #: 272

(m, 27H). ^{13}C NMR (101 MHzCDCl$_3$) δ 174.16, 173.52, 77.54, 77.22, 76.91, 74.48, 63.76, 59.10, 45.55, 42.02, 41.04, 38.75, 37.09, 37.02, 34.65, 34.36, 32.62, 30.71, 29.75, 29.72, 29.64, 29.62, 29.53, 29.48, 29.44, 29.38, 28.56, 28.45, 25.56, 25.23, 23.59, 23.23, 22.90, 22.86, 19.54, 19.03, 18.94. Molecular weight for $C_{49}H_{95}NO_6$ $(M+H)^+$ Calc. 794.2817, Found 794.7.

Compound 24: This compound was synthesized from 13 and 23 using a procedure analogous to that described for compound 15.

Yield: 0.567 g (30%). ^{1}H NMR (400 MHz, CDCl$_3$) δ 4.85 (p, J=6.1 Hz, 1H), 4.20-3.93 (m, 4H), 2.41-2.18 (m, 13H), 1.92-1.72 (m, 2H), 1.56 (ddd, J=27.4, 16.4, 5.8 Hz, 12H), 1.39 (s, 2H), 1.25 (s, 54H), 0.91 (dt, J=13.7, 6.4 Hz, 11H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.18, 173.51, 77.54, 77.23, 76.91, 74.50, 63.12, 59.10, 45.55, 34.81, 34.66, 34.38, 33.76, 32.67, 32.62, 32.45, 29.77, 29.73, 29.64, 29.49, 29.39, 26.42, 25.57, 25.24, 23.23, 22.89, 14.32. Molecular weight for $C_{47}H_{88}NO_6$ $(M+H)^+$ Calc. 762.6612, Found 762.5.

Example 31: Synthesis of Alcohol Components



Compound 2: Compound 2 was synthesized from 1 using a procedure analogous to that described in *Journal of the Organic Chemistry,* 2009, 1473.

^{1}H NMR (400 MHz, CDCl$_3$) δ 3.66 (s, 3H), 2.23 (d, J=6.9 Hz, 2H), 1.84 (brs, 1H), 1.27 (d, J=11.5 Hz, 16H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 174.29, 51.49, 39.25, 35.22, 34.00, 32.24, 26.34, 22.77, 14.22.

Compound 3: To a suspension of $LiAlH_4$ (2.84 g, 74.9 mmol) in THF (85 mL) was added a solution of compound 2 (8.55 g, 37.4 mmol) in THF (25 mL). The reaction mixture was refluxed overnight. Aqueous workup then column chromatography gave pure compound 3 (7.35 g, 36.7 mmol, 98%) as a colorless oil.

^{1}H NMR (400 MHz, CDCl$_3$) δ 3.66 (t, J=7.0 Hz, 2H), 1.59-1.12 (m, 19H), 0.88 (t, J=6.9 Hz, 6H).

Compound 4: Tetrahydrolavandulol (10.1 g, 63.8 mmol) was treated with methansulfonyl chloride (6.38 mL) in CH_2Cl_2 (200 mL) and Et_3N (17.6 mL). Aqueous workup gave the crude mesylate, which was treated with KCN (4.98 g, 76.5 mmol) in EtOH (90 mL) and H_2O (10 mL). Aqueous workup then column chromatography gave pure compound 4 (8.36 g, 50.0 mmol, 72%) as a colorless oil.

^{1}H NMR (400 MHz, CDCl$_3$) δ 2.38-2.23 (m, 2H), 1.86-1.78 (m, 1H), 1.59-1.42 (m, 3H), 1.40-1.07 (m, 3H), 0.93-0.89 (m, 12H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 119.73, 41.69, 36.46, 30.10, 28.44, 28.33, 22.82, 22.59, 19.62, 19.11, 19.05.

Compound 6: The cyano derivative 4 was converted to the ethyl ester under acidic conditions to give compound 5 and the ester was reduced by $LiAlH_4$ in THF to give compound 6.

Compound 7: Tetrahydrolavandulol (98.1 g, 51.2 mmol) was oxidized with PCC (16.6 g, 76.8 mmol) in CH_2Cl_2 (200

Case 1:22-cv-00323-UNA Document 1-1 Filed 03/17/22 Page 254 of 357 PageID #: 273

mL). Aqueous workup then column chromatography gave pure compound 7 (6.19 g, 39.6 mmol, 77%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.60 (d, J=3.1 Hz, 1H), 2.05-1.79 (m, 1H), 1.71-1.36 (m, 4H), 1.23-1.04 (m, 2H), 1.02-0.82 (m, 12H).

Compound 9: To a solution of compound 7 (2.0 g, 12.8 mmol) in toluene (40 mL) and CH_2Cl_2 (18 mL) and was added 8 (3.96 g, 11.8 mmol). The mixture was heated at 70° C. overnight. Column chromatography gave pure compound 9 (1.40 g, 6.59 mmol, 51%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.77 (dd, J=15.6, 9.9 Hz, 1H), 5.76 (d, J=15.6 Hz, 1H), 3.73 (s, 3H), 1.97-1.83 (m, 1H), 1.72-1.64 (m, 1H), 1.54-1.40 (m, 2H), 1.37-1.22 (m, 1H), 1.18-0.97 (m, 2H), 0.94-0.78 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.19, 152.54, 121.70, 51.53, 49.66, 36.95, 31.76, 29.49, 28.29, 22.92, 22.54, 20.84, 19.24.

Compound 10: To a solution of compound 9 (1.0 g, 4.71 mmol) in MeOH (15 mL) was added Pd—C (125 mg). The mixture was stirred under H_2 atmosphere overnight. The mixture was filtered over Celite then evaporated to give pure compound 10 (924 mg, 4.31 mmol, 92%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67 (s, 3H), 2.41-2.16 (m, 2H), 1.74-1.57 (m, 2H), 1.57-1.42 (m, 2H), 1.33-1.02 (m, 5H), 0.88-0.83 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.78, 51.62, 43.71, 36.97, 32.69, 29.23, 28.56, 27.94, 25.92, 22.85, 22.79, 19.32, 19.19.

Compound 11: To a suspension of $LiAlH_4$ (444 mg, 11.7 mmol) in THF (12 mL) was added a solution of compound 10 (1.25 g, 5.83 mmol) in THF (8 mL). The reaction mixture was refluxed overnight. Aqueous workup gave the crude compound 11 (1.1 g) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.63 (t, J=6.7 Hz, 2H), 1.74-1.66 (m, 1H), 1.60-1.45 (m, 3H), 1.37-1.05 (m, 7H), 0.88-0.82 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.75, 44.00, 37.16, 31.22, 29.40, 28.61, 28.28, 26.62, 22.90, 22.82, 19.43, 19.28.

Example 32: Synthesis of Ester-Containing Lipids

Compound 26: Compound 25 (840 mg, 1.03 mmol) was stirred in TFA (9 mL) and CH_2Cl_2 (36 mL) for 3 h at room temperature. Evaporation of the solvents and co-evaporation with toluene 3 times gave compound 26.

Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5986, Found 706.4.

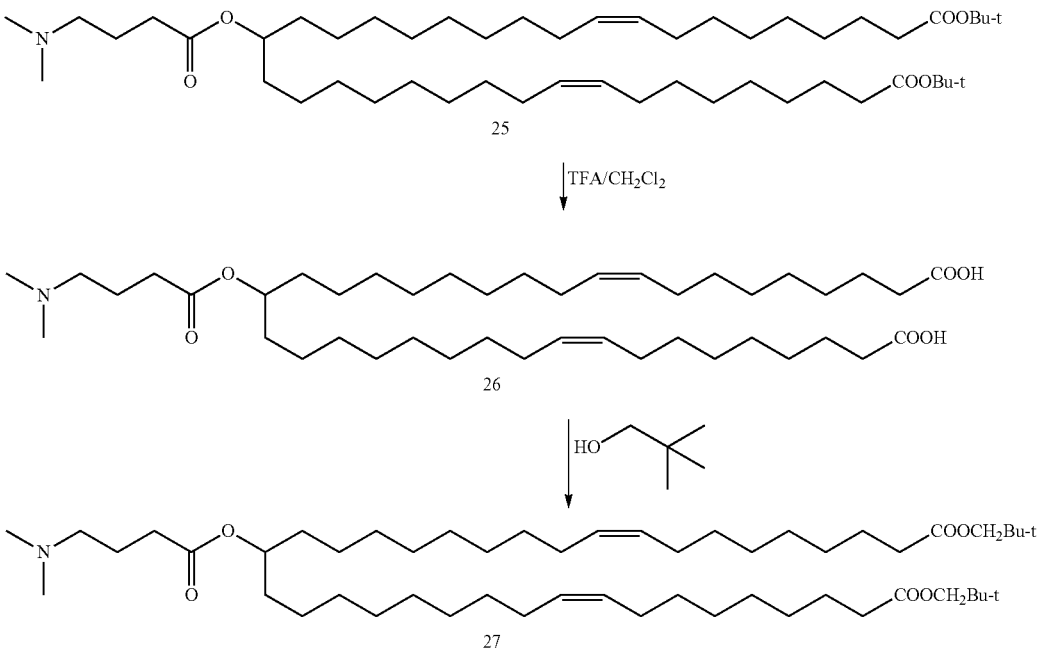
Compound 27: Compound 26 from the previous step was treated with 2,2-dimethylpropanol (363 mg, 4.12 mmol) in the presence of EDCI (592 mg, 3.09 mmol), DMAP (50 mg, 0.412 mmol) and DIEA (1.44 mL, 8.24 mmol) in CH_2Cl_2 (10 mL) for 14 h. Aqueous work-up then column chromatography gave compound 27 (575 mg, 0.679 mmol, 66%).

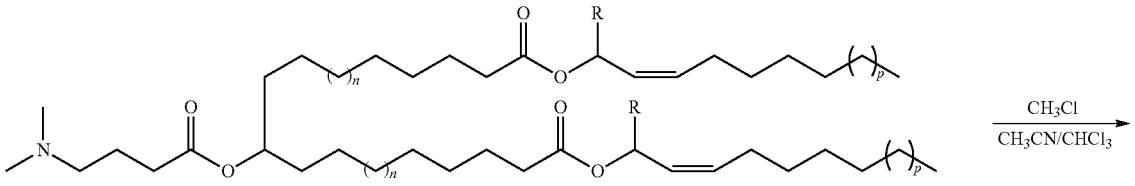
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40-5.28 (m, 4H), 4.91-4.81 (m, 1H), 3.76 (s, 4H), 2.34-2.27 (m, 8H), 2.22 (s, 6H), 2.03-1.97 (m, 8H), 1.83-1.26 (m, 50H), 0.94 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.14, 173.53, 130.09, 129.92, 74.41, 73.72, 59.12, 45.61, 34.60, 34.32, 32.64, 31.45, 29.93, 29.85, 29.71, 29.68, 29.48, 29.32, 29.28, 27.39, 27.33, 26.62, 25.52, 25.22, 23.32.

Molecular weight for $C_{53}H_{100}NO_6$ $(M+H)^+$ Calc. 846.7551, Found 846.5.

Example 33: Synthesis of Quaternary Lipids

A. The amino lipids synthesized in Examples 31 and 32 can be converted to the corresponding quaternary lipids as shown below by treatment with CH_3Cl in CH_3CN and $CHCl_3$.





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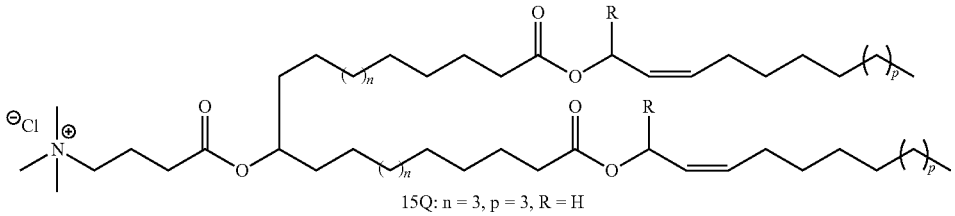
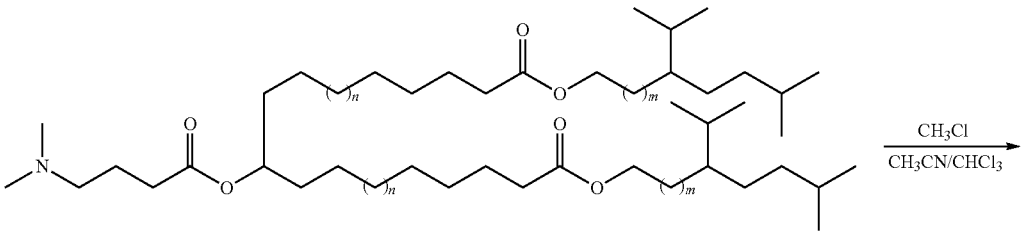
24: n = 3, p = 1, R = Me



15Q: n = 3, p = 3, R = H
24Q: n = 3, p = 1, R = Me



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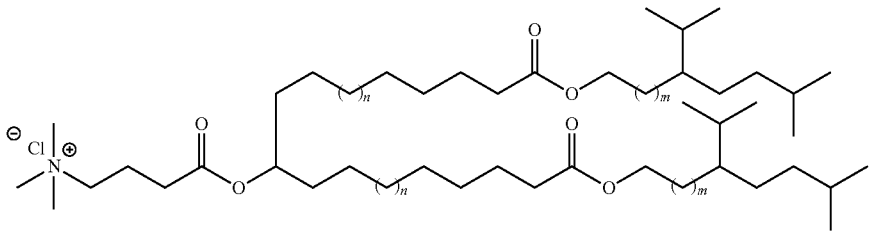
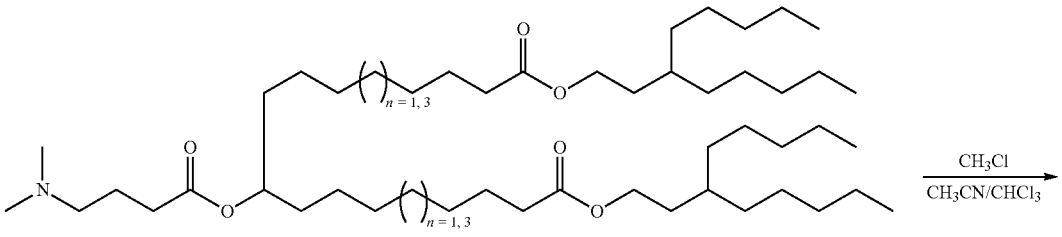
19: m = 2, n = 1
20: m = 2, n = 3
21: m = 1, n = 1
22: m = 1, n = 3

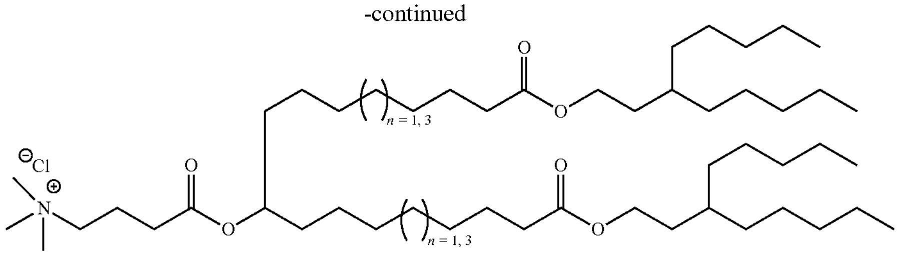


16Q: m = 0, n = 3
19Q: m = 2, n = 1
20Q: m = 2, n = 3
21Q: m = 1, n = 1
22Q: m = 1, n = 3

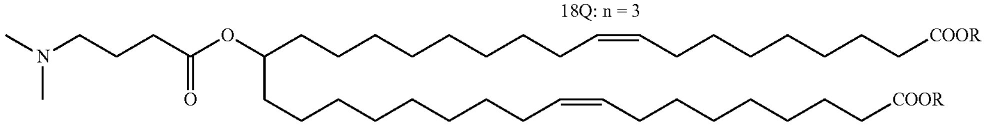


17: n = 1
18: n = 3

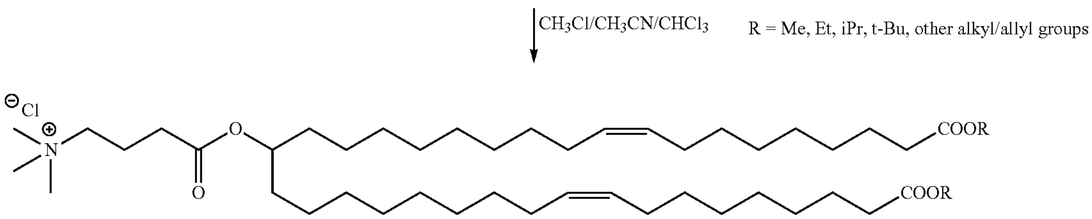
-continued



17Q: n = 1
18Q: n = 3



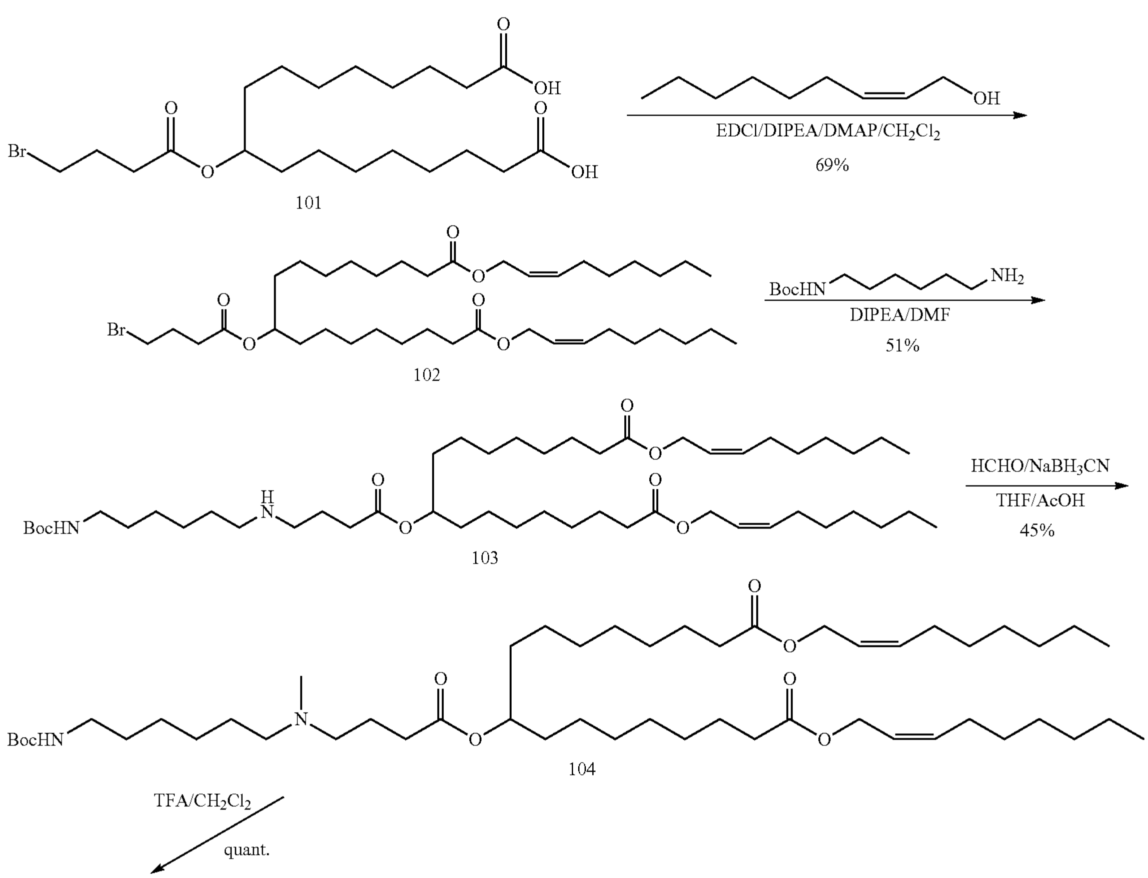
27T



27TQ

B. Synthesis of BODIPY-Lipid Conjugates

Synthesis of BODIPY-labeled lipid



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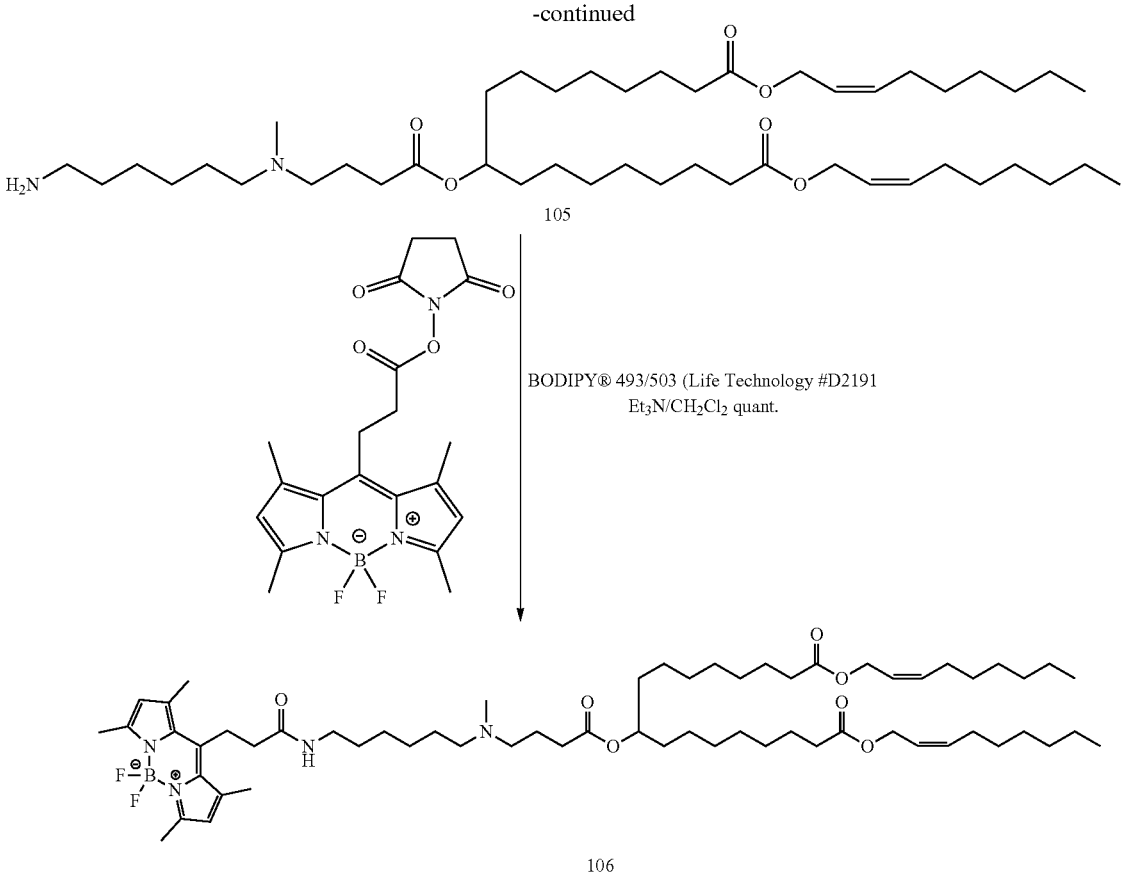


Compound 102: To a solution of compound 101 (2.00 g, 4.30 mmol) and cis-2-nonen-1-ol (1.81 mL, 10.7 mmol) in CH_2Cl_2 (20 mL) were added diisopropylethylamine (3.00 mL, 17.2 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.06 g, 10.7 mmol) and DMAP (106 mg, 0.868 mmol). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with saturated $NaHCO_3$ aq. (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% EtOAc in Hexane) to give compound 102 (2.11 g, 2.96 mmol, 69%, R_f=0.45 developed with 10% EtOAc in Hexane).

^1H NMR (500 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.54-5.49 (m, 2H), 4.89-4.84 (m, 1H), 4.62 (d, J=6.5 Hz, 4H), 3.46 (t, J=6.5 Hz, 2H), 2.48 (t, J=7.3 Hz, 2H), 2.30 (t, J=7.5 Hz, 4H), 2.20-2.14 (m, 2H), 2.12-2.04 (m, 4H), 1.63-1.60 (m, 4H), 1.51-1.50 (m, 4H), 1.37-1.27 (m, 32H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.90, 172.45, 135.58, 123.51, 74.74, 60.36, 34.47, 34.24, 32.93, 32.91, 31.83, 29.54, 29.48, 29.31, 29.21, 29.01, 28.03, 27.70, 25.43, 25.08, 22.76, 14.23.

Molecular weight for $C_{39}H_{69}BrNaO_6$ $(M+Na)^+$ Calc. 735.42, Found 735.2.

Compound 103: To a solution of 102 (2.11 g, 2.96 mmol) in DMF (20 mL) was added a solution of N-Boc-1,6-diaminohexane (670 mg, 3.10 mmol) in DMF (20 mL) at 0° C. The mixture was stirred for 18 hours at room temperature. Then additional N-Boc-1,6-diaminohexane (160 mg, 0.740 mmol) in DMF (1 mL) was added and the mixture was stirred for 12 hour. The reaction was quenched by adding saturated $NaHCO_3$ aq. (100 mL) then extracted with Et_2O (150 mL×3). The organic layer was separated and dried over anhydrous $MgSO_4$. After filtration and concentration, the crude was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2, R_f=0.24) to give 103 (1.28 g, 1.51 mmol, 51%).

^1H NMR (400 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.55-5.50 (m, 2H), 4.88-4.81 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 4.54 (brs, 1H), 3.11-3.08 (m, 2H), 2.67-2.59 (m, 4H), 2.35 (t, J=7.4 Hz, 2H), 2.29 (t, J=7.6 Hz, 4H), 2.10-2.07 (m, 4H), 1.84-1.81 (m, 4H), 1.63-1.57 (m, 4H) 1.50-1.47 (m, 8H), 1.44 (s, 9H), 1.38-1.27 (m, 34H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.90, 173.53, 135.57, 123.50, 74.49, 60.36, 49.82, 49.29, 40.64, 34.47, 34.24, 32.68, 31.83, 30.16, 29.89, 29.54, 29.50, 29.33, 29.23, 29.01, 28.58, 27.69, 27.11, 26.80, 25.44, 25.37, 25.09, 22.76, 14.23.

Molecular weight for $C_{50}H_{93}N_2O_8$ $(M+H)^+$ Calc. 849.69, Found 849.5.

Compound 104: To a solution of 103 (1.16 g, 1.37 mmol) in THF (20 mL) were added formaldehye (37 wt. % in H_2O, 0.306 mL, 4.11 mmol), sodium cyanoborohydride (1 M solution in THF, 2.06 mL, 2.06 mmol) and acetic acid (0.008 mL, 0.137 mmol) at 0° C. The mixture was stirred at room temperature for 17 hours. The reaction was quenched by adding saturated $NaHCO_3$ aq. (50 mL) then extracted with Et_2O (100 mL×3). The organic layer was separated and dried over anhydrous $MgSO_4$. After filtration and concentration, the crude was purified by silica gel column chromatography (8% MeOH in CH_2Cl_2, R_f=0.46) to give 104 (531 mg, 0.615 mmol, 45%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.66-5.60 (m, 2H), 5.53-5.47 (m, 2H), 4.86-4.80 (m, 1H), 4.61-4.59 (m, 5H), 3.12-3.07 (m, 2H), 2.89-2.78 (m, 4H), 2.62 (s, 3H), 2.40 (t, J 6.8 Hz, 2H), 2.28 (t, J=7.4 Hz, 4H), 2.11-2.06 (m, 4H), 1.99-1.92 (m, 2H), 1.69-1.27 (m, 57H), 0.87 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.86, 172.45, 156.18, 135.55, 123.45, 75.24, 60.32, 56.68, 55.83, 40.72, 40.36, 34.40, 34.09, 31.79, 31.29, 29.92, 29.49, 29.41, 29.26, 29.17, 28.96, 28.55, 27.65, 26.49, 26.30, 25.41, 25.02, 24.79, 22.71, 20.12, 14.19.

Molecular weight for $C_{51}H_{95}N_2O_8$ $(M+H)^+$ Calc. 863.71, Found 863.6.

Compound 105: To a solution of compound 104 (525 mg, 0.608 mmol) in CH_2Cl_2 (8 mL) was added trifluoroacetic acid (2 mL) at 0° C. The reaction mixture was stirred at 0° C. for 1 hour and at room temperature for 3 hours. The reaction mixture was evaporated and co-evaporated with toluene 3 times then dried in vacuo overnight to give compound 105 (603 mg, 0.603 mmol calculated as 2 TFA salt, quantitatively, R_f=0.24 developed with 8% MeOH in CH_2Cl_2).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (brs, 1H), 5.68-5.61 (m, 2H), 5.55-5.49 (m, 2H), 4.87-4.81 (m, 1H), 4.62 (d, J=6.8 Hz, 4H), 4.28 (brs, 3H), 3.20-3.02 (m, 6H), 2.82 (d, J=4.0 Hz, 3H), 2.45-2.40 (m, 2H), 2.30 (t, J=7.4 Hz, 4H), 2.12-2.00 (m, 6H), 1.78-1.22 (m, 52H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.04, 172.08, 161.84, 161.47, 135.63, 123.44, 117.60, 114.71, 75.56, 60.41, 55.69, 55.27, 39.94, 39.64, 34.44, 34.06, 31.82, 30.72, 29.53, 29.43, 29.28, 29.19, 29.00, 27.69, 26.58, 25.42, 25.27, 25.05, 24.60, 23.06, 22.75, 19.00, 14.22.

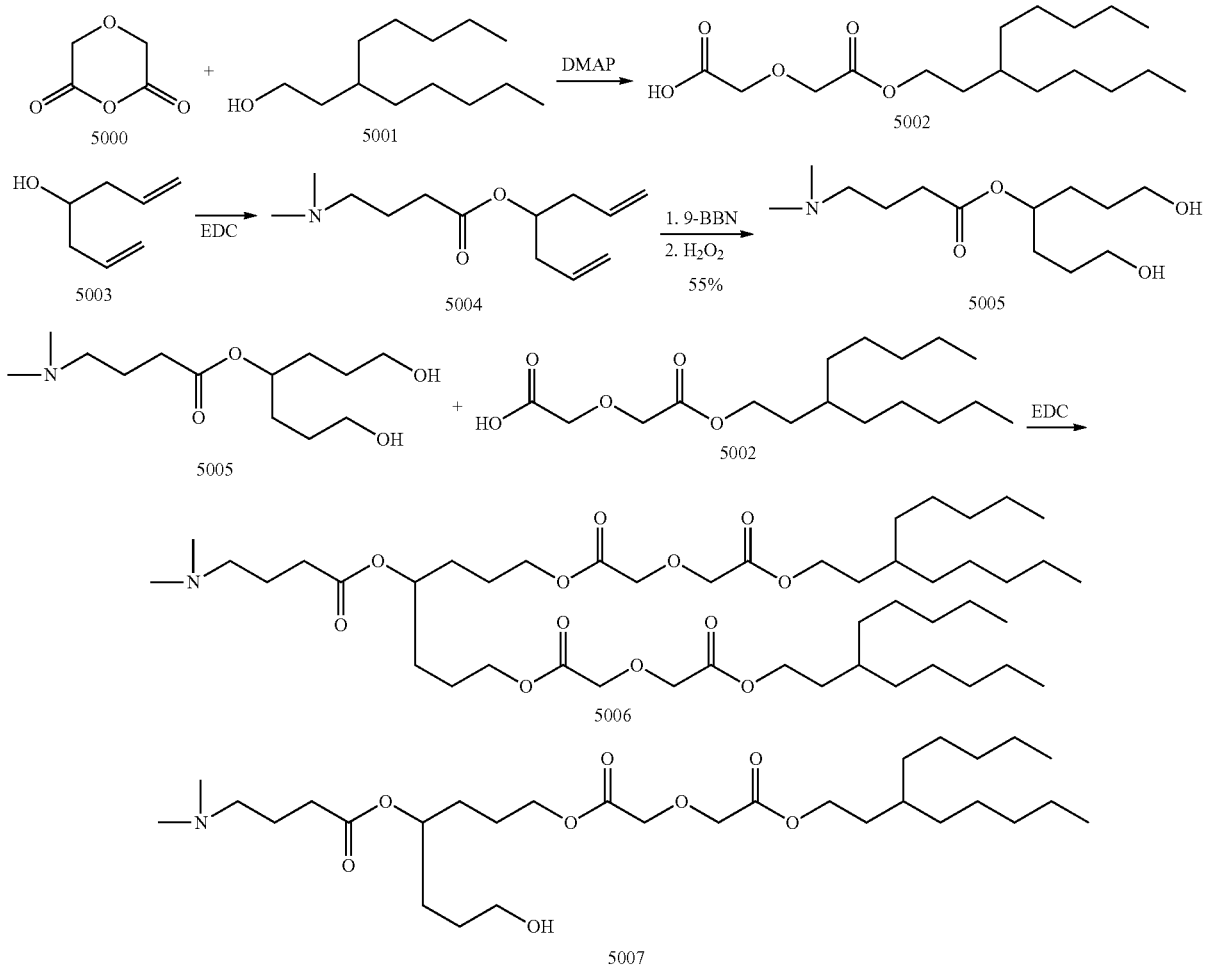
Molecular weight for $C_{46}H_{87}N_2O_6$ $(M+H)^+$ Calc. 763.66, Found 763.4.

Compound 106: To a solution of 105 (23.8 mg, 0.0240 mmol, calculated as 2TFA salt) in CH_2Cl_2 (1 mL) and Et_3N (0.050 mL, 0.360 mmol) was added a solution of BODIPY® 493/503 (10 mg, 0.0240 mmol, Life Technology #D2191) in CH_2Cl_2 (2 mL). The reaction mixture was stirred for 1 h. The reaction mixture was loaded onto silica gel column chromatography and eluted with 0-5% MeOH in CH_2Cl_2. The product color fractions were collected (5% MeOH in CH_2Cl_2, R_f=0.36) to give 106 (26 mg, 0.024 mmol, quantitatively).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.05 (s, 2H), 5.67-5.61 (m, 2H), 5.54-5.48 (m, 2H), 4.85-4.82 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 3.37-3.32 (m, 2H), 3.27-3.22 (m, 2H), 2.51-2.44 (m, 17H), 2.34-2.27 (m, 8H), 2.12-2.06 (m, 4H), 1.60-1.21 (m, 52H), 0.88 (t, J=6.8 Hz, 6H).

Molecular weight for $C_{62}H_{104}BF_2N_4O_7$ $(M+H)^+$ Calc. 1065.80, Found 1065.5.

Example 34: Multi-Ester Containing Lipids and Acetal Linked Lipids



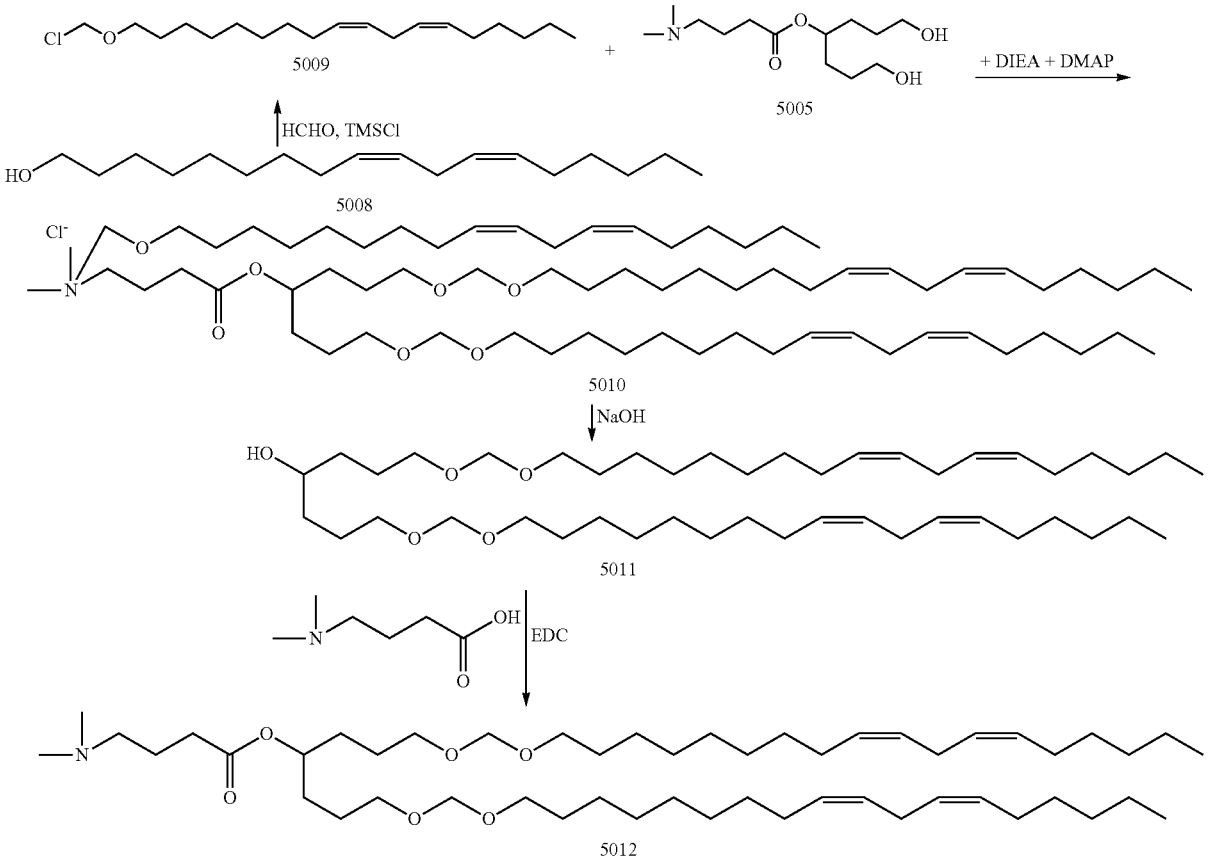
5000 5001 5002 5003 5004 5005 5005 5002 5006 5007

Synthesis of compound 5002: To a stirred solution of alcohol 5001 (1.0 g, 5.15 mmol), Glycolic anhydride 5000 (5.66 mmol) in DCM (20 mL) was added DMAP (1.26 g, 10.41 mmol) and stirred at room temperature for 48 h. The reaction mixture was concentrated followed by column purification gave the corresponding product 5002 (1.4 g, 86%) as DMAP salt. LCMS: Calculated: 316.22 (M^+), Found: 315.1 (M^+-1).

Synthesis of compound 5004: To a stirred solution of alcohol 5003 (5.0 g, 44.6 mmol), 4-(Dimethylamino)butyric acid hydrochloride (8.1 g, 48.3 mmol) and EDC (10.3 g, 53.6 mmol) in DCM (100 mL) was added DIEPA (23 g, 178.3 mmol) and stirred at room temperature overnight. After usual work up, the crude product was purified by column chromatography (9.0 g, 90%).

Synthesis of compound 5005: To a stirred solution of diene 5004 (4.0 g, 18 mmol) in 10 mL of THF was added 9-BBN and stirred overnight. To the above solution was added 6.6 mL of 3M NaOAc and 7.4 mL of 30% H_2O_2 at 0-5° C. The reaction mixture was stirred at room temperature overnight. After usual work up, the crude material was purified by column chromatography to get 5005 (2.6 g, 55%) as viscous oil. LCMS: Calculated: 261.19 (M^+), Found: 262.1 (M^++1).

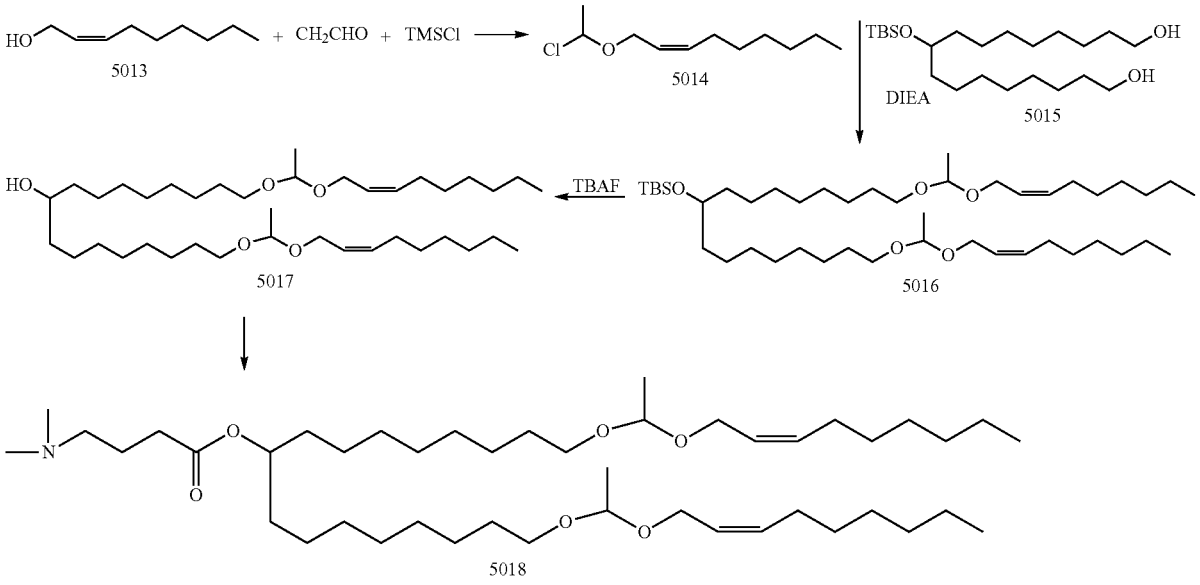
Synthesis of compound 5006 and 5007: To a stirred solution of diene 5005 (260 mg, 1 mmol), acid 5002 (1.0 g, 2.28 mmol), EDC (387 mg, 2 mmol) in 10 mL of DCM was added DIEA (516 mg, 4 mmol) and stirred overnight. After usual work up, the crude material was purified by column chromatography to get 5006 (0.1 g, 12%) and 5007 (0.2 g, 36%). LCMS for compound 5006: Calculated: 857.62 (M^+), Found: 858.5 (M^{30}+1), 880.5 (M^++Na). LCMS for compound 5007: Calculated: 559.4 (M^+), Found: 560.4 (M^{30}+1).



Synthesis of compound 5011: To a stirred solution of alcohol 5008 (2.66 g 10 mmol) in 5 mL of Chlorotrimethylsilane was added paraformaldehyde (0.3 g, 10 mmol) and stirred at room temperature overnight. The excess Chlorotrimethylsilane was evaporated followed by drying under reduced pressure gave the corresponding product 5009 and used for next step without purification. The compound 5009 was added dropwise to the solution of diol (261 mg, 1 mmol), DIEA (2.5 g, 19.4 mmol) and DMAP (20 mg, 0.16 mmol) in DCM (10 mL) and stirred overnight. Concentration of the solvent gave the crude product 5010, which was dissolved in 5 mL of THF and 2 mL of 1N NaOH was added and stirred for 2 days at room temperature. After usual work up, the crude material was purified by column chromatography to get the corresponding product 5011 (200 mg, 28%). LCMS for compound 5010: Calculated: 1131.95 (M^+), Found: 1096.98 (M^+-Cl^-). LCMS for compound 5011: Calculated: 704.63 (M^+), Found: 727.5 (M^++Na).

Synthesis of compound 5012: To a stirred solution of alcohol 5011 (200 mg, 0.284 mmol), 4-(Dimethylamino) butyric acid hydrochloride (103 mg, 0.57 mmol), EDC (109 mg, 0.57 mmol) in 10 mL of DCM was added DIEA (294 mg, 4 mmol) and stirred overnight. After usual work up, the crude material was purified by column chromatography to get 5012 (190 mg, 85%). LCMS for compound 5012: Calculated: 817.72 (M^+), Found: 818.5 (M^++Na).

519 520



Synthesis of compound 5016: To a stirred solution of alcohol 5013 (1.0 g 7.03 mmol) in 5 mL of Chlorotrimethylsilane was added acetaldehyde (0.3 g, 7.03 mmol) and stirred at room temperature for 2 h. The excess Chlorotrimethylsilane was evaporated followed by drying under reduced pressure gave the corresponding product 5014 and used for next step without purification. The compound 5014 was added dropwise to the solution of diol 5015 (223 mg, 0.55 mmol), DIEA (2 mL g, 11.5 mmol) and DMAP (20 mg, 0.16 mmol) in DCM (10 mL) and stirred overnight. 10 mL of water was added followed by extraction with DCM (3×30 mL), washed with water, saturated $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was used for the next step without purification. LCMS for compound 5016: Calculated: 738.66 (M^+), Found: 761.5 (M^++Na).

Synthesis of compound 5017: To a stirred solution of alcohol 5016 in 5 mL of THF was added 0.54 mL of 1M TBAF in THF (0.54 mmol) and stirred for 2 days at room temperature. After usual work up, the crude material was purified by column chromatography to get 5017. However, it contains some inseparable impurity and hence used for next step without further purification. LCMS for compound 5017: Calculated: 624.57 (M^+), Found: 647.5 (M^++Na).

Synthesis of compound 5018: To a stirred solution of alcohol 5017 (0.55 mmol), 4-(Dimethylamino)butyric acid hydrochloride (116 mg, 0.64 mmol), EDC (123 mg, 0.64 mmol) in 10 mL of DCM was added DIEA (165 mg, 1.28 mmol) and stirred for 2 days. After usual work up, the crude material is purified by column chromatography (0-10% MeOH in 1% Et_3N containing DCM) to get 5018 (300 mg, 75% from 5015). LCMS for compound 5018: Calculated: 737.65 (M^+), Found: 738.6 (M^{30}+1), 760.5 (M^++Na^+).

Example 35: Preparation of Lipid Nanoparticles

The cationic lipids described herein are used to formulate liposomes containing the AD-1661 duplex (shown in the table below) using an in-line mixing method as described in International Publication No. WO 2010/088537, which is incorporated by reference in its entirety. The lipid nanoparticles had the formulation shown in the table below.

Component	Mole Percentage (Based on 100% of the lipid components in the LNP)
Cationic lipid	50%
Distearoylphosphatidylcholine (DSPC)	10%
Cholesterol	38.5%
1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) (with an average PEG molecular weight of 2000)	1.5%
siRNA (AD-1661)	—

The siRNA AD-1661 duplex has the sequence shown below.

Duplex	Sequence 5'-3'	SEQ ID NO:	Target
AD-1661	GGAfUfCAfUfCfUfCAAGfUfCfUfUAfCdTsdT	1	FVII
	GfUAAGAfCfUfUGAGAfUGAfUfCfCdTsdT	2	

Lower case is 2'OMe modification and Nf is a 2'F modified nucleobase, dT is deoxythymidine, s is phosphothioate

The lipid nanoparticles was prepared as follows. Cationic lipid, DSPC, cholesterol, and PEG-DMG in the ratio recited in the table above were solubilized in ethanol at a total lipid concentration of 25 mg/mL.

A siRNA stock solution was prepared by solubilizing the siRNA AD-1661 in a low pH acetate or citrate buffer (pH=4) at 0.8 mg/mL.

The stock solutions should be completely clear and the lipids should be completely solubilized before combining with the siRNA. Therefore, if it was determined appropriate, the stock solutions were heated to completely solubilize the lipids.

The individual stock solutions were combined by pumping each solution to a T-junction (i.e., by in-line mixing).

Specifically, the ethanol solution (at 5 ml/min, via 0.01 in. PEEK tube) and aqueous buffer solution (at 15 mL/min, via 0.02 in. PEEK tube) were mixed through a T-junction (PEEK Tee body, IDEX).

After the T-junction a single tubing is placed where the combined stream will emit. Ethanol is removed and exchanged for PBS by dialysis. The lipid formulations are then concentrated using centrifugation or diafiltration to an appropriate working concentration.

Lipid nanoparticles containing the cationic lipids listed in the table in Example 36 were prepared as described above.

Example 36: Efficacy of Lipid Nanoparticles

Factor VII (FVII), a prominent protein in the coagulation cascade, is synthesized in the liver (hepatocytes) and secreted into the plasma. FVII levels in plasma can be determined by a simple, plate-based colorimetric assay. As such, FVII represents a convenient model for determining siRNA-mediated downregulation of hepatocyte-derived proteins.

Test formulations of the lipid nanoparticles prepared in Example 35 were initially assessed for their FVII knockdown in female 7-9 week old, 15-25 g, female C57Bl/6 mice at 0.1, 0.3, 1.0 and 5.0 mg/kg with 3 mice per treatment group. All studies included animals receiving either phosphate-buffered saline (PBS, control group) or a benchmark formulation. Formulations were diluted to the appropriate concentration in PBS immediately prior to testing. Mice were weighed and the appropriate dosing volumes calculated (10 μl/g body weight). Test and benchmark formulations as well as PBS (for control animals) were administered intravenously via the lateral tail vein. Animals were anesthetised 24 hours later with an intraperitoneal injection of ketamine/xylazine and 500-700 μl of blood was collected by cardiac puncture into serum separator tubes (BD Microtainer). Blood was centrifuged at 2,000×g for 10 minutes at 15° C. and serum was collected and stored at −70° C. until analysis. Serum samples were thawed at 37° C. for 30 minutes, diluted in PBS and aliquoted into 96-well assay plates. Factor VII levels were assessed using a chromogenic assay (Biophen FVII kit, Hyphen BioMed) according to the manufacturer's instructions and absorbance was measured in a microplate reader equipped with a 405 nm wavelength filter. Plasma FVII levels were quantified and ED_{50} values (dose resulting in a 50% reduction in plasma FVII levels compared to control animals) were calculated using a standard curve generated from a pooled sample of serum from control animals. Those formulations of interest showing high levels of FVII knockdown (ED_{50}<<0.1 mg/kg) were re-tested in independent studies at a lower dose range to confirm potency and establish ED_{50} levels.

The following table shows ED_{50} values for some of the cationic lipids described herein. Two asterisks (**) indicates an ED_{50} value between 0.001 and 0.10. One asterisk (*) indicates an ED_{50} value greater than 0.10.

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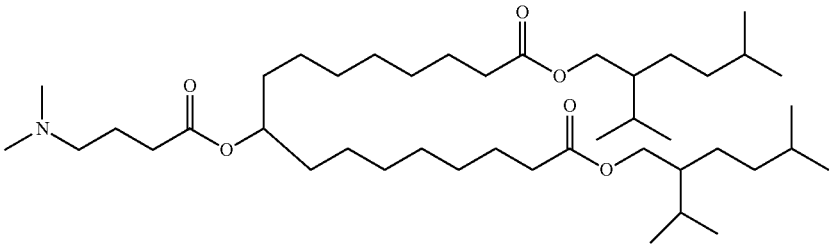
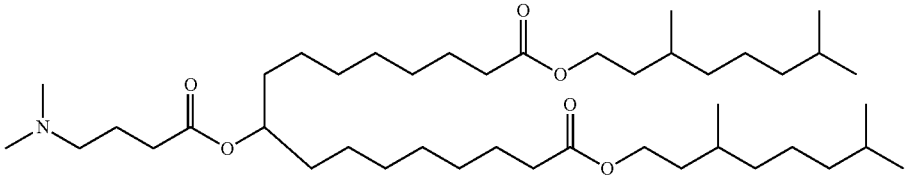
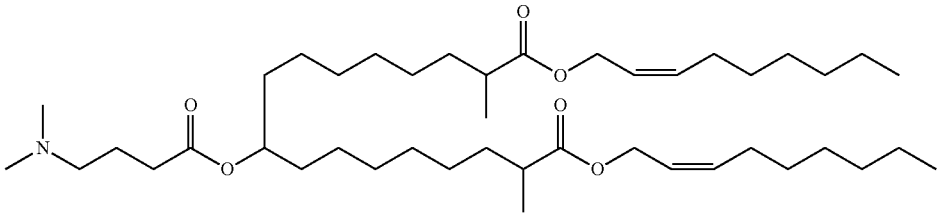
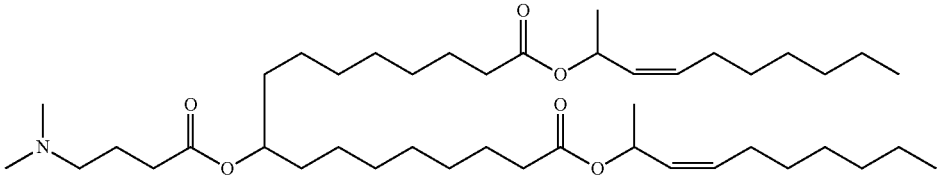
**	
**	
**	
**	

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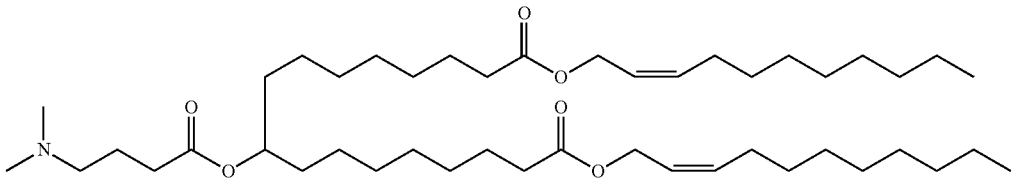
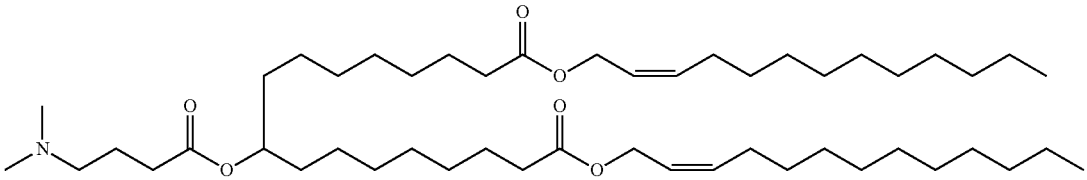
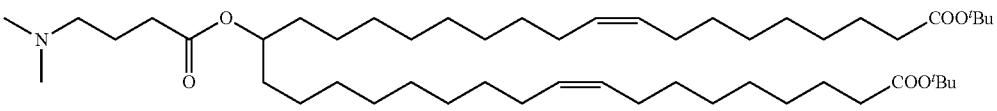
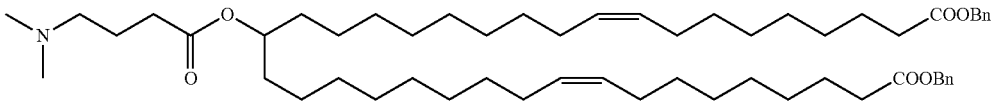
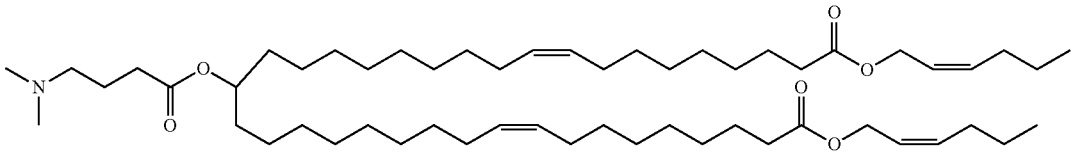
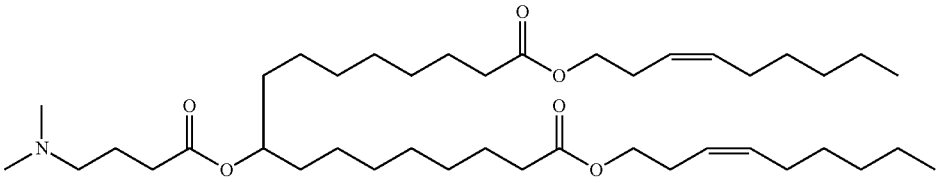
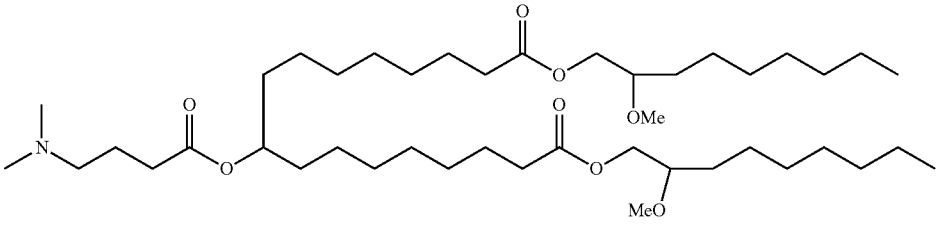
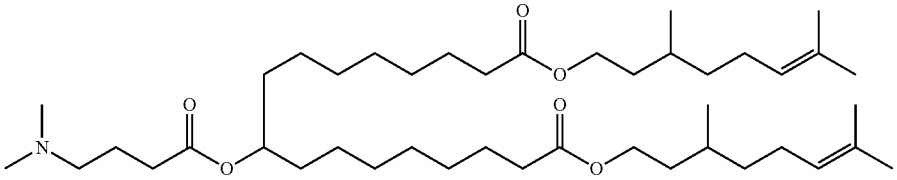
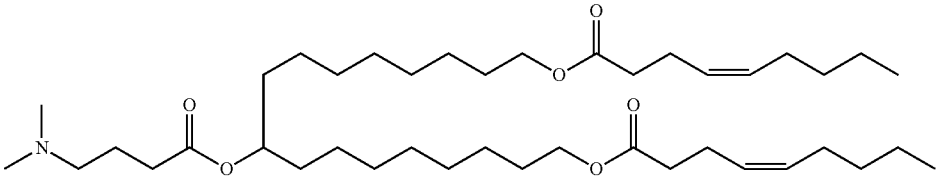
**	
**	
**	
**	
*	
**	
*	
**	
**	

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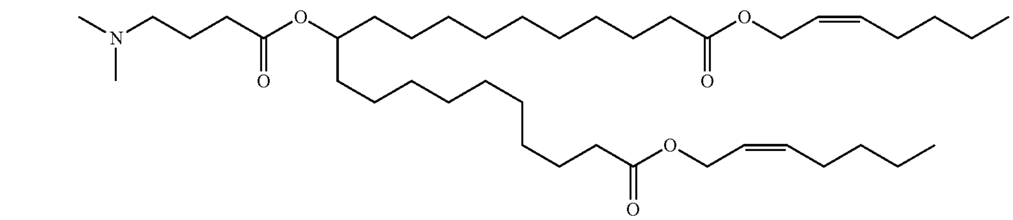
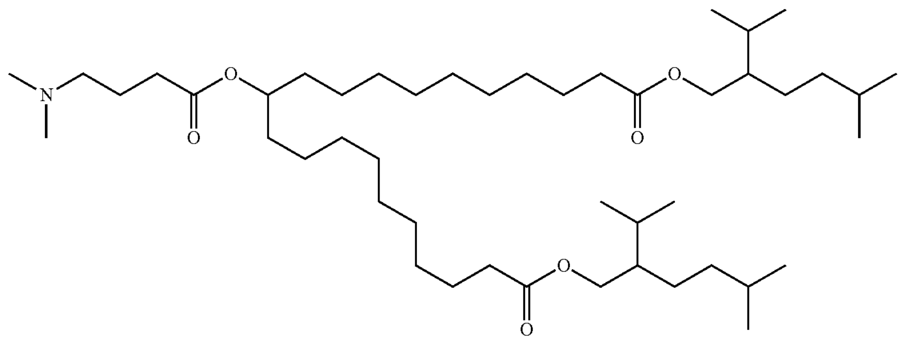
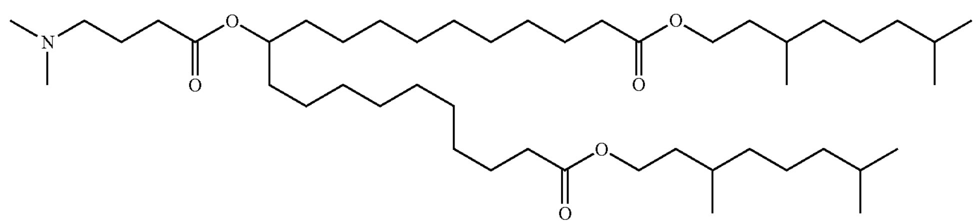
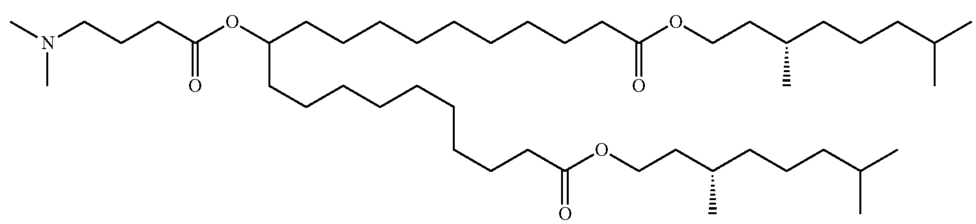
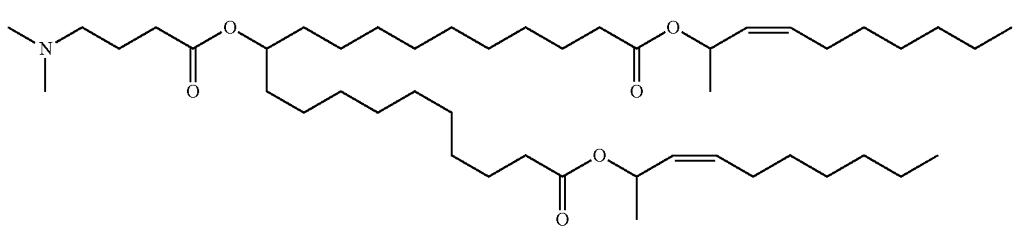
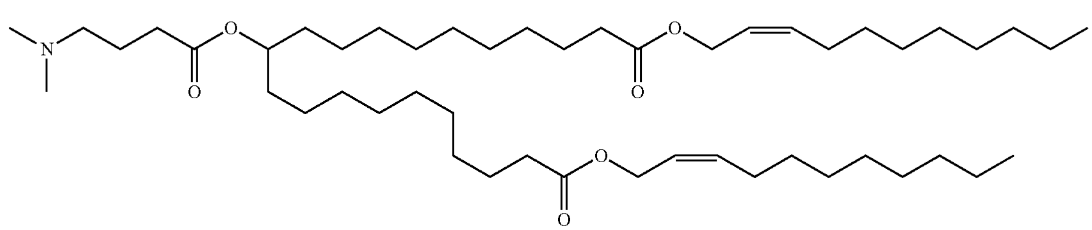
**	
**	
**	
**	
**	
**	

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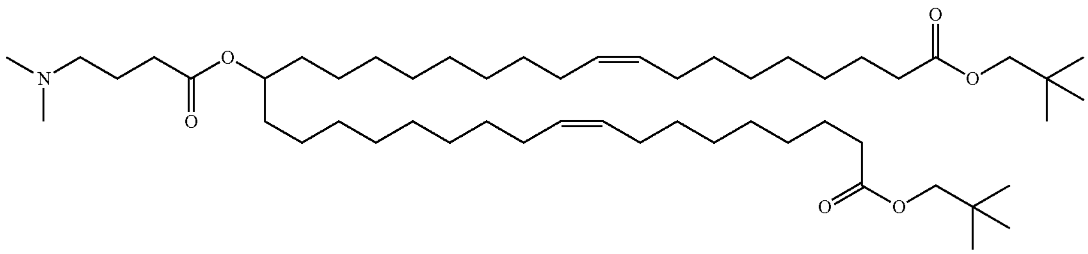
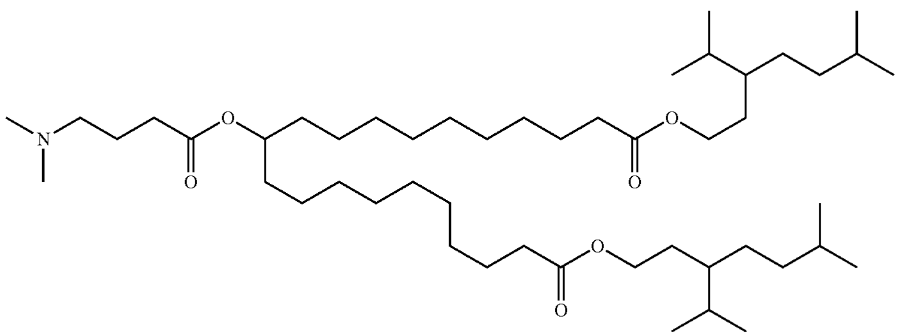
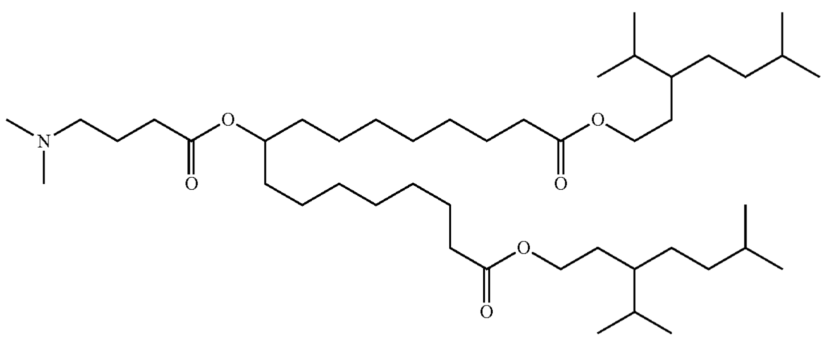
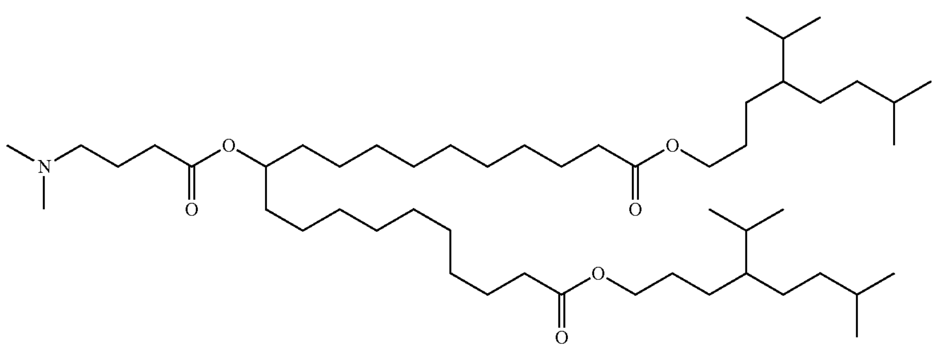
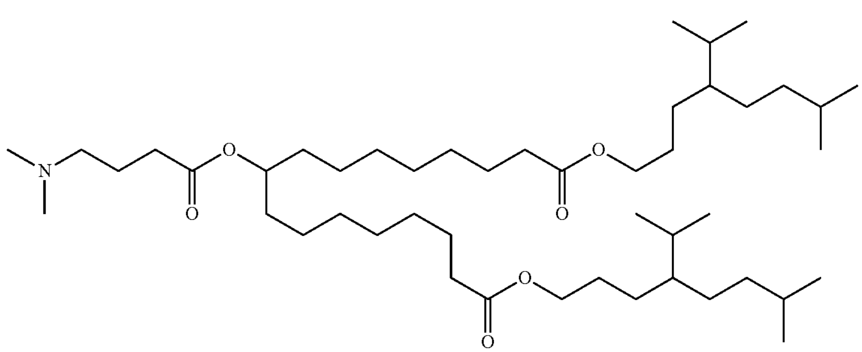
**	
**	
**	
**	
**	

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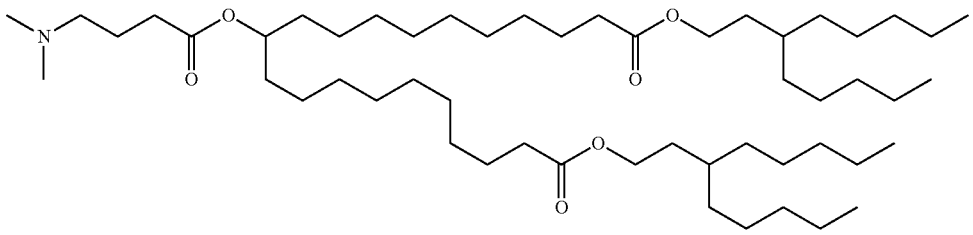
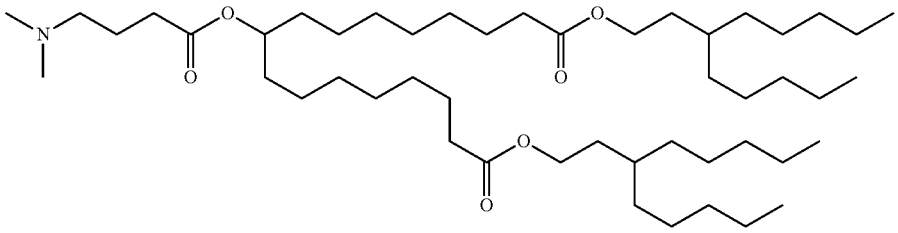
**	
**	

Example 37: Hydrophobicity and Stability

The log P values for the biodegrabable cationic lipids listed in the table below were calculated using the software available at http://www.molinspiration.com/services/logp.html from Molinspiration Cheminformatics of Slovensky Grob, Slovak Republic.

Furthermore, the HPLC retention time for each biodegradable cationic lipid was measured in lipid nanoparticles prepared from them. The lipid nanoparticles were prepared as described in Example 35 using AD-1661 as the payload. The retention times are reported in the table below relative to the retention time for cholesterol.

The HPLC buffer used was a mixture of two solutions (Solution #1 and Solution #2).

Solution #1: 80% methanol/20% 10 mM NH_4HCO_3

Solution #2: 80% methanol/20% isopropanol

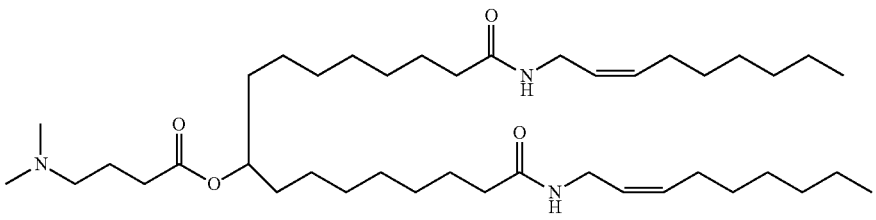
The ratios of the two solutions in the mixture changed over time as indicated in the table below.

Time (mm)	Solution #1 (vol %)	Solution #2 (vol %)
0	70	30
4	10	90
6	10	90
6.1	70	30
8	70	30

The size of the lipid nanoparticles was measured before and after undergoing dialysis overnight. In general, greater changes in lipid nanoparticle size are indicative of lesser stability.

Dynamic laser light scattering was used to determine the lipid nanoparticle size (expressed as the intensity weighted diameter) with a Zetasizer (Malvern Instruments, Inc. of Westborough, Mass.). All measurements were made at 532 nm wavelength at the scattering angle of 173° using normal resolution mode as the analysis model.

The results of these experiments are provided in the table below.

Cationic Lipid	logP	t(lipid) – t(chol)	LNPs Size (nm) change
	9.647	−1.4	170 -> 260

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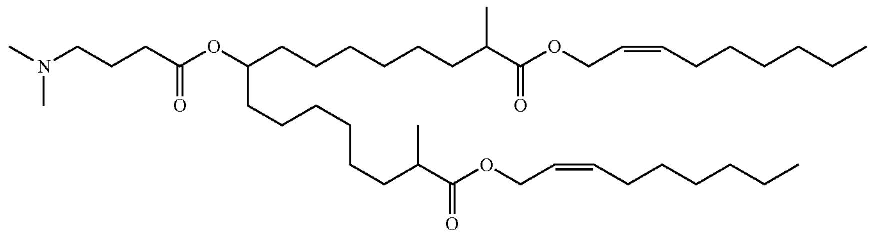
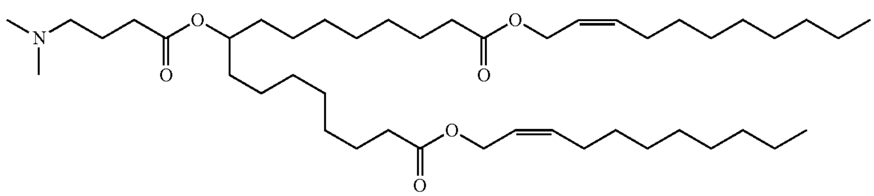
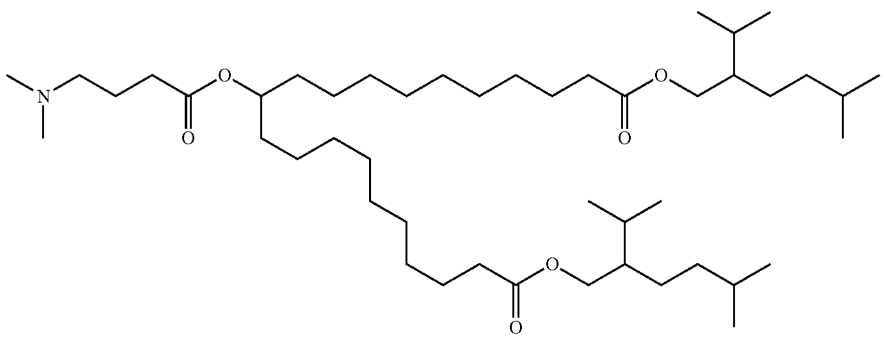
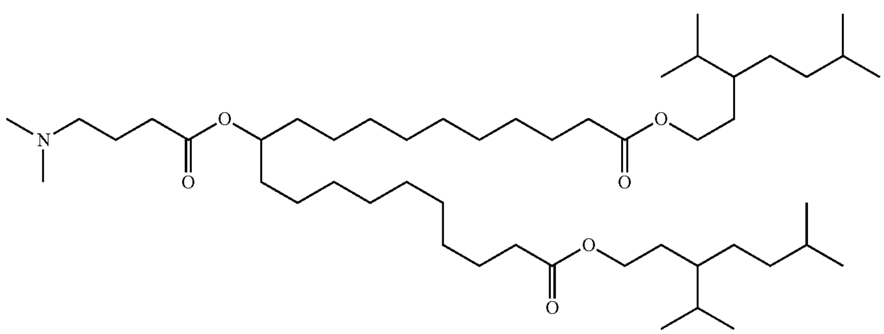
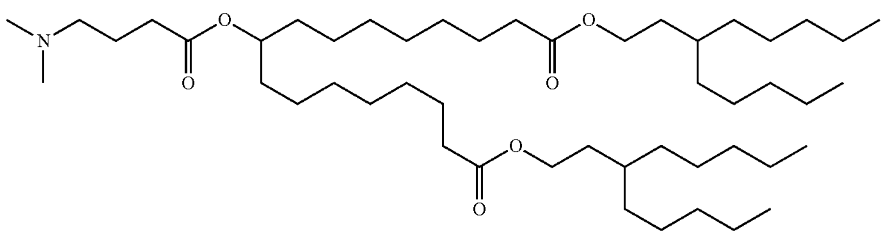
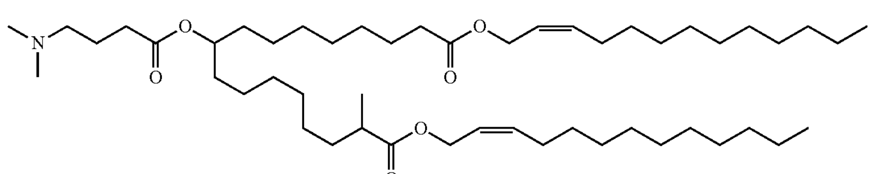
Cationic Lipid	logP	t(lipid) − t(chol)	LNPs Size (nm) change
	9.972	0.848	73 -> 77
	10.093	1.44	60 -> 67
	10.201	1.751	59 -> 60
	10.259	2.106	
	10.313	2.365	56 -> 56
	10.315	2.219	68 -> 67

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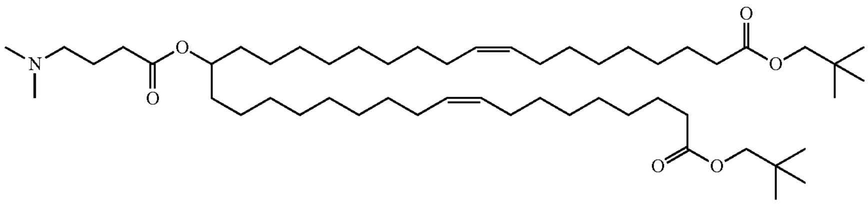
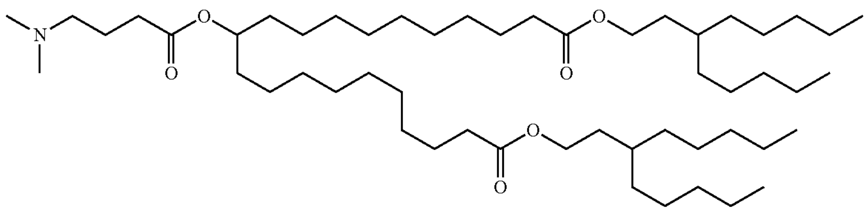
Cationic Lipid	logP	t(lipid) − t(chol)	LNPs Size (nm) change
	10.416	2.707	
	10.495	3.178	

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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Case: 23-2357 Document: 1 Page: 368 Filed: 12/04/2023

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What is claimed is:

1. A cationic lipid comprising a primary group and two biodegradable hydrophobic tails, wherein (a) the primary group includes a head group and a central moiety to which both the biodegradable hydrophobic tails and the head group are directly bonded, wherein the primary group has a protonatable group having a pK_a of from about 4 to about 11, (b) the cationic lipid has an in vivo half life ($t_{1/2}$) of less than about 3 hours in the liver, (c) the cationic lipid has a logP value of at least 10.1, and (d) each biodegradable hydrophobic tail has the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain),

wherein in at least one biodegradable hydrophobic tail,

(i) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl group, where the branching occurs at the α-position relative to the biodegradable group;

(ii) the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms;

(iii) the at least one biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the biodegradable group, and R^{13} is a branched C_{10}-C_{20} alkyl; and

(iv) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26.

2. The cationic lipid of claim **1**, wherein the central moiety is selected from the group consisting of a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central heterocyclic group, and a central heteroaryl group.

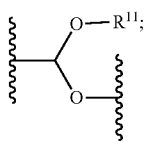
3. The cationic lipid of claim **1**, wherein the biodegradable group is —OC(O)—.

4. The cationic lipid of claim **1**, wherein the biodegradable group is —C(O)O—.

5. The cationic lipid of claim **1**, wherein the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms from the first carbon atom after the primary group to a terminus of the tail.

6. The cationic lipid of claim **1**, wherein

each biodegradable group is independently selected from the group consisting of —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



each occurrence of R^3 and R^4 is, independently, H, halogen, OH, alkyl, alkoxy, —NH_2, R^{10}, alkylamino, or dialkylamino;

each occurrence of R^5 is, independently, H or alkyl;

each occurrence of R^{10} is, independently, selected from polyethylene glycol (PEG) and polymers based on

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poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly [N-(2-hydroxypropyl)methacrylamide] and poly (amino acid)s, wherein (i) the PEG or polymer is linear or branched, (ii) the PEG or polymer is polymerized by n subunits, (iii) n is a number-averaged degree of polymerization between 10 and 200 units and (iv) the compound of formula has at most two R^{10} groups; and

R^{11} is a C_2-C_8 alkyl or C_2-C_8 alkenyl.

7. The cationic lipid of claim **1**, wherein in both biodegradable hydrophobic tails,

(i) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl group, where the branching occurs at the α-position relative to the biodegradable group;

(ii) the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms;

(iii) the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the biodegradable group, and R^{13} is a branched C_{10}-C_{20} alkyl; and

(iv) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26.

8. The cationic lipid of claim **1**, wherein the cationic lipid has a logP value of at least about 10.2.

9. The cationic lipid of claim **1**, wherein the cationic lipid has a t_{lipid}–t_{chol} value of at least about 1.75.

10. The cationic lipid of claim **1**, wherein the cationic lipid has a pKa value of from about 4 to about 7.

11. The cationic lipid of claim **10**, wherein the cationic lipid has a pKa value of from about 6 to about 6.5.

12. The cationic lipid of claim **1**, wherein the cationic lipid has a logP value of at least about 10.1 and/or a t_{lipid}–t_{chol} value of at least about 1.4.

13. A cationic lipid comprising (i) a head group, (ii) two hydrophobic tails, each of the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), and (iii) a linker group bound to the head group and the hydrophobic tails, wherein the cationic lipid has:

(i) a log P value of at least 10.1;

(ii) a pKa of from about 4 to about 7;

(iii) for at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms;

(iv) for at least one hydrophobic tail, the total number of carbon atoms in the hydrophobic tail is from 21 to 26;

(v) for at least one hydrophobic tail, the number of carbon atoms between the linker group and the biodegradable group in the hydrophobic tail ranges from about 5 to about 10;

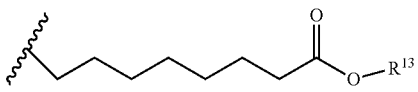
(vi) for at least one hydrophobic tail, the total number of carbon atoms between the linker group and a terminus of the hydrophobic tail is from about 15 to about 20;

(vii) for at least one hydrophobic tail, the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl or branched alkenyl group; and

(viii) when formulated as a lipid nanoparticle, the cationic lipid has an in vivo half life ($t_{1/2}$) in the liver of less than about 3 hours.

14. The cationic lipid of claim **1**, wherein the branched alkyl group has only one carbon atom which is bound to three other carbon atoms.

15. The cationic lipid of claim **1**, wherein the at least one biodegradable hydrophobic tail has the formula



where R^{13} is a branched alkyl group having from 13 to 17 carbon atoms, and the total carbon length of the tail from the first carbon to a terminus of the tail is at most 20.

16. A method of delivering a nucleic acid molecule comprising administering to a subject a lipid particle comprising:

(i) a nucleic acid molecule,

(ii) a cationic lipid according to claim **1**, and

(iii) a PEG lipid.

17. The method of claim **16**, wherein the pKa of the cationic lipid is 6.0 to 7.0.

18. A cationic lipid comprising a primary group and two biodegradable hydrophobic tails, wherein

the primary group comprises (i) a head group that optionally comprises a primary, secondary, or tertiary amine, and (ii) a central moiety to which the head group and the two biodegradable hydrophobic tails are directly bonded;

the central moiety is a central carbon or nitrogen atom;

each biodegradable hydrophobic tail independently has the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), wherein the biodegradable group is —OC(O)— or —C(O)O—;

for at least one biodegradable hydrophobic tail, the terminal hydrophobic chain in the biodegradable hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the biodegradable group and the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the biodegradable group, R^{13} is a branched C_{10}-C_{20} alkyl, and the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26;

in at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms; and

the lipid has a pKa in the range of about 4 to about 11 and a logP of at least 10.1.

19. The cationic lipid of claim **18**, wherein the biodegradable group is —OC(O)—.

20. The cationic lipid of claim **18**, wherein the biodegradable group is —C(O)O—.

21. The cationic lipid of claim **18**, wherein both biodegradable hydrophobic tails have the formula —R^{12}-M^1-R^{13}.

22. The cationic lipid of claim **18**, wherein the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms from the first atom after the central moiety to a terminus of the tail.

23. The cationic lipid of claim **18**, wherein the lipid has a pKa of from about 5 to about 7 when incorporated into a lipid particle.

24. The cationic lipid of claim **18**, wherein, in at least one hydrophobic tail, the number of carbon atoms between the central moiety and the biodegradable group in the hydrophobic tail ranges from 5 to 10.

25. The cationic lipid of claim **18**, wherein, in at least one hydrophobic tail, the total number of carbon atoms between the central moiety and a terminus of the hydrophobic tail ranges from 15 to 20.

26. The cationic lipid of claim **18**, wherein in at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by from 8 to 12 carbon atoms.

27. The cationic lipid of claim **26**, wherein in at least one hydrophobic tail, the biodegradable group is separated from a terminus of the hydrophobic tail by 8 carbon atoms.

28. The cationic lipid of claim **18**, wherein in both biodegradable hydrophobic tails,

(i) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl group, where the branching occurs at the α-position relative to the biodegradable group;

(ii) the biodegradable group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms;

(iii) the biodegradable hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the biodegradable group, and R^{13} is a branched C_{10}-C_{20} alkyl; and

(iv) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26.

* * * * *

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(12) **United States Patent**
Maier et al.

(10) **Patent No.: US 11,382,979 B2**
(45) **Date of Patent: *Jul. 12, 2022**

(54) **BIODEGRADABLE LIPIDS FOR THE DELIVERY OF ACTIVE AGENTS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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C07F 5/02	(2006.01)
C07C 327/32	(2006.01)
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C07C 229/12	(2006.01)
C07C 327/28	(2006.01)
C07C 31/125	(2006.01)
A61K 31/713	(2006.01)
C07C 323/58	(2006.01)
C07C 251/38	(2006.01)
C07C 211/10	(2006.01)
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C07D 317/30	(2006.01)
C07C 211/09	(2006.01)
C07C 217/08	(2006.01)
C07D 295/14	(2006.01)
A61K 31/7088	(2006.01)

(Continued)

(52) **U.S. Cl.**

CPC ***A61K 47/18*** (2013.01); ***A61K 9/1272*** (2013.01); ***A61K 9/5123*** (2013.01); ***A61K 31/7088*** (2013.01); ***A61K 31/713*** (2013.01); ***A61K 31/7105*** (2013.01); ***C07C 31/125*** (2013.01); ***C07C 211/09*** (2013.01); ***C07C 211/10*** (2013.01); ***C07C 211/11*** (2013.01); ***C07C 217/08*** (2013.01); ***C07C 229/12*** (2013.01); ***C07C 235/06*** (2013.01); ***C07C 251/38*** (2013.01); ***C07C 323/12*** (2013.01); ***C07C 323/58*** (2013.01); ***C07C 327/22*** (2013.01); ***C07C 327/28*** (2013.01); ***C07C 327/32*** (2013.01); ***C07D 207/32*** (2013.01); ***C07D 233/54*** (2013.01); ***C07D 295/08*** (2013.01); ***C07D 295/12*** (2013.01); ***C07D 295/14*** (2013.01); ***C07D 317/30*** (2013.01); ***C07F 5/022*** (2013.01)

(58) **Field of Classification Search**

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See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a cationic lipid having one or more biodegradable groups located in a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid. These cationic lipids may be incorporated into a lipid particle for delivering an active agent, such as a nucleic acid. The invention also relates to lipid particles comprising a neutral lipid, a lipid capable of reducing aggregation, a cationic lipid of the present invention, and optionally, a sterol. The lipid particle may further include a therapeutic agent such as a nucleic acid.

30 Claims, No Drawings

Specification includes a Sequence Listing.

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Case 23-2357 Document: 14 Page: 372 Filed: 12/04/2023

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continuation of application No. 16/520,183, filed on Jul. 23, 2019, now Pat. No. 11,071,784, which is a continuation of application No. 14/677,801, filed on Apr. 2, 2015, now Pat. No. 10,369,226, which is a continuation of application No. 13/708,383, filed on Dec. 7, 2012, now Pat. No. 9,061,063.

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(51) **Int. Cl.**

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C07D 295/12	(2006.01)
A61K 31/7105	(2006.01)

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BIODEGRADABLE LIPIDS FOR THE DELIVERY OF ACTIVE AGENTS

This application is a continuation of U.S. patent application Ser. No. 17/302,311, filed Apr. 29, 2021, which is a continuation of Ser. No. 16/520,183, filed Jul. 23, 2019, now U.S. Pat. No. 11,071,784, which is a continuation of U.S. patent application Ser. No. 14/677,801, filed Apr. 2, 2015, now U.S. Pat. No. 10,369,226, which is a continuation of U.S. patent application Ser. No. 13/708,383, filed Dec. 7, 2012, now U.S. Pat. No. 9,061,063, which claims the benefit of U.S. Provisional Application No. 61/568,133, filed Dec. 7, 2011, and U.S. Provisional Application No. 61/623,274, filed Apr. 12, 2012, each of which is hereby incorporated by reference.

TECHNICAL FIELD

The present invention relates to biodegradable lipids and to their use for the delivery of active agents such as nucleic acids.

BACKGROUND

Therapeutic nucleic acids include, e.g., small interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, immune stimulating nucleic acids, antisense, antagomir, antimir, microRNA mimic, supermir, U1 adaptor, and aptamer. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both in vitro and in vivo models. In addition, siRNA constructs are currently being evaluated in clinical studies.

However, two problems currently faced by siRNA or miRNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind the protein RISC when administered systemically as the free siRNA or miRNA. Lipid nanoparticles formed from cationic lipids with other lipid components, such as cholesterol and PEG lipids, and oligonucleotides (such as siRNA and miRNA) have been used to facilitate the cellular uptake of the oligonucleotides.

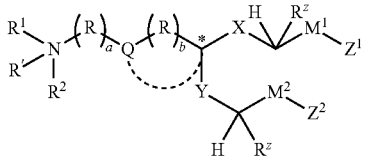
There remains a need for improved cationic lipids and lipid nanoparticles for the delivery of oligonucleotides. Preferably, these lipid nanoparticles would provide high drug:lipid ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to the patient.

SUMMARY

The present invention relates to a cationic lipid and PEG lipid suitable for forming nucleic acid-lipid particles. Each of the cationic and PEG lipids of the present invention includes one or more biodegradable groups. The biodegradable groups are located in a lipidic moiety (e.g., a hydrophobic chain) of the cationic or PEG lipid. These cationic and PEG lipids may be incorporated into a lipid particle for delivering an active agent, such as a nucleic acid (e.g., an siRNA). The incorporation of the biodegradable group(s) into the lipid results in faster metabolism and removal of the lipid from the body following delivery of the active agent to a target area. As a result, these lipids have lower toxicity than similar lipids without the biodegradable groups.

In one embodiment, the cationic lipid is a compound of formula (I), which has a branched alkyl at the alpha position adjacent to the biodegradable group (between the biodegradable group and the tertiary carbon):

Formula (I)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl);

with respect to R^1 and R^2,

(i) R^1 and R^2 are each, independently, optionally substituted alkyl, alkenyl, alkynyl, cycloalkylalkyl, heterocycle, or R^{10};

(ii) R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocylic ring; or

(iii) one of R^1 and R^2 is optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, or heterocycle, and the other forms a 4-10 member heterocyclic ring or heteroaryl (e.g., a 6-member ring) with (a) the adjacent nitrogen atom and (b) the $(R)_a$ group adjacent to the nitrogen atom;

each occurrence of R is, independently, $—(CR^3R^4)—$;

each occurrence of R^3 and R^4 are, independently H, halogen, OH, alkyl, alkoxy, $—NH_2$, R^{10}, alkylamino, or dialkylamino (in one preferred embodiment, each occurrence of R^3 and R^4 are, independently H or C_1-C_4 alkyl);

each occurrence of R^{10} is independently selected from PEG and polymers based on poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein (i) the PEG or polymer is linear or branched, (ii) the PEG or polymer is polymerized by n subunits, (iii) n is a number-averaged degree of polymerization between 10 and 200 units, and (iv) wherein the compound of formula has at most two R^{10} groups (preferably at most one R^{10} group);

the dashed line to Q is absent or a bond;

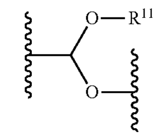
when the dashed line to Q is absent then Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —C(O)N(R^4)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —O—N=C(R^5)—, —C(R^5)=N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)=N—O—C(O)—; or

when the dashed line to Q is a bond then (i) b is 0 and (ii) Q and the tertiary carbon adjacent to it (C*) form a substituted or unsubstituted, mono- or bi-cyclic heterocyclic group having from 5 to 10 ring atoms (e.g., the heteroatoms in the heterocyclic group are selected from O and S, preferably O);

each occurrence of R^5 is, independently, H or alkyl (e.g. C_1-C_4 alkyl);

X and Y are each, independently, alkylene or alkenylene (e.g., C_4 to C_{20} alkylene or C_4 to C_{20} alkenylene);

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



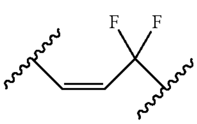
(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

each occurrence of R^z is, independently, C_1-C_8 alkyl (e.g., methyl, ethyl, isopropyl, n-butyl, n-pentyl, or n-hexyl);

a is 1, 2, 3, 4, 5 or 6;

b is 0, 1, 2, or 3; and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g., ).

The R'R^1R^2N—(R)$_a$-Q-(R)$_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—(R)$_a$-Q-(R)$_b$— is $(CH_3)_2$N—$(CH_2)_3$—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2$N—$(CH_2)_3$—C(CH_3)═N—O—.

In one embodiment, R^1 and R^2 are both alkyl (e.g., methyl).

In a further embodiment, a is 3. In another embodiment, b is 0.

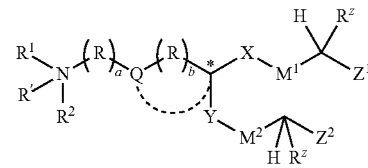
In a further embodiment, a is 3, b is 0 and R is —CH_2—. In yet a further embodiment, a is 3, b is 0, R is —CH_2— and Q is —C(O)O—. In another embodiment, R^1 and R^2 are methyl, a is 3, b is 0, R is —CH_2— and Q is —C(O)O—.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

In further embodiments, M^1 and M^2 are each, independently, —OC(O)— or —C(O)O—. For example, in one embodiment, M^1 and M^2 are each —C(O)O—.

In another embodiment, the cationic lipid is a compound of formula (II), which has a branched alkyl at the alpha position adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 o Z^2):

Formula (II)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl);

with respect to R^1 and R^2,

(i) R^1 and R^2 are each, independently, optionally substituted alkyl, alkenyl, alkynyl, cycloalkylalkyl, heterocycle, or R^{10};

(ii) R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocylic ring; or

(iii) one of R^1 and R^2 is optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, or heterocycle, and the other forms a 4-10 member heterocyclic ring or heteroaryl (e.g., a 6-member ring) with (a) the adjacent nitrogen atom and (b) the $(R)_a$ group adjacent to the nitrogen atom;

each occurrence of R is, independently, —(CR^3R^4)—;

each occurrence of R^3 and R^4 are, independently H, halogen, OH, alkyl, alkoxy, —NH_2, R^{10}, alkylamino, or dialkylamino (in one preferred embodiment, each occurrence of R^3 and R^4 are, independently H or C_1-C_4 alkyl);

each occurrence of R^{10} is independently selected from PEG and polymers based on poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein (i) the PEG or polymer is linear or branched, (ii) the PEG or polymer is polymerized by n subunits, (iii) n is a number-averaged degree of polymerization between 10 and 200 units, and (iv) wherein the compound of formula has at most two R^{10} groups (preferably at most one R^{10} group);

the dashed line to Q is absent or a bond;

when the dashed line to Q is absent then Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —C(O)N(R^4)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —O—N═C(R^5)—, —C(R^5)═N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)═N—O—C(O)—; or

when the dashed line to Q is a bond then (i) b is 0 and (ii) Q and the tertiary carbon adjacent to it (C*) form a substituted or unsubstituted, mono- or bi-cyclic heterocyclic group having from 5 to 10 ring atoms (e.g., the heteroatoms in the heterocyclic group are selected from O and S, preferably O);

each occurrence of R^5 is, independently, H or alkyl;

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group

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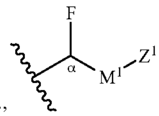
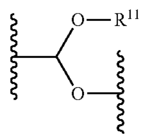


);

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi$(R^5)_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



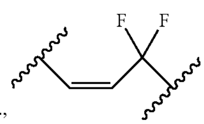
(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

each occurrence of R^z is, independently, C_1-C_8 alkyl (e.g., methyl, ethyl, isopropyl);

a is 1, 2, 3, 4, 5 or 6;

b is 0, 1, 2, or 3; and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein (i) the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g.,


)

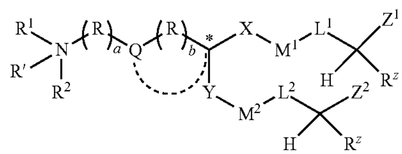
and (ii) the terminus of at least one of Z^1 and Z^2 is separated from the group M^1 or M^2 by at least 8 carbon atoms.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—C(CH_3)═N—O—.

In another embodiment, the cationic lipid is a compound of formula (III), which has a branching point at a position that is 2-6 carbon atoms (i.e., at the beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position (ζ)) adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (III)



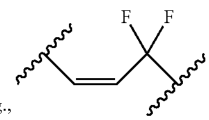
or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, R^z, a, and b are defined as in formula (I);

L^1 and L^2 are each, independently, C_1-C_5 alkylene or C_2-C_5 alkenylene;

X and Y are each, independently, alkylene (e.g., C_4 to C_{20} alkylene or C_6-C_8 alkylene) or alkenylene (e.g., C_4 to C_{20} alkenylene); and

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2

(e.g.,


),

and with the proviso that the terminus of at least one of Z^1 and Z^2 is separated from the group M^1 or M^2 by at least 8 carbon atoms.

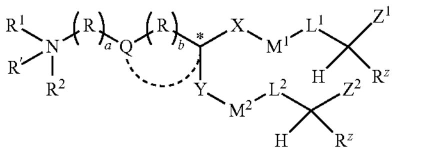
In one embodiment, L^1 and L^2 are each —CH_2—. In another embodiment, L^1 and L^2 are each —$(CH_2)_2$—. In one embodiment, L^1 and L^2 are each —$(CH_2)_3$—. In yet another embodiment, L^1 and L^2 are each —$(CH_2)_4$—. In yet another embodiment, L^1 and L^2 are each —$(CH_2)_5$—. In yet another embodiment, L^1 and L^2 are each —CH_2—CH═CH—. In a preferred embodiment, L^1 and L^2 are each —CH_2— or —$(CH_2)_2$.

In one embodiment, X and Y are each, independently —$(CH_2)_n$ wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_7$—. In another exemplary embodiment, X and Y are —$(CH_2)_8$—. In yet another exemplary embodiment, X and Y are —$(CH_2)_9$—.

The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—C(CH_3)═N—O—.

In another embodiment, the cationic lipid is a compound of formula (IIIA), which has a branching point at a position that is 2-6 carbon atoms (i.e., at the beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position (ζ) from the biodegradable groups M^1 and M^2 (i.e., between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (IIIA)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

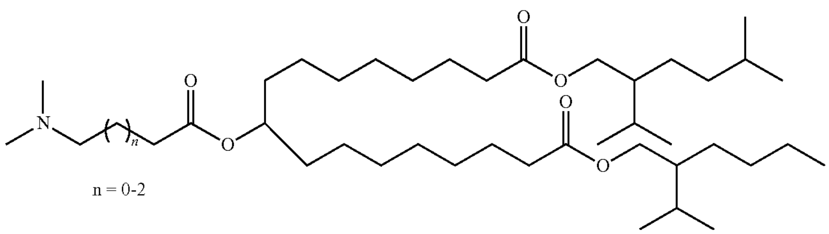
each R^z is, independently, C_1-C_8 alkyl (e.g., C_3-C_6 alkyl or C_2-C_3 alkyl);

L^1 and L^2 are each, independently, C_1-C_5 alkylene (e.g., C_2-C_3 alkylene) or C_2-C_5 alkenylene;

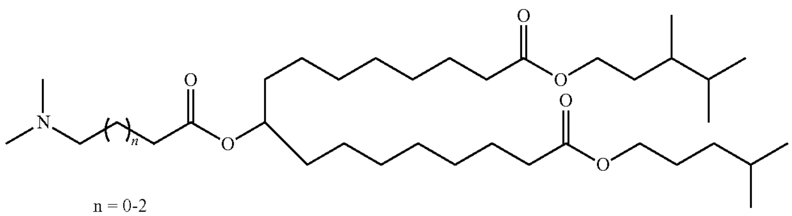
X and Y are each, independently, alkylene (e.g., C_4 to C_{20} alkylene or C_7-C_9 alkylene) or alkenylene (e.g., C_4 to C_{20} alkenylene or C_7-C_9 alkenylene); and

Z^1 and Z^2 are each, independently, C_1-C_8 alkyl (e.g., C_1-C_6 alkyl, such as C_1, C_3 or C_5 alkyl) or C_2-C_8 alkenyl (such as C_2-C_6 alkenyl);

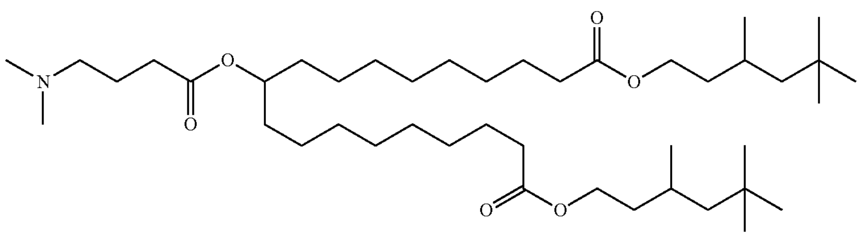
wherein said cationic lipid is not one selected from:

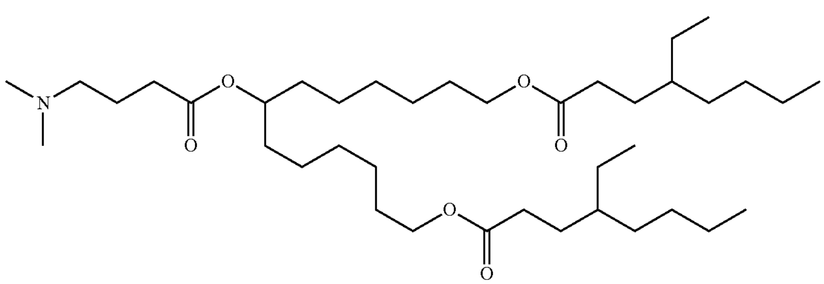


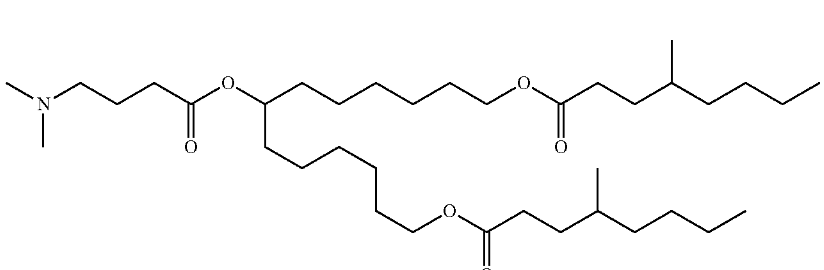
n = 0-2



n = 0-2







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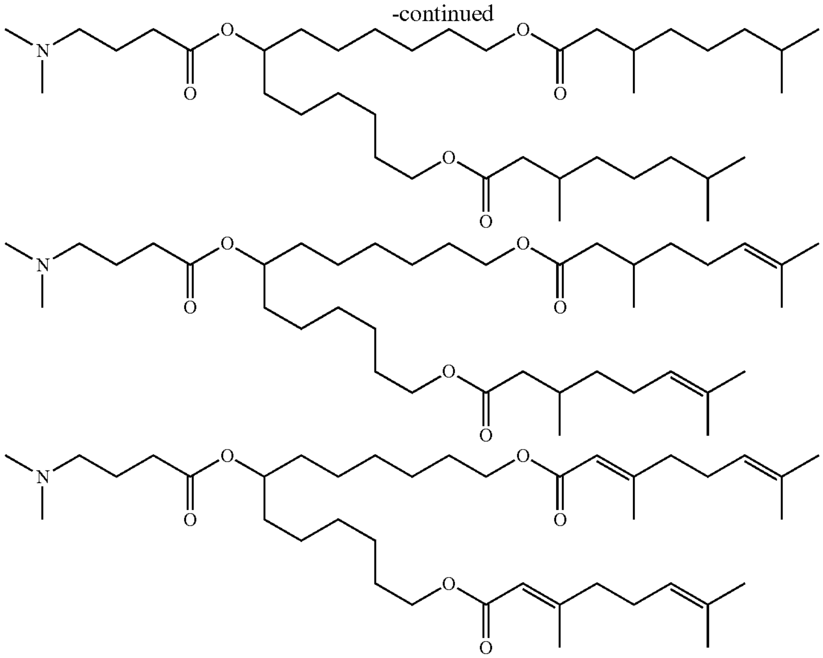


In one embodiment, L^1 and L^2 are each —$(CH_2)_2$—. In another embodiment, L^1 and L^2 are each —$(CH_2)_3$—.

In one embodiment, X and Y are each, independently —$(CH_2)_n$ wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, 4 to 12 or 7-9. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_7$—. In yet another exemplary embodiment, X and Y are —$(CH_2)_9$.

In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and L^2).

The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—$C(CH_3)$═N—O—.

In one preferred embodiment, Z^1 and Z^2 are branched alkyl or branched alkenyl groups.

In one embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 alkyl (such as a C_3-C_6 alkyl). In another embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 branched alkyl (such as a C_3-C_6 branched alkyl). In yet another embodiment of formula (IIIA), Z^1, Z^2, and each R^z are C_3-C_8 straight alkyl (such as a C_3-C_6 straight alkyl).

In one embodiment of formula (IIIA), the branching point is at the second position (the β-position) from the biodegradable groups M^1 and M^2 in each tail. Z^1, Z^2, and each R^z can be C_3-C_8 alkyl (e.g., a C_3-C_6 alkyl), such as a C_3-C_8 branched alkyl (e.g., a C_3-C_6 branched alkyl) or a C_3-C_8 straight alkyl (e.g., a C_3-C_6 straight alkyl). In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and/or L^2).

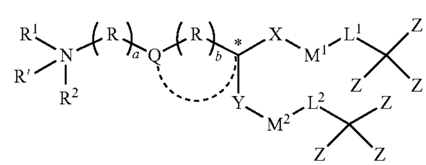
In one embodiment of formula (IIIA), the branching point is at the third position (the γ-position) from the biodegradable groups M^1 and M^2 in each tail. Z^1, Z^2, and each R^z can be C_3-C_8 alkyl (e.g., a C_3-C_6 alkyl), such as a C_3-C_8 branched alkyl (e.g., a C_3-C_6 branched alkyl) or a C_3-C_8 straight alkyl (e.g., a C_3-C_6 straight alkyl). In one preferred embodiment, M^1 and M^2 are —C(O)O— (where the carbonyl group in M^1 and M^2 is bound to the variable X, and the oxygen atom in M^1 and M^2 is bound to the variable L^1 and/or L^2).

In one embodiment of formula (IIIA), the branching point is at the third position (the γ-position) from the biodegradable groups M^1 and M^2 in each tail.

In another embodiment of formula (IIIA), M^1 and/or M^2 are not —O(C(O)— (where the oxygen atom in M^1 and/or M^2 is bound to the variable X, and the carbonyl in M^1 and/or M^2 is bound to the variable L^1 and/or L^2). In yet another embodiment of formula (IIIA), Z^1, Z^2, and R^z are not C_3-C_{10} cycloalkyl(C_1-C_6 alkyl).

In another embodiment, the cationic lipid is a compound of formula (IV), which has a branching point at a position that is 2-6 carbon atoms (i.e., at beta (β), gamma (γ), delta (δ), epsilon (ε) or zeta position (ζ) adjacent to the biodegradable group (between the biodegradable group and the terminus of the tail, i.e., Z^1 or Z^2):

Formula (IV)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, R^z, a, and b are defined as in formula (I);

L^1 and L^2 and are each, independently, C_1-C_5 alkylene or C_2-C_5 alkenylene;

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X and Y are each, independently, alkylene or alkenylene (e.g., C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene); and

each occurrence of Z is independently C_1-C_4 alkyl (preferably, methyl).

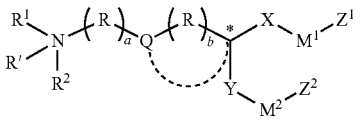
For example, in one embodiment, -L^1-$C(Z)_3$ is —$CH_2C(CH_3)_3$. In another embodiment, -L^1-$C(Z)_3$ is —$CH_2CH_2C(CH_3)_3$.

In one embodiment, the total carbon atom content of each tail (e.g., —X-M^1-L^1-$C(Z)_3$ or —Y-M^2-L^2-$C(Z)_3$) is from about 17 to about 26. For example, the total carbon atom content can be from about 19 to about 26 or from about 21 to about 26.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

In one embodiment, the cationic lipid is a compound of formula (V), which has an alkoxy or thioalkoxy (i.e., —S-alkyl) group substitution on at least one tail:

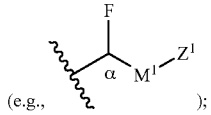
Formula (V)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

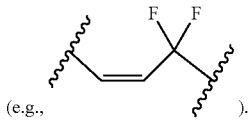
R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



(e.g.,);

Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein (i) the C_8-C_{14} alkyl or C_8-C_{14} alkenyl of at least one of Z^1 and Z^2 is substituted by one or more alkoxy (e.g., a C_1-C_4 alkoxy such as —OCH_3) or thioalkoxy (e.g., a C_1-C_4 thioalkoxy such as —SCH_3) groups, and (ii) the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2



(e.g.,).

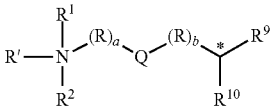
In one embodiment, the alkoxy substitution on Z^1 and/or Z^2 is at the beta position from the M^1 and/or M^2 group.

In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

The R'R^1R^2N—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2$N—$(CH_2)_3$—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2$N—$(CH_2)_3$—$C(CH_3)$=N—O—.

In one embodiment, the cationic lipid is a compound of formula (VIA), which has one or more fluoro substituents on at least one tail at a position that is either alpha to a double bond or alpha to a biodegradable group:

Formula (VIA)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R^1, R^2, R, a, and b are as defined with respect to formula (I);

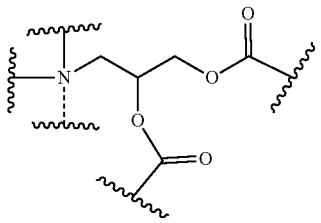
Q is absent or is —O—, —NH—, —S—, —C(O)—, —C(O)O—, —OC(O)—, —$C(O)N(R^4)$—, —$N(R^5)$C(O)—, —S—S—, —OC(O)O—, —O—N=$C(R^5)$—, —$C(R^5)$=N—O—, —OC(O)$N(R^5)$—, —$N(R^5)C(O)N(R^5)$—, —$N(R^5)C(O)O$—, —C(O)S—, —C(S)O— or —$C(R^5)$=N—O—C(O)—;

R' is absent, hydrogen, or alkyl (e.g., C_1-C_4 alkyl); and

each of R^9 and R^{10} are independently C_{12}-C_{24} alkyl (e.g., C_{12}-C_{20} alkyl), C_{12}-C_{24} alkenyl (e.g., C_{12}-C_{20} alkenyl), or C_{12}-C_{24} alkoxy (e.g., C_{12}-C_{20} alkoxy) (a) having one or more biodegradable groups and (b) optionally substituted with one or more fluorine atoms at a position which is (i) alpha to a biodegradable group and between the biodegradable group and the tertiary carbon atom marked with an asterisk (*), or (ii) alpha to a carbon-carbon double bond and between the double bond and the terminus of the R^9 or R^{10} group; each biodegradable group independently interrupts the C_{12}-C_{24} alkyl, alkenyl, or alkoxy group or is substituted at the terminus of the C_{12}-C_{24} alkyl, alkenyl, or alkoxy group, wherein

(i) at least one of R^9 and R^{10} contains a fluoro group;

(ii) the compound does not contain the following moiety:



wherein ---- is an optional bond; and

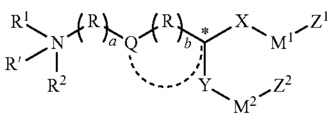
(iii) the terminus of R^9 and R^{10} is separated from the tertiary carbon atom marked with an asterisk (*) by a chain of 8 or more atoms (e.g., 12 or 14 or more atoms).

In one preferred embodiment, the terminus of R^9 and R^{10} is separated from the tertiary carbon atom marked with an asterisk (*) by a chain of 18-22 carbon atoms (e.g., 18-20 carbon atoms).

In another embodiment, the terminus of the R^9 and/or R^{10} has the formula —C(O)O—CF_3.

In another embodiment, the cationic lipid is a compound of formula (VIB), which has one or more fluoro substituents on at least one tail at a position that is either alpha to a double bond or alpha to a biodegradable group:

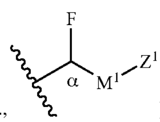
Formula (VIB)



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

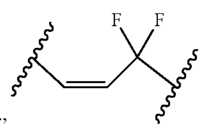
R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, M^1, M^2, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group

(e.g., );

and

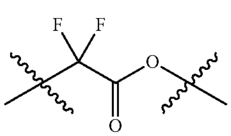
Z^1 and Z^2 are each, independently, C_8-C_{14} alkyl or C_8-C_{14} alkenyl, wherein said C_8-C_{14} alkenyl is optionally substituted by one or more fluorine atoms at a position that is alpha to a double bond

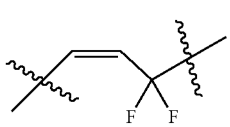
(e.g., ),

wherein at least one of X, Y, Z^1, and Z^2 contains a fluorine atom.

In one embodiment, at least one of Z^1 and Z^2 is substituted by two fluoro groups at a position that is either alpha to a double bond or alpha to a biodegradable group. In one embodiment, at least one of Z^1 and Z^2 has a terminal —CF_3 group at a position that is alpha to a biodegradable group (i.e., at least one of Z^1 and Z^2 terminates with an —C(O)OCF_3 group).

For example, at least one of Z^1 and Z^2 may include one or more of the following moieties:





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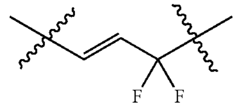
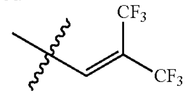
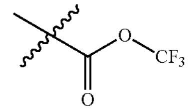




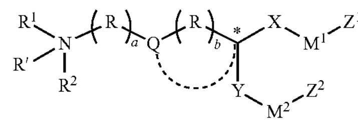


In one embodiment, X and Y are each, independently —$(CH_2)_n$, wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_7$—. In another exemplary embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

The R'R^1R^2N—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, R'R^1R^2N—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2$N—$(CH_2)_3$—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2$N—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2$N—$(CH_2)_3$—C(CH_3)═N—O—.

In one embodiment, the cationic lipid is a compound of formula (VII), which has an acetal group as a biodegradable group in at least one tail:

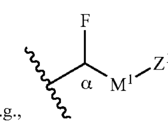
Formula (VII)



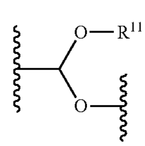
or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

R', R^1, R^2, R, R^3, R^4, R^{10}, Q, R^5, a, and b are defined as in formula (I);

X and Y are each, independently, alkylene (e.g., C_6-C_8 alkylene) or alkenylene, wherein the alkylene or alkenylene group is optionally substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group

(e.g., );

M^1 and M^2 are each, independently, a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)═N—, —N═C(R^5)—, —C(R^5)═N—O—, —O—N═C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



(wherein R^{11} is a C_4-C_{10} alkyl or C_4-C_{10} alkenyl));

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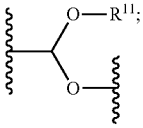
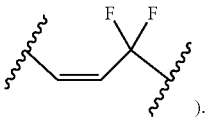
with the proviso that at least one of M^1 and M^2 is



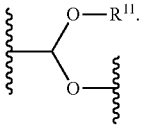
and

Z^1 and Z^2 are each, independently, C_4-C_{14} alkyl or C_4-C_{14} alkenyl, wherein the alkenyl group may optionally be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of Z^1 or Z^2 (e.g.,



).

In one embodiment, each of M^1 and M^2 is



In another embodiment, X and Y are each, independently —$(CH_2)_n$— wherein n is 4 to 20, e.g., 4 to 18, 4 to 16, or 4 to 12. In one embodiment, n is 4, 5, 6, 7, 8, 9, or 10. In one exemplary embodiment, X and Y are —$(CH_2)_6$—. In another embodiment, X and Y are —$(CH_2)_7$—. In yet another embodiment, X and Y are —$(CH_2)_9$—. In yet another embodiment, X and Y are —$(CH_2)_8$—.

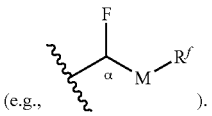
The $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— group can be any of the head groups described herein, including those shown in Table 1 below, and salts thereof. In one preferred embodiment, $R'R^1R^2N$—$(R)_a$-Q-$(R)_b$— is $(CH_3)_2N$—$(CH_2)_3$—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—NH—C(O)O—, $(CH_3)_2N$—$(CH_2)_2$—OC(O)—NH—, or $(CH_3)_2N$—$(CH_2)_3$—$C(CH_3)$=N—O—.

In another embodiment, the present invention relates to a cationic lipid or a salt thereof having:

(i) a central carbon atom,

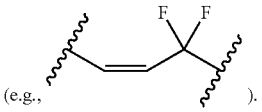
(ii) a nitrogen containing head group directly bound to the central carbon atom, and

(iii) two hydrophobic tails directly bound to the central carbon atom, wherein each hydrophobic tail is of the formula —R^e-M-R^f where R^e is a C_4-C_{14} alkyl or alkenyl, M is a biodegradable group, and R^f is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of —R^e-M-R^f is at most 20 atoms (i.e. the total length of the tail from the first carbon atom after the central carbon atom to a terminus of the tail is at most 20), and (ii) the group —R^e-M-R^f has at least 20 carbon atoms (e.g., at least 21 atoms). Optionally, the alkyl or alkenyl group in R^e may be substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group



(e.g.,).

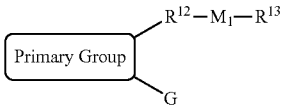
Also, optionally, the alkenyl group in R^f may be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of R^f



(e.g.,).

In one embodiment, the cationic lipid of the present invention (such as of formulas I-VII) has assymetrical hydrophobic groups (i.e., the two hydrophobic groups have different chemical formulas). For example, the cationic lipid can have the formula:

Formula (VIII)

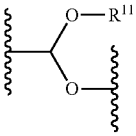


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

G is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl (e.g., a n-C_8 alkyl n-C_9 alkyl, or n-C_{10} alkyl);

R^{12} is a branched or unbranched alkylene or alkenylene (e.g., C_6-C_{20} alkylene or C_6-C_{20} alkenylene such as C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene);

M_1 is a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —$C(R^5)$=N—, —N=$C(R^5)$—, —$C(R^5)$=N—O—, —O—N=$C(R^5)$—, —C(O)(NR^5)—, —$N(R^5)$C(O)—, —C(S)(NR^5)—, —$N(R^5)$C(O)—, —$N(R^5)$C(O)N(R^5)—, —OC(O)O—, —OSi$(R^5)_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or

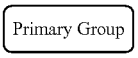


(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

R^3 and R^4 are defined as in formula (I);

each occurrence of R^5 is, independently, H or alkyl (e.g., C_1-C_4 alkyl);

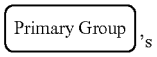
R^{13} is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl;



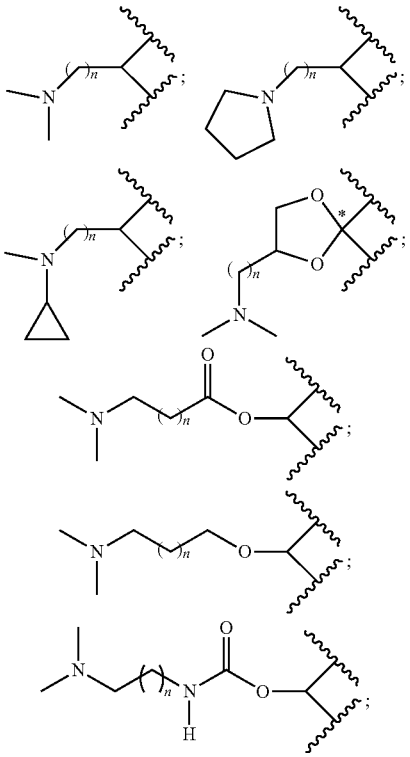
comprises a protonatable group having a pK_a of from about 4 to about 13, more preferably from about 5 to about 8 (e.g. from about 5 to about 7, or from about 5 to about 6.5, or from about 5.5 to about 6.5, or from about 6 to about 6.5).

In one embodiment, the primary group includes (i) a head group, and (ii) a central moiety (e.g., a central carbon atom) to which both the hydrophobic tails are directly bonded. Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group.

Representative



include, but are not limited to,



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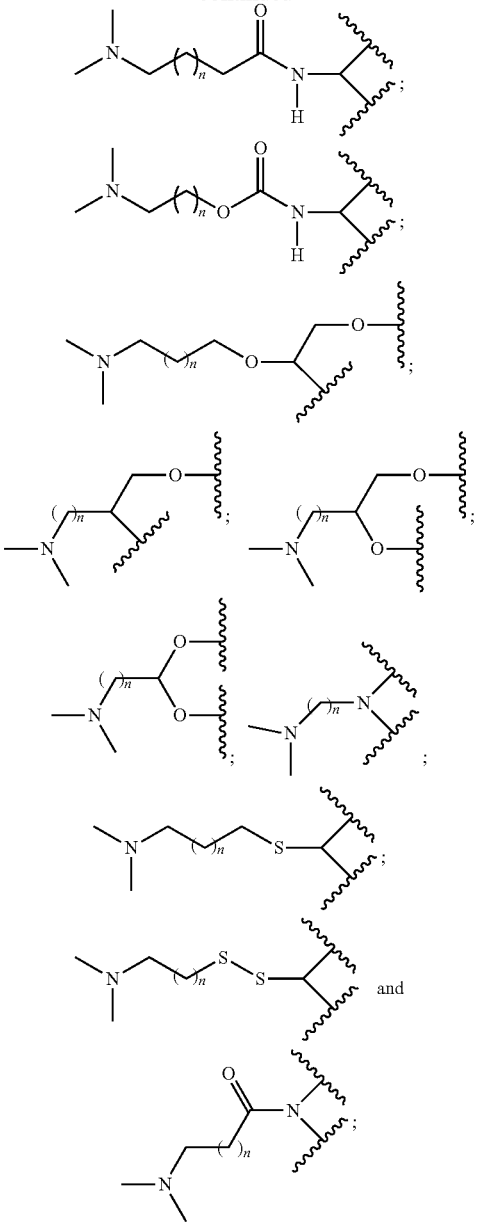
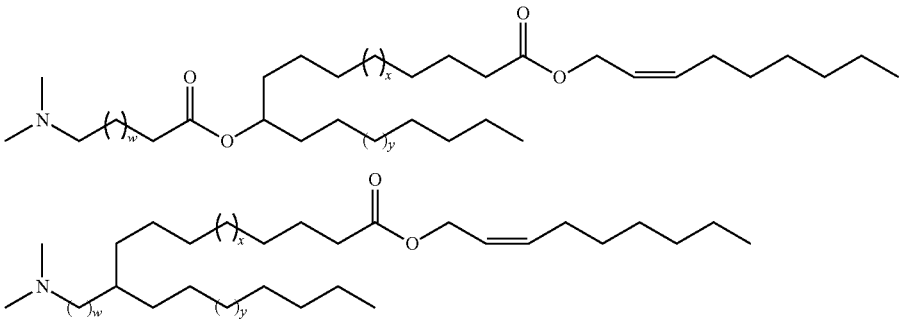


and

where n is 0-6.

Representative asymmetrical cationic lipids include:



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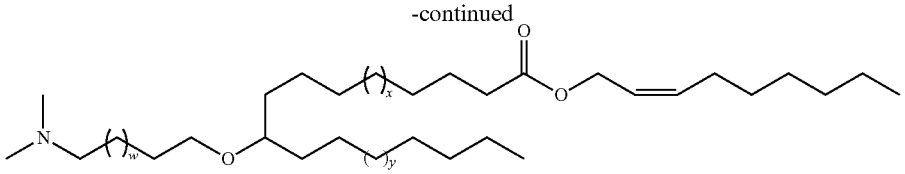
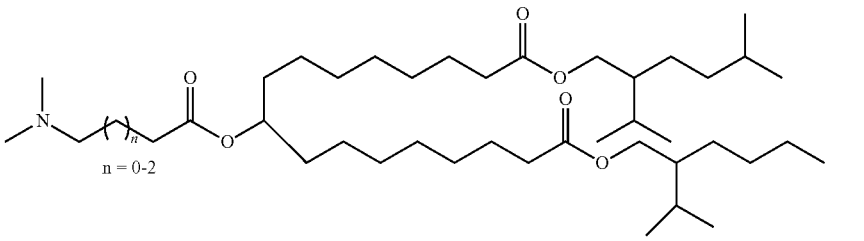


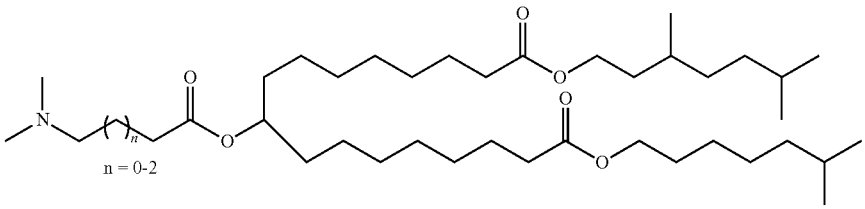
wherein w is 0, 1, 2, or 3; and x and y are each independently 1, 2, 3, 4, 5, 6, or 7.

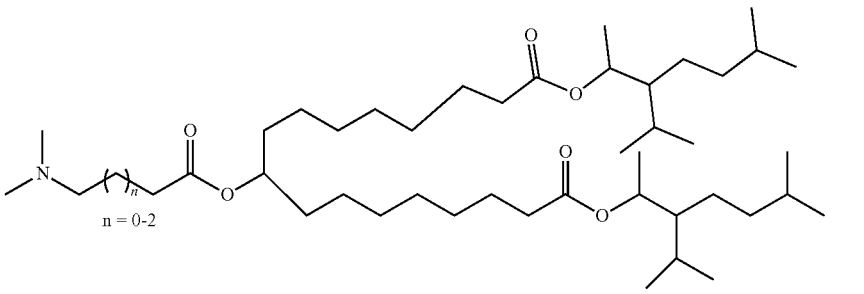
In a preferred embodiment of the aforementioned biodegradable cationic lipids, the biodegradable cationic lipid has a log P value of at least 10.1 (as calculated by the software available at http://www.molinspiration.com/services/logp.html from Molinspiration Cheminformatics of Slovensky Grob, Slovak Republic). More preferably, the log P value is at least 10.2 or 10.3.

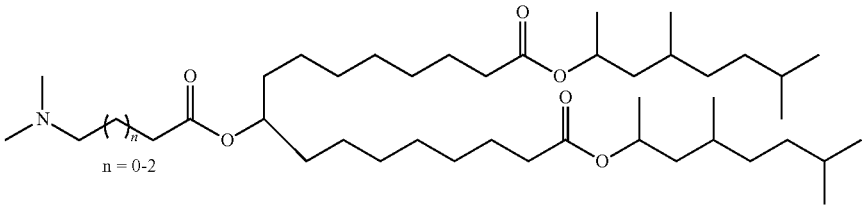
In another preferred embodiment of the aforementioned biodegradable cationic lipids, the biodegradable cationic lipid in the lipid nanoparticle has a HPLC retention time (relative to the retention time of cholesterol in the lipid nanoparticle), hereafter referred to as t_{lipid}–t_{chol}, of at least 1.4. (The HPLC parameters are provided in the examples below. Unless otherwise specified, the formulation of the lipid nanoparticle used is that described in Example 31). More preferably, the t_{lipid}–t_{chol} value is at least 1.75, 2.0, or 2.25.

In another embodiment, the biodegradable cationic lipid of the present invention is not one selected from:









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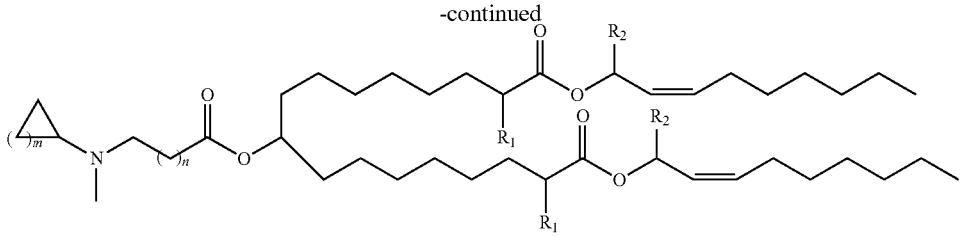
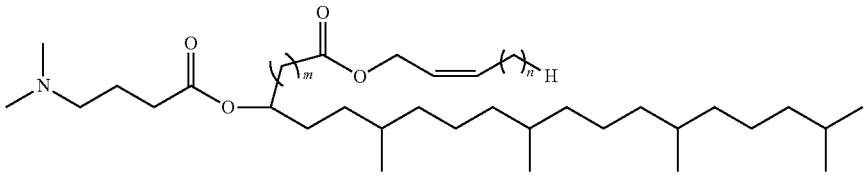
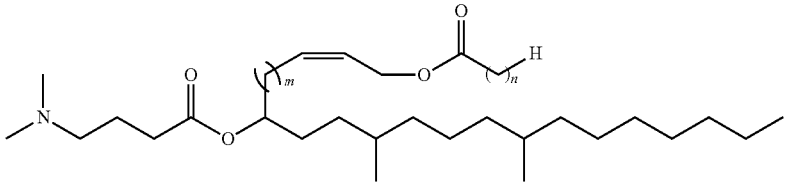


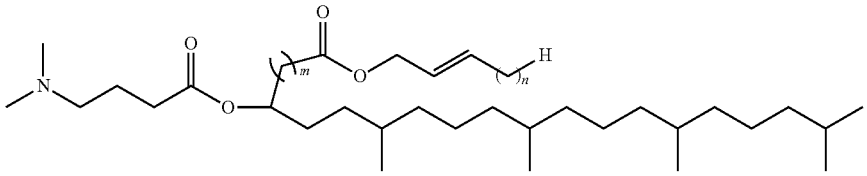
m = 1-6; n = 0-3
$R_1 = R_2$ = Me, Et, iPr etc.



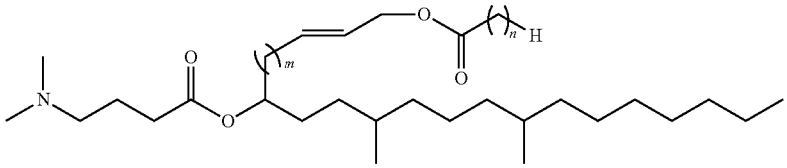
where m and n are integers, and m+n=13



where m and n are integers, and m+n=13



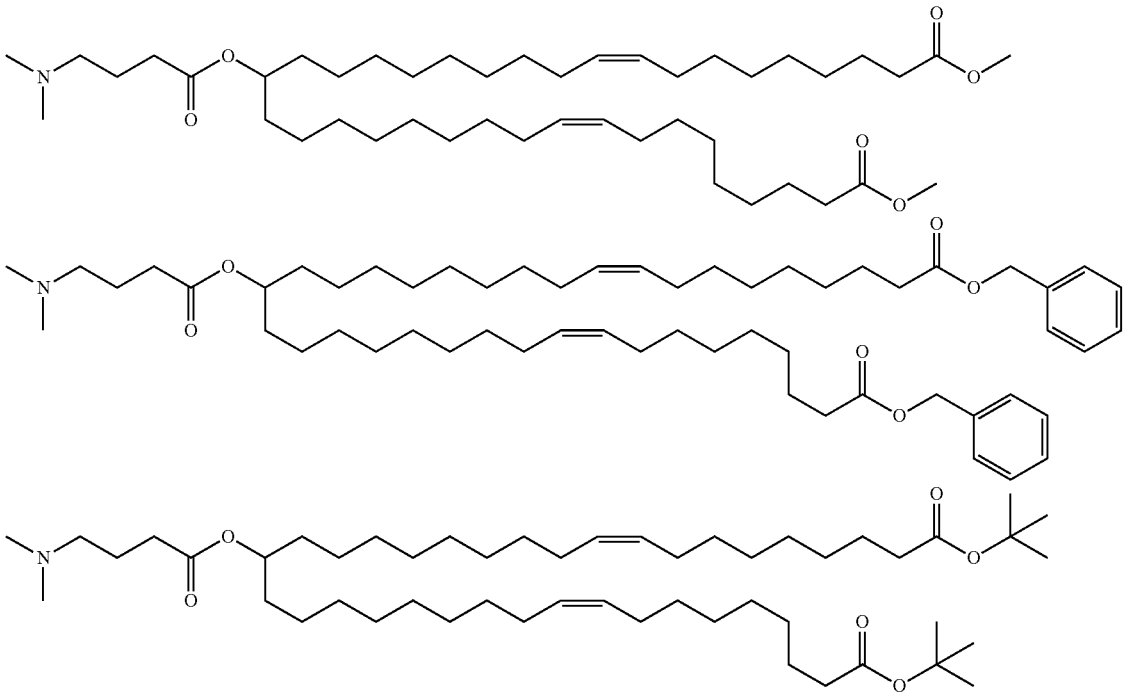
where m and n are integers, and m+n=13



where m and n are integers, and m+n=13

In yet another embodiment, the biodegradable cationic lipid is not one selected from those disclosed in International Publication No. WO 2011/153493 and U.S. Patent Publication No. 2012/0027803, both of which are hereby incorporated by reference.

Yet another embodiment is a biodegradable cationic lipid having (i) a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and (2) one or more biodegradable groups (such as an ester group) located in the mid- or distal section of a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid, with the proviso that the compound is not selected from



In another embodiment, the biodegradable cationic lipid is not one selected from those disclosed in International Publication No. WO 2011/153493 and U.S. Patent Publication No. 2012/0027803, both of which are hereby incorporated by reference. The incorporation of the biodegradable group(s) into the cationic lipid results in faster metabolism and removal of the cationic lipid from the body following delivery of the active pharmaceutical ingredient to a target area. In a preferred embodiment, the cationic lipid includes a branched alkyl or branched alkenyl group in its biodegradable group(s). In another preferred embodiment, the cationic lipid has a log P of at least 10.2 or 10.3. In yet another preferred embodiment, the cationic lipid has a t_{lipid}–t_{chol}, of at least 1.75, 2.0, or 2.25. The cationic lipid preferably has a pKa of from about 4 to about 7 (such as 6.0 to 6.5).

In one embodiment, the cationic lipid having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4 comprises (a) a head group (preferably a nitrogen containing head group, such as the head groups described herein), (b) at least two hydrophobic tails, each of the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), and (c) a linker group (for instance, a single central carbon atom) which is bound to the head group and the hydrophobic tails. The cationic lipid preferably has one, two, three, four or more of the properties listed below:

(i) a pKa of from about 4 to about 7 (such as 6.0 to 6.5);

(ii) in at least one hydrophobic tail (and preferably all hydrophobic tails), the biodegradable group is separated from the terminus of the hydrophobic tail by from about 6 to about 12 carbon atoms (for instance, 6 to 8 carbon atoms or 8 to 12 carbon atoms),

(iii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the chain length from the linker group to the terminus of the hydrophobic tail is at most 21 (e.g., at most 20, or from about 17 to about 21, from about 18 to about 20, or from about 16 to about 18) (The atom(s) in the linker group are not counted when calculating the chain length);

(iv) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms in the hydrophobic tail is from about 17 to about 26 (such as from about 19 to about 26, or from about 21 to about 26);

(v) for at least one hydrophobic tail (and preferably all hydrophobic tails), the number of carbon atoms between the linker group and the biodegradable group ranges from about 5 to about 10 (for example, 6 to 10, or 7 to 9);

(vi) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms between the linker group and the terminus of the hydrophobic tail is from about 15 to about 20 (such as from 16 to 20, 16 to 18, or 18 to 20);

(vii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the total number of carbon atoms between the biodegradable group and the terminus of the hydrophobic tail is from about 12 to about 18 (such as from 13 to 25);

(viii) for at least one hydrophobic tail (and preferably all hydrophobic tails), the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl or alkenyl group, for example, where the branching occurs at the α, β, γ, or δ position on the hydrophobic chain relative to the biodegradable group;

(ix) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid has an in vivo half life ($t_{1/2}$) in the liver of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours;

(x) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from the liver in mice with a greater than 10-fold reduction in lipid levels relative to C_{max} within the first 24 hours post-dose;

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(xi) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from the spleen in mice with an equal or greater than 10-fold reduction in lipid levels relative to C_{max} within the first 168 hours post-dose; and

(xii) when formulated as a lipid nanoparticle (such as in Example 35), the cationic lipid is eliminated from plasma with a terminal plasma half-life ($t_{1/2\beta}$) in rodents and non-human primates of 48 hours or shorter.

The present invention embodies compounds having any combination of some or all of the aforementioned properties. These properties provide a cationic lipid which remains intact until delivery of an active agent, such as a nucleic acid, after which cleavage of the hydrophobic tail occurs in vivo. For instance, the compounds can have all of properties (i) to (viii) (in addition to the log P or t_{lipid}–t_{chol} value). In another embodiment, the compounds have properties (i), (ii), (iii), and (viii). In yet another embodiment, the compounds have properties (i), (ii), (iii), (v), (vi), and (viii).

Another embodiment is a method of preparing a cationic lipid comprising:

(a) designing a cationic lipid having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii)); and

(b) synthesizing the cationic lipid of step (a). The cationic lipid in step (a) may comprises (a) a head group (preferably a nitrogen containing head group, such as the head groups described herein), (b) at least two hydrophobic tails, each of the formula -(hydrophobic chain)-(biodegradable group)-(hydrophobic chain), and (c) a linker group (for instance, a single central carbon atom) which is bound to the head group and the hydrophobic tails. Step (a) may comprise:

(a)(i) preparing one or more cationic lipids having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii);

(a) (ii) screening the cationic lipids to determine their efficacy and/or toxicity in lipid nanoparticles; and

(a)(iii) selecting a cationic lipid for synthesis.

Yet another embodiment is a method of designing a cationic lipid comprising:

(a) selecting a cationic lipid having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii)); and

(b) optionally,

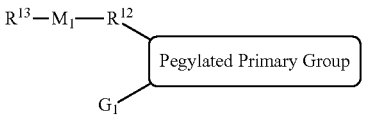
(i) preparing one or more cationic lipids having a log P value of at least 10.1 and/or a t_{lipid}–t_{chol}, of at least 1.4, and optionally also having one, two, three, four, or more properties from the list above (i.e., properties (i)-(xii);

(ii) screening the cationic lipids to determine their efficacy and/or toxicity in lipid nanoparticles; and

(iii) optionally, selecting a cationic lipid for further development or use.

In one embodiment, the PEG lipid has the formula:

Formula (IX)

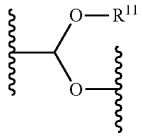


wherein

G_1 is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl (e.g., a n-C_8 alkyl n-C_9 alkyl, or n-C_{10} alkyl); or G_1 is —R^{12}-M_1-R^{13};

R^{12} is a branched or unbranched alkylene or alkenylene (e.g., C_6-C_{20} alkylene or C_6-C_{20} alkenylene such as C_{12}-C_{20} alkylene or C_{12}-C_{20} alkenylene);

M_1 is a biodegradable group (e.g., —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



(wherein R^{11} is a C_2-C_8 alkyl or alkenyl));

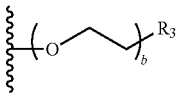
R^3 and R^4 are defined as in formula (I);

each occurrence of R^5 is, independently, H or alkyl (e.g., C_1-C_4 alkyl);

R^{13} is branched or unbranched C_3-C_{15} alkyl, alkenyl or alkynyl;

Pegylated Primary Group

comprises a PEG moiety, such as



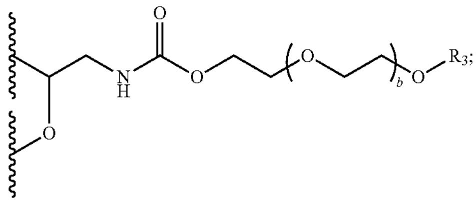
moiety wherein b is an integer from 10 to 1,000 (e.g., 5-100, 10-60, 15-50, or 20-45); R^3 is —H, —R^c, or —OR^c; and R^c is —H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, or heterocyclyl.

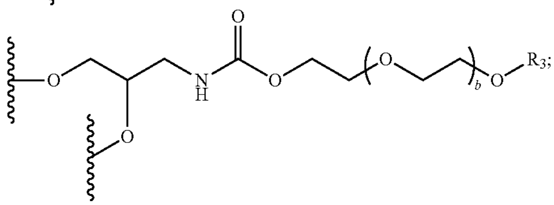
In one embodiment, the pegylated primary group includes (i) a head group having a PEG moiety, and (ii) a central moiety (e.g., a central carbon atom) to which both the hydrophobic tails are directly bonded. Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group.

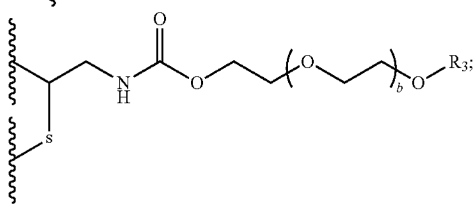
Representative

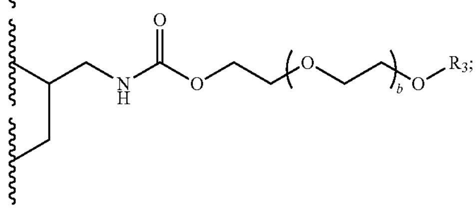
Pegylated Primary Group's

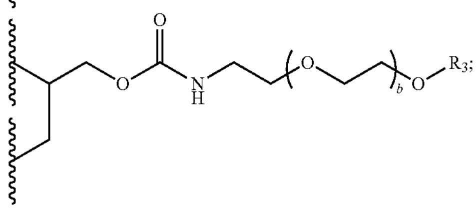
include, but are not limited to,

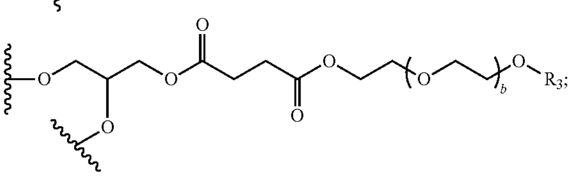


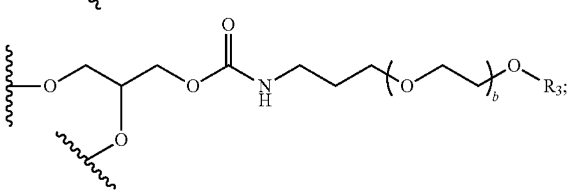


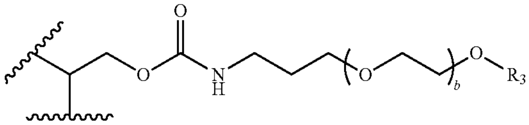










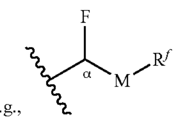


where b is 10-100 (e.g., 20-50 or 40-50)

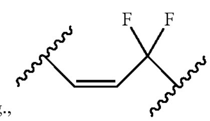
Another embodiment of the present invention is a PEG lipid (or a salt thereof) having:

(i) a pegylated primary group including a head group which includes a PEG moiety (e.g., having from 10 to 1000 repeating units such as ethoxy units)), and

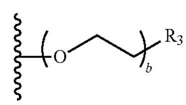
(iii) one or more hydrophobic tails (preferably, two hydrophobic tails) directly bound to the pegylated primary group, wherein at least one hydrophobic tail is of the formula —R^e-M-R^f where R^e is a C_4-C_{14} alkyl or alkenyl, M is a biodegradable group, and R^f is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of —R^e-M-R^f is at most 20 atoms (i.e. the total length of the tail from the first carbon atom after the central carbon atom to a terminus of the tail is at most 20), and (ii) the group —R^e-M-R^f has at least 20 carbon atoms (e.g., at least 21 atoms). Optionally, the alkyl or alkenyl group in R^e may be substituted with one or two fluorine atoms at the alpha position to the M^1 or M^2 group

(e.g., 

Also, optionally, the alkenyl group in R^f may be substituted with one or two fluorine atoms at the alpha position to a double bond which is between the double bond and the terminus of R^f

(e.g., ).

In one embodiment, the pegylated primary group includes (i) a head group having a PEG moiety, and (ii) a central moiety (e.g., a central carbon atom) to which the hydrophobic tails are directly bound. The PEG moiety may have 5-100, 10-60, 15-50, or 20-45 repeating units. For example, the PEG moiety may have the formula



moiety wherein b is an integer from 10 to 1,000 (e.g., 5-100, 10-60, 15-50, or 20-45); R^3 is —H, —R^c, or —OR^c; and R^c is —H, alkyl (e.g., C_1-C_4 alkyl), acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, or heterocyclyl.

Yet another embodiment is a lipid particle that includes a cationic lipid and/or PEG lipid of the present invention. In one embodiment, the lipid particle includes a cationic lipid of the present invention (e.g., of one of formulas (I)-(VIII)). In another embodiment, the lipid particle includes a PEG lipid of the present invention (e.g., of formula (IX)). In yet another embodiment, the lipid particle includes a cationic lipid of the present invention and a PEG lipid of the present invention.

In a preferred embodiment, the lipid particle includes a neutral lipid, a lipid capable of reducing aggregation, a cationic lipid, and optionally, a sterol (e.g., cholesterol). Suitable neutral lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), POPC, DOPE, and SM. Suitable lipids capable of reducing aggregation include, but are not limited to, a PEG lipid, such as PEG-DMA, PEG-DMG, and those of the present invention (e.g., of formula (IX)) or a combination thereof.

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The lipid particle may further include an active agent (e.g., a therapeutic agent). The active agent can be a nucleic acid such as a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, or a ribozyme. In a preferred embodiment, the nucleic acid is a siRNA. In another preferred embodiment, the nucleic acid is a miRNA.

In another embodiment, the lipid particle includes a cationic lipid of the present invention, a neutral lipid and a sterol. The lipid particle may further include an active agent, such as a nucleic acid (e.g., an siRNA or miRNA).

In yet another embodiment, the lipid particle includes a PEG lipid of the present invention, a cationic lipid, a neutral lipid, and a sterol. The lipid particle may further include an active agent, such as a nucleic acid (e.g., an siRNA or miRNA).

The lipid particles described herein may be lipid nanoparticles.

Yet another embodiment of the invention is a pharmaceutical composition which includes a lipid particle of the present invention and a pharmaceutically acceptable carrier.

In one embodiment, the cationic lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

In another embodiment, the PEG lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

In another embodiment, the present invention relates to a method of delivering a nucleic acid molecule comprising administering a nucleic lipid particle comprising (i) the nucleic acid molecule and (ii) a cationic lipid and/or a PEG lipid of the present invention. In one embodiment, the cationic lipid and/or a PEG lipid remains intact until delivery of the nucleic acid molecule after which cleavage of the hydrophobic tail occurs in vivo.

Yet another aspect is a method of modulating the expression of a target gene in a cell by providing to the cell a lipid particle of the present invention. The active agent can be a nucleic acid selected from a plasmid, an immuno stimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme. In a preferred embodiment, the nucleic acid is a siRNA or miRNA.

Yet another aspect is a method of treating a disease or disorder characterized by the overexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a nucleic acid selected from an siRNA, a microRNA, and an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense oligonucleotide includes a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof. In a preferred embodiment, the nucleic acid is a siRNA or miRNA.

Yet another aspect is a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

Yet another aspect is a method of inducing an immune response in a subject by providing to the subject a pharmaceutical composition wherein the active agent is an immunostimulatory oligonucleotide.

Yet another aspect is a transfection agent that includes the composition or lipid particles described above, where the composition or lipid particles include a nucleic acid. The agent, when contacted with cells, can efficiently deliver nucleic acids to the cells. Yet another aspect is a method of delivering a nucleic acid to the interior of a cell, by obtaining or forming a composition or lipid particles described above, and contacting the composition or lipid particles with a cell.

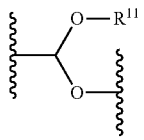
DETAILED DESCRIPTION

In one aspect, the present invention relates to a lipid particle that includes a neutral lipid, a lipid capable of reducing aggregation (e.g., a PEG lipid), a cationic lipid, and optionally a sterol. In certain embodiments, the lipid particle further includes an active agent (e.g., a therapeutic agent). Various exemplary embodiments of these lipids, lipid particles and compositions comprising the same, and their use to deliver therapeutic agents and modulate gene and protein expression are described in further detail below.

The Cationic Lipid

In one embodiment, the cationic lipid is a compound of any one of Formulas I-VIII. The following disclosure represents various embodiments of the compounds described above, including the compounds of Formulas I-VIII.

In one embodiment, M^1 and M^2 are each, independently: —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —C(O)(NR^5)—, —N(R^5)C(O)—, —C(S)(NR^5)—, —N(R^5)C(O)—, —N(R^5)C(O)N(R^5)—, —OC(O)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, —OC(O)(CR^3R^4)C(O)—, or



(wherein R^{11} is a C_2-C_8 alkyl or alkenyl).

In another embodiment, M^1 and M^2 are each, independently: —OC(O)—, —C(O)—O—, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —O—C(O)O—, —C(O)N(R^5)—, —N(R^5)C(O)—, —C(O)S—, —SC(O)—, —C(S)O—, —OC(S)—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

In yet another embodiment, M^1 and M^2 are each, independently: —C(O)—O—, —OC(O)—, —C(R^5)=N—, —C(R^5)=N—O—, —O—C(O)O—, —C(O)N(R^5)—, —C(O)S—, —C(S)O—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

In another embodiment, M^1 and M^2 are each —C(O)O—.

In one embodiment, R^1 and R^2 are each, individually, optionally substituted alkyl, cycloalkyl, cycloalkylalkyl, or heterocycle. In one embodiment, R^1 is alkyl and R^2 is alkyl, cycloalkyl or cycloalkylalkyl. In one embodiment, R^1 and R^2 are each, individually, alkyl (e.g., C_1-C_4 alkyl, such as methyl, ethyl, or isopropyl). In one embodiment, R^1 and R^2 are both methyl. In another embodiment, R^1 and R^2, together with the nitrogen atom to which they are attached, form an optionally substituted heterocylic ring (e.g., N-methylpiperazinyl). In another embodiment, one of R^1 and R^2 is

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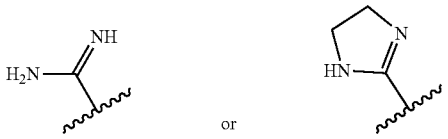


(e.g., R^1 is one of the two aforementioned groups and R^2 is hydrogen).

In one embodiment, R' is hydrogen or alkyl. In another embodiment, R' is hydrogen or methyl. In one embodiment, R' is absent. In one embodiment, R' is absent or methyl.

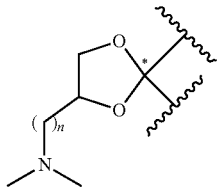
For cationic lipid compounds which contain an atom (e.g., a nitrogen atom) that carries a positive charge, the compound also contains a negatively charged counter ion. The counterion can be any anion, such as an organic or inorganic anion. Suitable examples of anions include, but are not limited to, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, α-glycerophosphate, halide (e.g., chloride), sulfate, nitrate, bicarbonate, and carbonate. In one embodiment, the counterion is a halide (e.g., Cl).

In one embodiment each R is, independently, —(CR^3R^4)—, wherein R^3 and R^4 are each, independently, H or alkyl (e.g., C_1-C_4 alkyl). For example, in one embodiment each R is, independently, —(CHR^4)—, wherein each R^4 is, independently H or alkyl (e.g., C_1-C_4 alkyl). In another embodiment, each R is, independently, —CH_2—, —$C(CH_3)_2$— or —CH(iPr)- (where iPr is isopropyl). In another embodiment, each R is —CH_2—.

In another embodiment R^5 is, in each case, hydrogen or methyl. For example, R^5 can be, in each case, hydrogen.

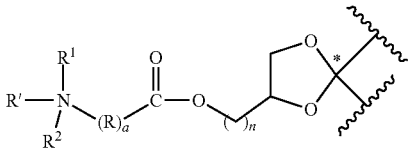
In one embodiment, Q is absent, —C(O)O—, —OC(O)—, —C(O)N(R^5)—, —N(R^5)C(O)—, —S—S—, —OC(O)O—, —C(R^5)=N—O—, —OC(O)N(R^5)—, —N(R^5)C(O)N(R^5)—, —N(R^5)C(O)O—, —C(O)S—, —C(S)O— or —C(R^5)=N—O—C(O)—. In one embodiment, Q is —C(O)O—.

In one embodiment, the dashed line to Q is absent, b is 0 and R'R^1R^2N—$(R)_a$-Q- and the tertiary carbon adjacent to it (C*) form the following group:



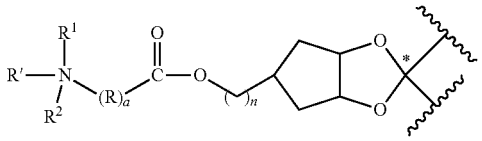
where n is 1 to 4 (e.g., n is 2).

In one embodiment, the dashed line to Q is absent, b is 0 and R'R^1R^2N—$(R)_a$-Q- and the tertiary carbon adjacent to it form the following group:

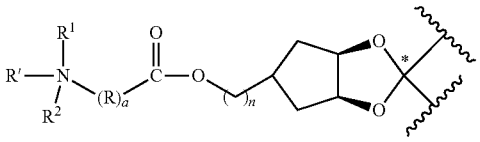


where n is 1 to 4 (e.g., n is 2), and R^1, R^2, R, a, and b are as defined with respect to formula (I). In one embodiment, a is 3.

In one embodiment, the dashed line to Q is absent, b is 0 and R'R^1R^2N—$(R)_a$-Q- and the tertiary carbon adjacent to it form the following group:



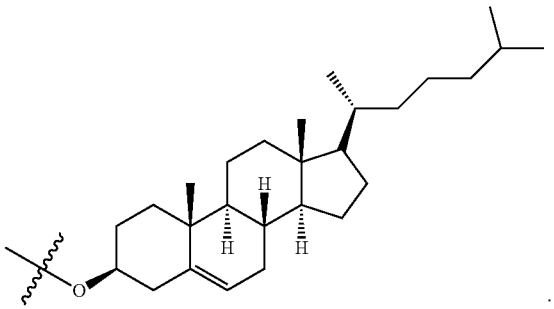
where n is 1 to 4 (e.g., n is 2), and R^1, R^2, R, a, and b are as defined with respect to formula (I). In one embodiment, a is 0. For example, the group can be:



In one embodiment, b is 0. In another embodiment, a is 2, 3, or 4 and b is 0. For example, in one embodiment, a is 3 and b is 0. In another embodiment, a is 3, b is 0, and Q is —C(O)O—.

In certain embodiments, the biodegradable group present in the cationic lipid is selected from an ester (e.g., —C(O)O— or —OC(O)—), disulfide (—S—S—), oxime (e.g., —C(H)=N—O— or —O—N=C(H)—), —C(O)—O—, —OC(O)—, —C(R^5)=N—, —N=C(R^5)—, —C(R^5)=N—O—, —O—N=C(R^5)—, —O—C(O)O—, —C(O)N(R^5), —N(R^5)C(O)—, —C(S)(NR^5)—, (NR^5)C(S)—, —N(R^5)C(O)N(R^5)—, —C(O)S—, —SC(O)—, —C(S)O—, —OC(S)—, —OSi(R^5)$_2$O—, —C(O)(CR^3R^4)C(O)O—, or —OC(O)(CR^3R^4)C(O)—.

A suitable cholesterol moiety for the cationic lipids of the present invention (including compounds of formulas I-VI) has the formula:



Additional embodiments include a cationic lipid having a head group, one or more hydrophobic tails, and a central moiety between the head group and the one or more tails. The head group can include an amine; for example an amine having a desired pK_a. The pK_a can be influenced by the structure of the lipid, particularly the nature of head group; e.g., the presence, absence, and location of functional groups such as anionic functional groups, hydrogen bond donor

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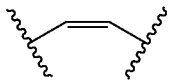
functional groups, hydrogen bond acceptor groups, hydrophobic groups (e.g., aliphatic groups), hydrophilic groups (e.g., hydroxyl or methoxy), or aryl groups. The head group amine can be a cationic amine; a primary, secondary, or tertiary amine; the head group can include one amine group (monoamine), two amine groups (diamine), three amine groups (triamine), or a larger number of amine groups, as in an oligoamine or polyamine. The head group can include a functional group that is less strongly basic than an amine, such as, for example, an imidazole, a pyridine, or a guanidinium group. The head group can be zwitterionic. Other head groups are suitable as well.

Representative central moieties include, but are not limited to, a central carbon atom, a central nitrogen atom, a central carbocyclic group, a central aryl group, a central hetrocyclic group (e.g., central tetrahydrofuranyl group or central pyrrolidinyl group) and a central heteroaryl group. Additionally, the central moiety can include, for example, a glyceride linker, an acyclic glyceride analog linker, or a cyclic linker (including a spiro linker, a bicyclic linker, and a polycyclic linker). The central moiety can include functional groups such as an ether, an ester, a phosphate, a phosphonate, a phosphorothioate, a sulfonate, a disulfide, an acetal, a ketal, an imine, a hydrazone, or an oxime. Other central moieties and functional groups are suitable as well.

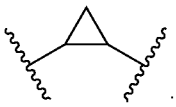
In one embodiment, the cationic lipid is a racemic mixture. In another embodiment, the cationic lipid is enriched in one diastereomer, e.g. the cationic lipid has at least 95%, at least 90%, at least 80% or at least 70% diastereomeric excess. In yet another embodiment, the cationic lipid is enriched in one enantiomer, e.g. the lipid has at least 95%, at least 90%, at least 80% or at least 70% enantiomer excess. In yet another embodiment, the cationic lipid is chirally pure, e.g. is a single optical isomer. In yet another embodiment, the cationic lipid is enriched for one optical isomer.

Where a double bond is present (e.g., a carbon-carbon double bond or carbon-nitrogen double bond), there can be isomerism in the configuration about the double bond (i.e. cis/trans or E/Z isomerism). Where the configuration of a double bond is illustrated in a chemical structure, it is understood that the corresponding isomer can also be present. The amount of isomer present can vary, depending on the relative stabilities of the isomers and the energy required to convert between the isomers. Accordingly, some double bonds are, for practical purposes, present in only a single configuration, whereas others (e.g., where the relative stabilities are similar and the energy of conversion low) may be present as inseparable equilibrium mixture of configurations.

In some cases, a double-bonded unsaturation is replaced by a cyclic unsaturation. The cyclic unsaturation can be a cycloaliphatic unsaturation, e.g., a cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl group. In some cases, the cyclic group can be a polycyclic group, e.g., a bicyclic group or tricyclic group. A bicyclic group can be bridged, fused, or have a spiro structure. In some cases, a double bond moiety can be replaced by a cyclopropyl moiety, e.g.,



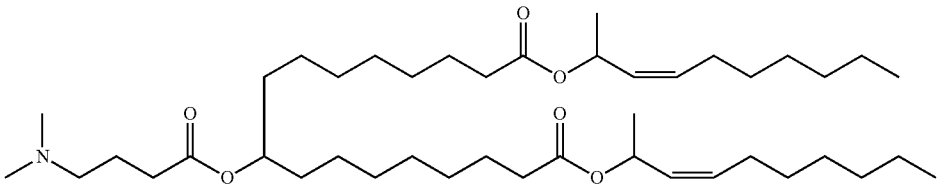
can be replaced by



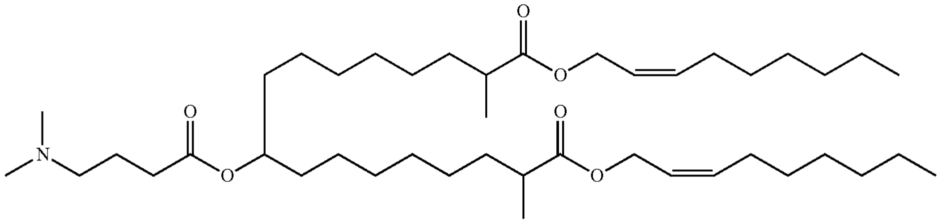
The cationic lipid includes one or more biodegradable groups. The biodegradable group(s) include one or more bonds that may undergo bond breaking reactions in a biological environment, e.g., in an organism, organ, tissue, cell, or organelle. Functional groups that contain a biodegradable bond include, for example, esters, dithiols, and oximes. Biodegradation can be a factor that influences the clearance of the compound from the body when administered to a subject. Biodegredation can be measured in a cell based assay, where a formulation including a cationic lipid is exposed to cells, and samples are taken at various time points. The lipid fractions can be extracted from the cells and separated and analyzed by LC-MS. From the LC-MS data, rates of biodegradation (e.g., as $t_{1/2}$ values) can be measured.

For example, the compound

(Compound 1)

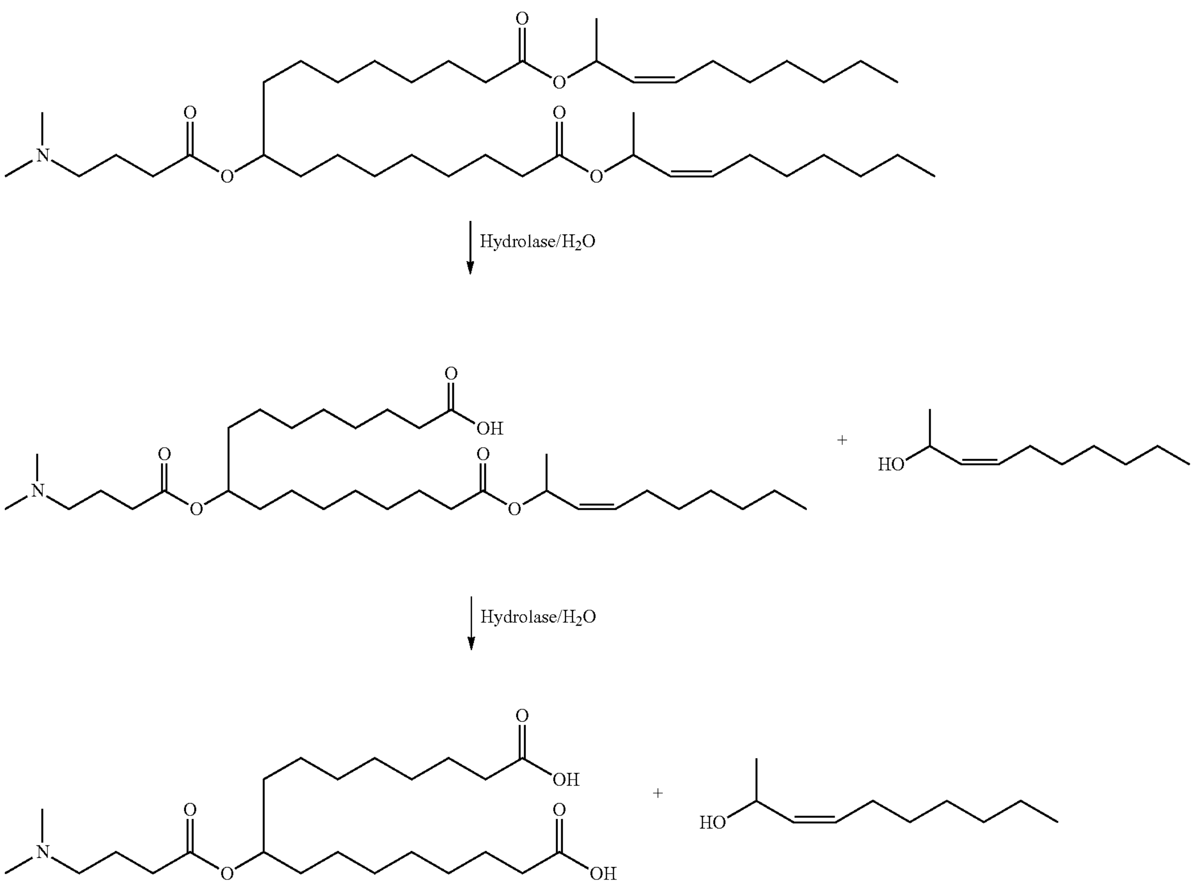


includes an ester linkage in each aliphatic chain, which can undergo hydrolysis in a biological environment, for example, when exposed to, e.g., a lipase or an esterase. The structure of the compound, of course, influences the rate at which the compound undergoes biodegradation. Thus, a compound where the methyl substituent is on the other side of the biodegradable group such as



would be expected to exhibit a different rate of biodegradation. Greater effects on that rate would be expected from changes in the structure of the compound at the site of hydrolysis. One modification that can influence the rate of hydrolysis, and thereby influence the rate of biodegradation and clearance from a subject's body, is to make the leaving group of the hydrolysis reaction have a secondary, rather than primary, alcohol.

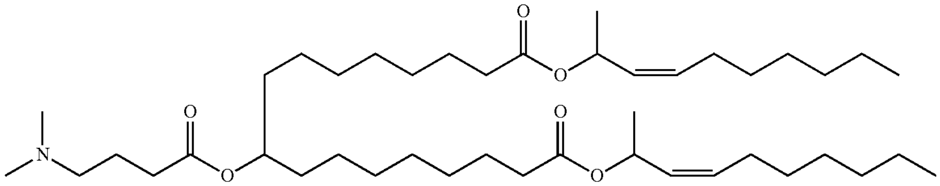
For example, without wishing to be bound by theory, Compound 1 shown above may be metabolized as shown in the scheme below:



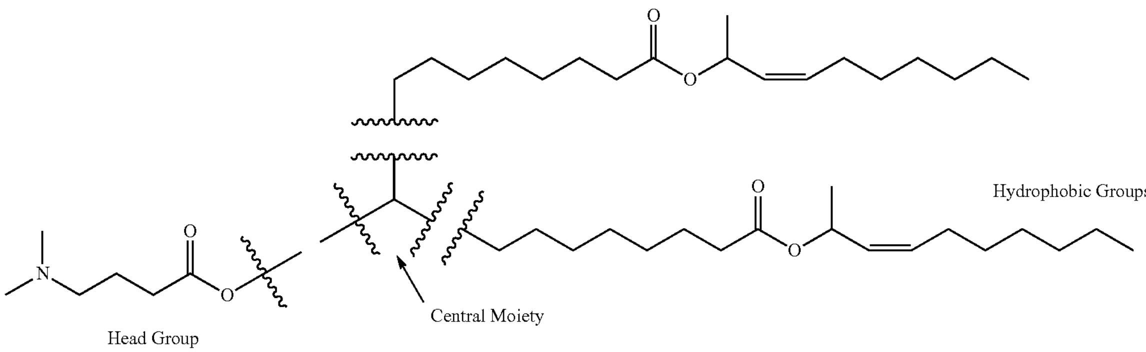
In one embodiment, a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours. The cationic lipid preferably remains intact, or has a half-life sufficient to form a stable lipid nanoparticle which effectively delivers the desired active pharmaceutical ingredient (e.g., a nucleic acid) to its target but thereafter rapidly degrades to minimize any side effects to the subject. For instance, in mice, the cationic lipid preferably has a $t_{1/2}$ in the spleen of from about 1 to about 7 hours.

In another embodiment, a cationic lipid of any of the embodiments described herein containing a biodegradable group or groups has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 10% (e.g., less than about 7.5%, less than about 5%, less than about 2.5%) of that for the same cationic lipid without the biodegrable group or groups.

Some cationic lipids can be conveniently represented as a hydrophobic group combined via a central moiety (such as a carbon atom) with a headgroup. By way of example, the compound:



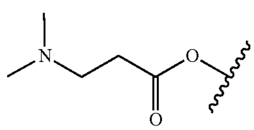
can be thought of as a combination of a headgroup, a central moiety, and two hydrophobic groups as follows:

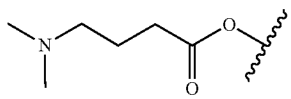


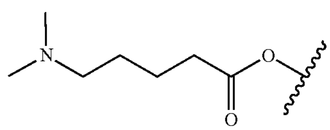
The present invention includes compounds composed of any combination of the head and hydrophobic groups listed below (in combination with a central moiety (such as a central carbon atom).

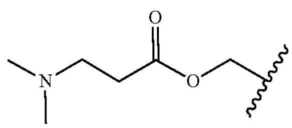
Some suitable head groups include those depicted in Table 1A:

Table 1A









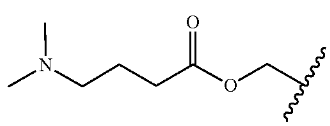
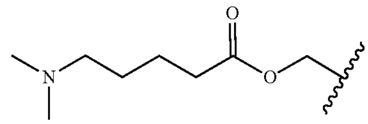
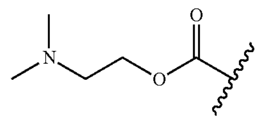
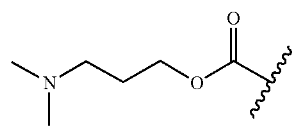
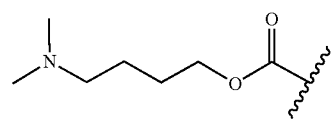


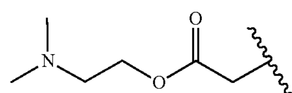
Table 1A-continued

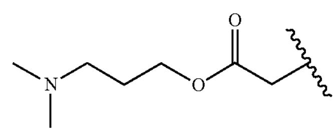












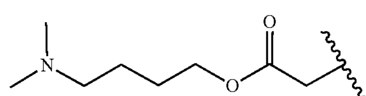


Table 1A-continued

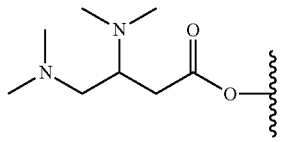
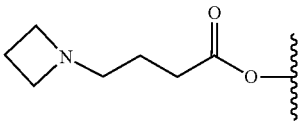
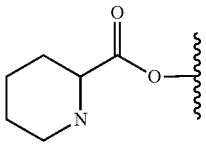
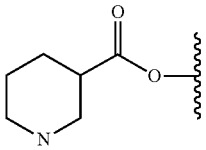
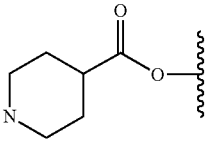
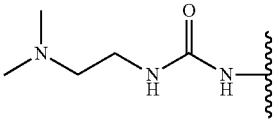
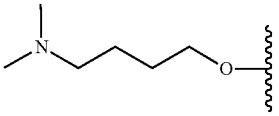
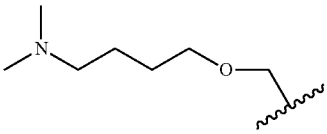
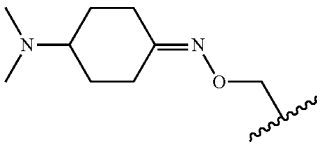
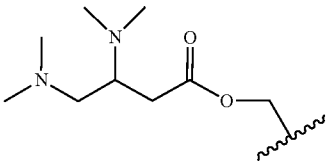











Table 1A-continued

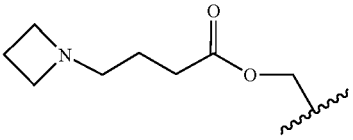
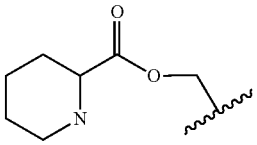
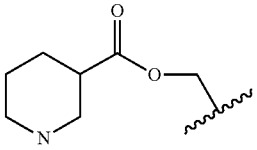
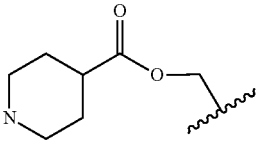
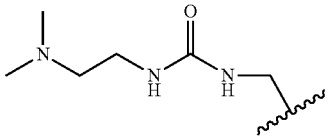
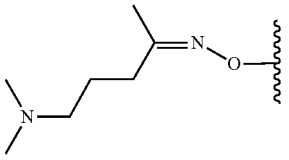
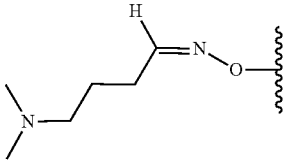
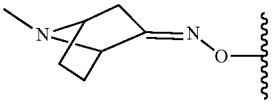
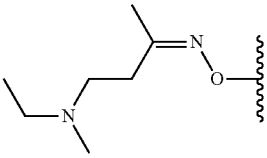
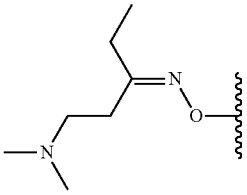
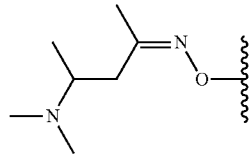
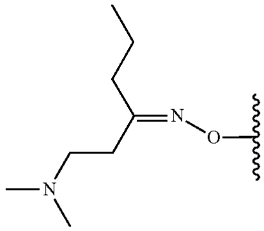
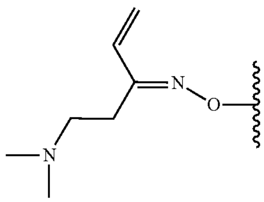
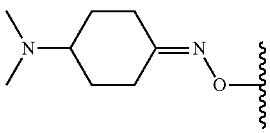











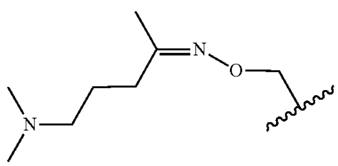
Table 1A-continued

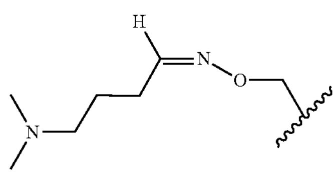


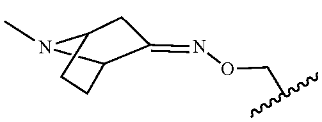


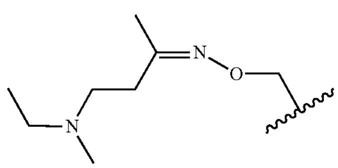












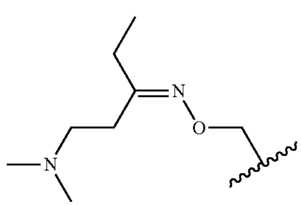
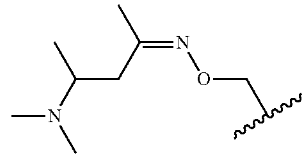
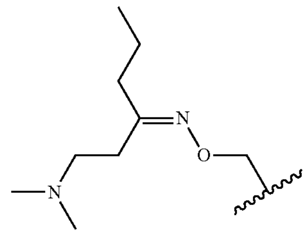
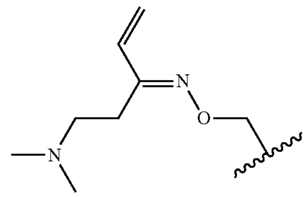
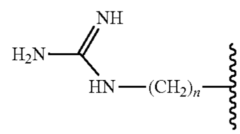


Table 1A-continued

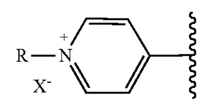




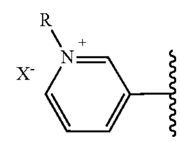




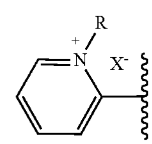
(where n is 0-5)



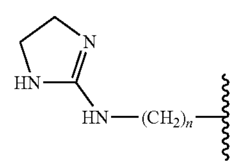
R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)



R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)



R = H, alkyl (e.g., methyl)
X = halogen (e.g., Cl)

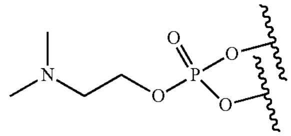


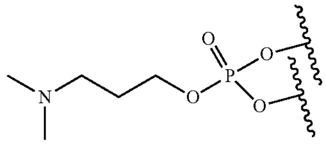
(where n is 0-5)

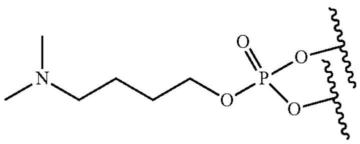
Suitable primary groups include, but are not limited to, those that are a combination of a head group from table 1A

with a central carbon atom. Other suitable primary groups include those in table 1B below:

Table 1B







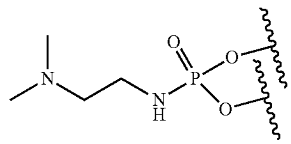
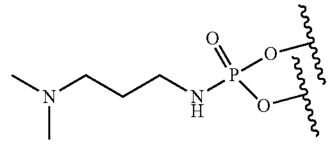
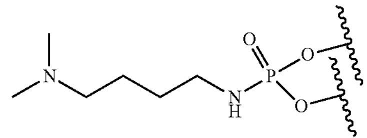
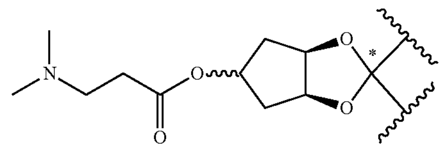
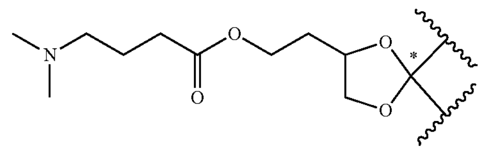


Table 1B-continued



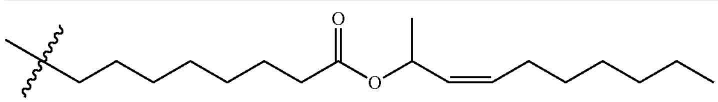


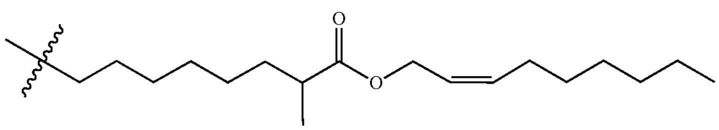


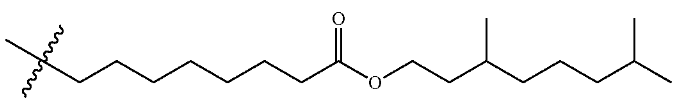


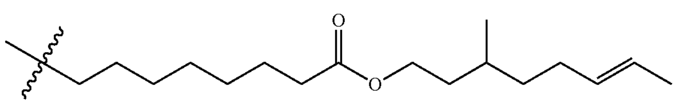
Some suitable hydrophobic tail groups include those depicted in Table 1C:

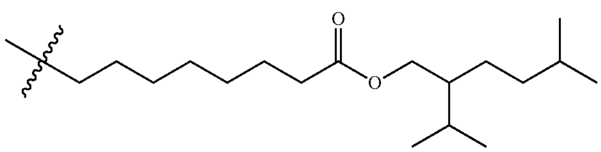
Table 1C

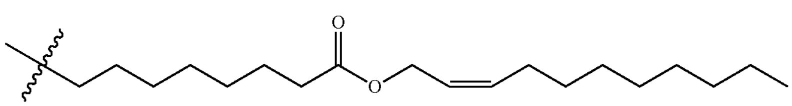












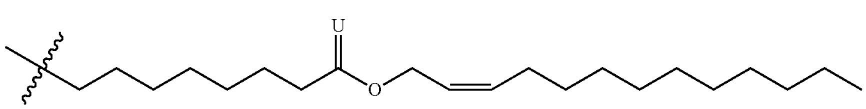
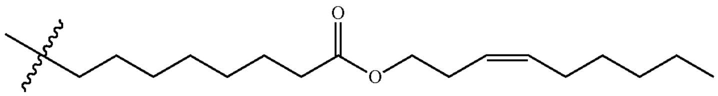
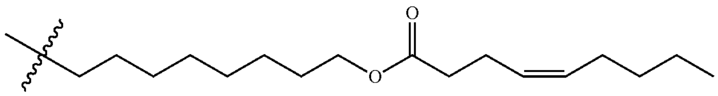
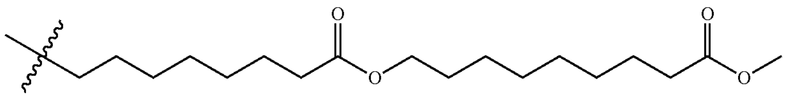
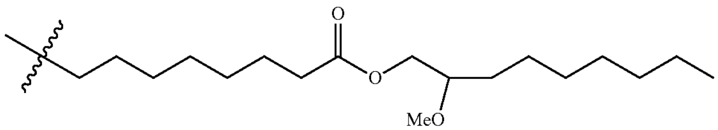


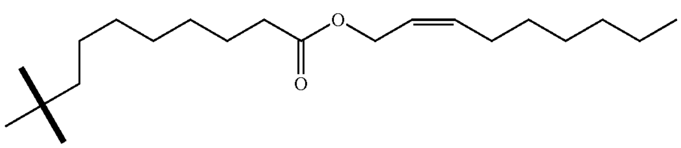
Table 1C-continued

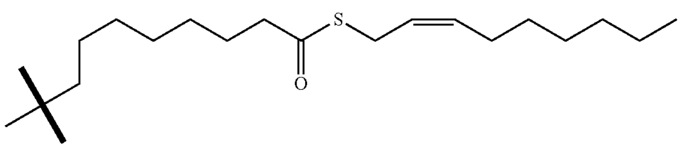


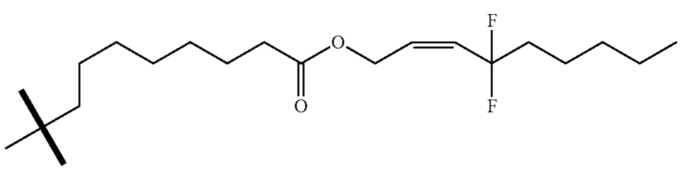


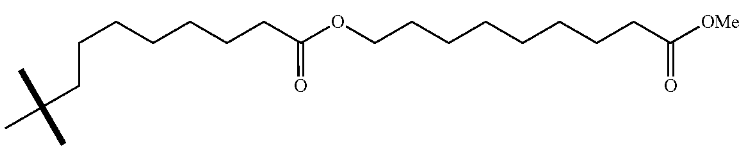


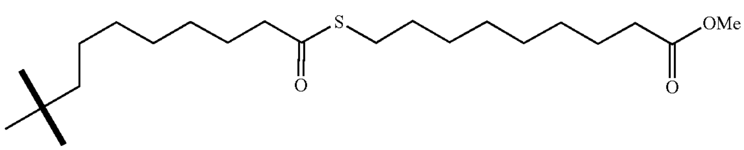


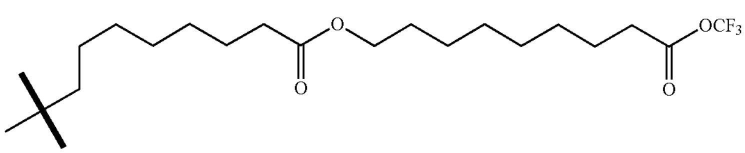












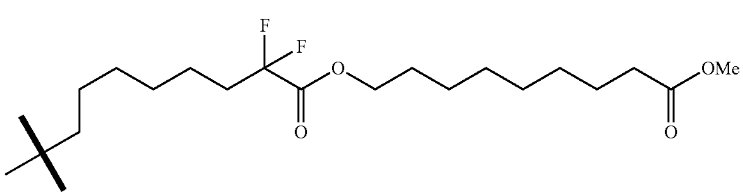
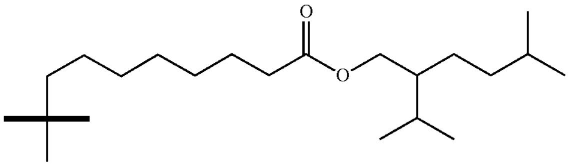
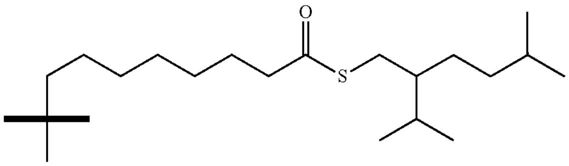
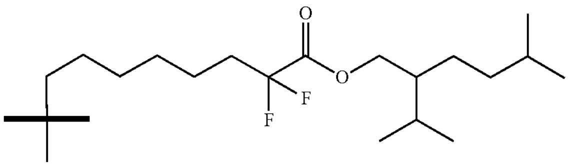
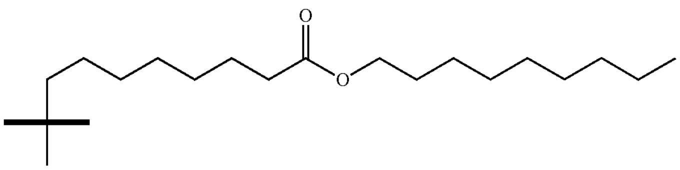


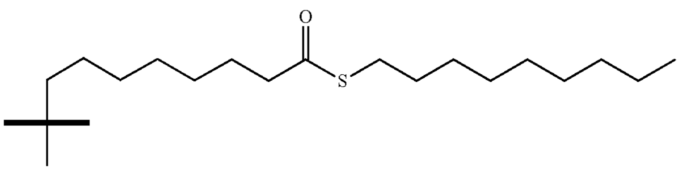
Table 1C-continued

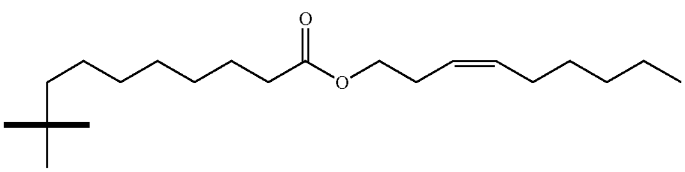


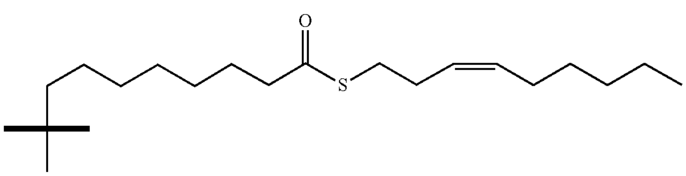


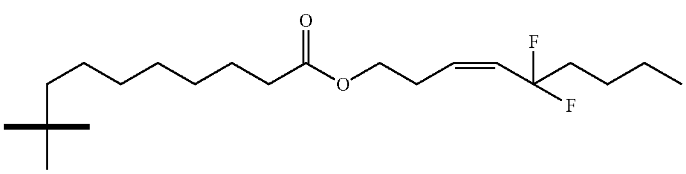












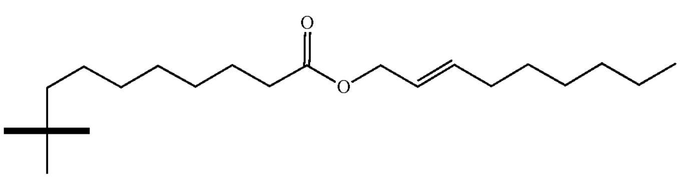
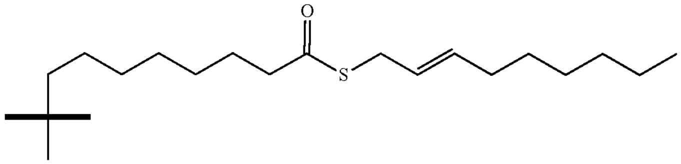
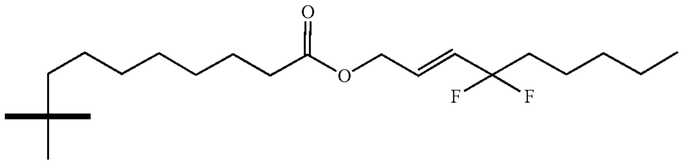
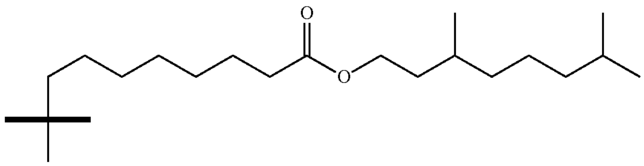
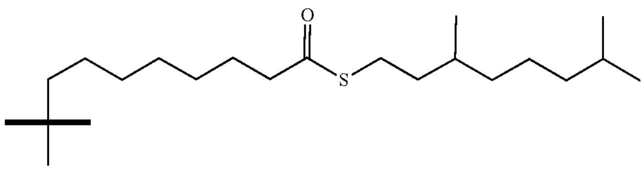


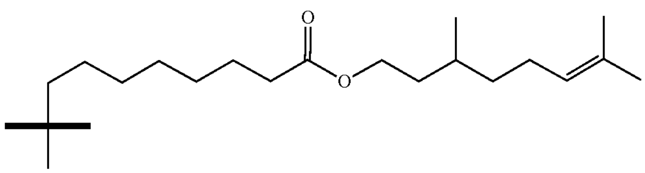
Table 1C-continued

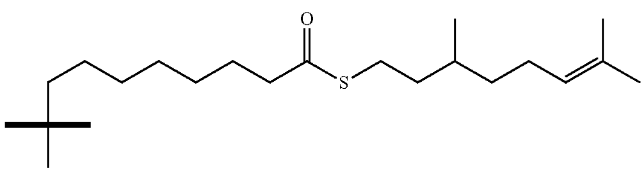


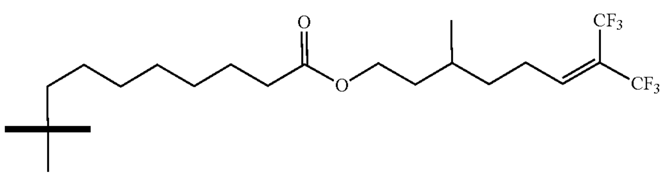


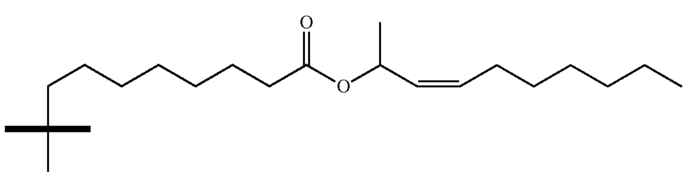












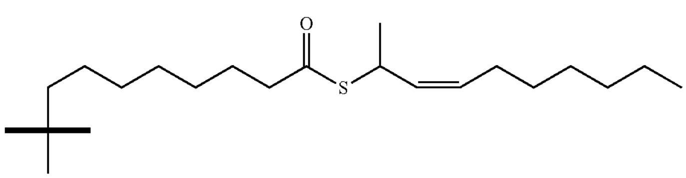
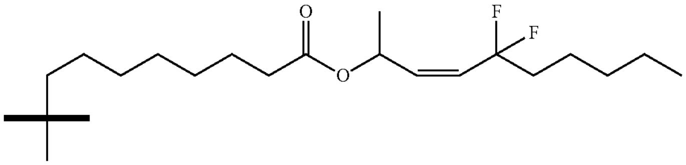
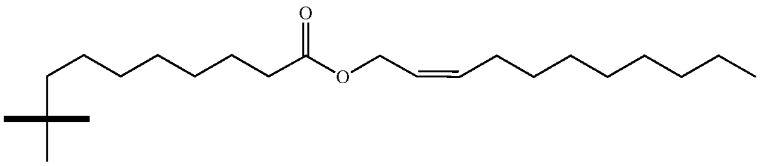
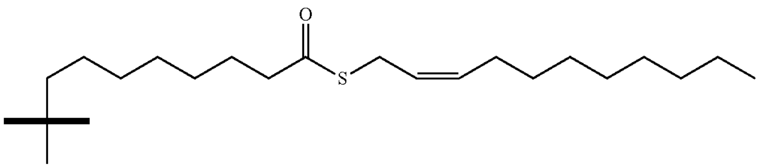
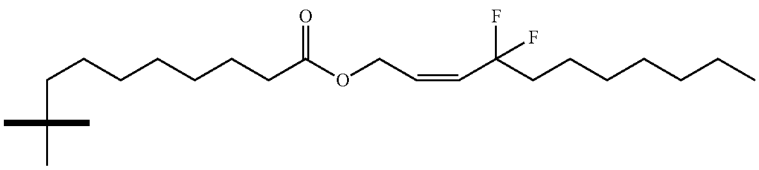


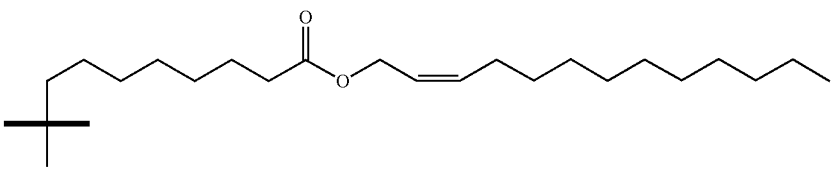
Table 1C-continued

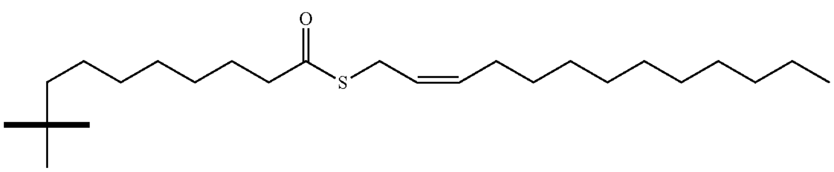


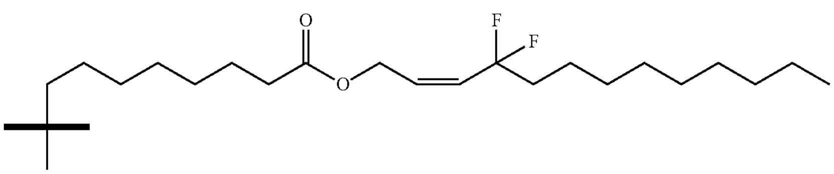


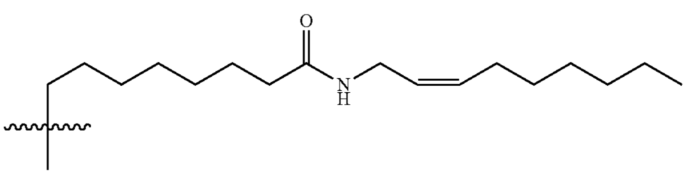












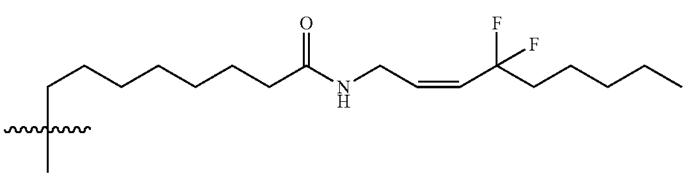
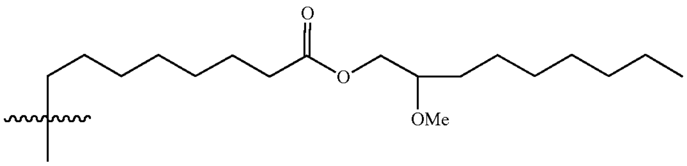
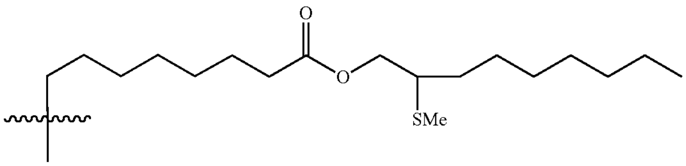
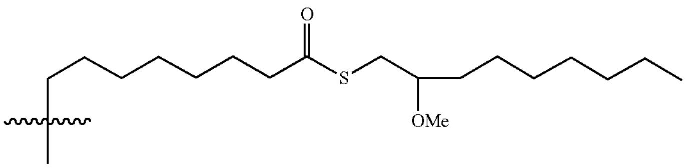
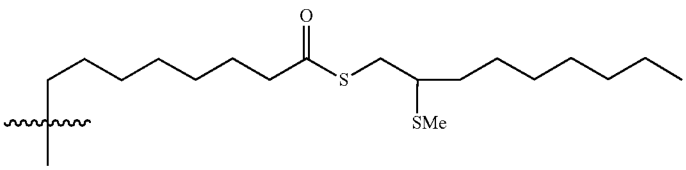


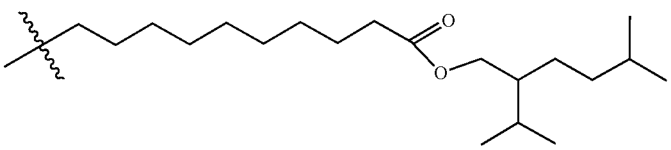
Table 1C-continued

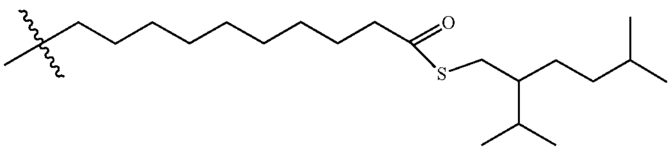


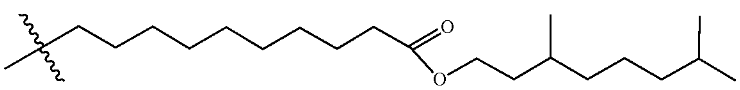


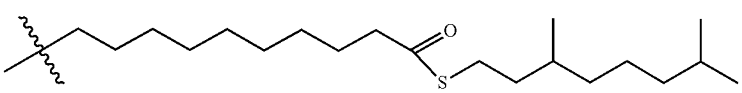


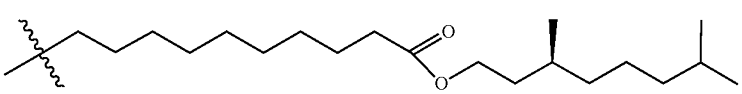


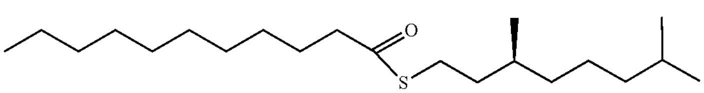












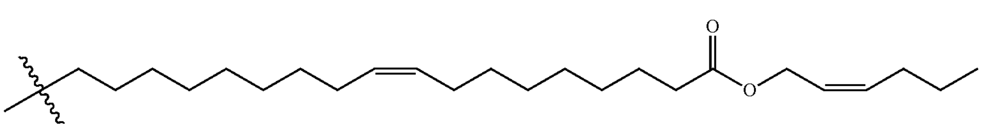
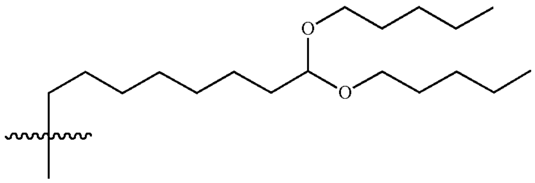
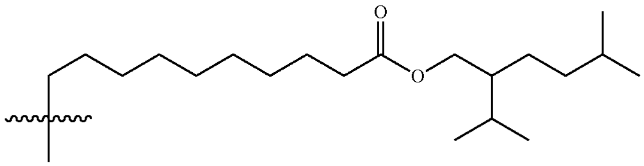


Table 1C-continued



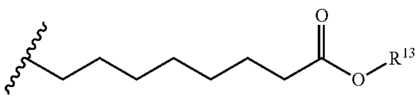


Other suitable tail groups includes those of the formula —R^{12}-M^1-R^{13} where R^{12} is a C_4-C_{14} alkyl or C_4-C_{14} alkenyl, M^1 is a biodegradable group as defined above, and R^{13} is a branched alkyl or alkenyl (e.g., a C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl), such that (i) the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms (i.e., the total length of the tail from the first carbon after the tertiary carbon (marked with an asterisk) to a terminus of the tail is at most 21), and (ii) the group —R^{12}-M^1-R^{13} has at least 20 carbon atoms (e.g., at least 21 or 22 carbon atoms).

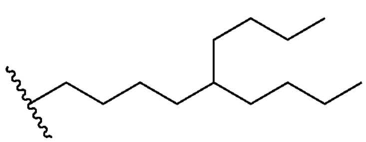
In one preferred embodiment, the chain length of —R^{12}-M^1-R^{13} is at most 21 (e.g., at most 20). For example, the chain length can be from about 17 to about 24 or from about 18 to about 20.

In one embodiment, the total carbon atom content of each tail (—R^{12}-M^1-R^{13}) is from about 17 to about 26. For example, the total carbon atom content can be from about 19 to about 26 or from about 21 to about 26.

In one embodiment, the tail has the formula:

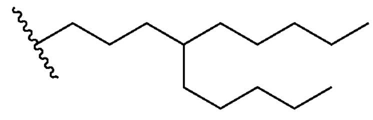


where R^{13} is an alkyl or alkenyl group having from about 13 to about 17 carbon atoms, and the total carbon length of the tail from the first carbon (the leftmost carbon atom above) to a terminus of the tail is at most 20. Preferably, the tail has from about 22 to about 26 carbon atoms. In one embodiment, the maximum length of R^{13} from its attachment point to the ester group of the compound is 12 carbon atoms (e.g., the maximum length can be 11 carbon atoms). In one preferred embodiment, the branch in the alkyl or alkenyl group is at the δ-position or later from the point of attachment of R^{13} to the ester group. Suitable R^{13} groups include, but are not limited to

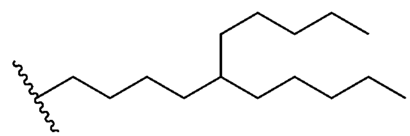


C13 (C21)
Length: C9 (18)

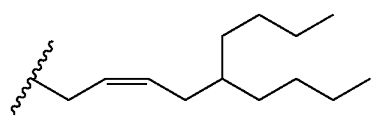
-continued



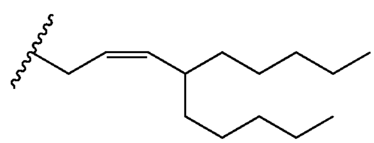
C14 (C22)
Length: C9 (18)



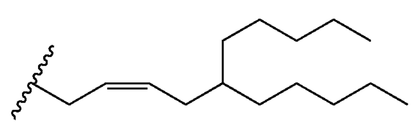
C15 (C23)
Length: C10 (19)



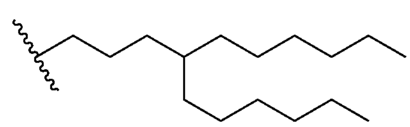
C13 (C21)
Length: C9 (18)



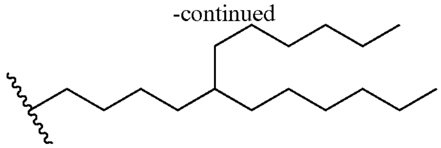
C14 (C22)
Length: C9 (18)



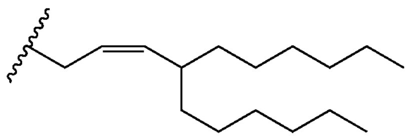
C15 (C23)
Length: C10 (19)



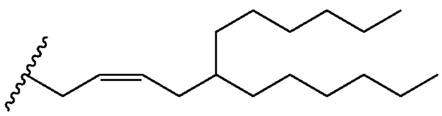
C16 (C24)
Length: C10 (19)



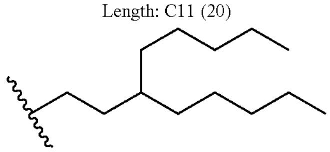
C17 (C25)
Length: C11 (20)



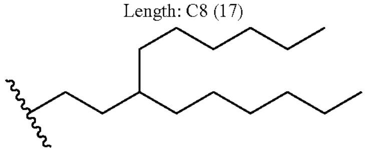
C16 (C24)
Length: C10 (19)



C17 (C25)
Length: C11 (20)

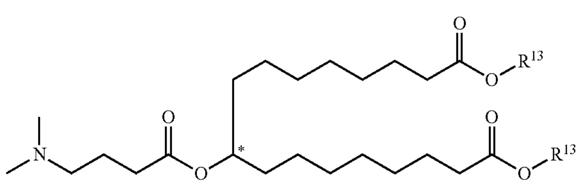


C13 (C21)
Length: C8 (17)



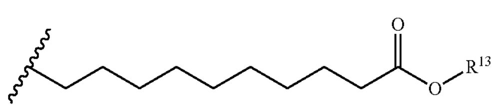
C15 (C23)
Length: C9 (18)

For example, the cationic lipid can be

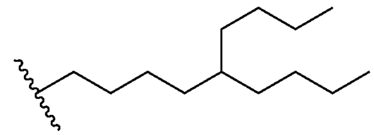


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), where R^{13} is selected from the groups mentioned above.

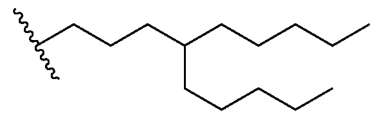
Another example is a tail of the formula



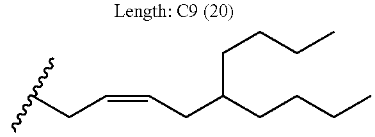
where R^{13} is an alkyl or alkenyl group having from about 13 to about 15 carbon atoms, and the total carbon length of the tail from the first carbon (i.e., the leftmost carbon atom, which is attached to a tertiary carbon) to a terminus of the tail is at most 20. Preferably, the tail has from about 24 to about 26 carbon atoms. In one embodiment, the maximum length of R^{13} from its attachment point to the ester group of the compound is 10 carbon atoms (e.g., the maximum length can be 9 carbon atoms). In one preferred embodiment, the branch in the alkyl or alkenyl group is at the δ-position or later from the point of attachment of R^{13} to the ester group. Suitable R^{13} groups include, but are not limited to



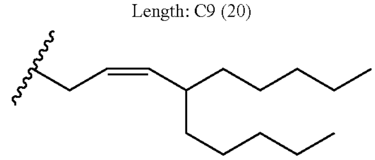
C13 (C23)
Length: C9 (20)



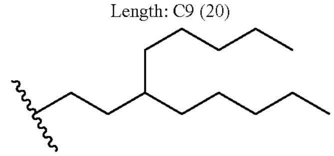
C14 (C24)
Length: C9 (20)



C13 (C23)
Length: C9 (20)

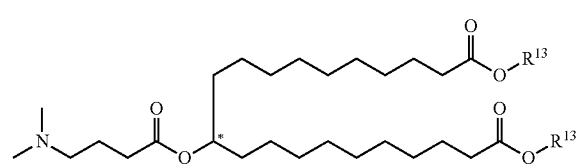


C14 (C24)
Length: C9 (20)



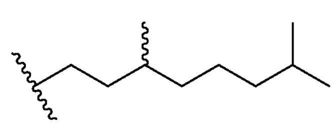
C13 (C24)
Length: C8 (19)

For example, the cationic lipid can be

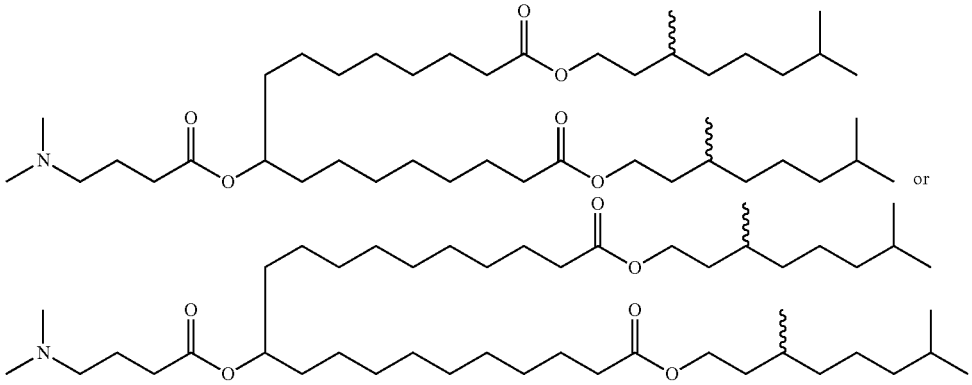


or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), where R^{13} is selected from the groups above.

The R^{13} group may be derived from a natural product, such as dihydrocitgronellol, lavandulol, phytol, or dihydrophytol. In one embodiment, the R^{13} group in the tails above is a dihydrocitronellol group (either as a racemic group or a chirally pure group):

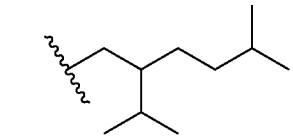


For example, the cationic lipid having a dihydroitronellol group can be

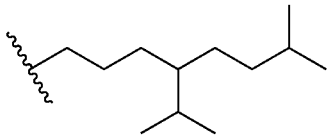


or a salt thereof.

In another embodiment, the R^{13} group in the tails above is a lavandulol group or a homolog of it as shown below:

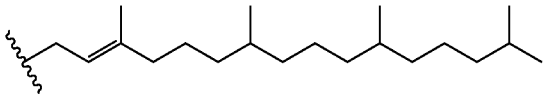


Lavandulol

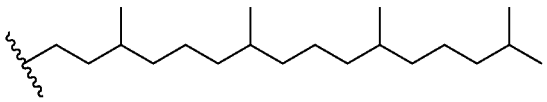


homolog

In another embodiment, the R^{13} group in the tails above is a phytol or dihydrophytol group:

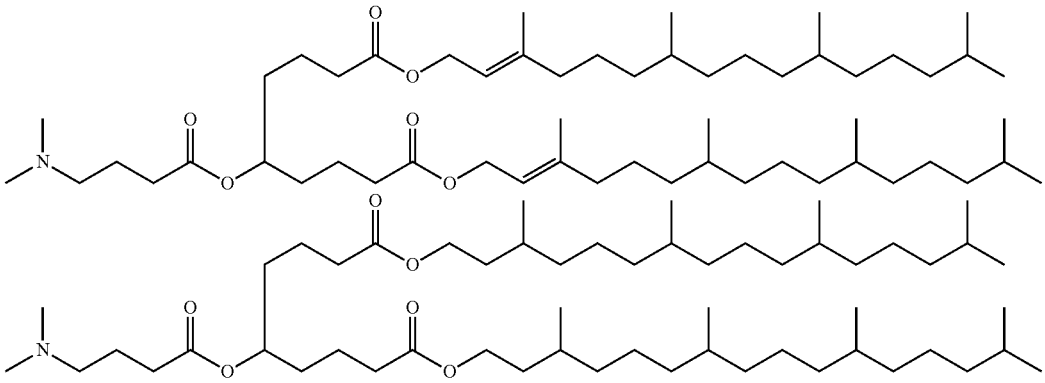


Phytol

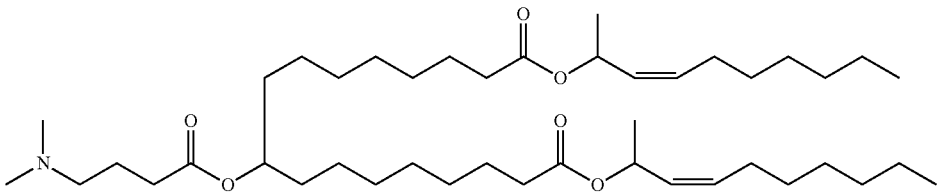


Dihydrophytol

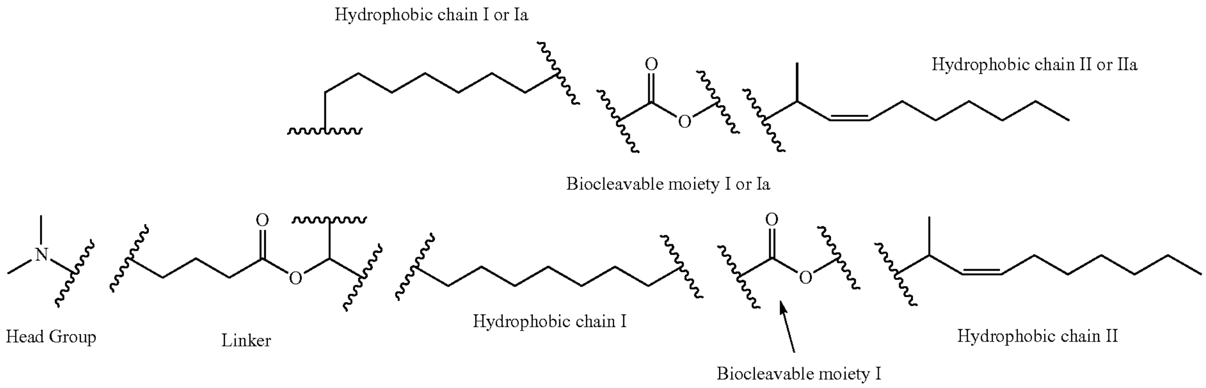
For instance, the cationic lipid can be:



A cationic lipid of the formula:



can also be thought of as a combination of a headgroup, a linker moiety, and two parts of the hydrophobic chains as follows:



Various headgroups, linker moieties, and hydrophobic chains I and II are listed below. The present invention includes compounds composed of any combination of the head, linker, hydrophobic chain I, and hydrophobic chain II groups listed below.

TABLE 2A

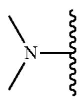
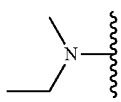
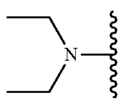
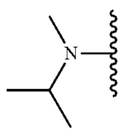
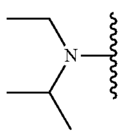
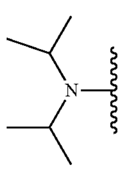
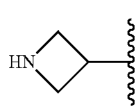
Representative headgroups








TABLE 2A-continued

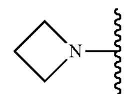
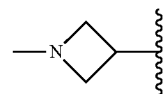
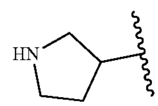
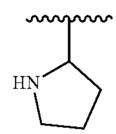
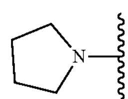
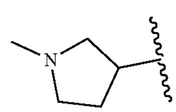
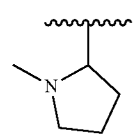
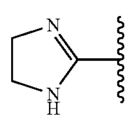
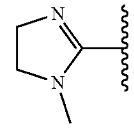
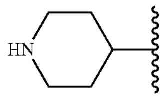
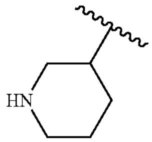
Representative headgroups









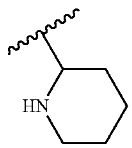
TABLE 2A-continued

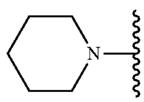
Representative headgroups

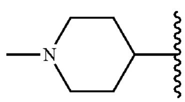


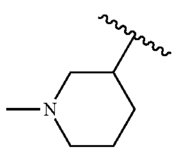


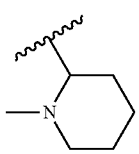


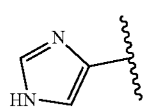


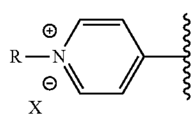




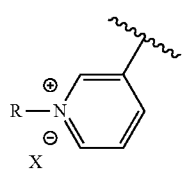








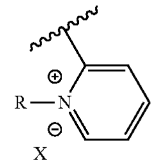
R = H, alkyl; X = halogen



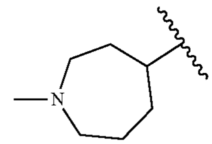
R = H, alkyl; X = halogen

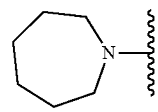
TABLE 2A-continued

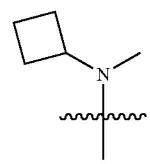
Representative headgroups

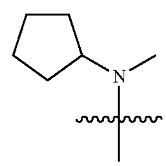


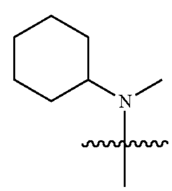
R = H, alkyl; X = halogen

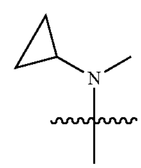


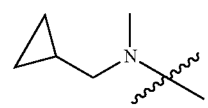


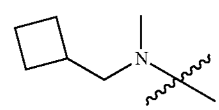












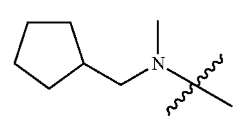
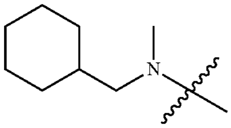
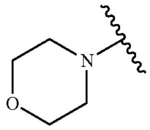
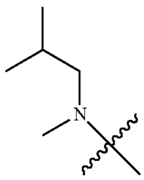


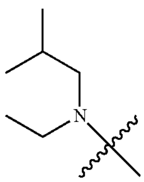
TABLE 2A-continued

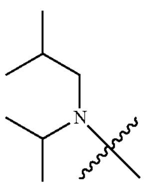
Representative headgroups

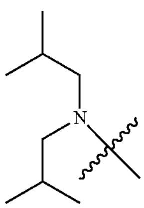


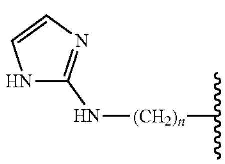




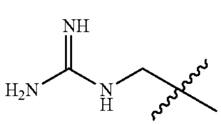








(where n is 0-5)



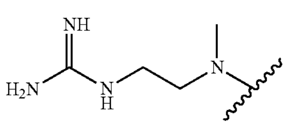
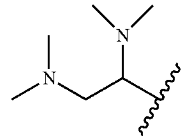
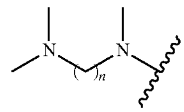


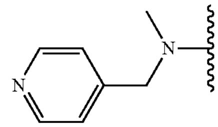
TABLE 2A-continued

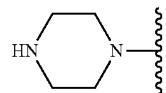
Representative headgroups

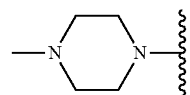


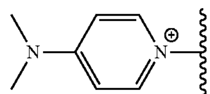


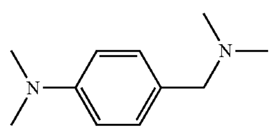
n = 0-6

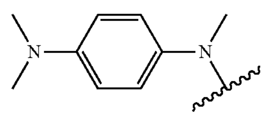


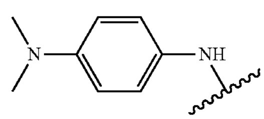


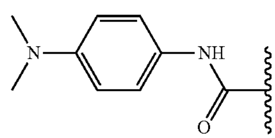


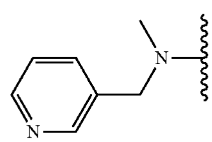












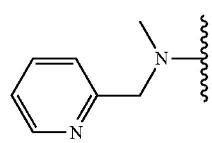
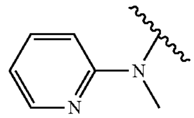
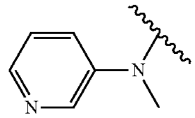


TABLE 2A-continued

Representative headgroups





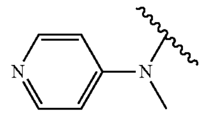
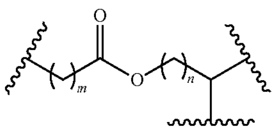
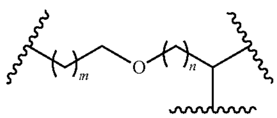


TABLE 2B

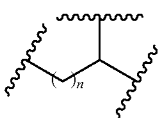
Representative linker groups



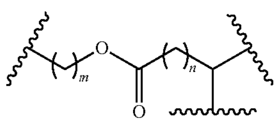
m = 1-5; n = 0-3



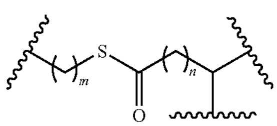
m = 1-5; n = 0-3



n = 0-3



m = 1-5; n = 0-3



m = 1-5; n = 0-3

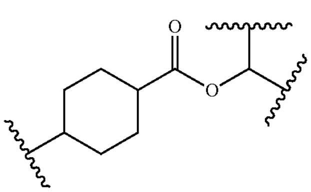
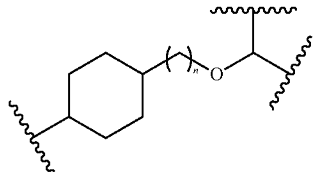
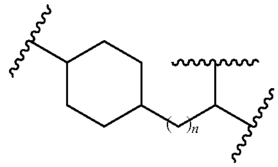


TABLE 2B-continued

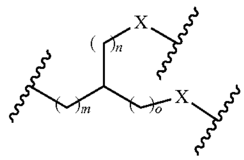
Representative linker groups



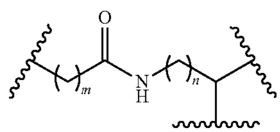
n = 0-3



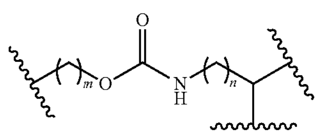
n = 0-3



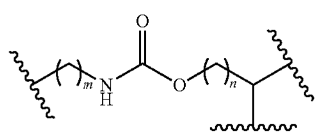
m = 1-4; n/o = 0-3
X = O or S



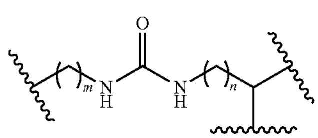
m = 0-5; n = 0-3



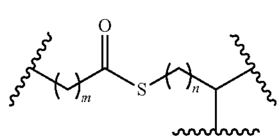
m = 0-5; n = 0-3



m = 0-5; n = 0-3



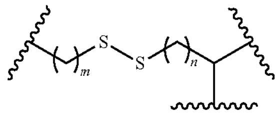
m = 0-5; n = 0-3



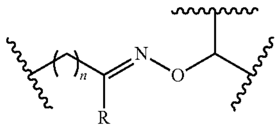
m = 0-5; n = 0-3

TABLE 2B-continued

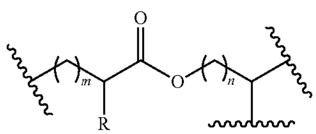
Representative linker groups



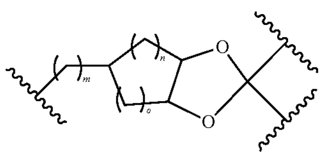
m = 0-5; n = 0-3



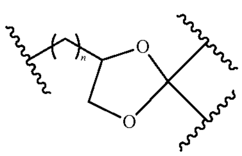
n = 0-5



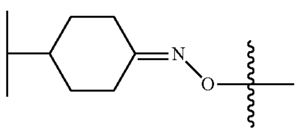
m = 1-4; n/o = 0-3
R = COOH, COOMe,
COOEt, CN, CONH2
CONHMe

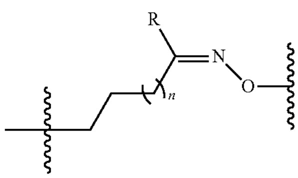


m = 1-4; n/o = 1-3

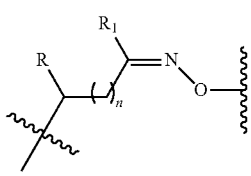


n = 1-5





n = 0-5
R = H, Me, Et, Pr, allyl

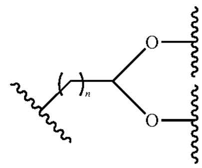


n = 0-5

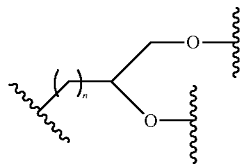
TABLE 2B-continued

Representative linker groups

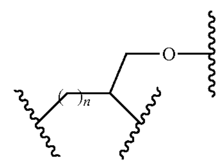
R = H, Me, Et, Pr, allyl
R1= Me, Et, Pr, allyl



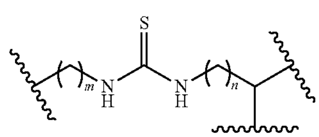
n = 0-6



n = 0-6



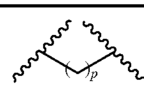
n = 0-6



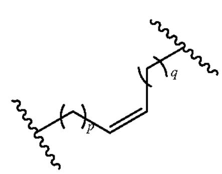
m = 0-5; n = 0-3

TABLE 2C

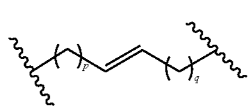
Representative hydrophobic chain I and/or Ia, and combination thereof



p = 0-15



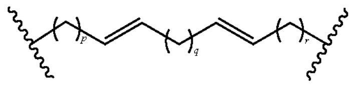
p = 0-15, q = 0-15



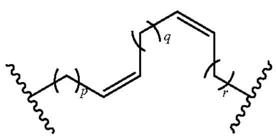
p = 0-15, q = 0-15

TABLE 2C-continued

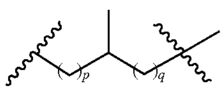
Representative hydrophobic chain I and/or Ia, and combination thereof



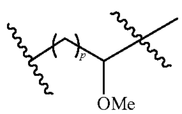
p = 0-15, q = 1-4, r = 0-15



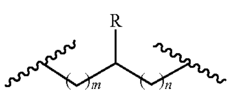
p = 0-15, q = 1-4, r = 0-15



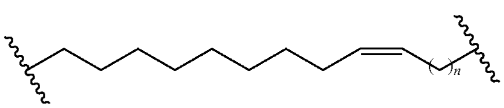
p = 0-15, q = 0-6



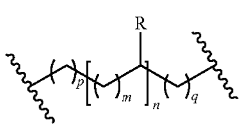
p = 0-15



m = 0-4; n = 0-4;
R = Me, Et, Pr, iPr, bu, iBu



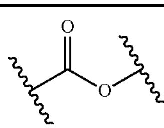
n = 1-7



m = 1-4, n = 1-10, p = 0-15, q = 0-15
R = Me, Et, OMe

TABLE 2D

Representative biodegradable moieties I and/or Ia and combinations thereof



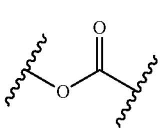
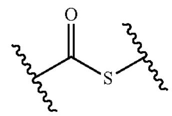
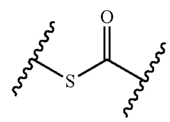
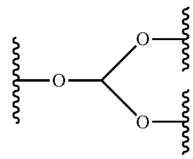


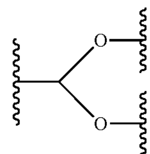
TABLE 2D-continued

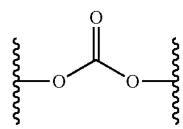
Representative biodegradable moieties I and/or Ia and combinations thereof

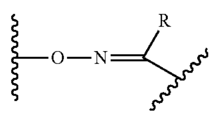


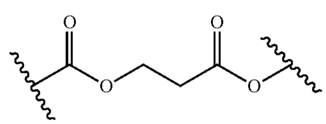


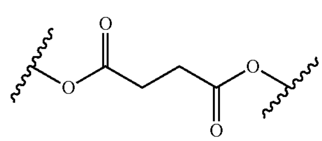


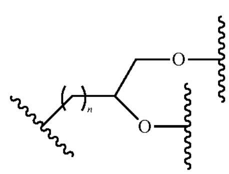




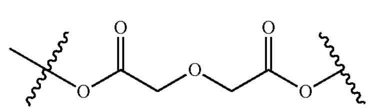








n = 0-6



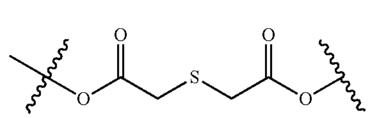
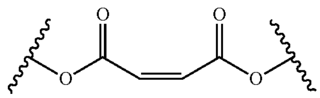
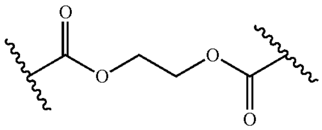
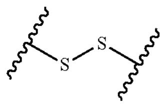


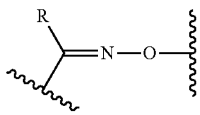
TABLE 2D-continued

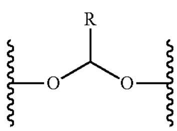
Representative biodegradable moieties I and/or Ia and combinations thereof



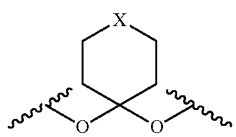








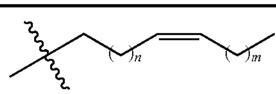
R = H, Me, Et, cyclic alkyl, alicylic, aromatic



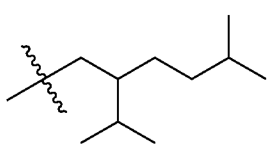
X = CH_2, O. S

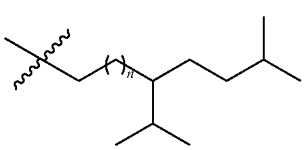
TABLE 2E

Representative hydrophobic chain II and/or IIa and combinations thereof



n = 0-6; m = 0-16





n = 0-6

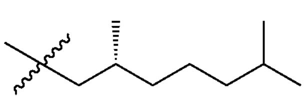
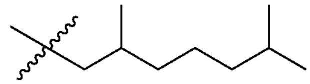
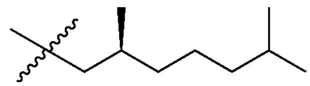
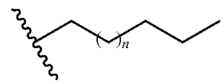


TABLE 2E-continued

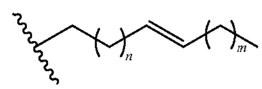
Representative hydrophobic chain II and/or IIa and combinations thereof



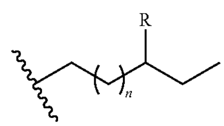




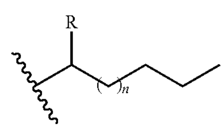
n = 0-8



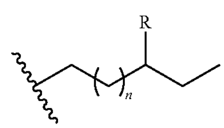
n = 0-8; m = 0-6



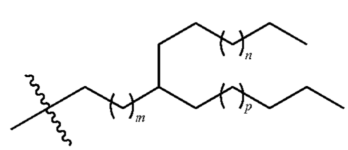
n = 0-8
R = OMe, Me, Et, n-Pr, n-Bu



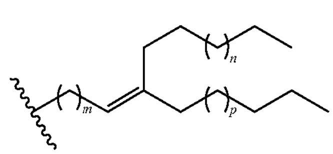
n = 0-8
R = OMe, Me, Et, Pr



n = 0-8
R = OMe, Me, Et, Pr



m = 0-6; n = 0-6; p = 0-6



m = 0-6; n = 0-6; p = 0-6

TABLE 2E-continued

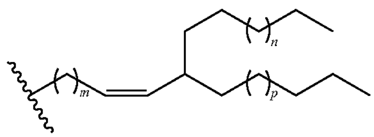
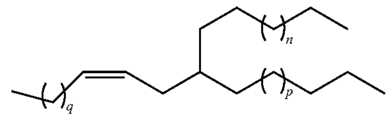
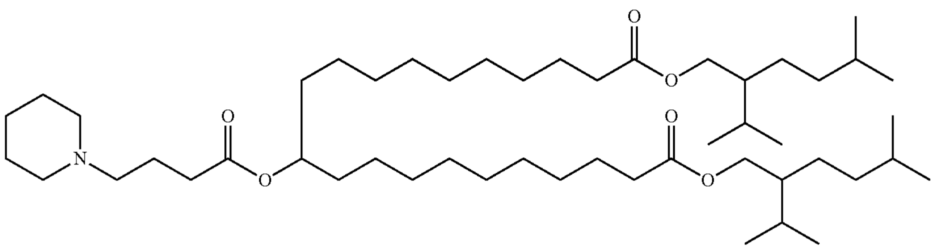
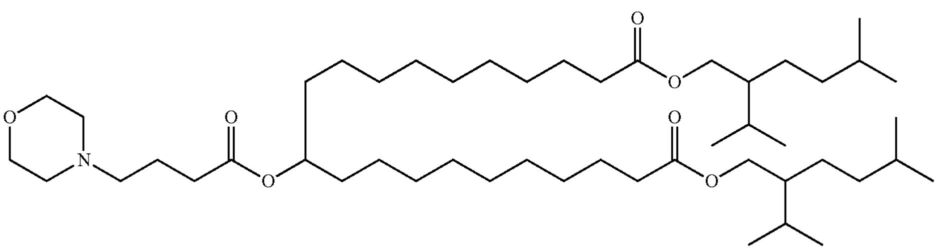
Representative hydrophobic chain II and/or IIa and combinations thereof
 m = 0-6; n = 0-6; p = 0-6

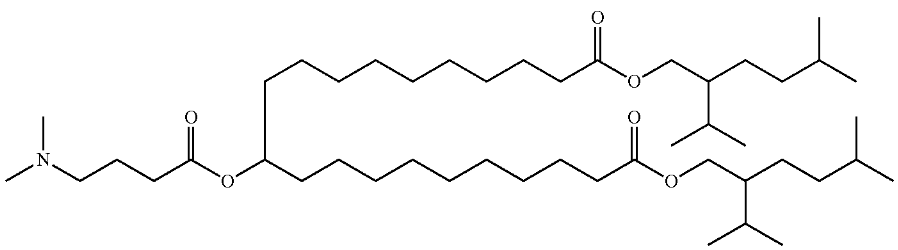
TABLE 2E-continued

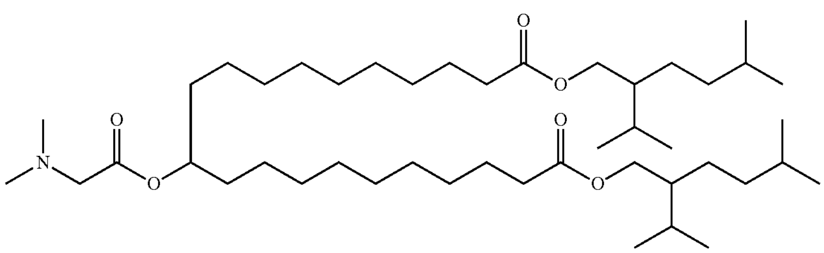
Representative hydrophobic chain II and/or IIa and combinations thereof
 m = 0-6; n = 0-6; p = 0-6; q = 0-6;

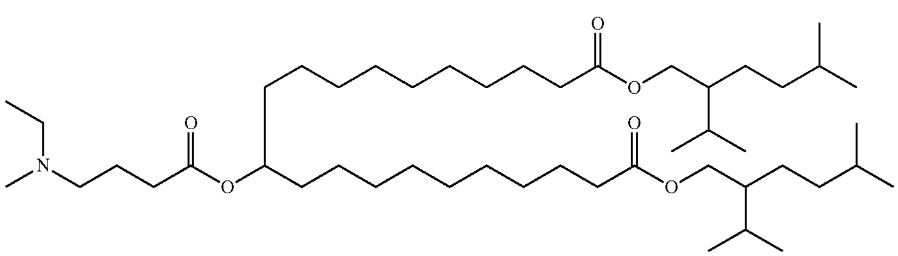
Other cationic lipids of the present invention include those in Table 3 below. Each asymmetric carbon atom in the compounds below can be either chirally pure (R or S) or racemic. These cationic lipids as well as those in the working examples (such as Examples 36 and 37) are suitable for forming nucleic acid-lipid particles.











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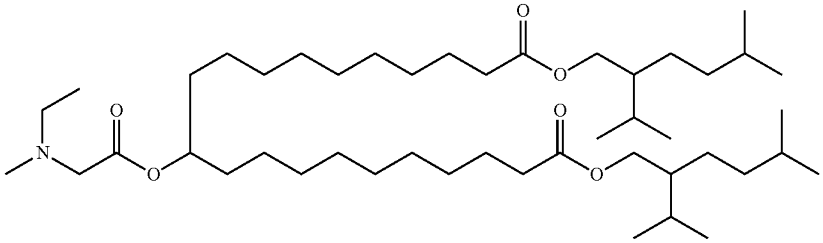

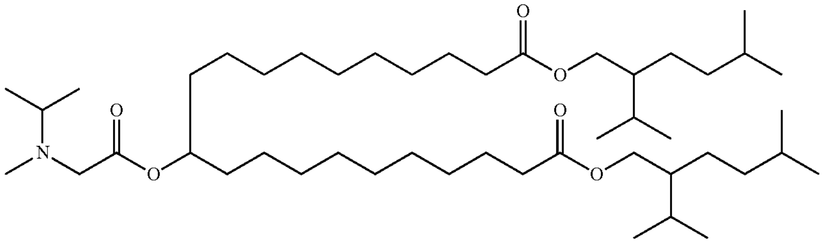

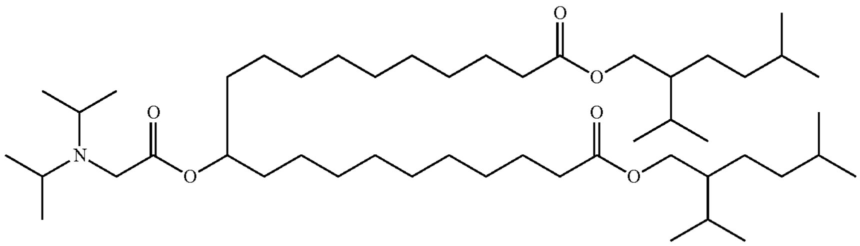

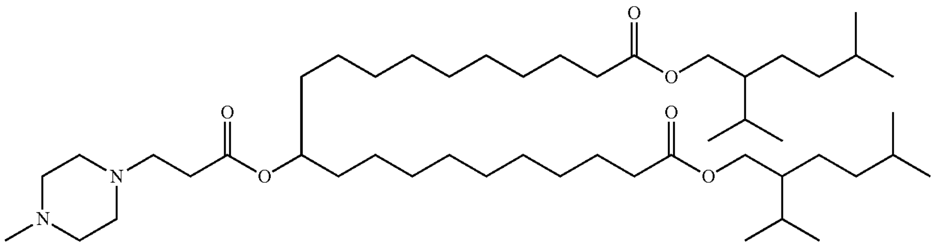

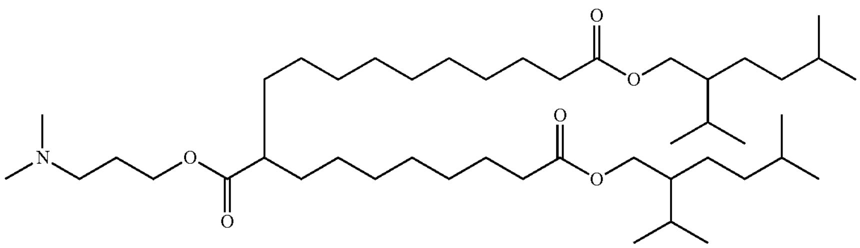

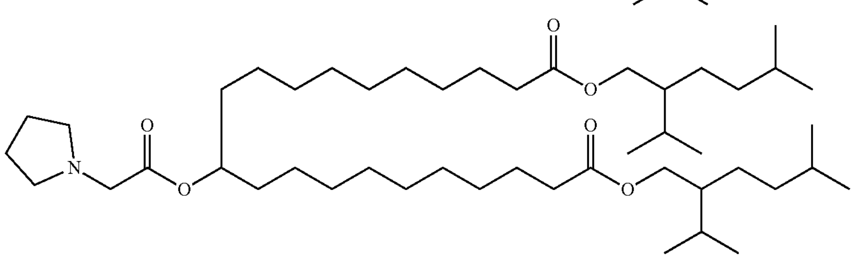

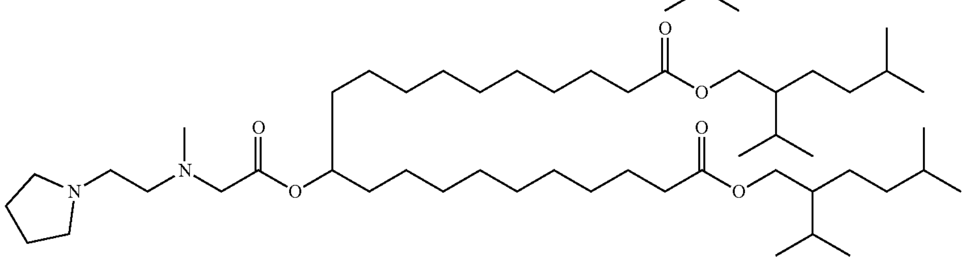

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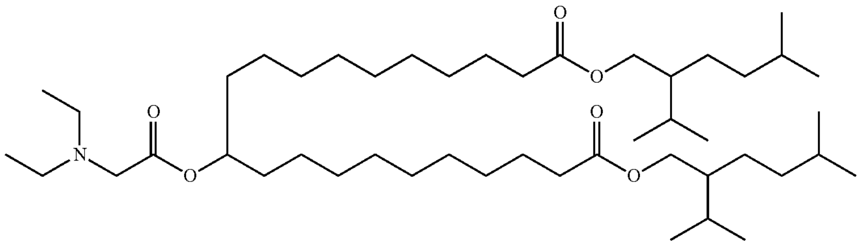

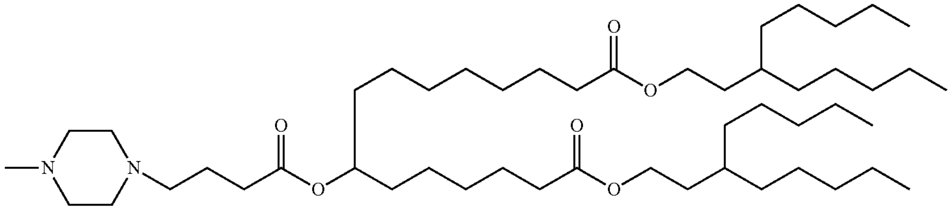

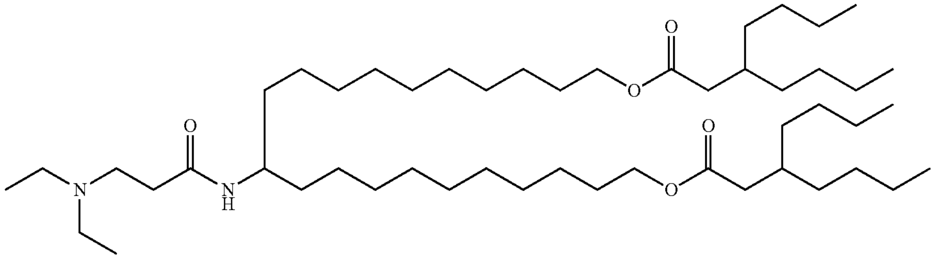

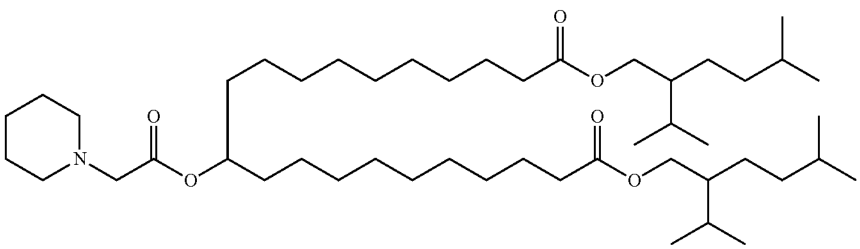

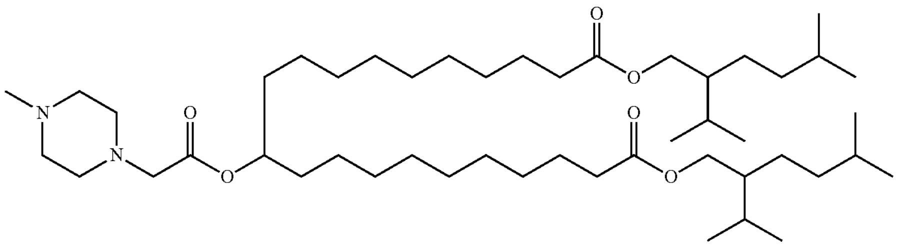

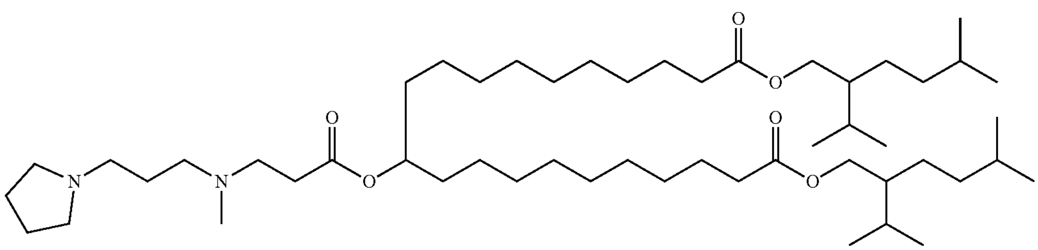

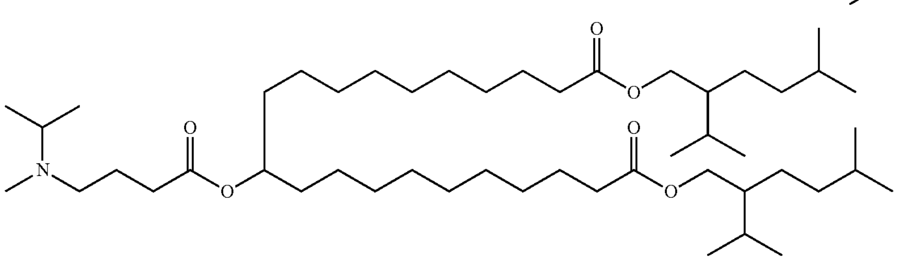

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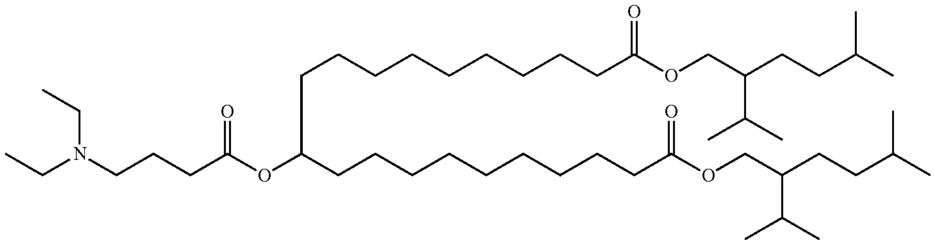

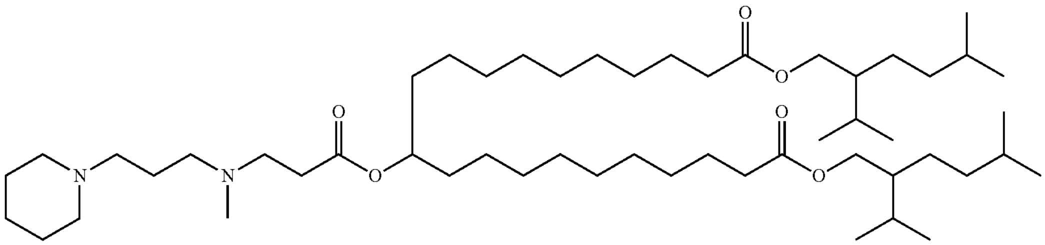

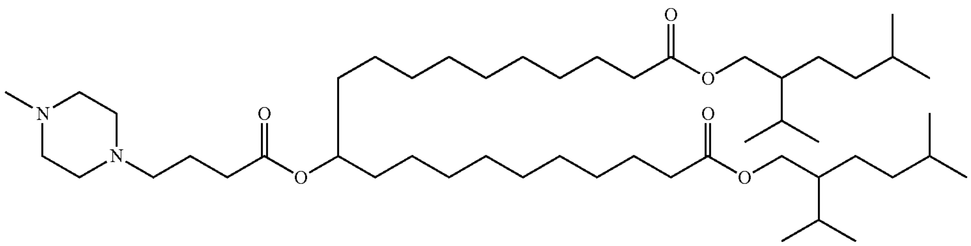

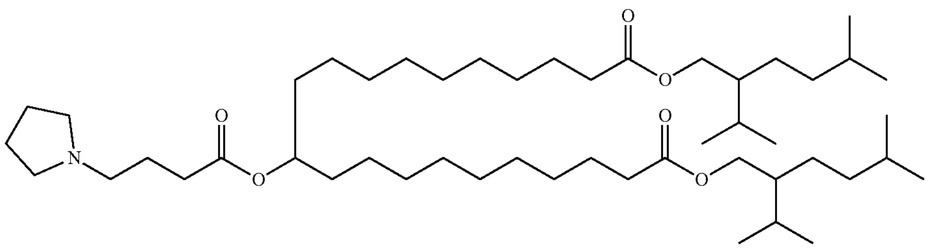

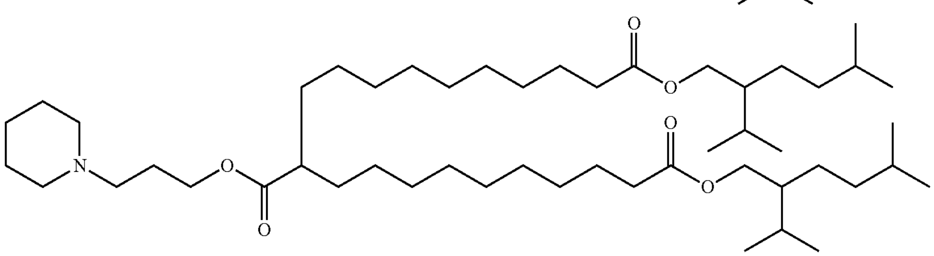

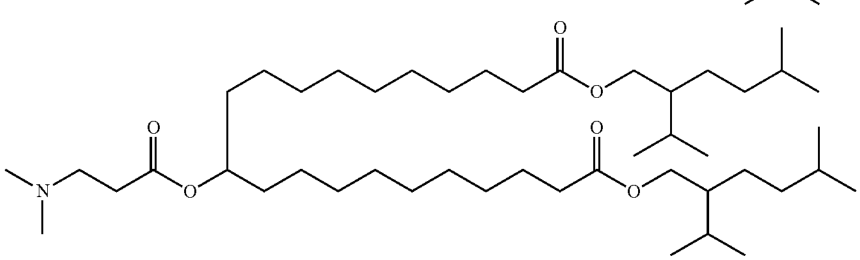

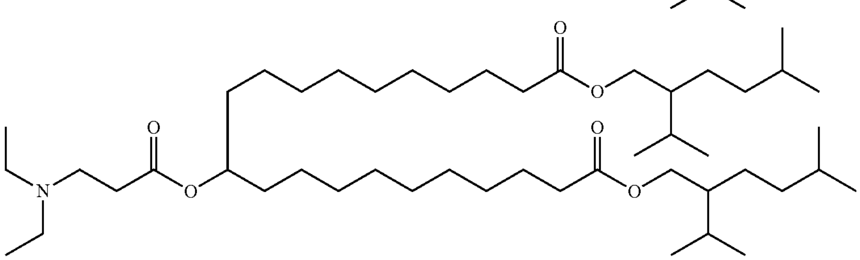

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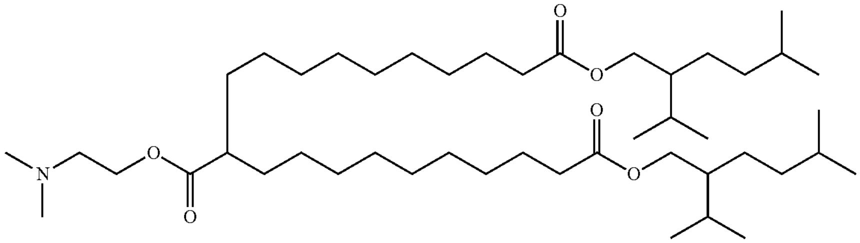

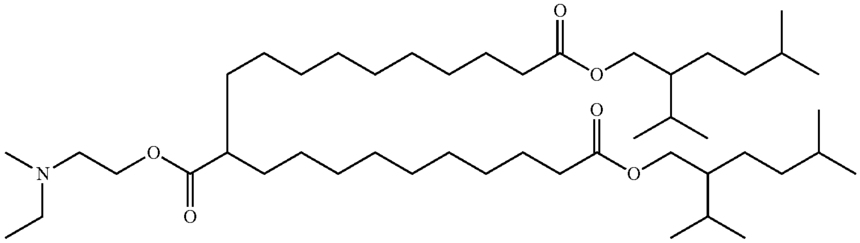

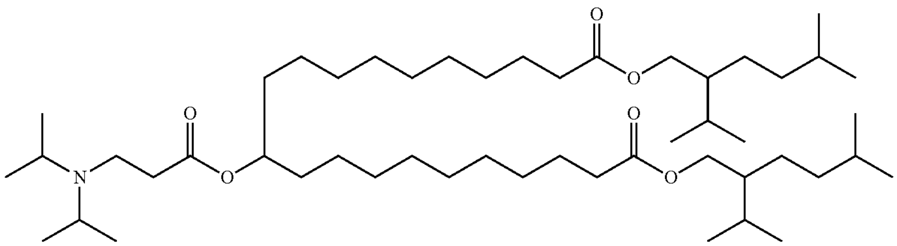

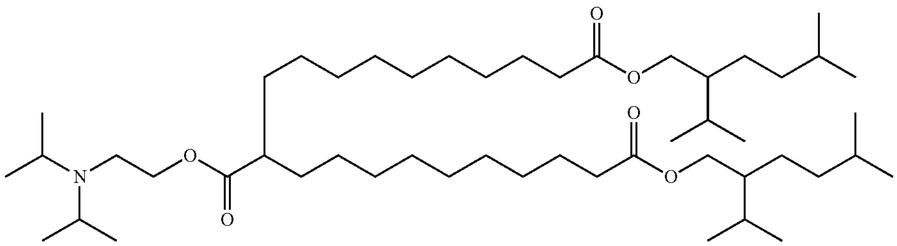

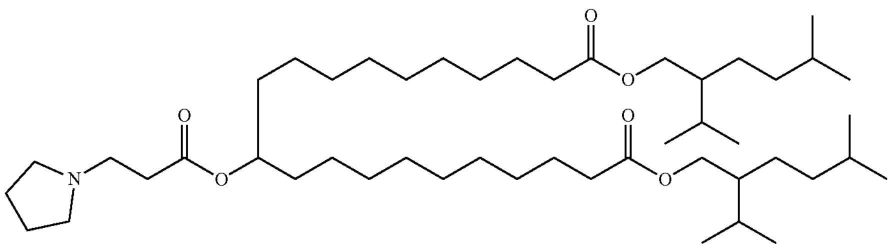

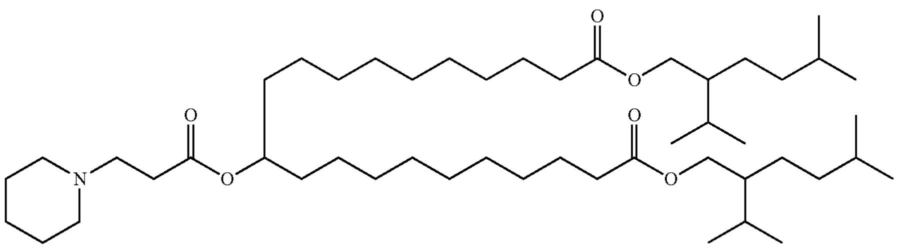

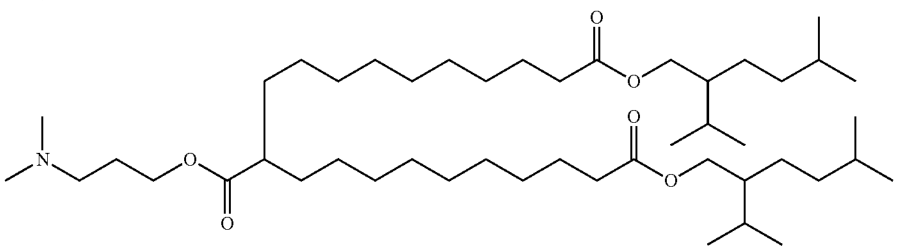

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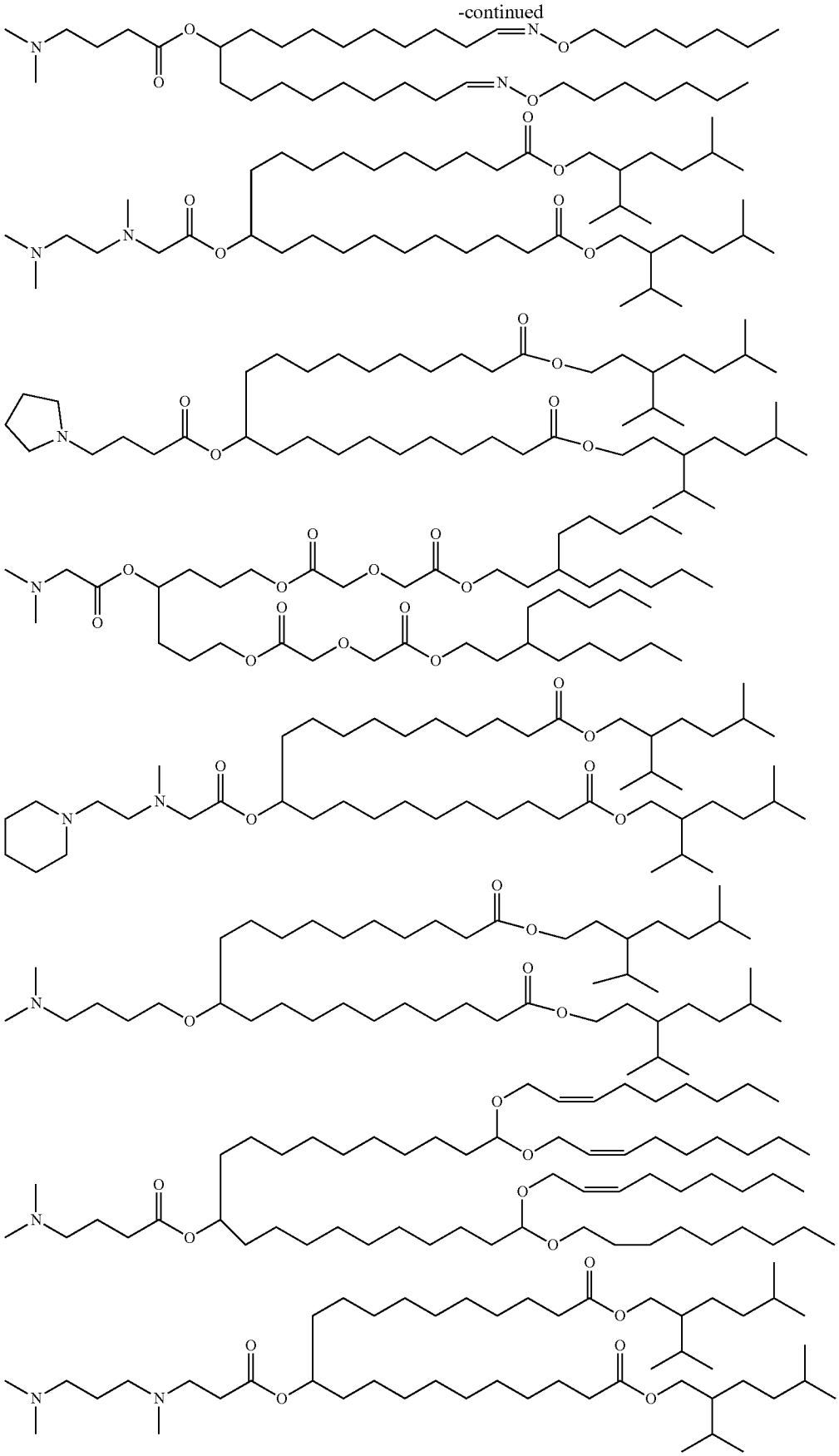

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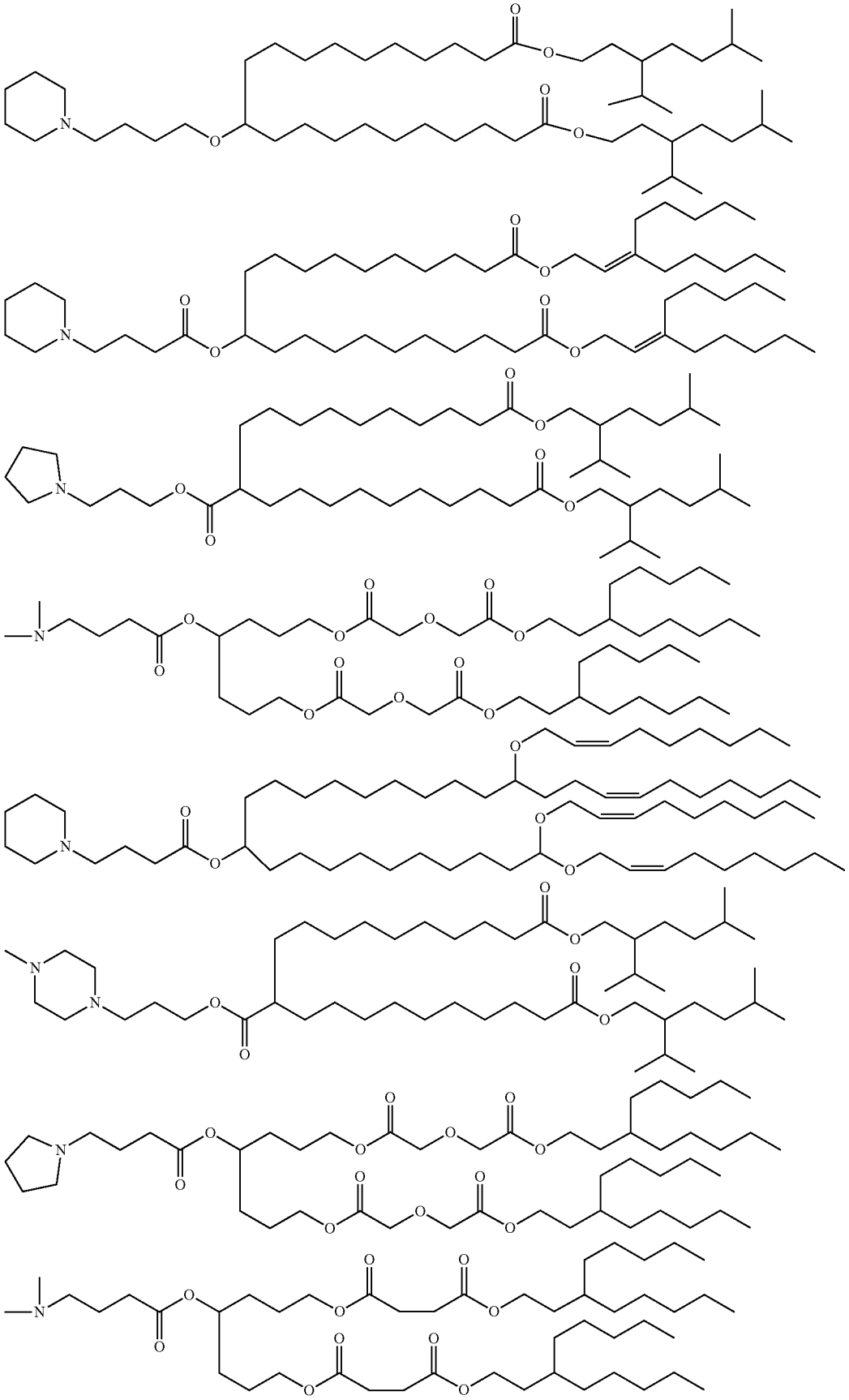

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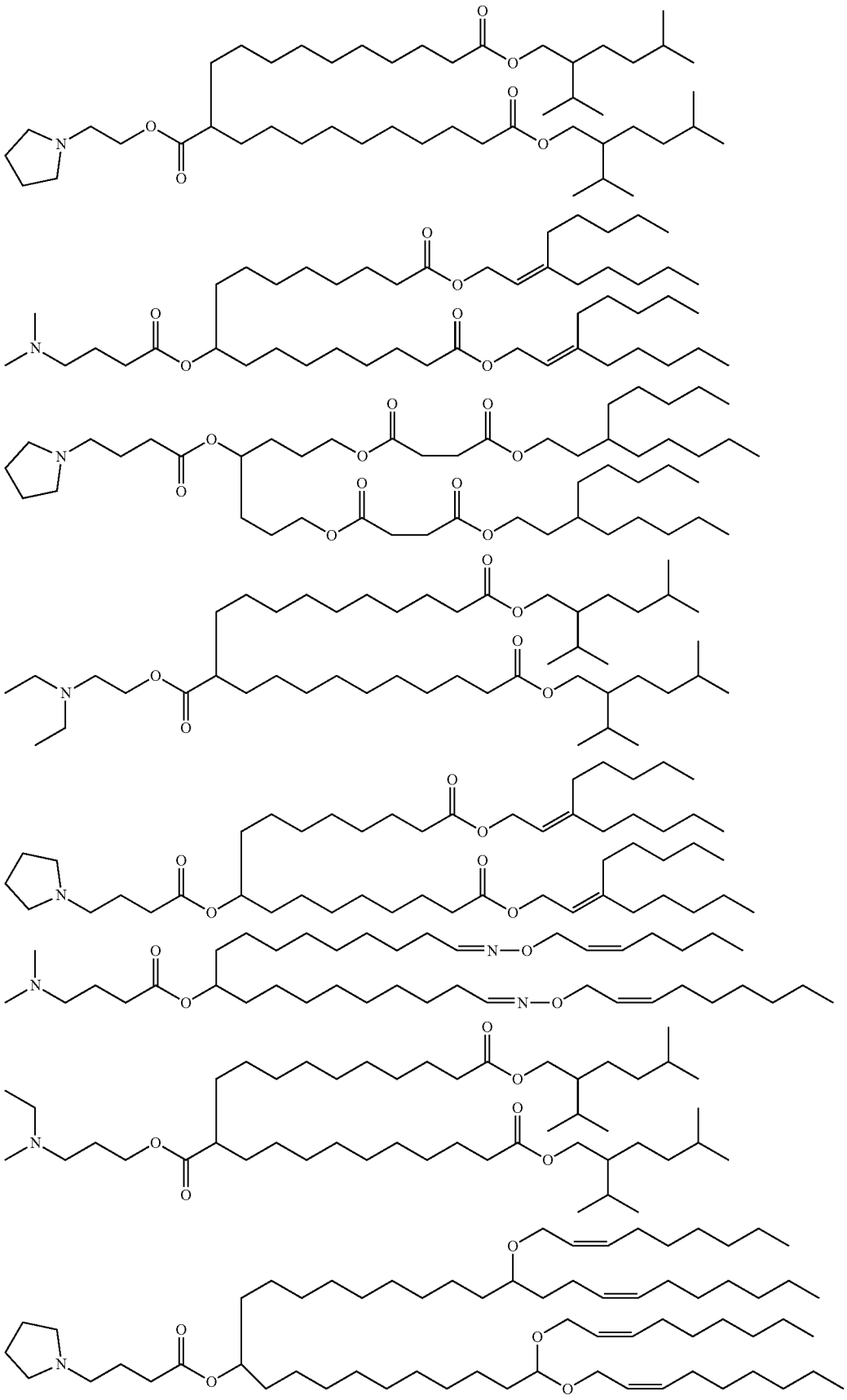

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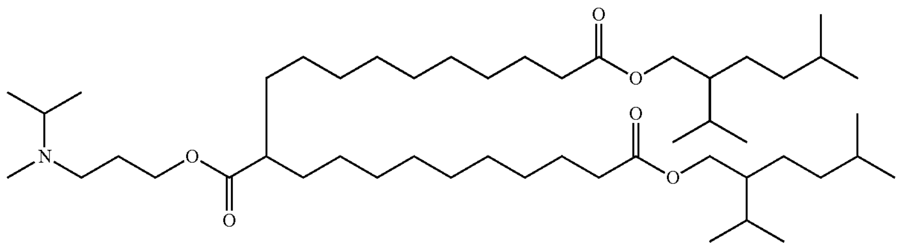

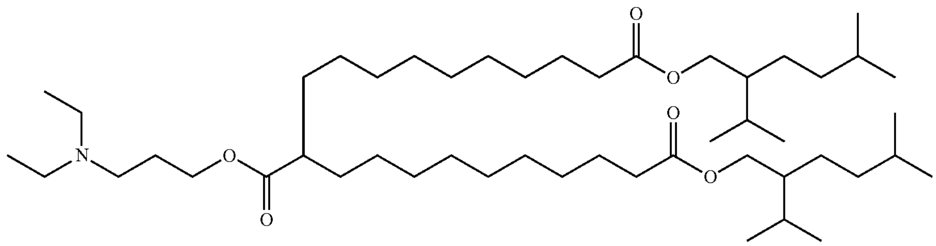

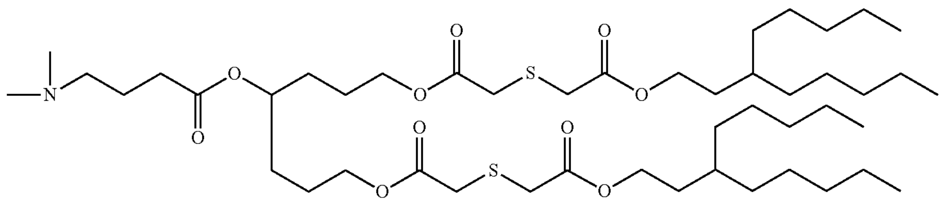

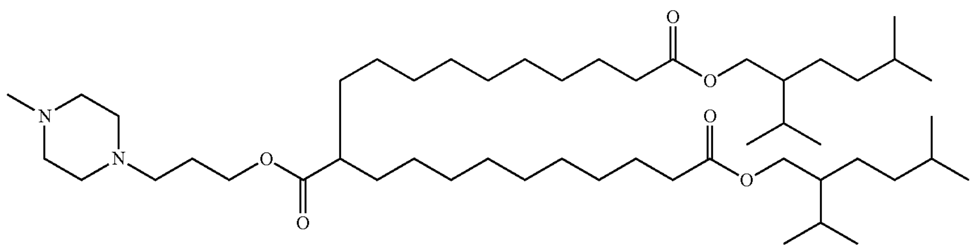

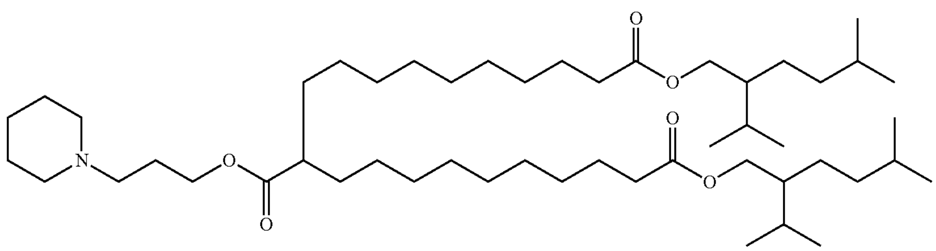

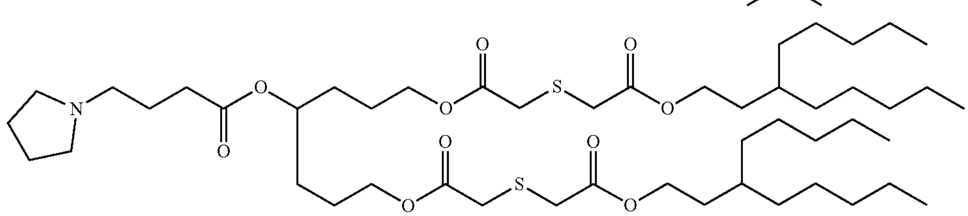

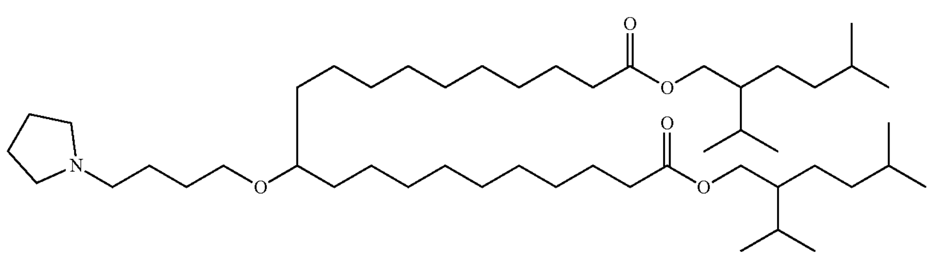

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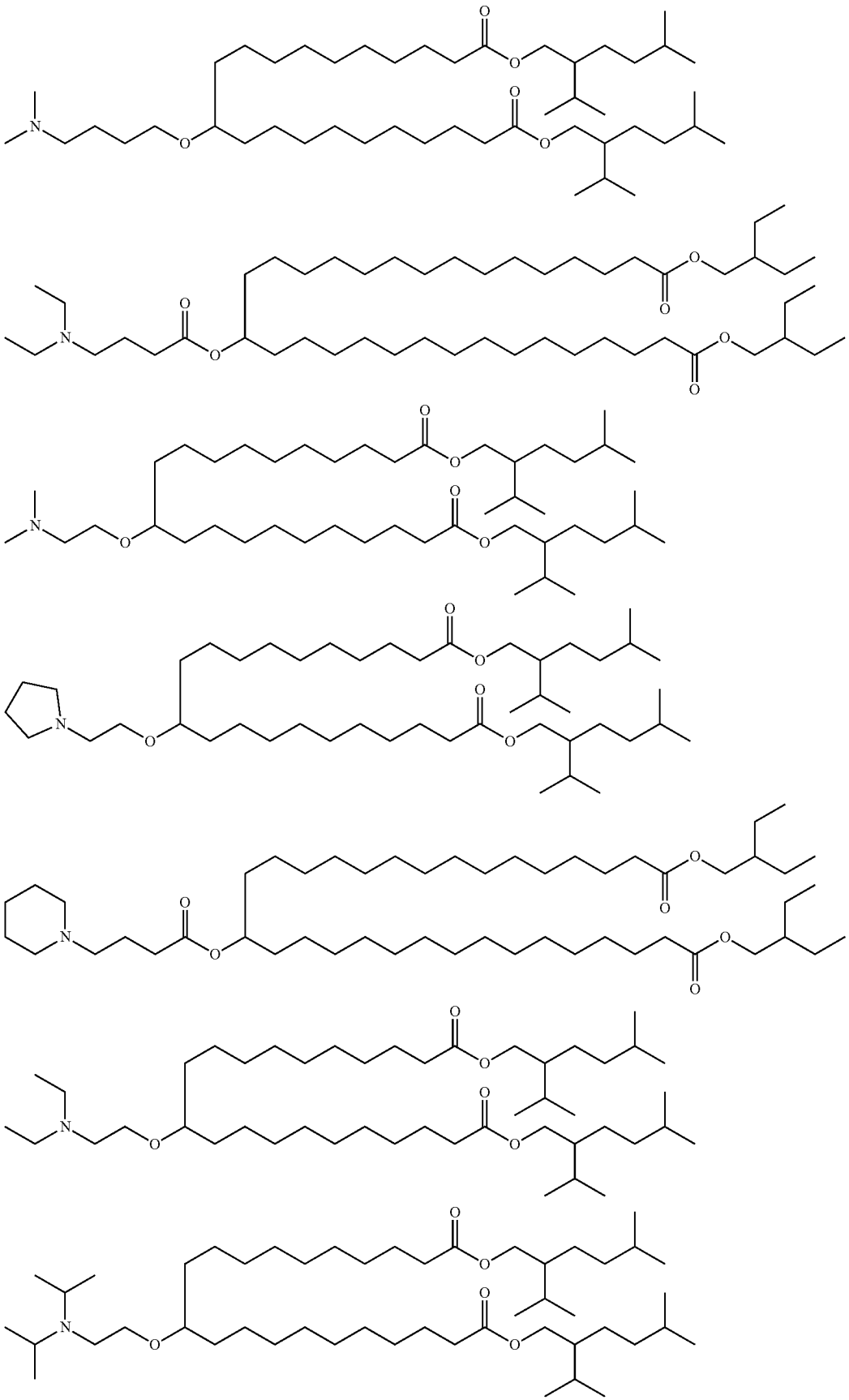

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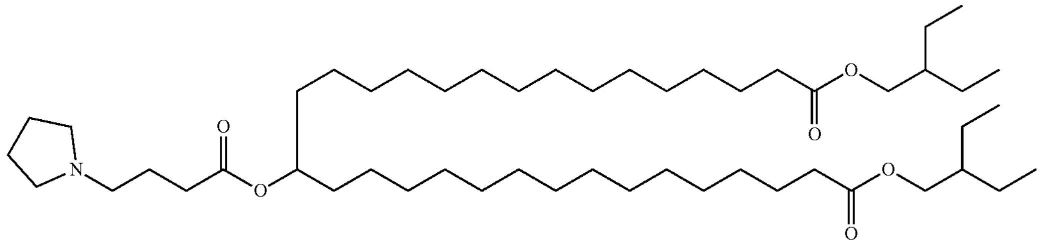

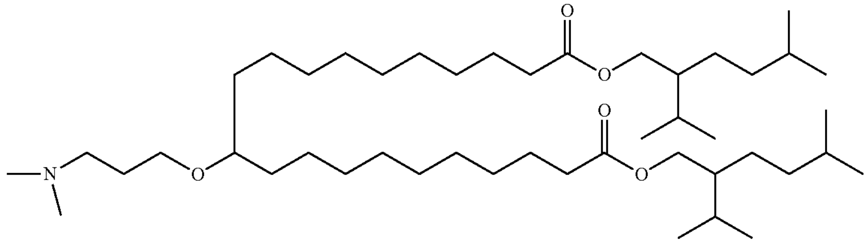

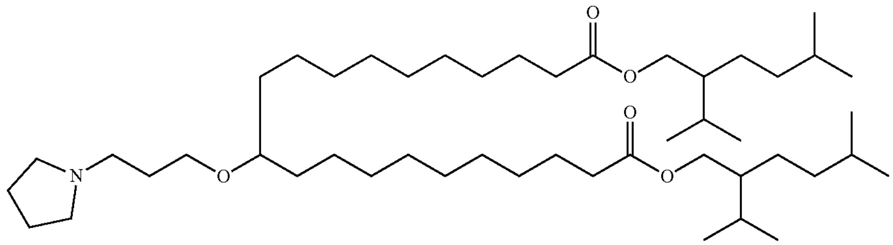

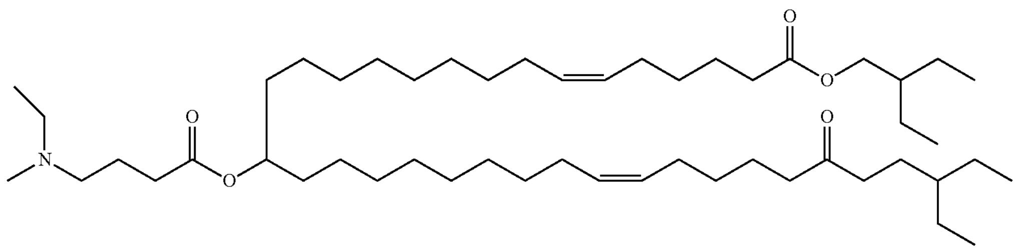

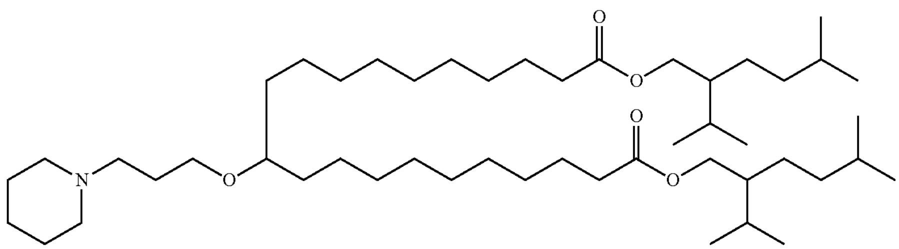

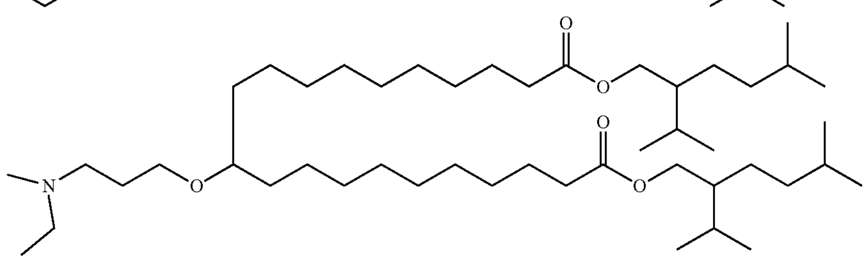

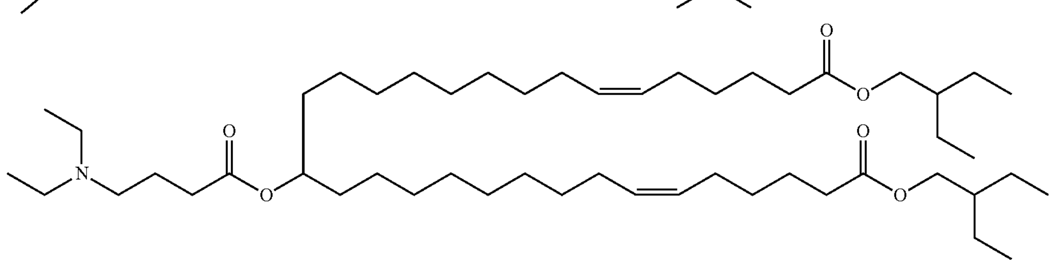

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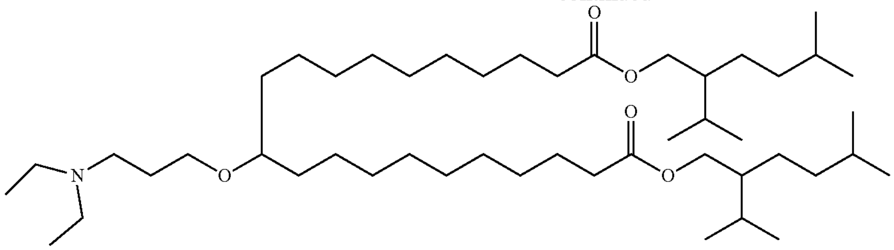

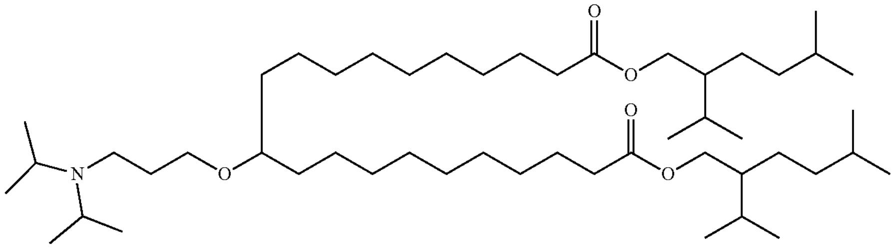

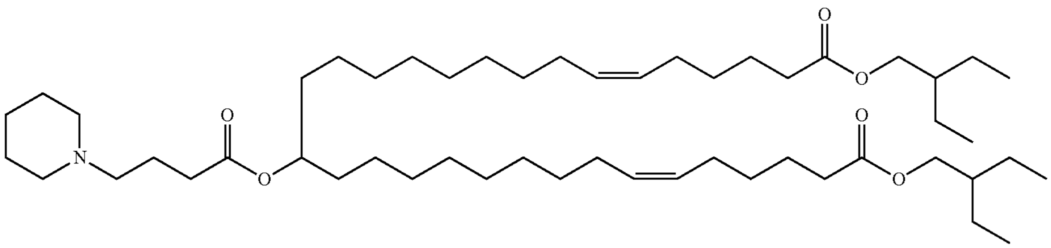

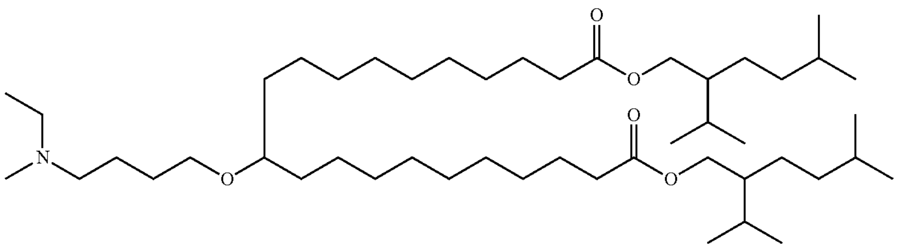

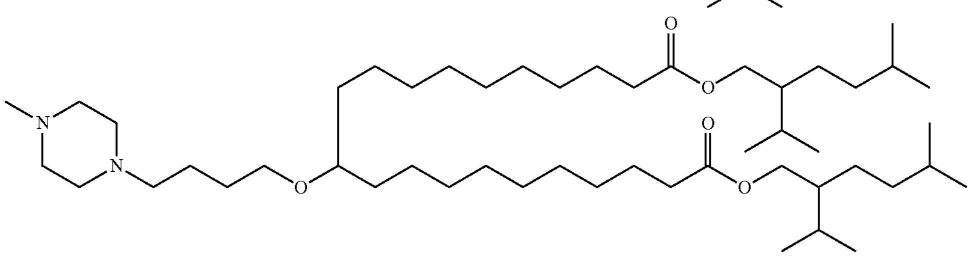

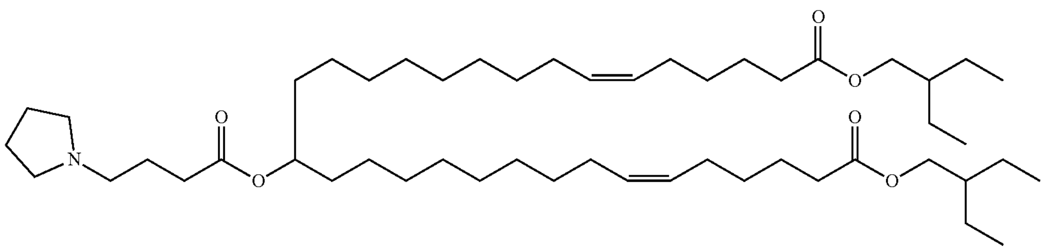

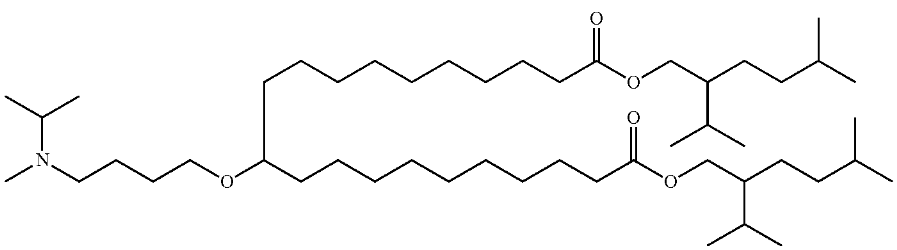

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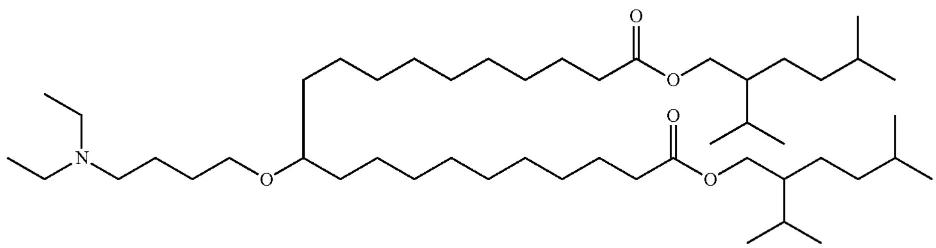

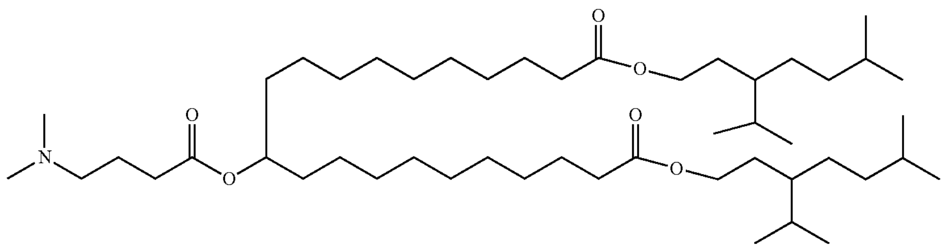

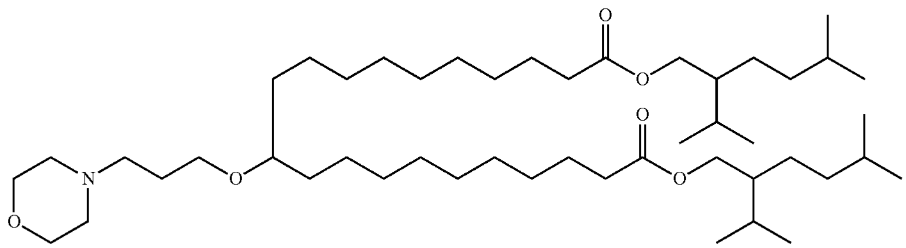

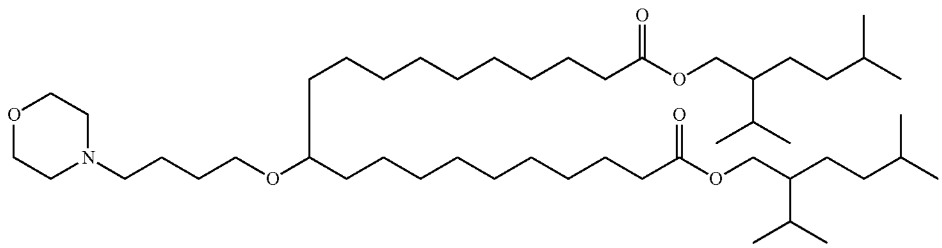

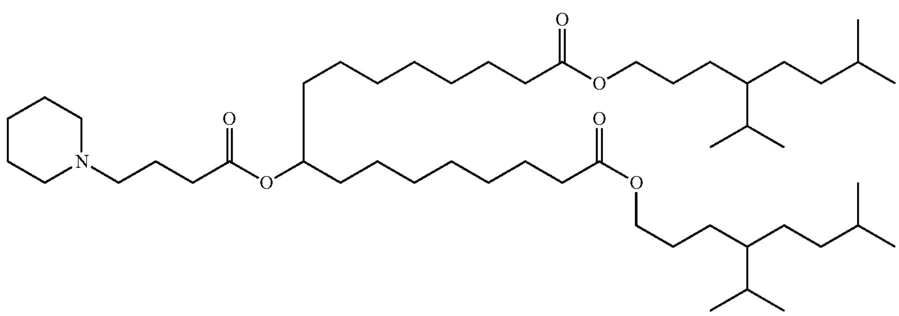

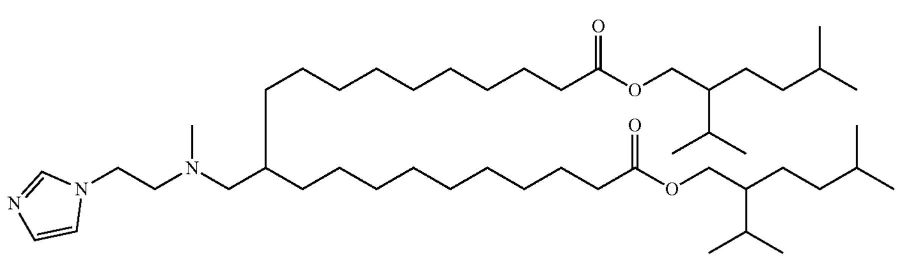

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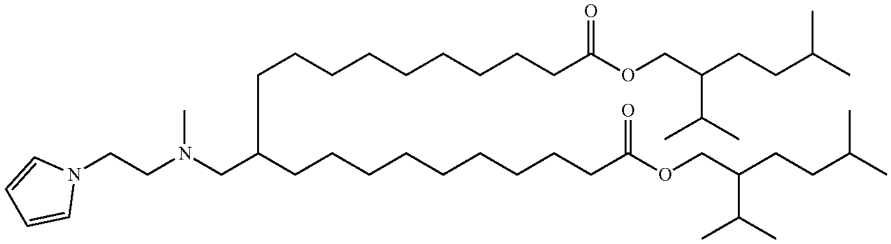

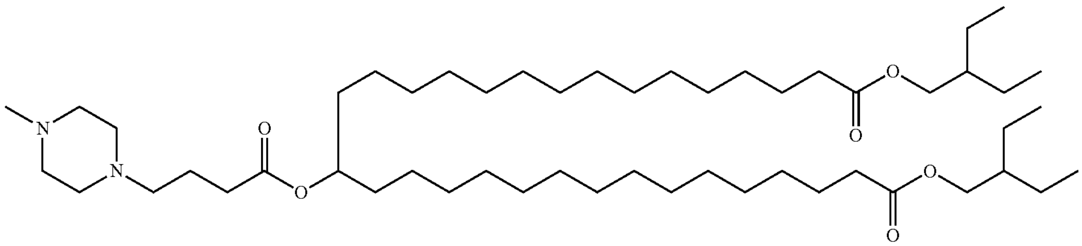

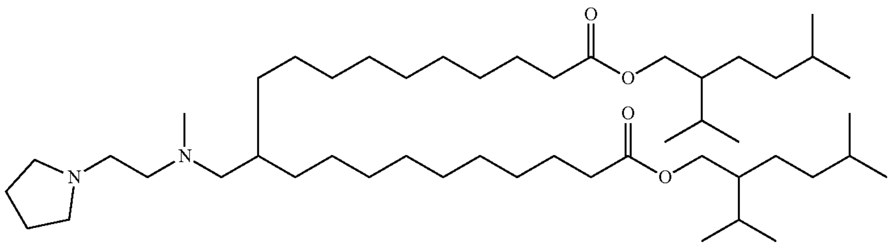

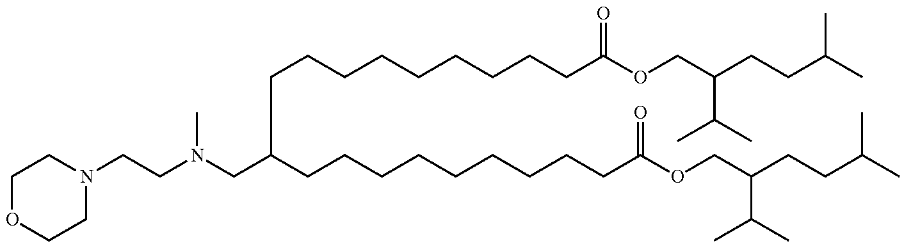

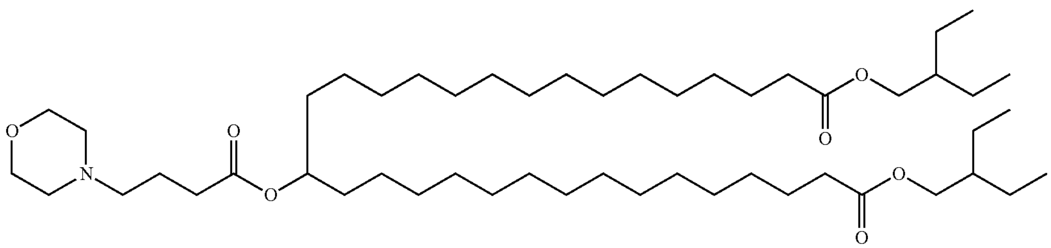

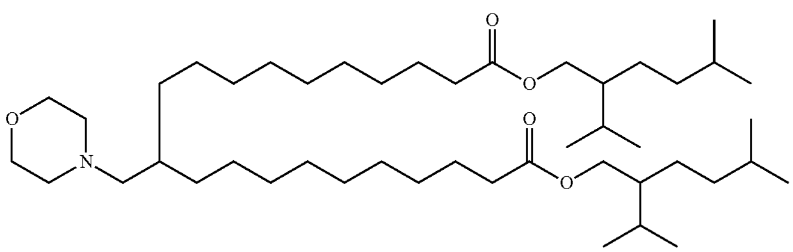

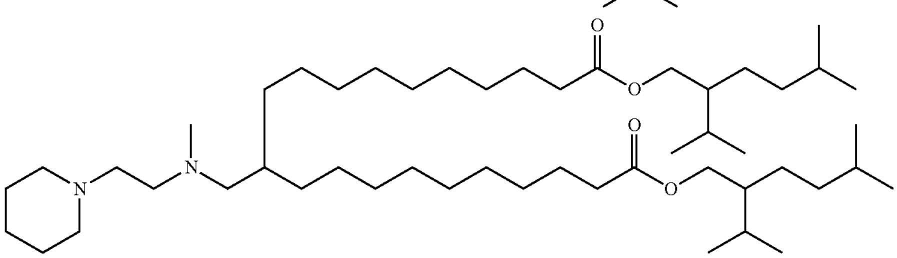

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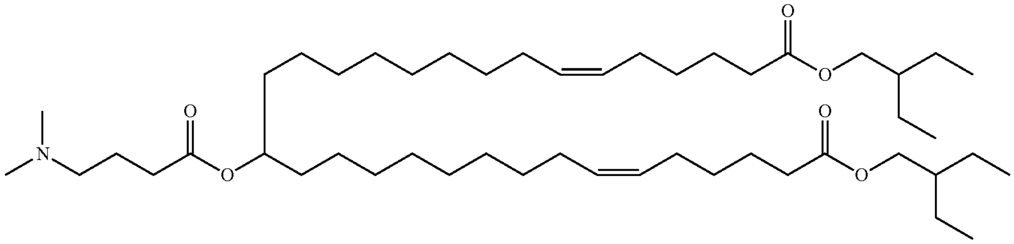

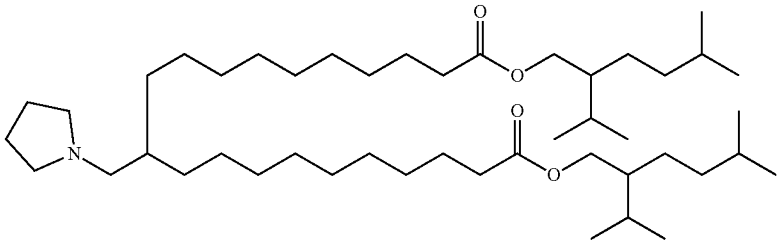

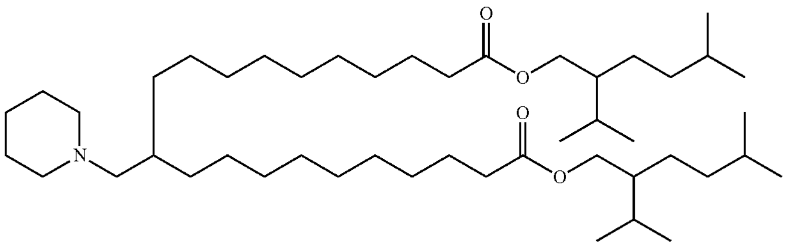

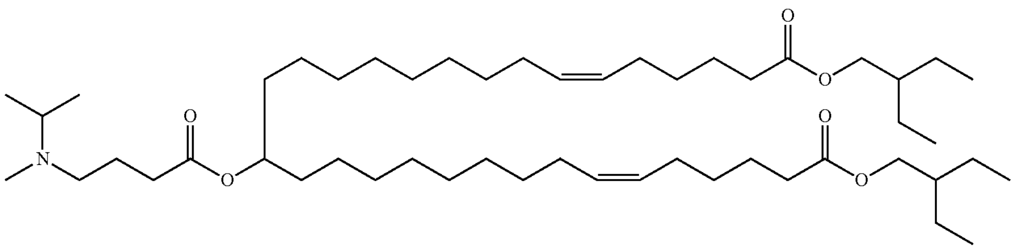

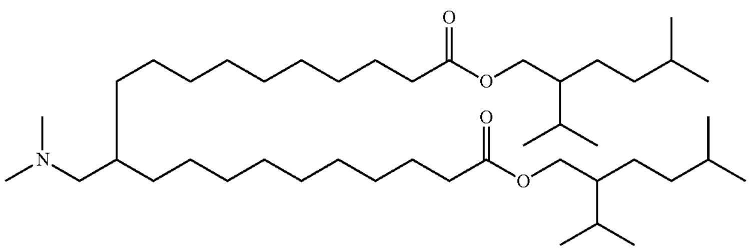

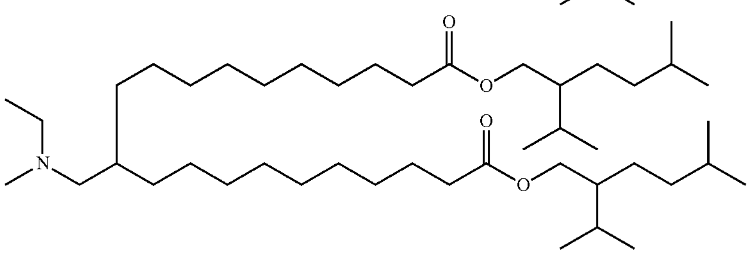

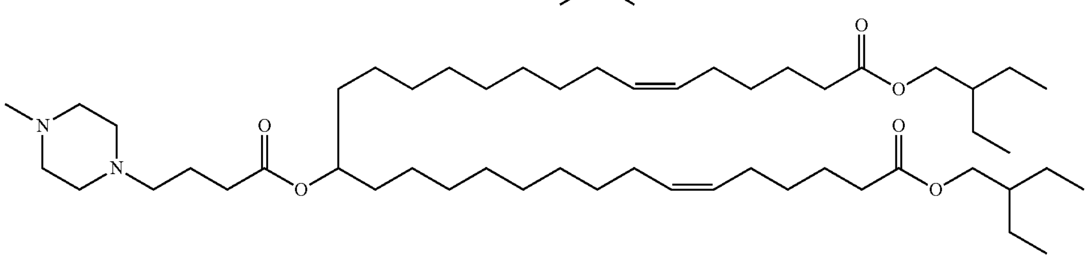

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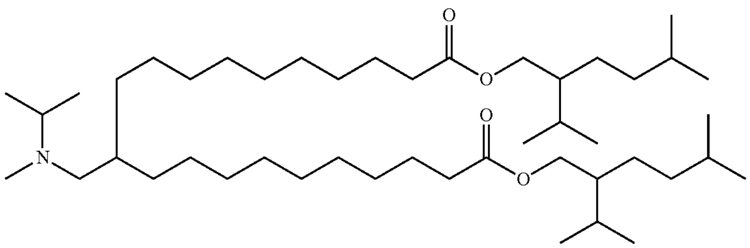

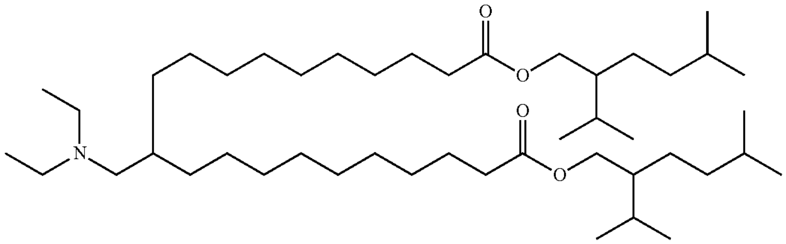

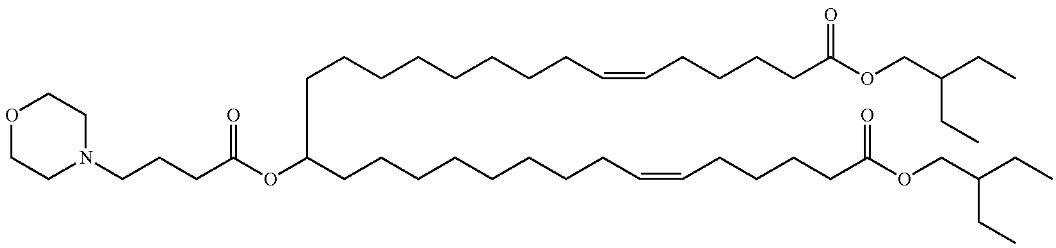

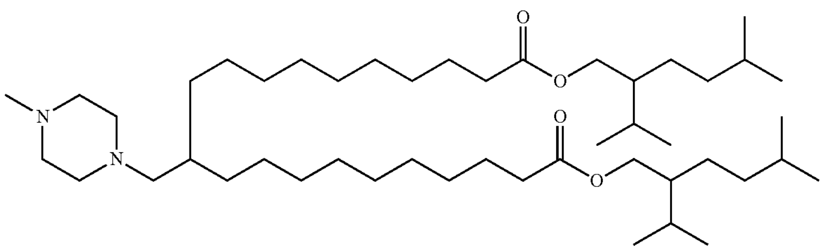

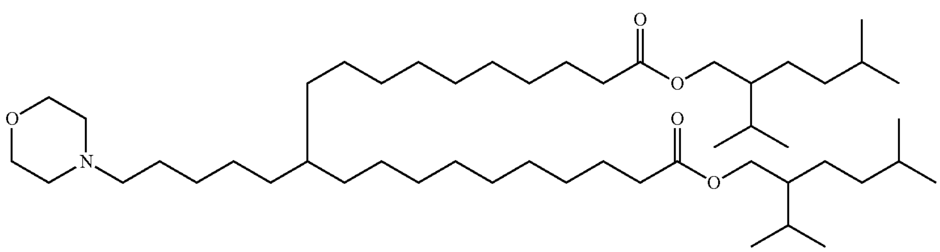

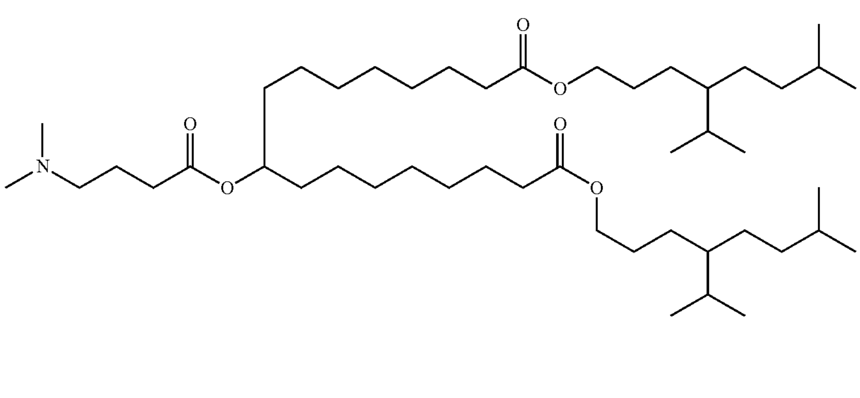

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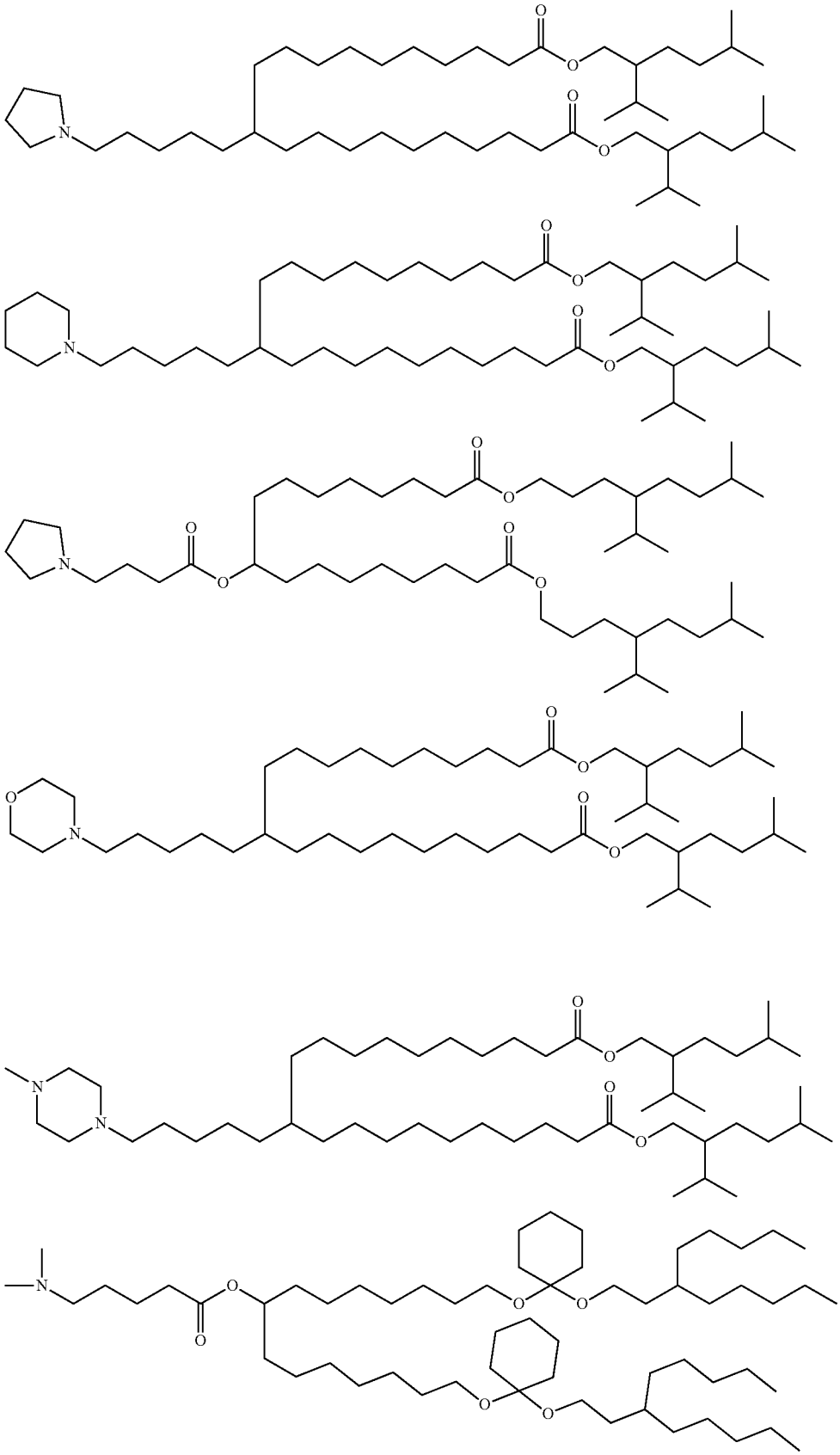

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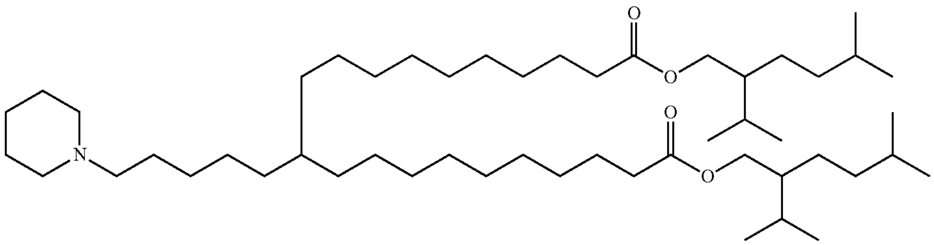

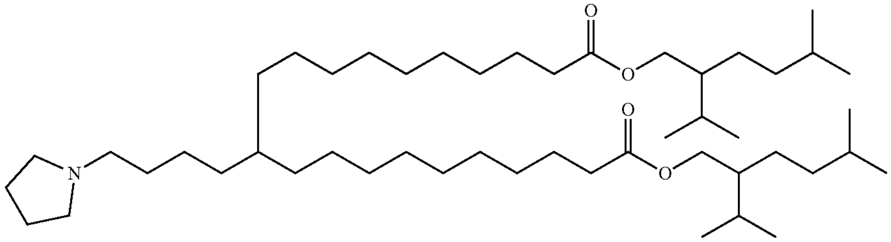

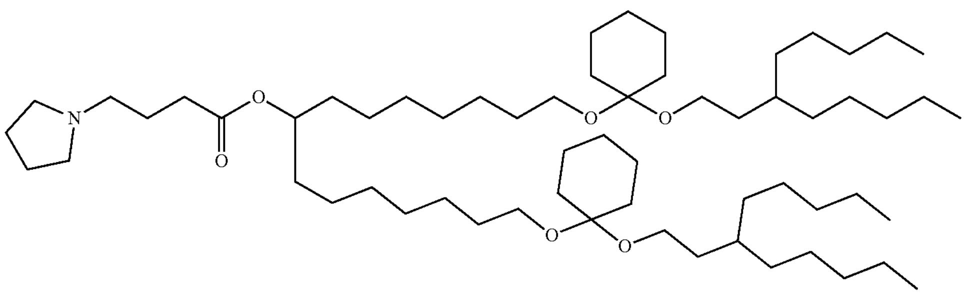

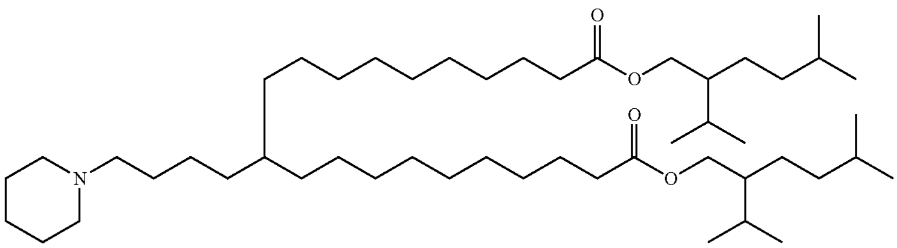

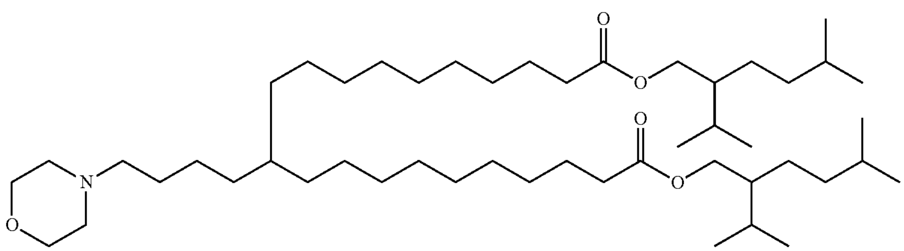

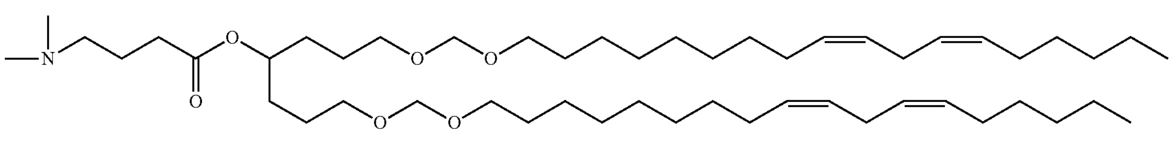

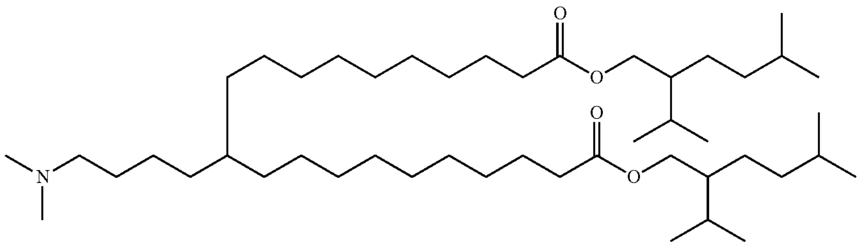

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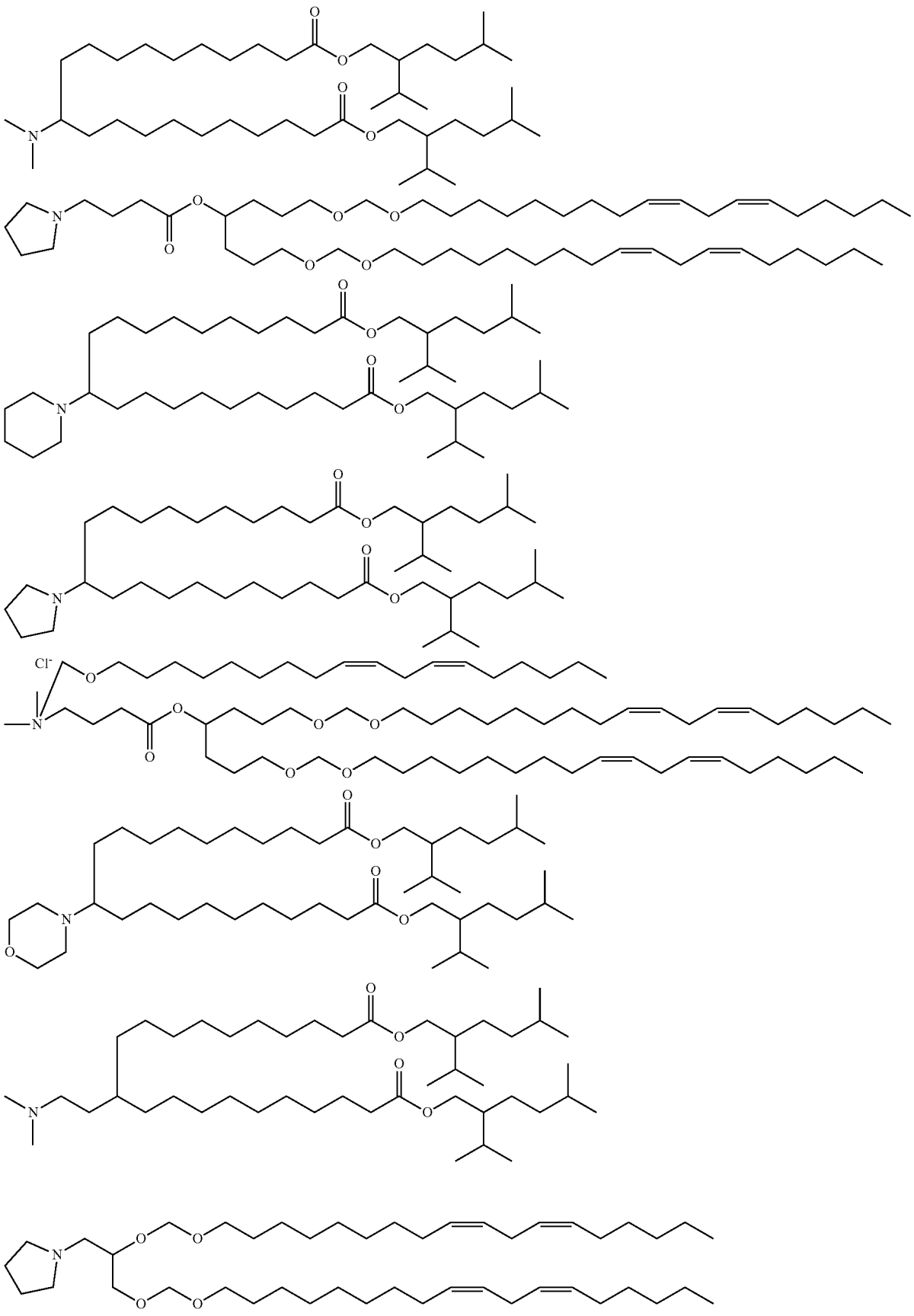

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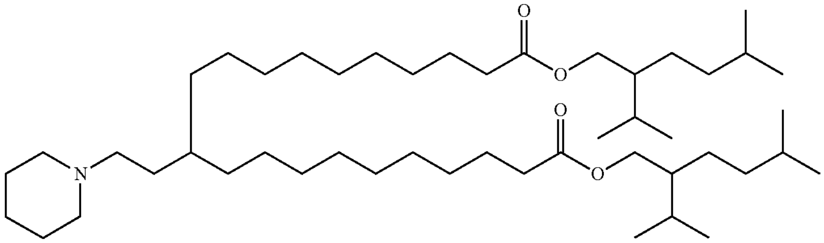

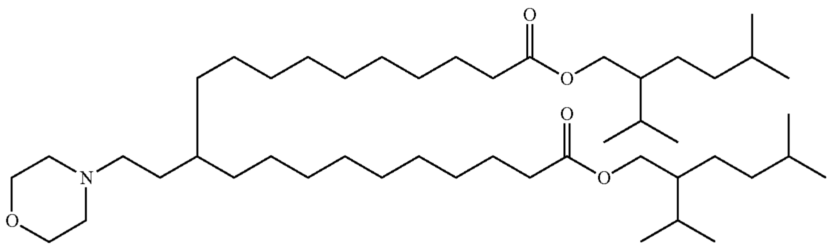

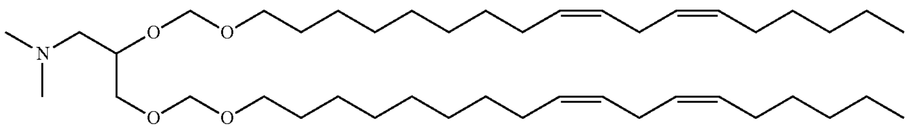

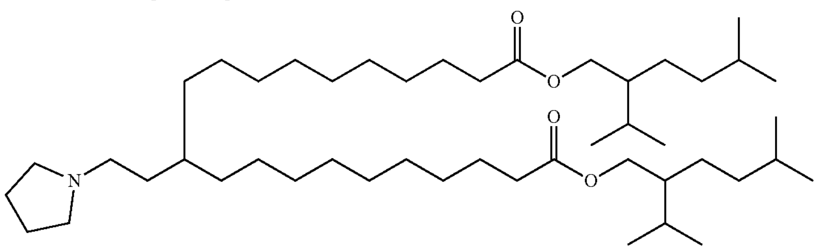

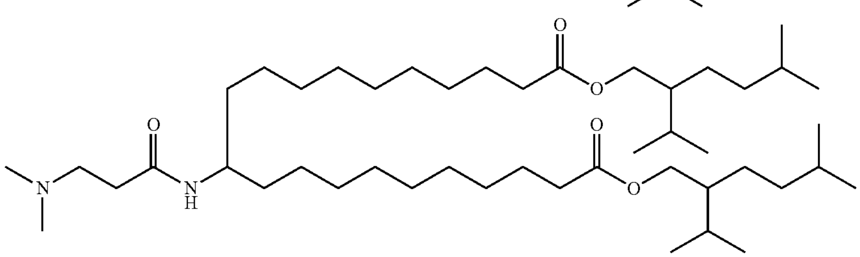

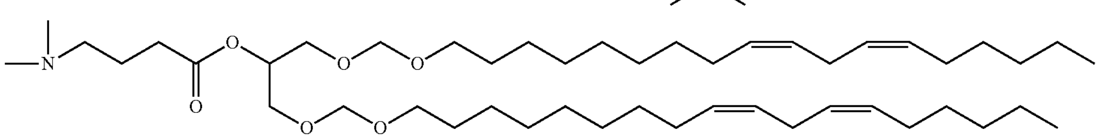

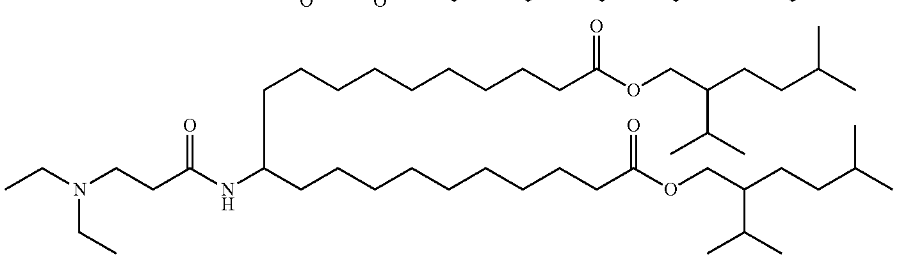

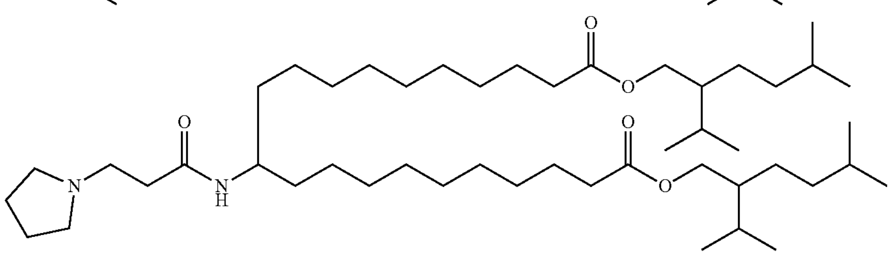

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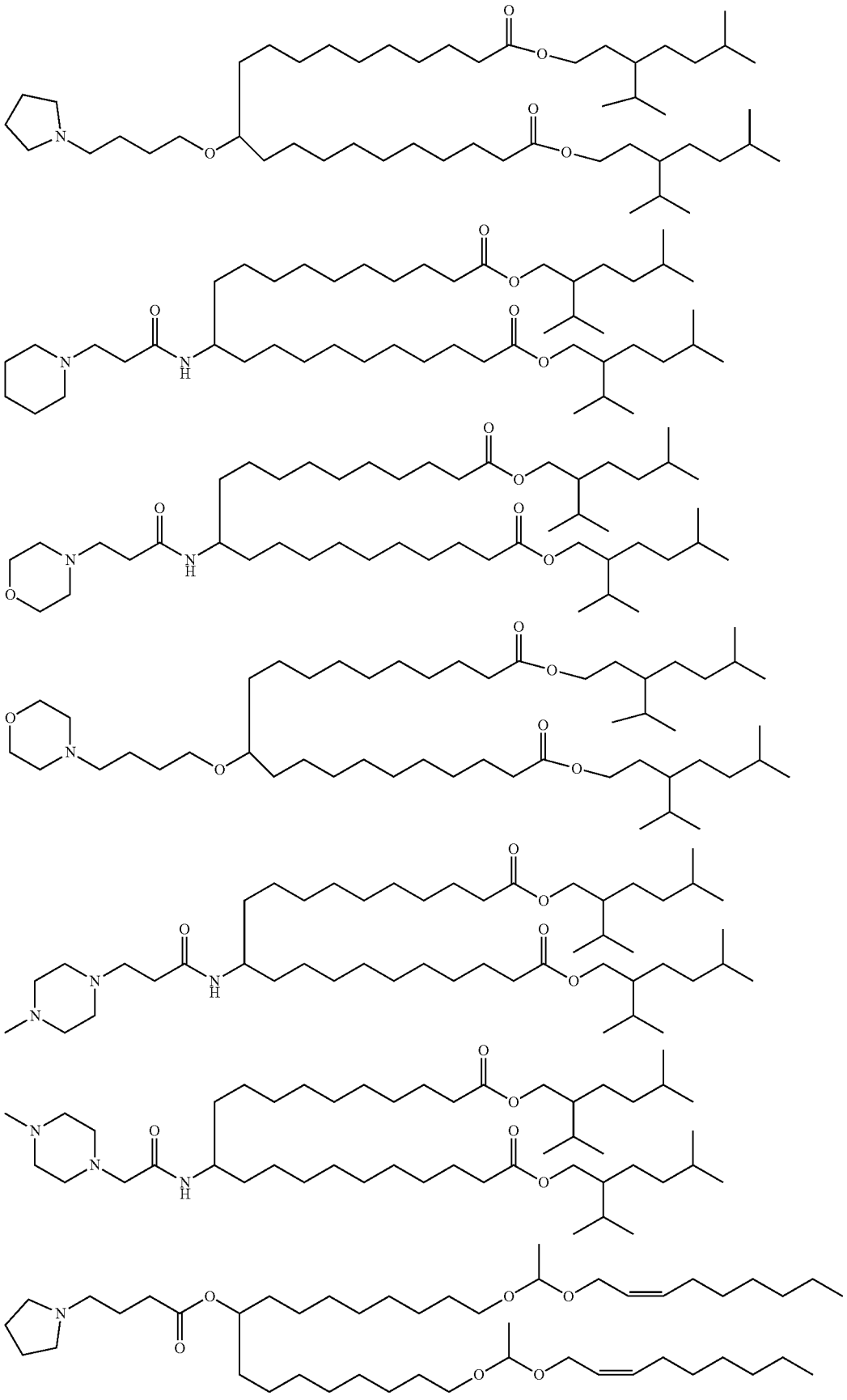

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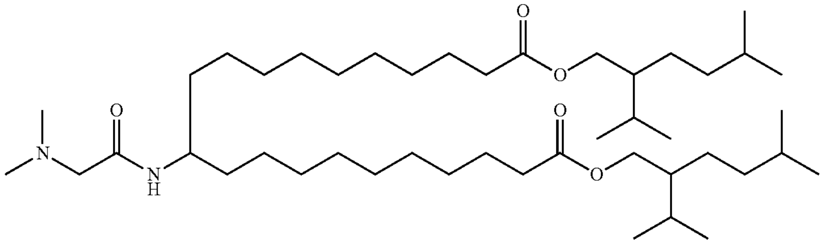

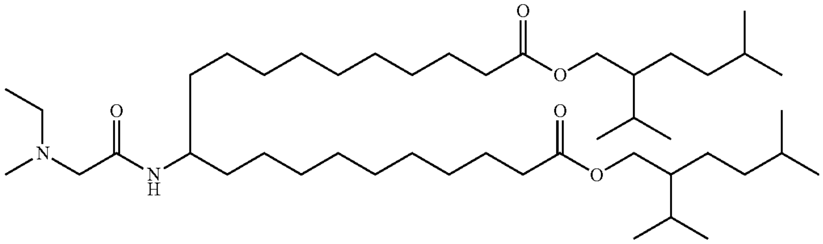

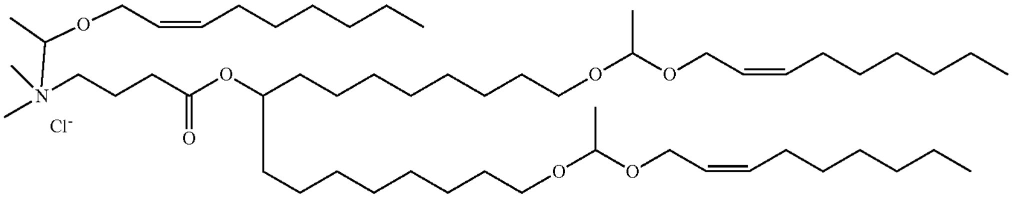

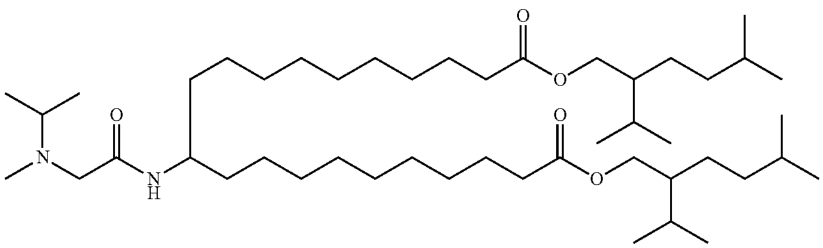

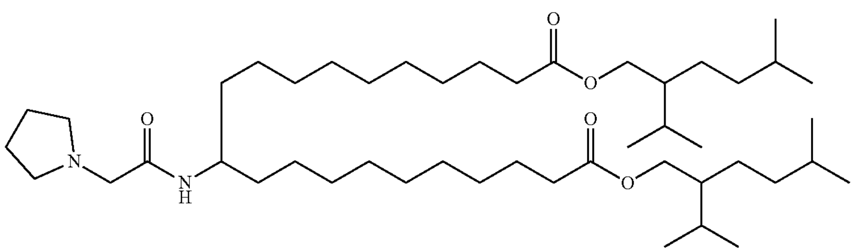

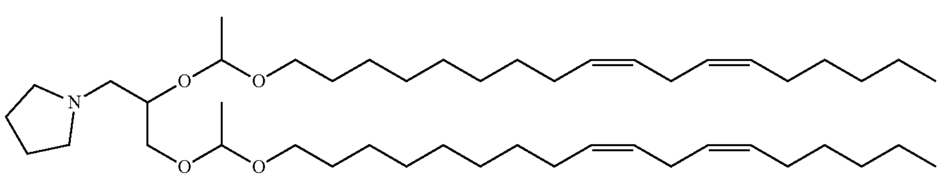

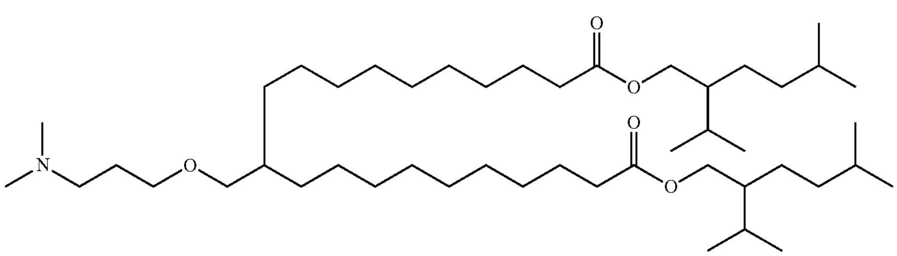

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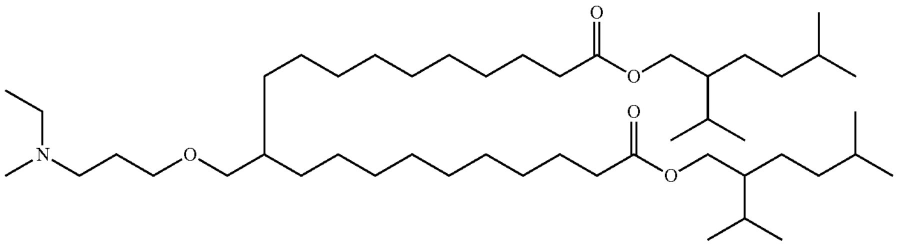

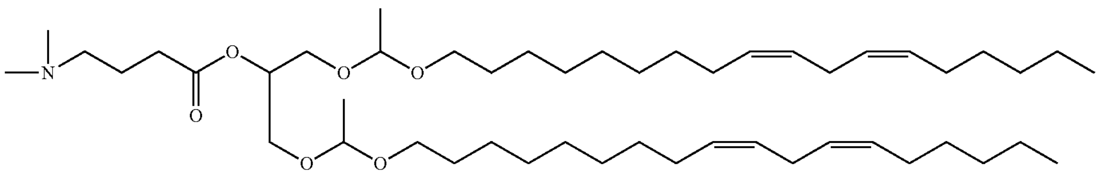

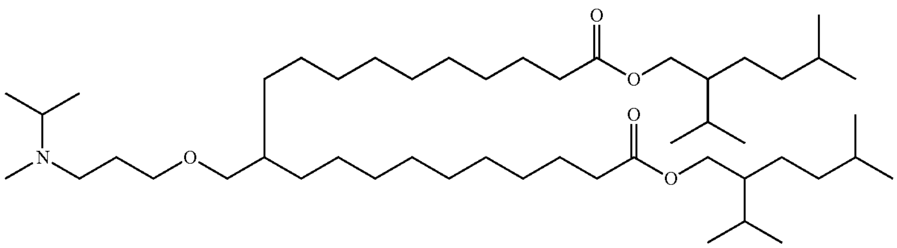

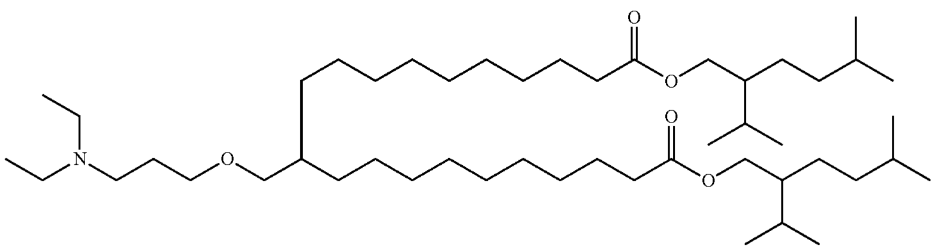

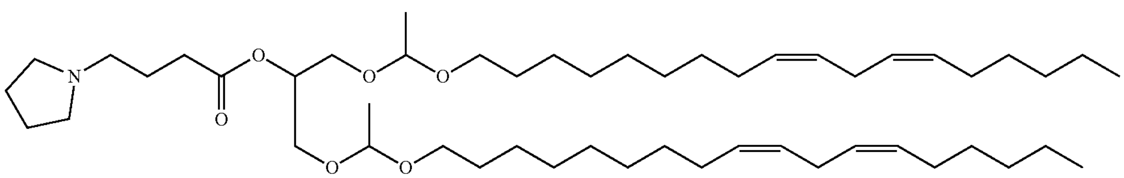

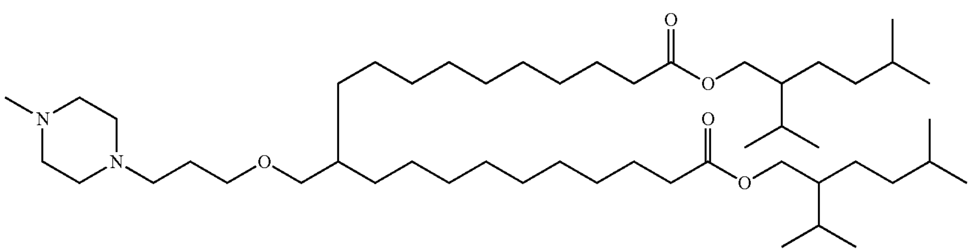

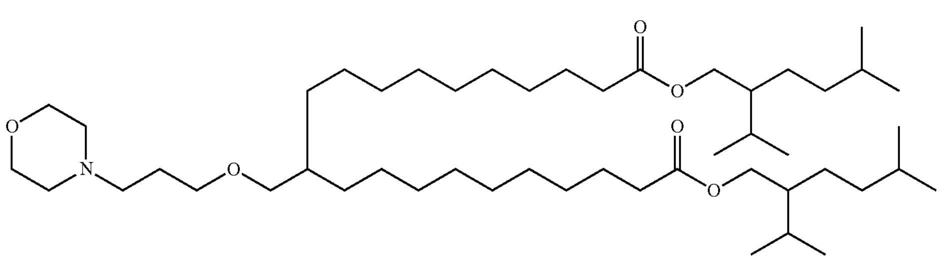

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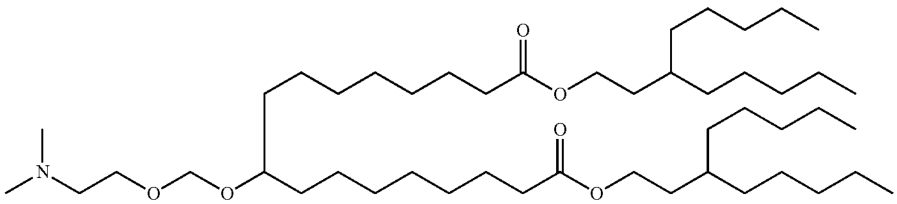

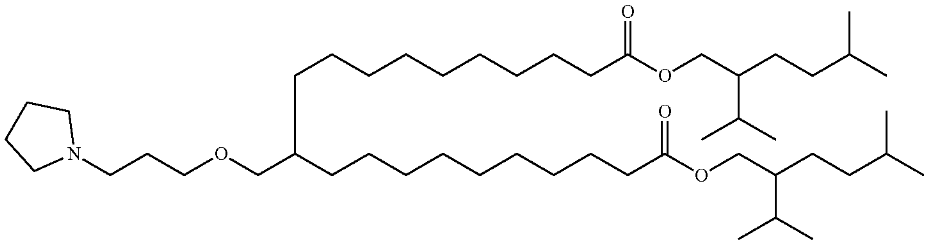

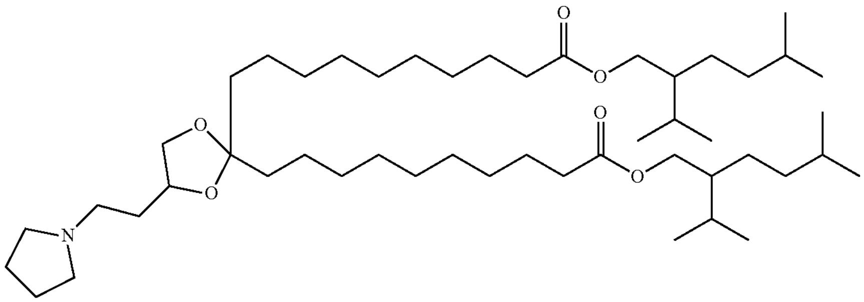

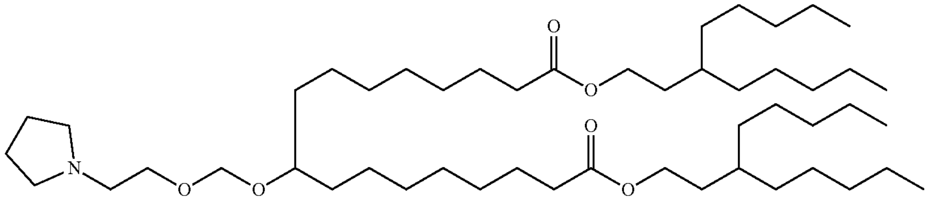

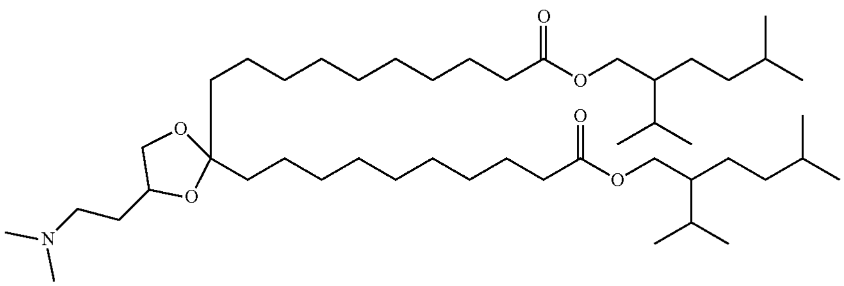

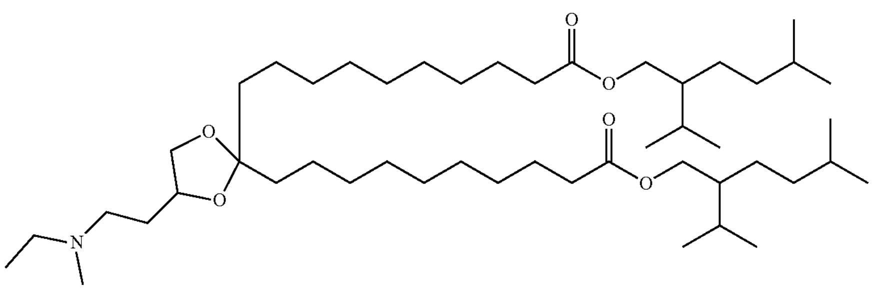

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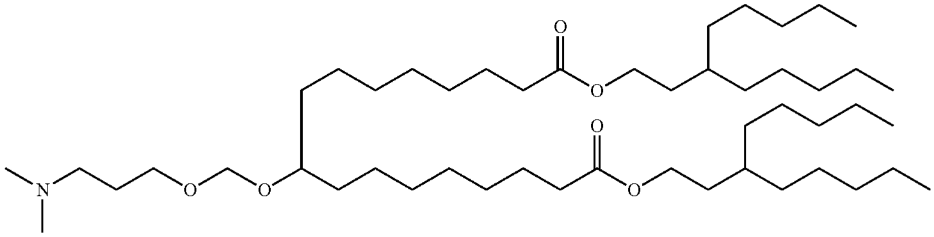

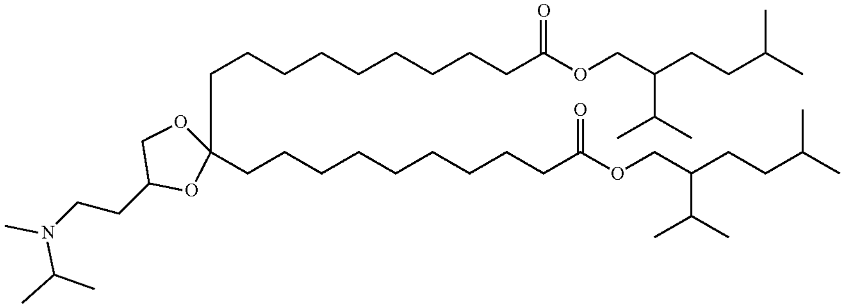

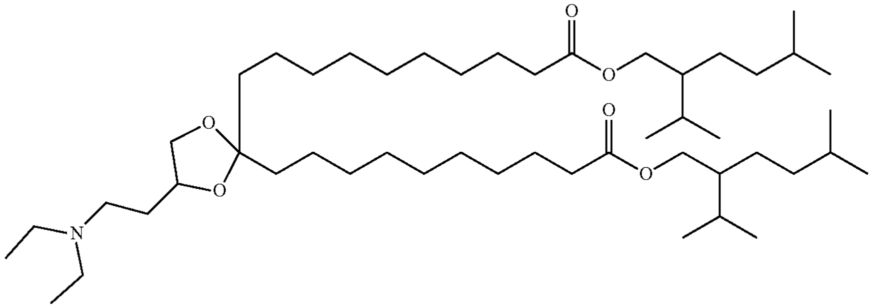

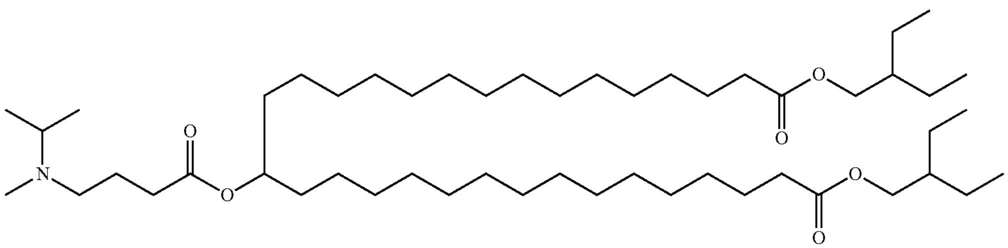

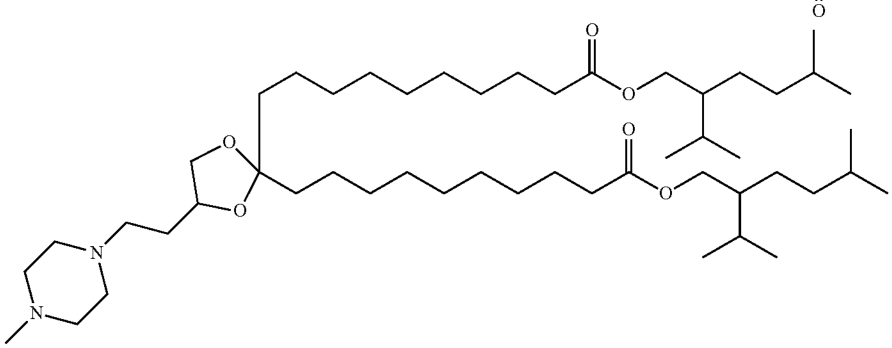

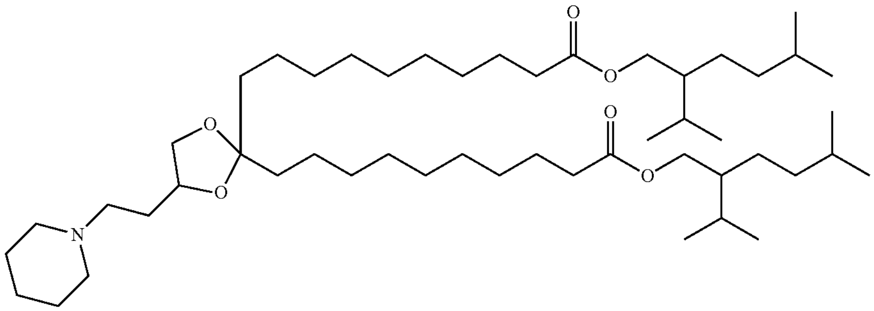

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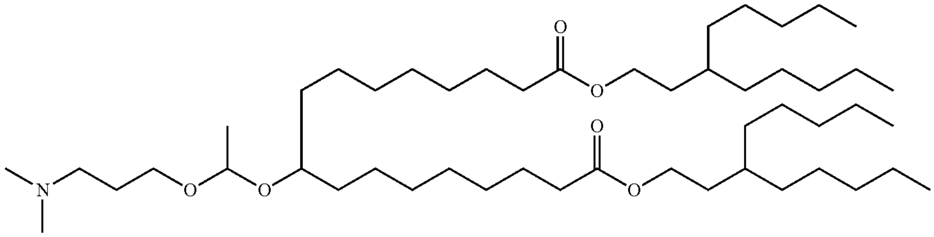

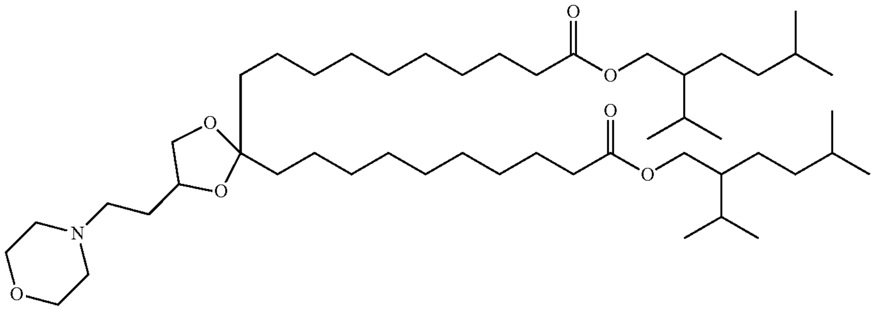

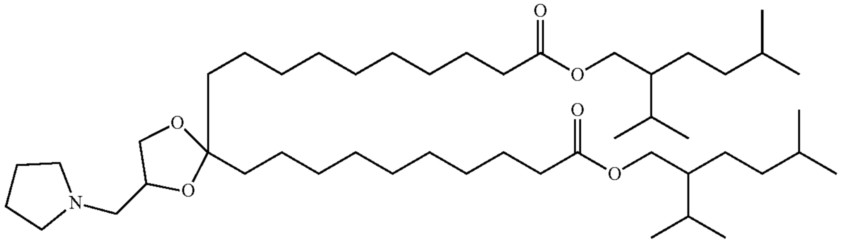

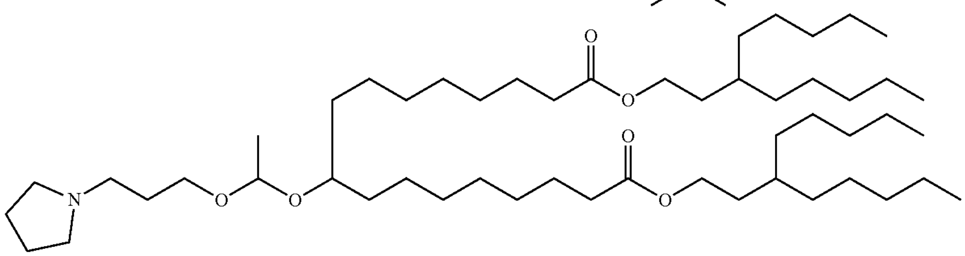

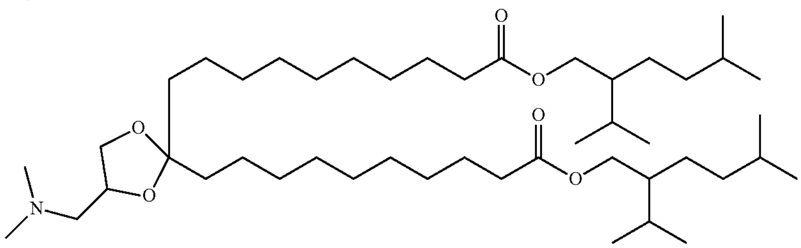

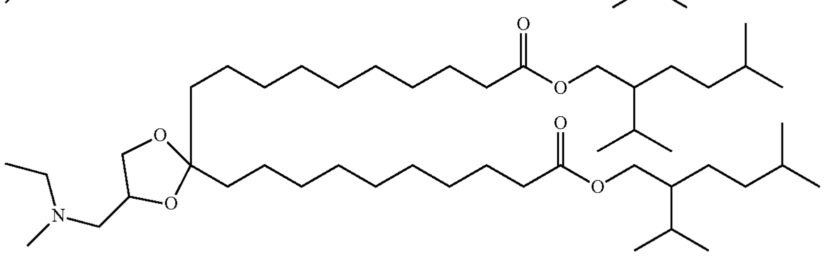

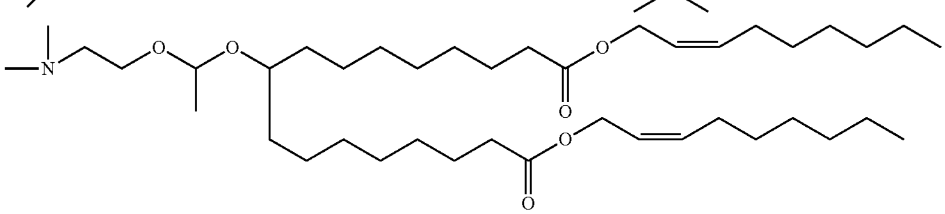

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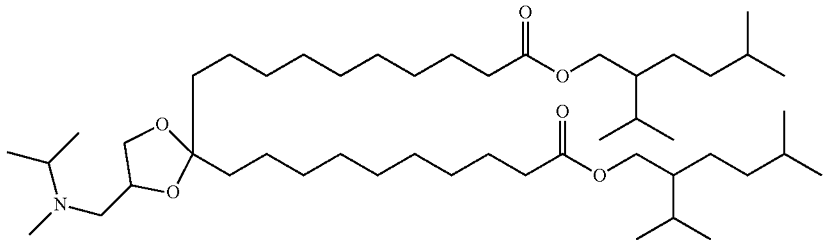

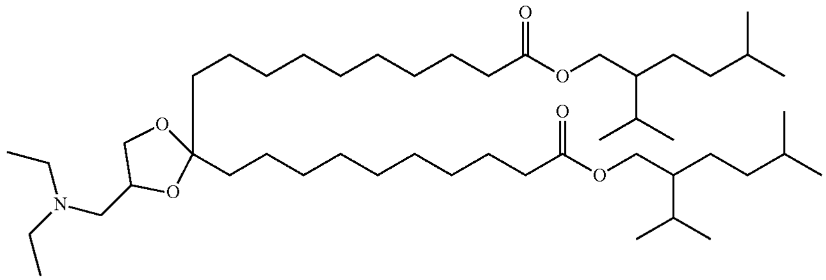

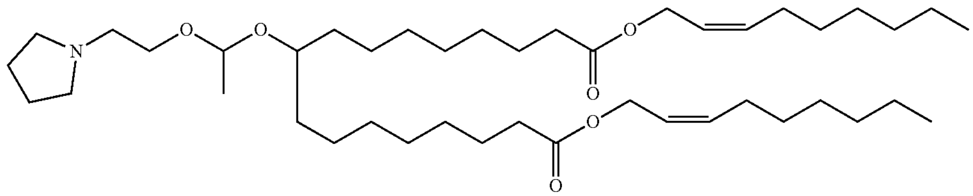

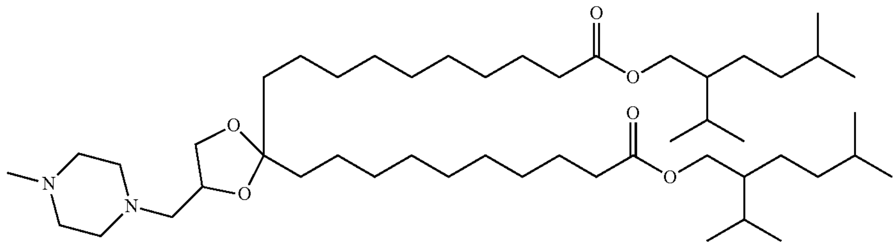

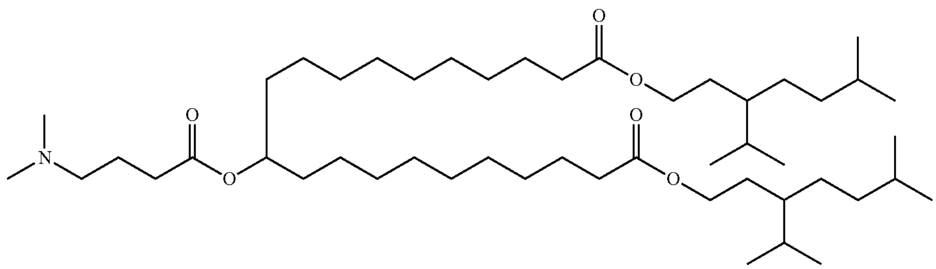

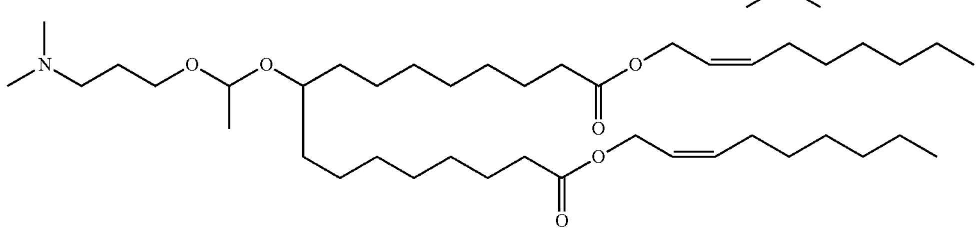

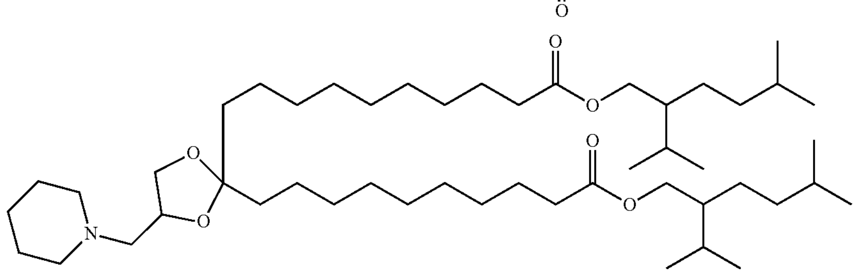

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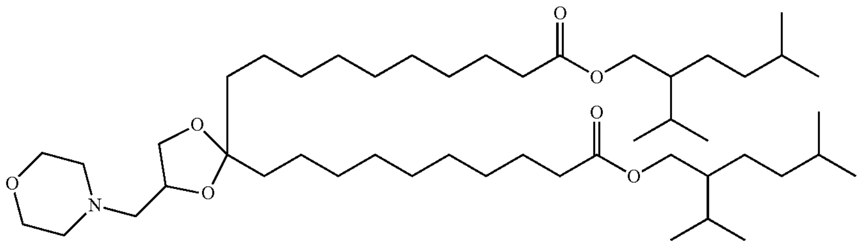

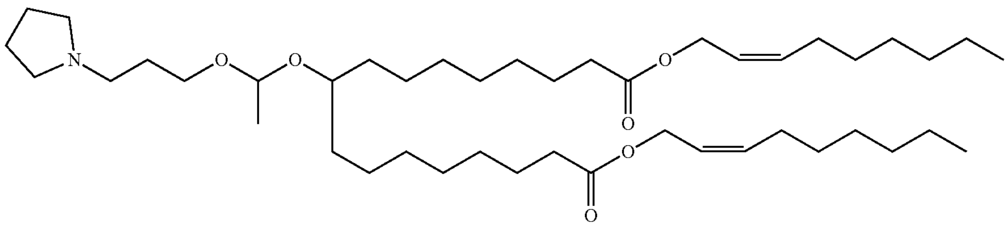

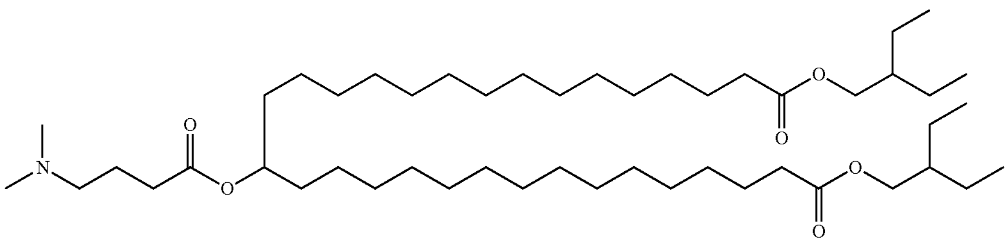

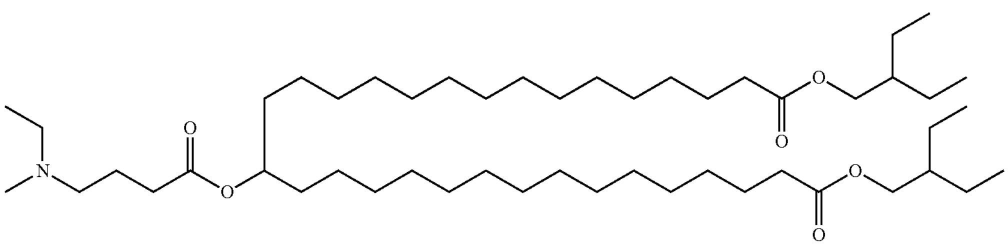

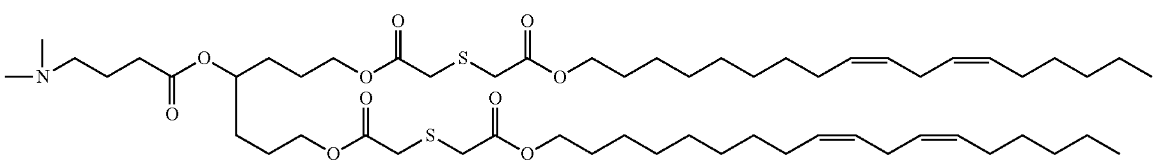

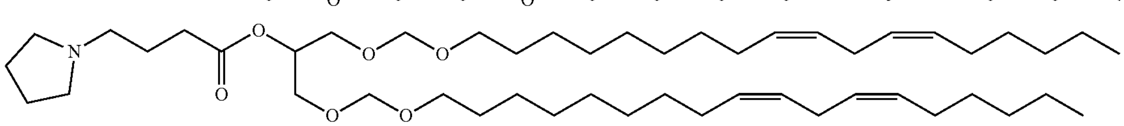

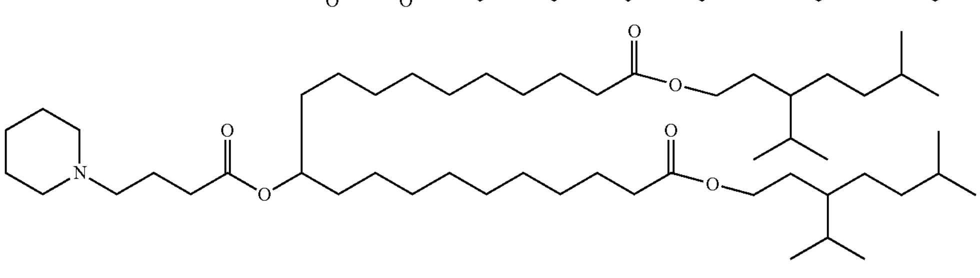

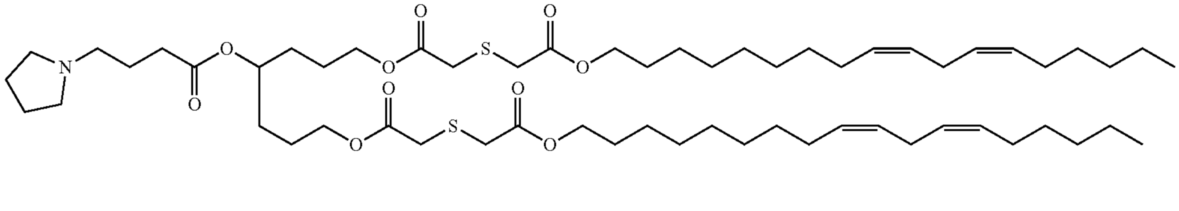

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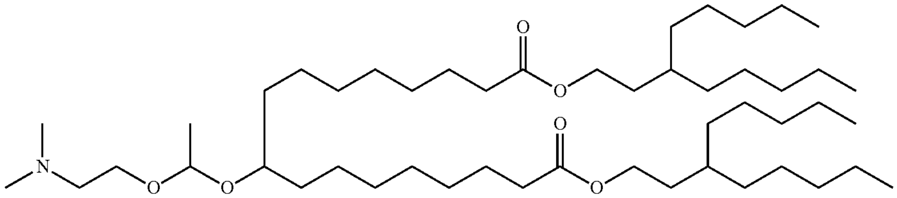

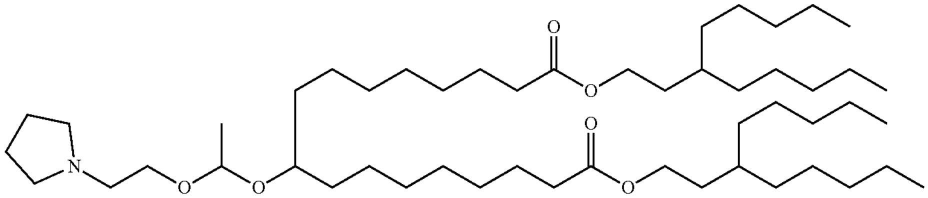

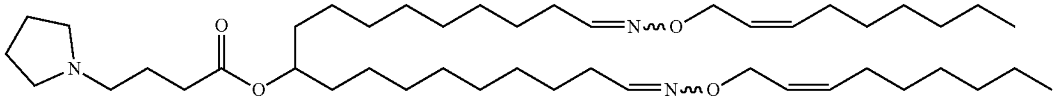

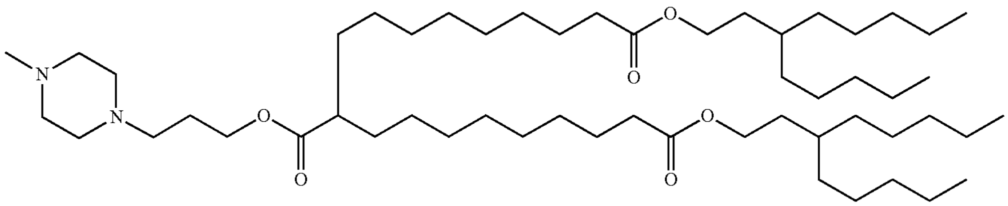

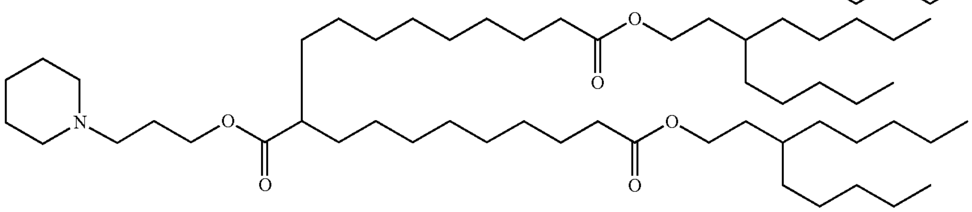

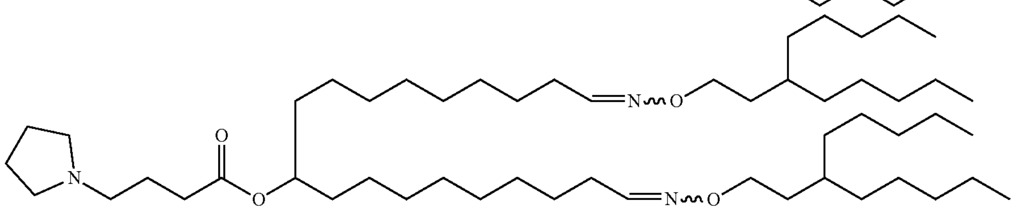

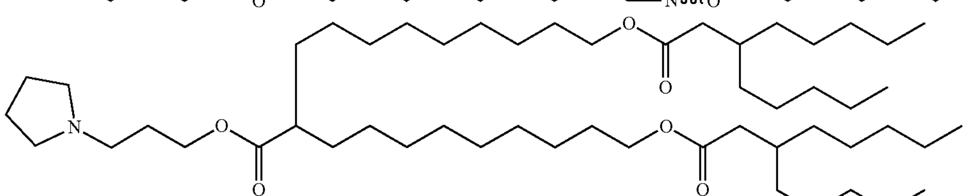

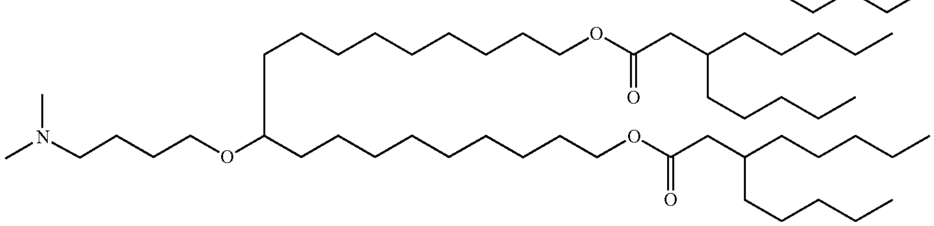

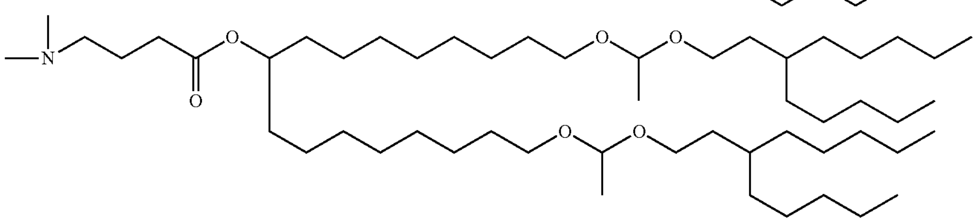

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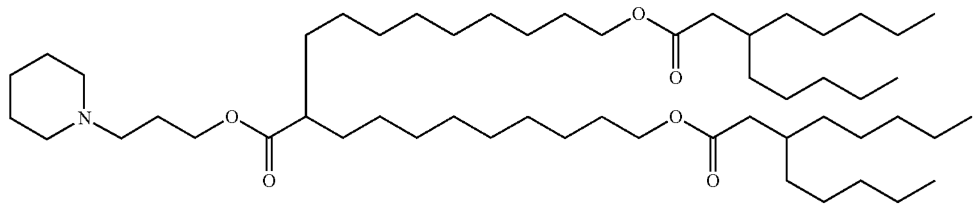

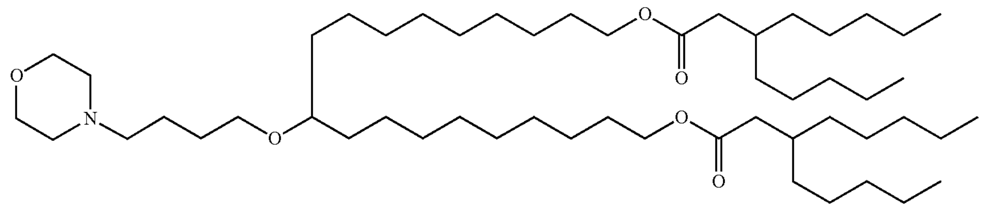

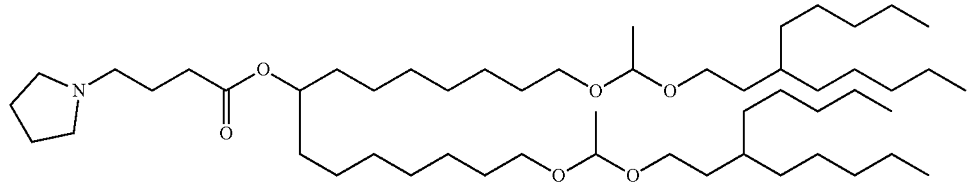

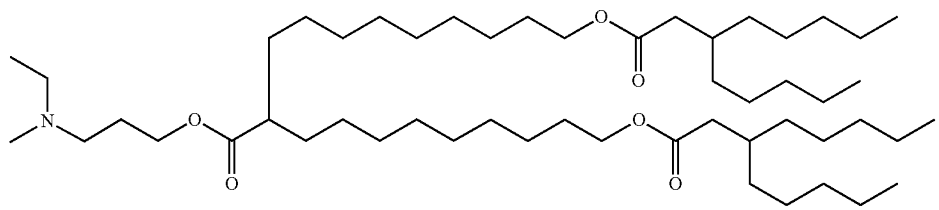

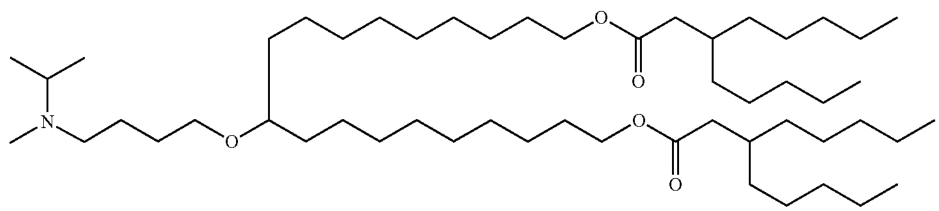

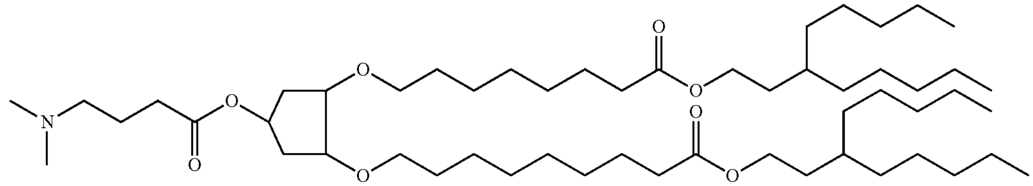

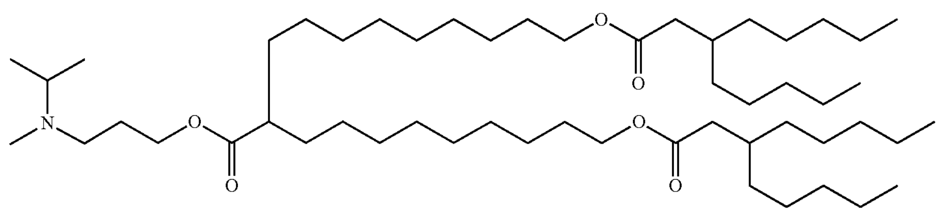

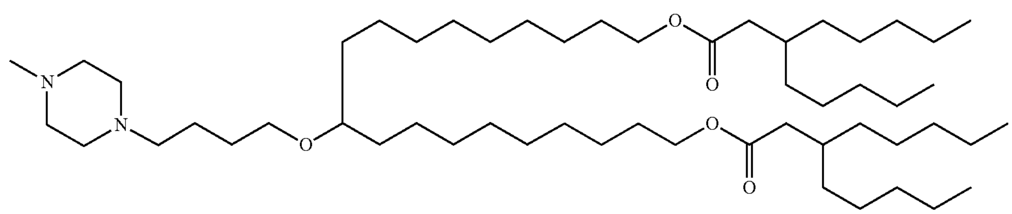

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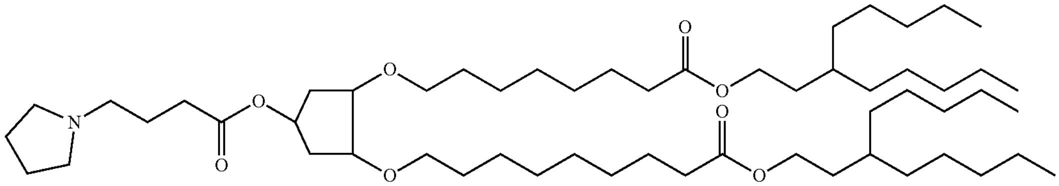

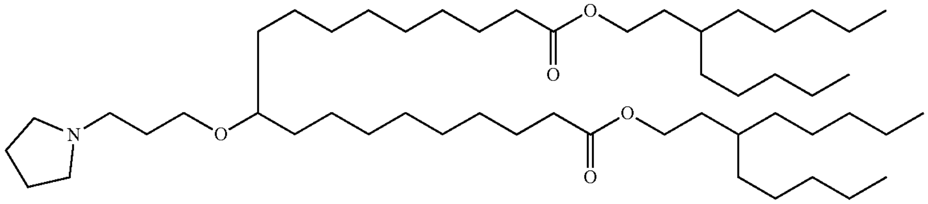

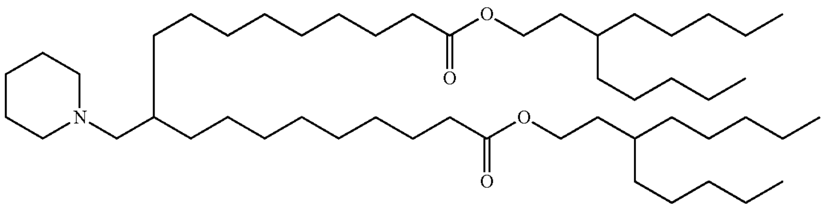

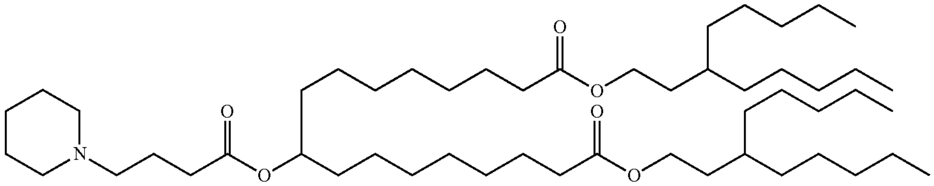

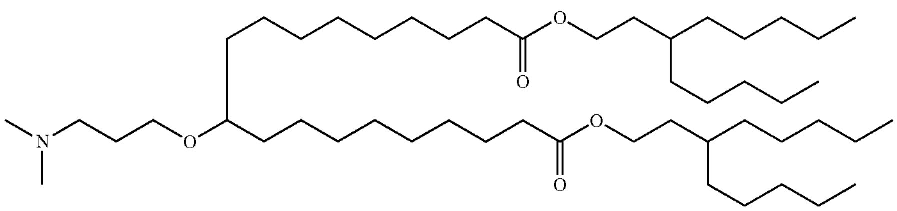

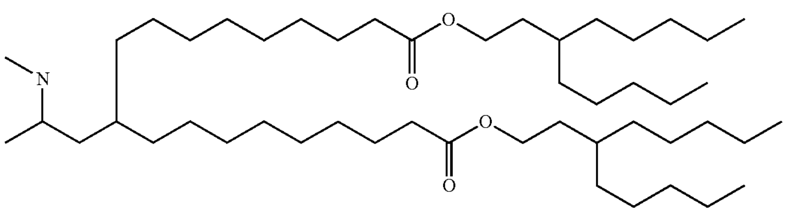

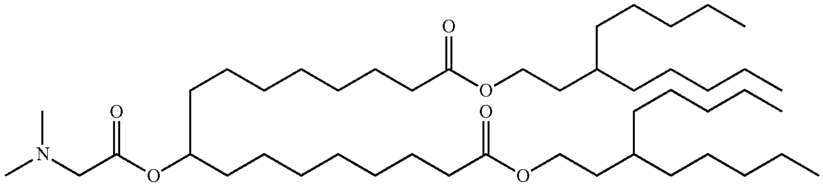

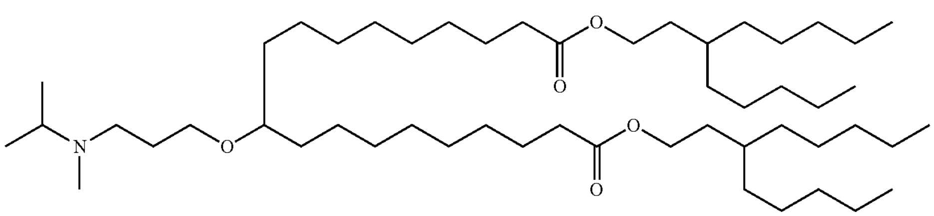

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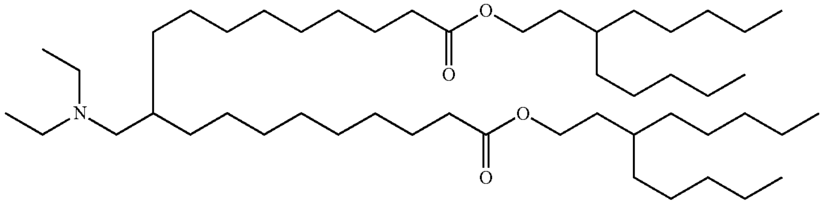

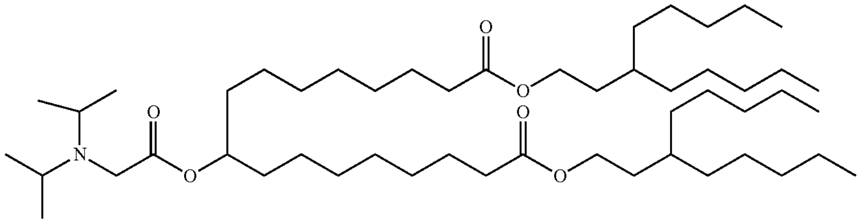

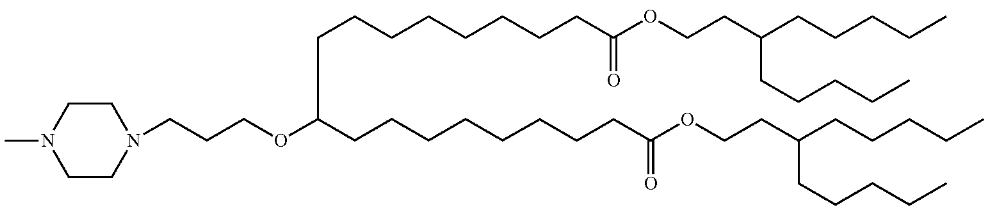

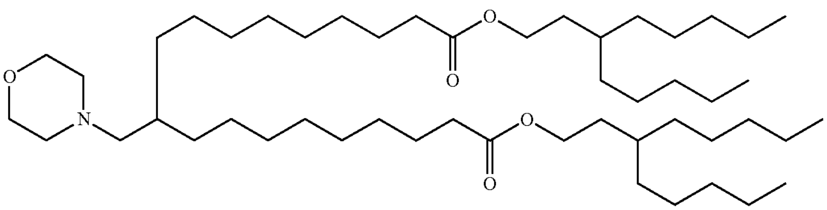

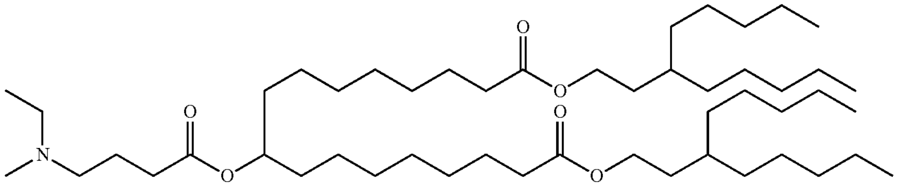

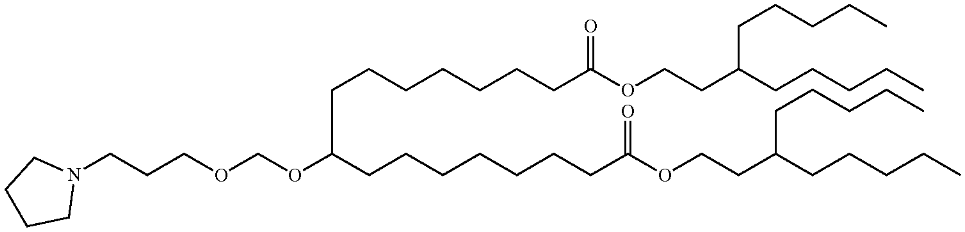

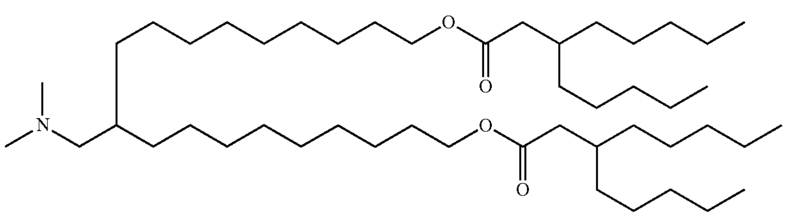

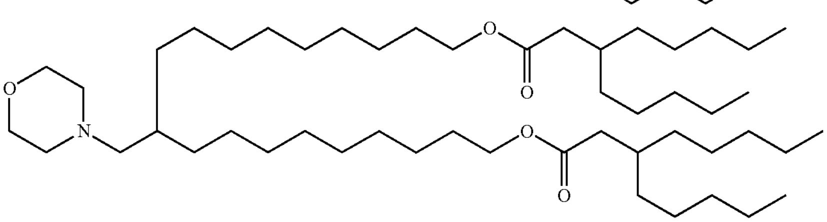

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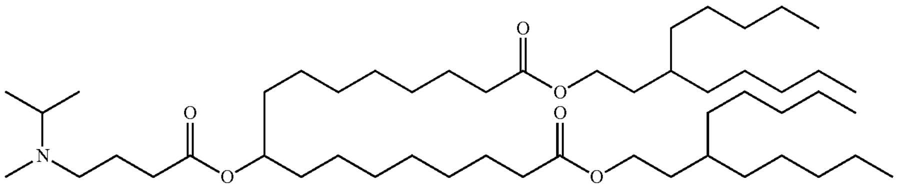

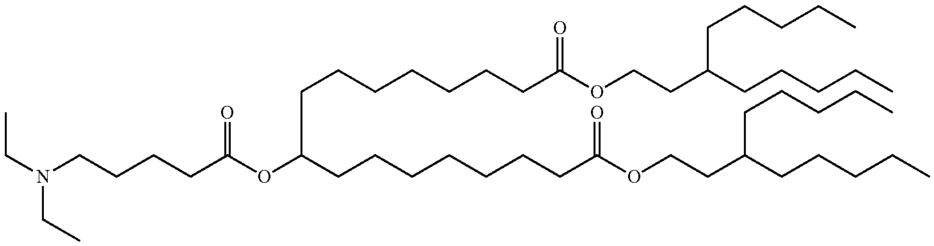

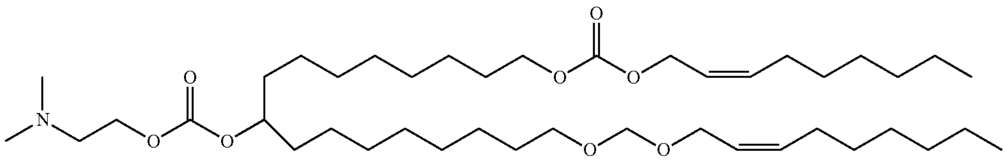

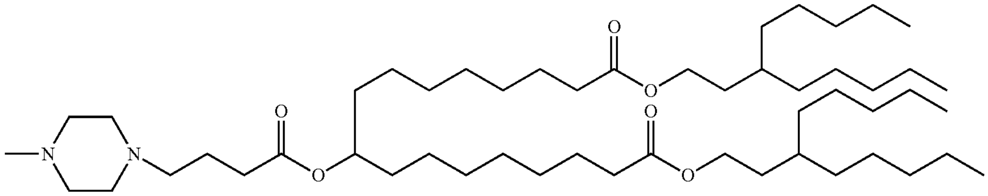

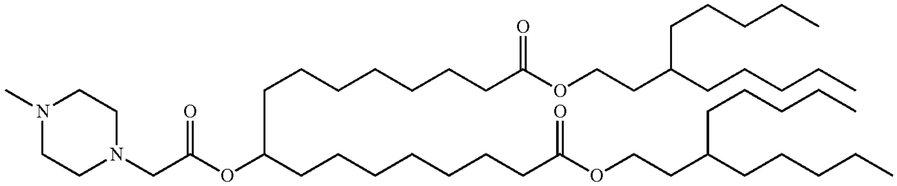

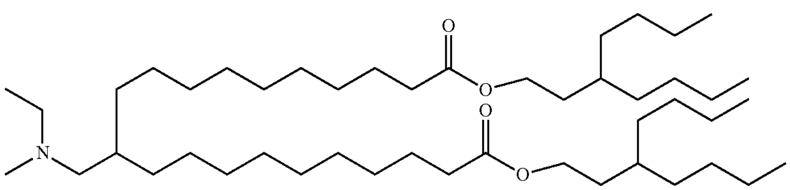

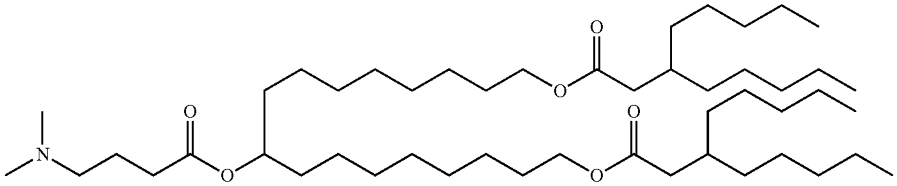

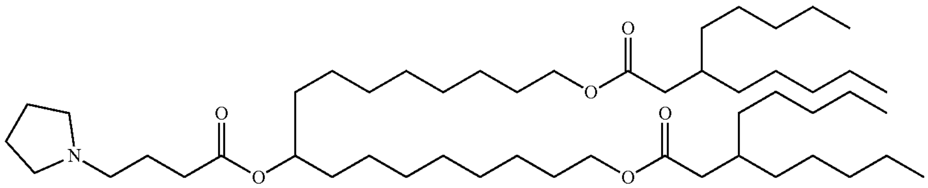

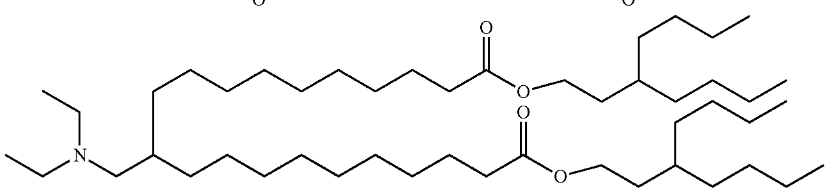

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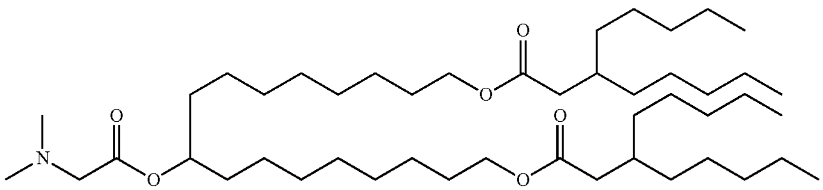

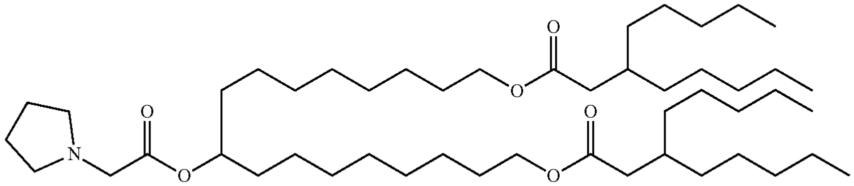

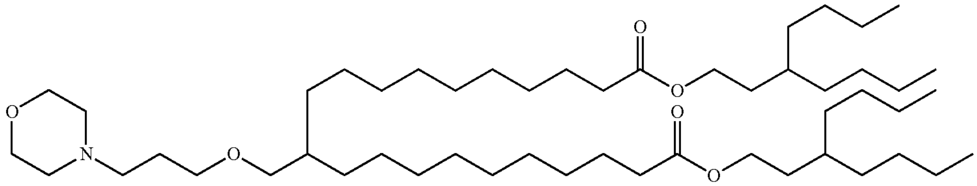

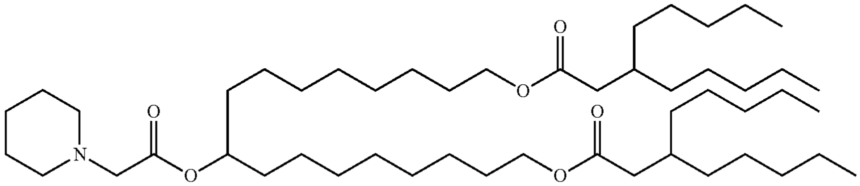

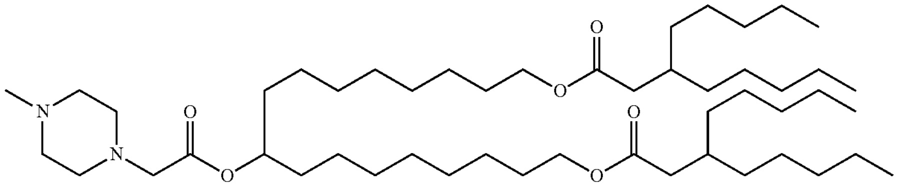

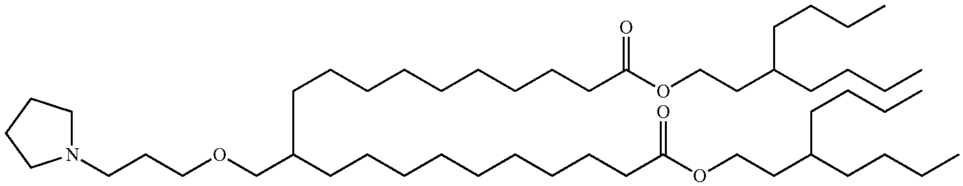

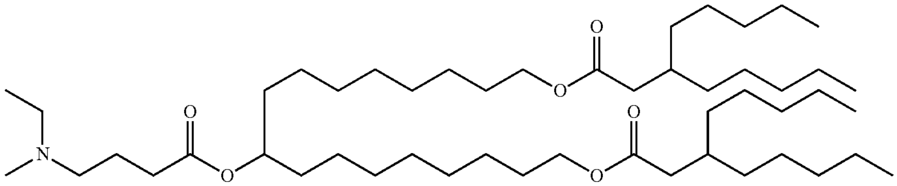

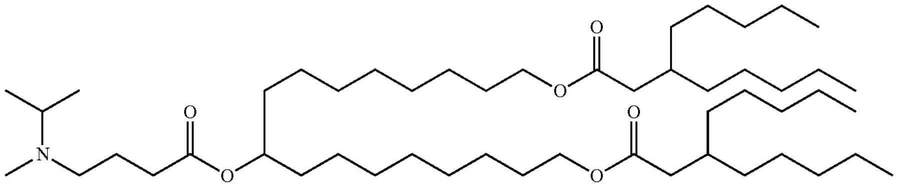

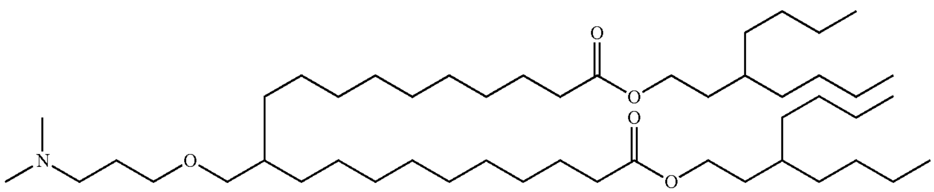

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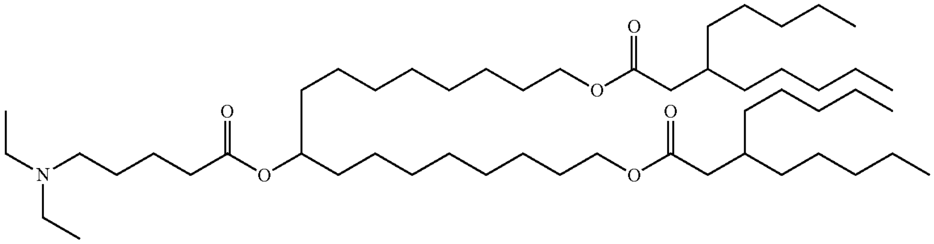

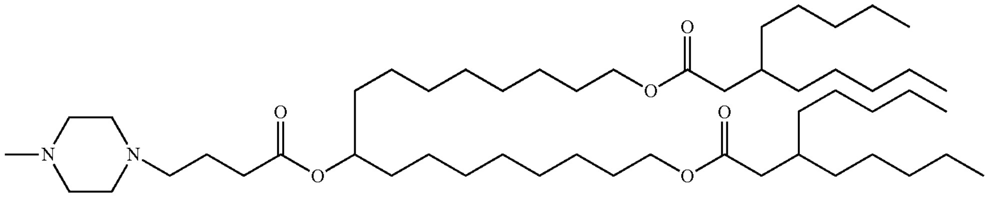

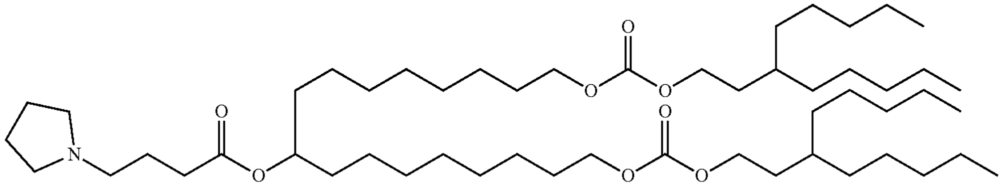

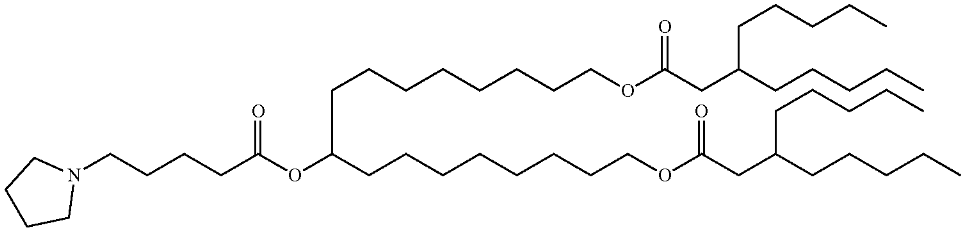

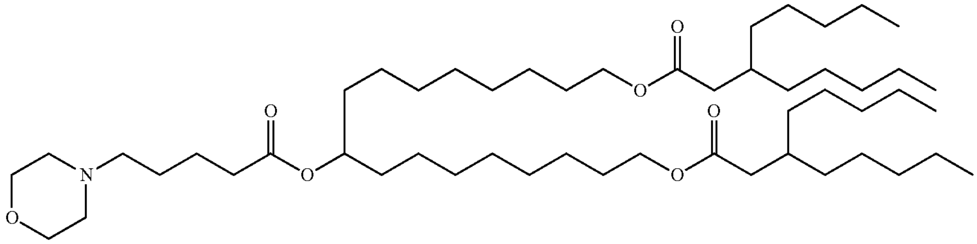

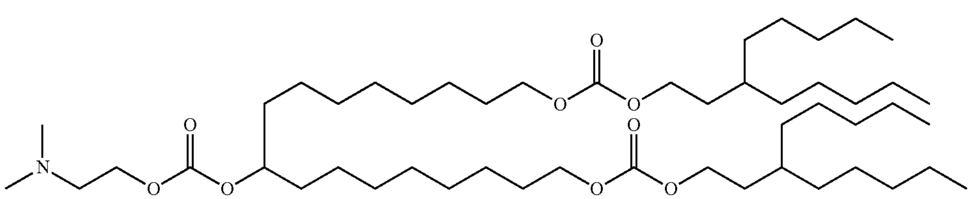

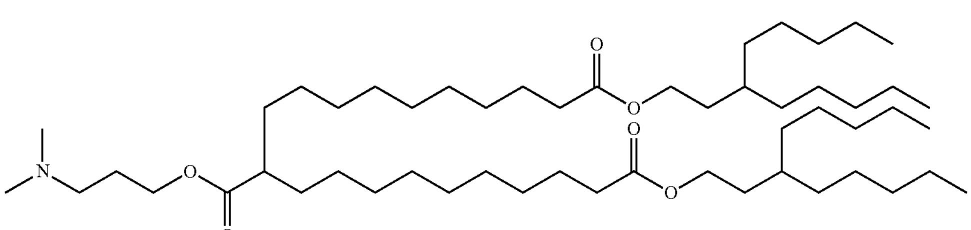

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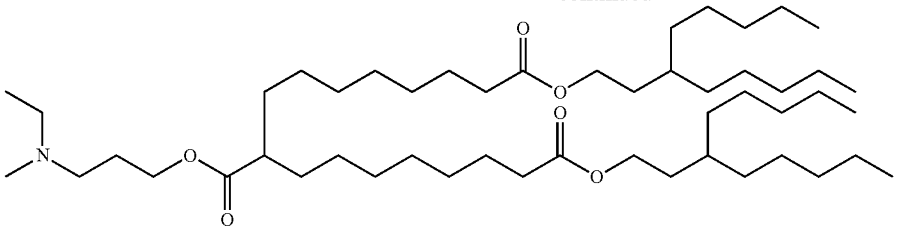

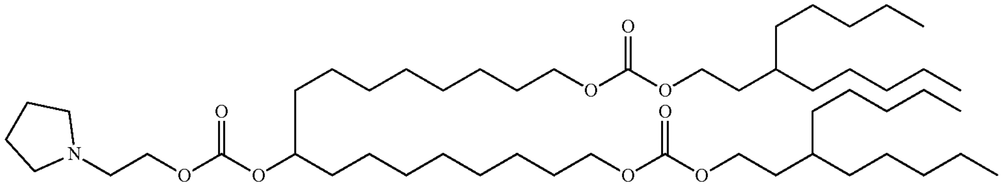

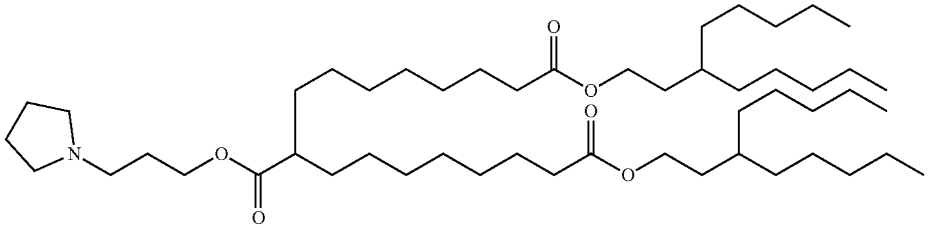

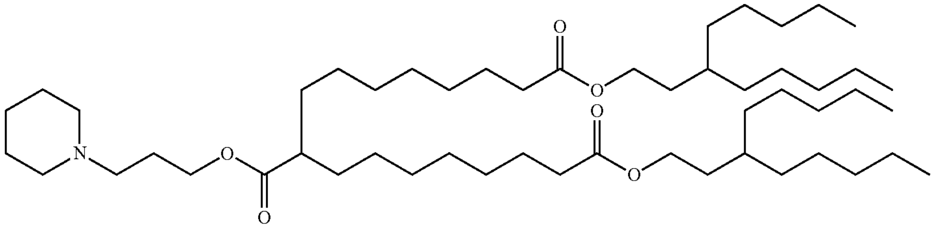

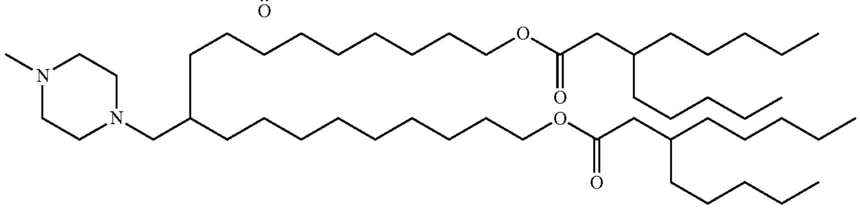

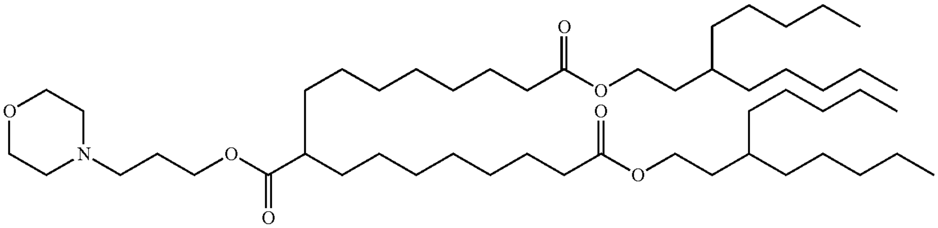

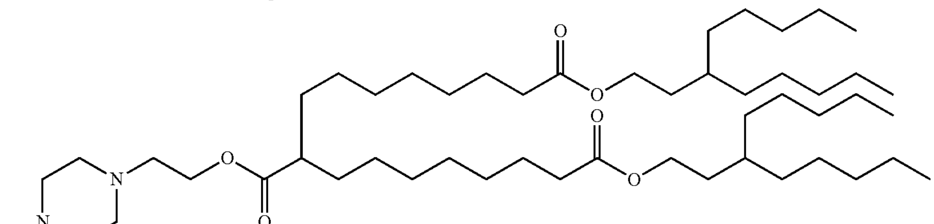

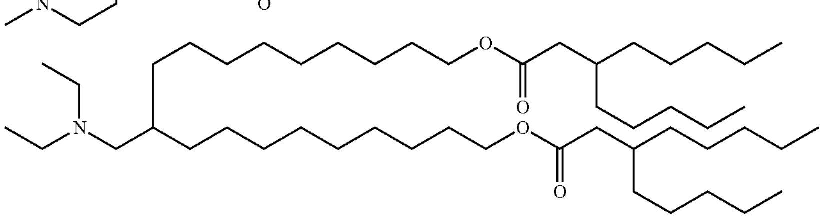

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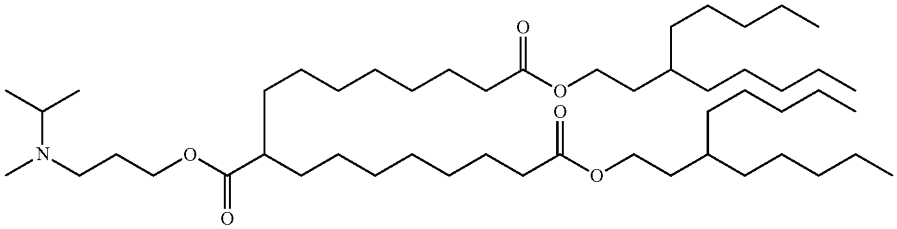

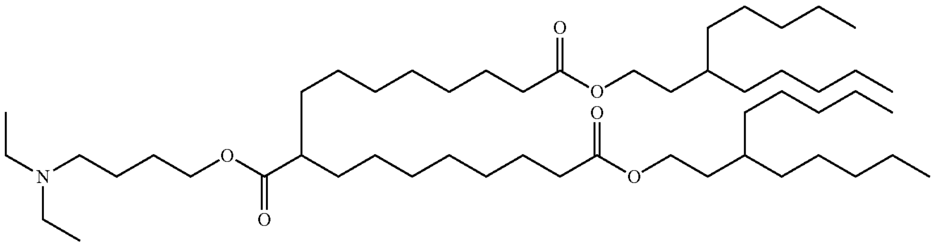

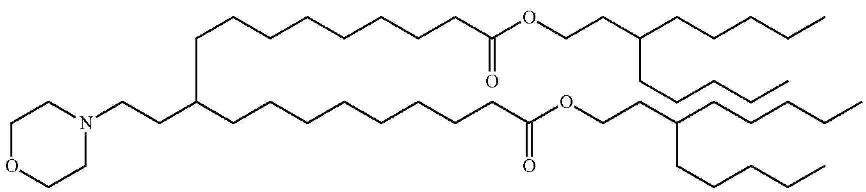

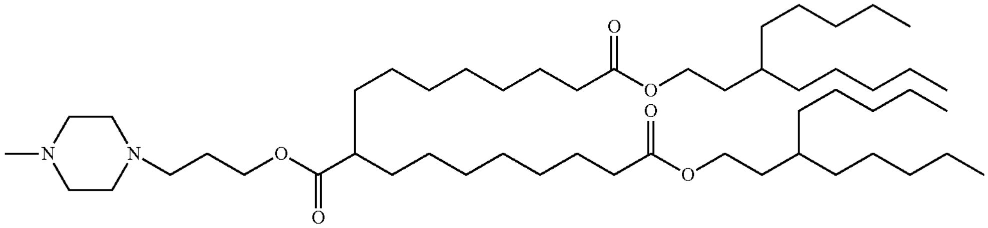

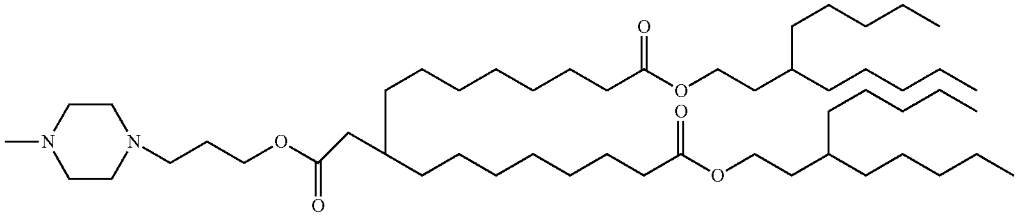

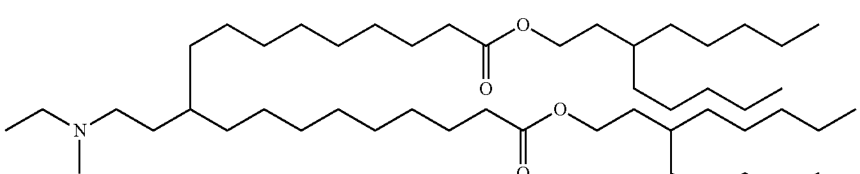

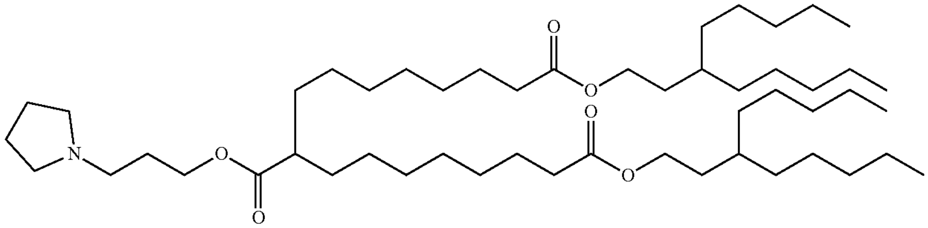

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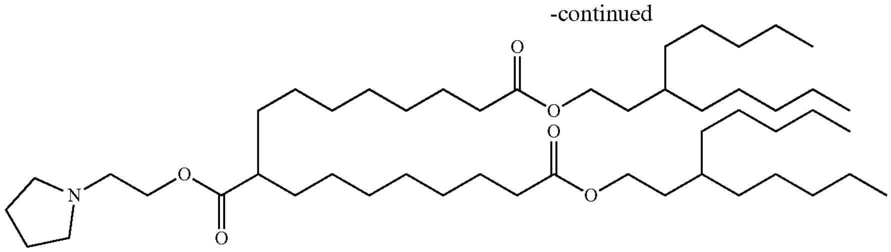

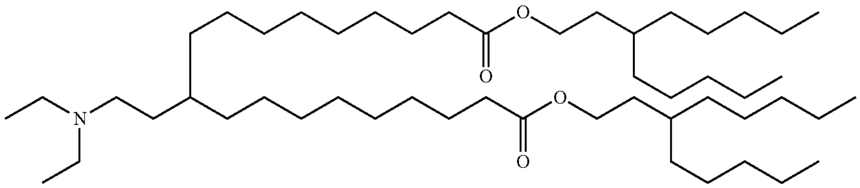

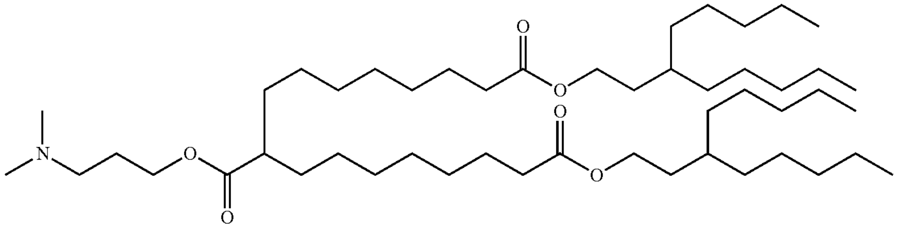

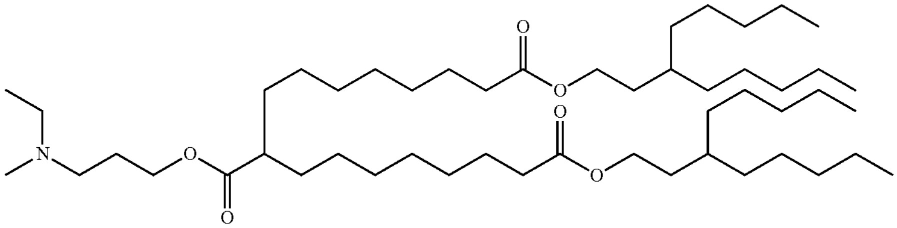

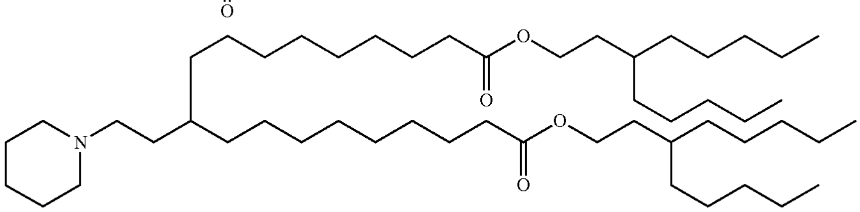

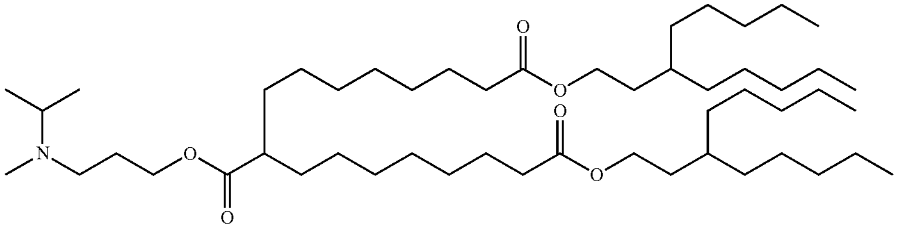

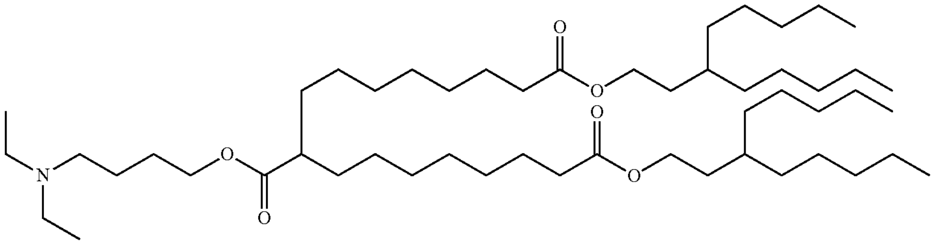

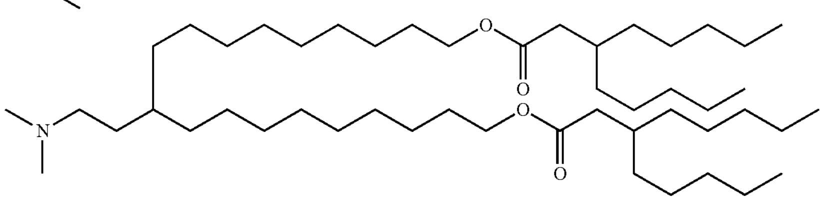

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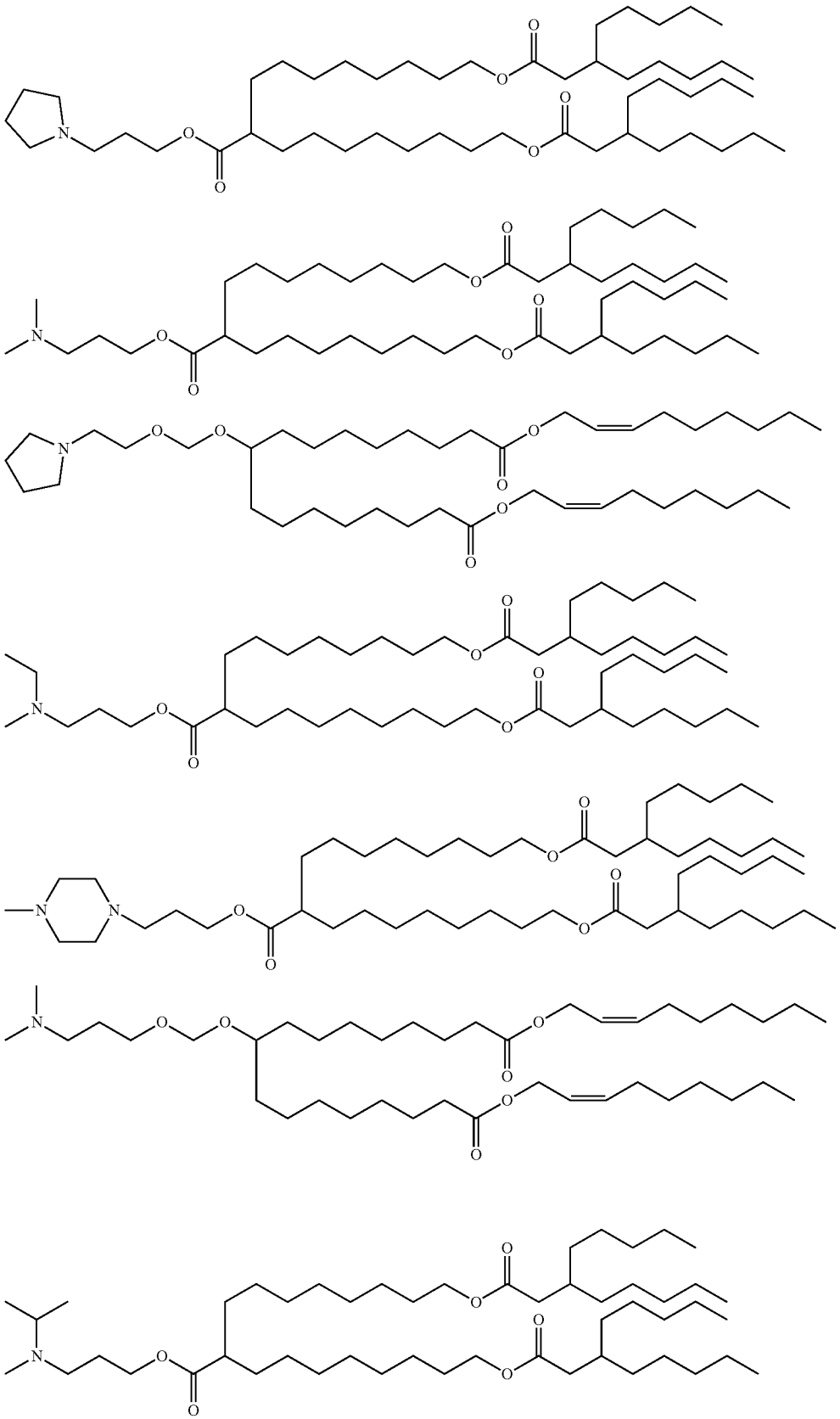

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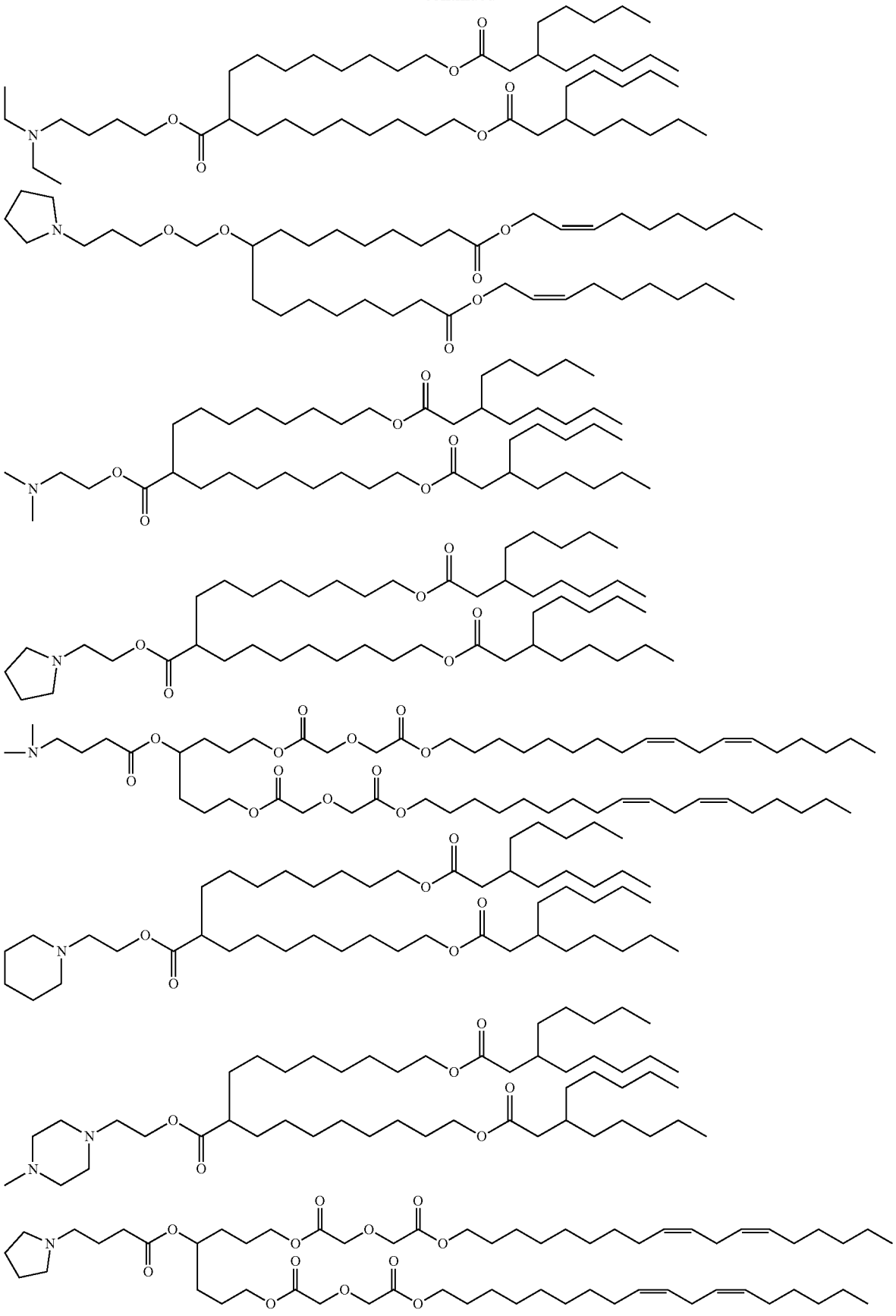

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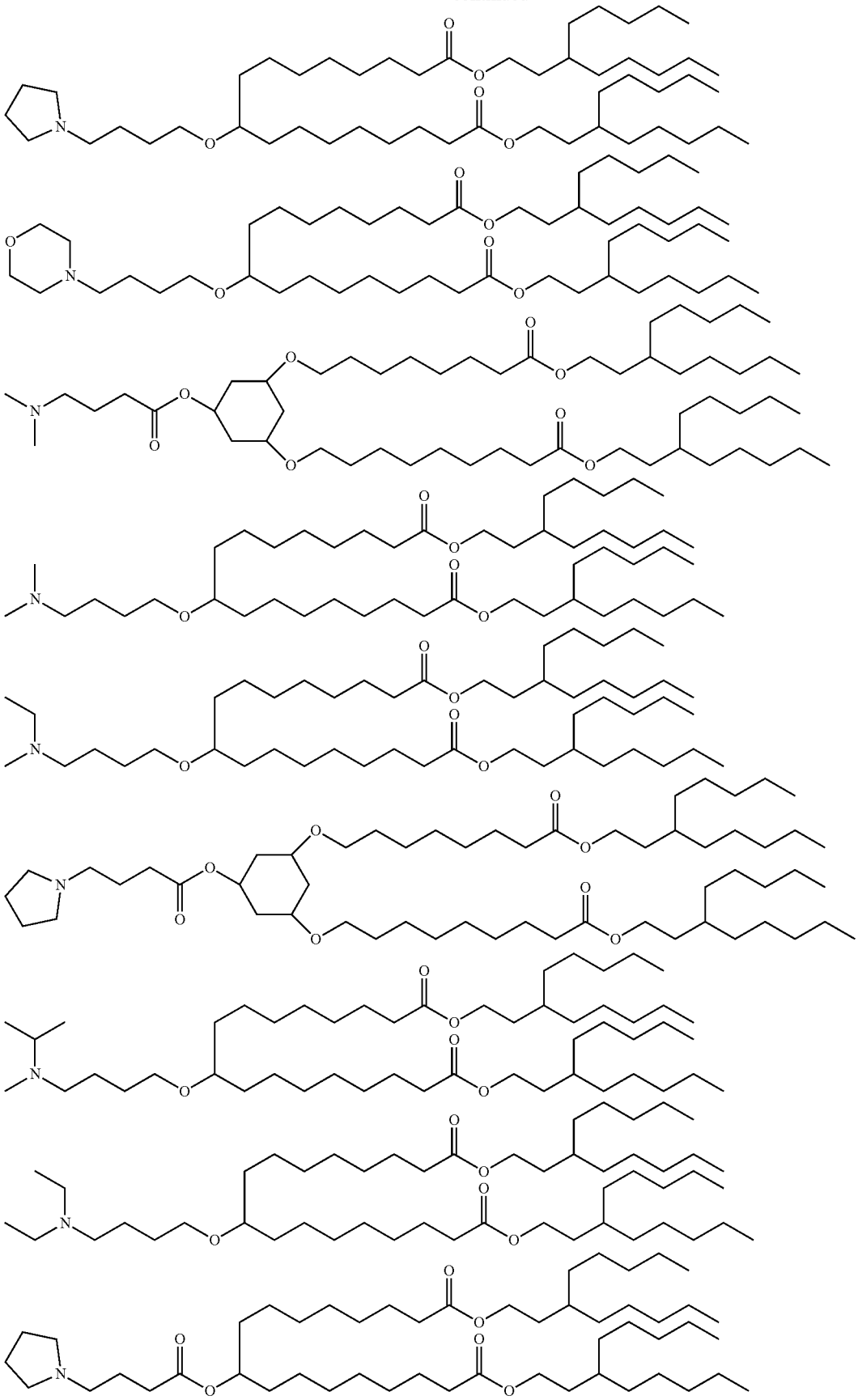

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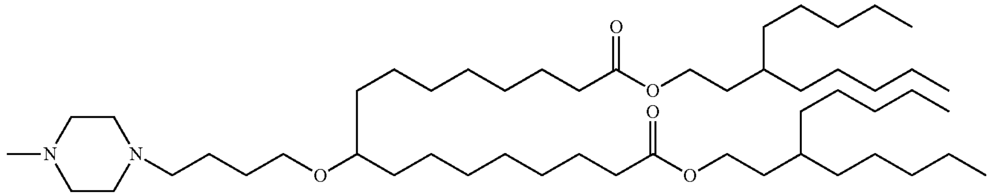

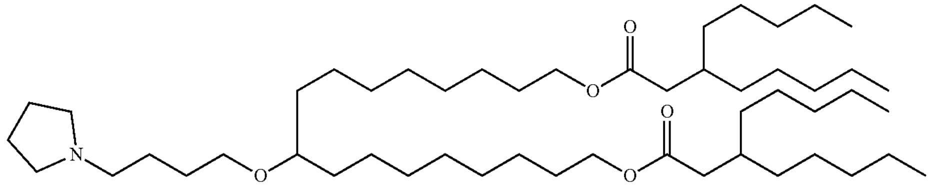

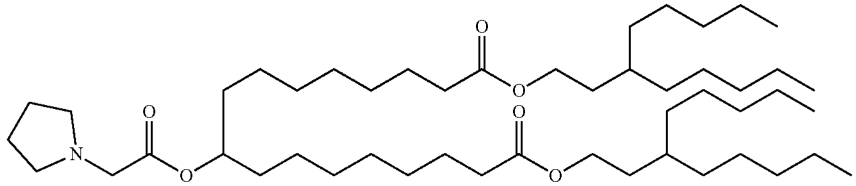

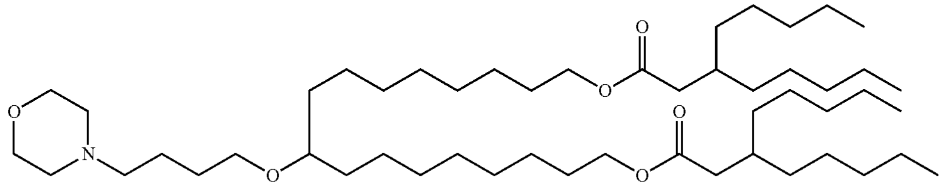

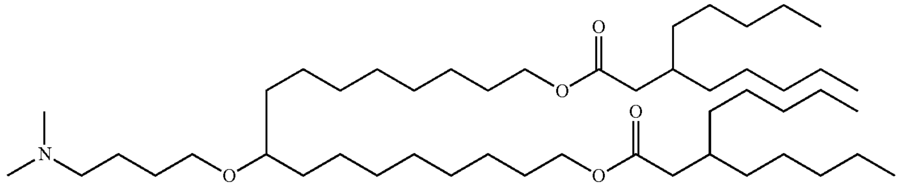

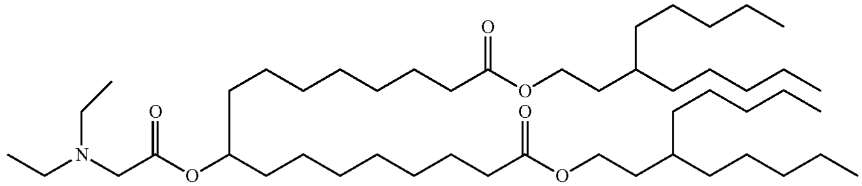

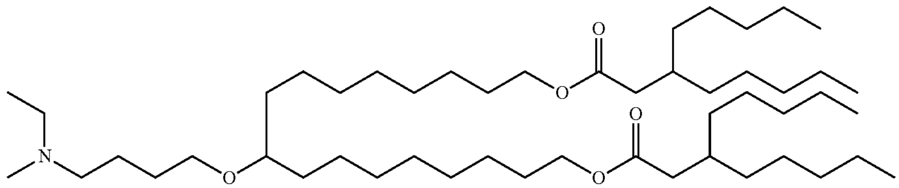

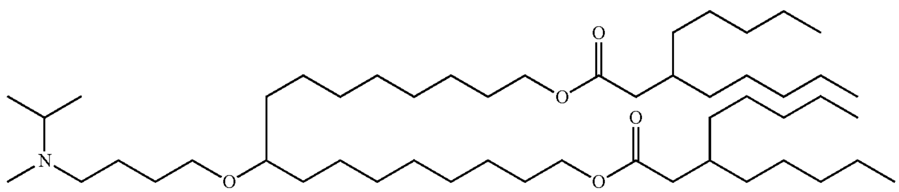

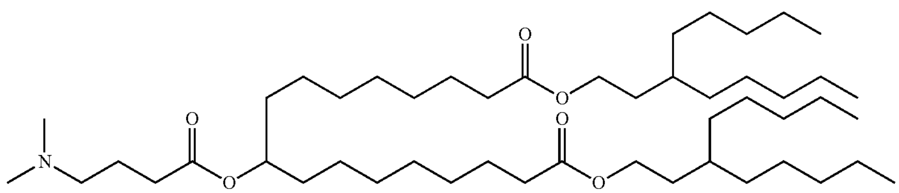

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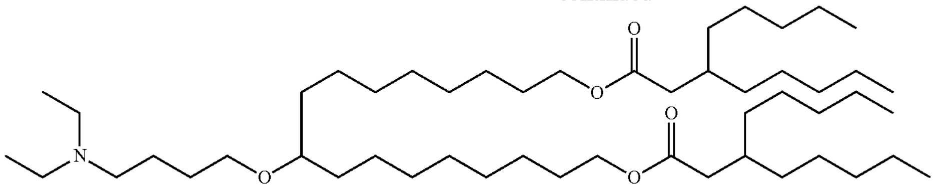
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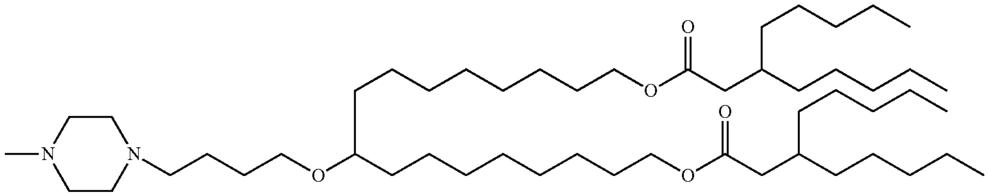
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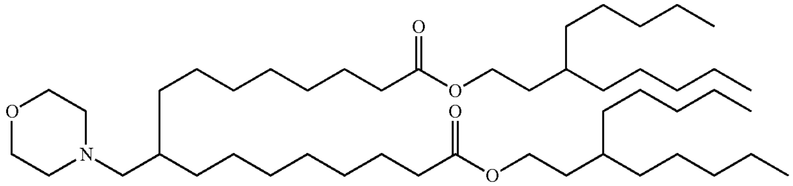
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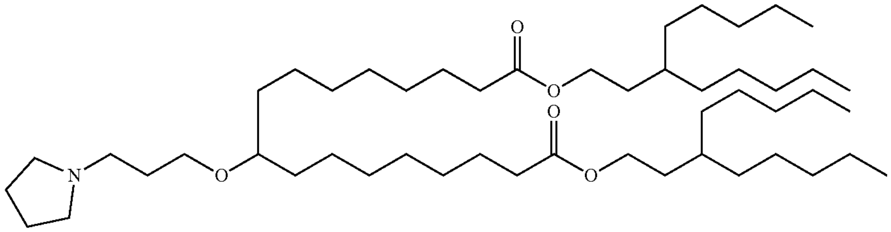
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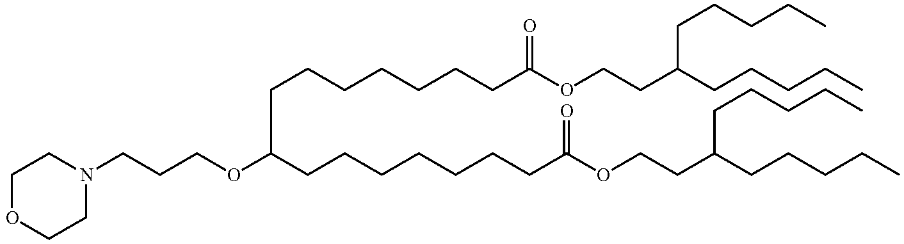
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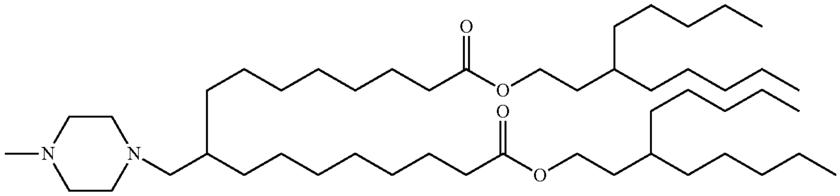
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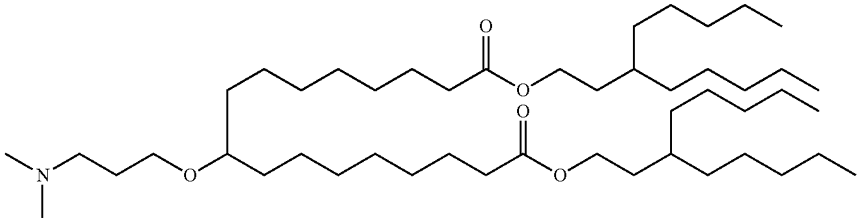
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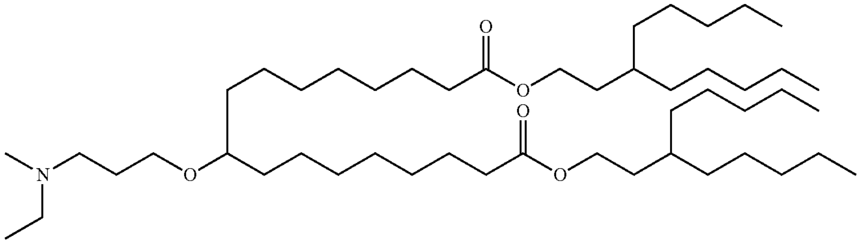
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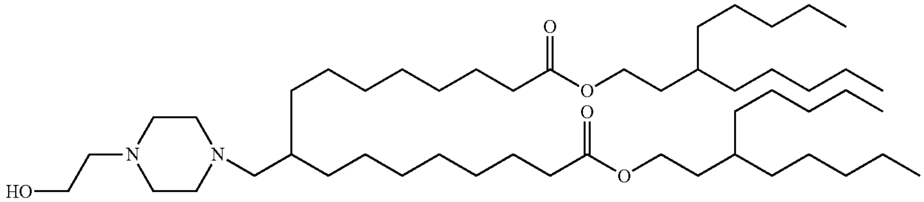

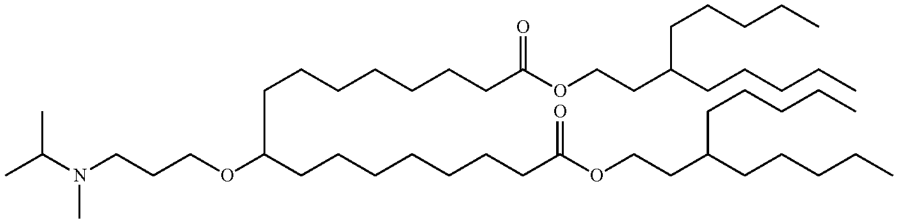

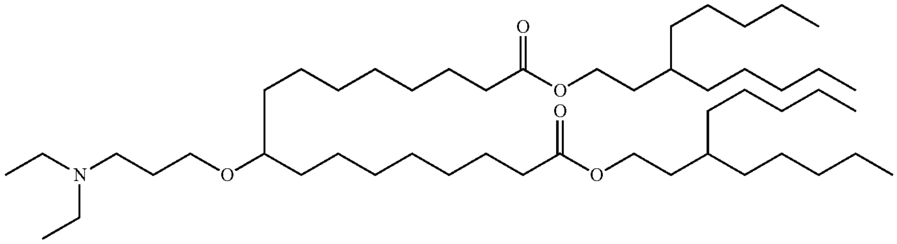

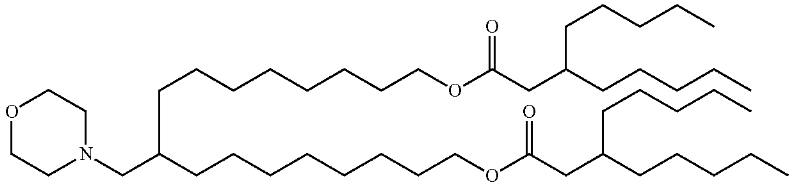

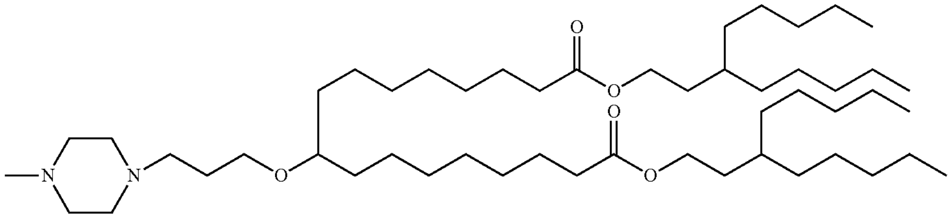

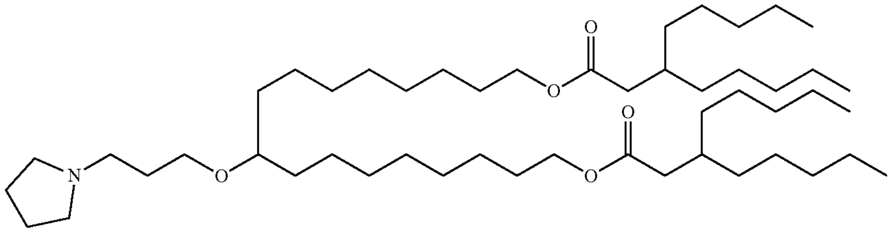

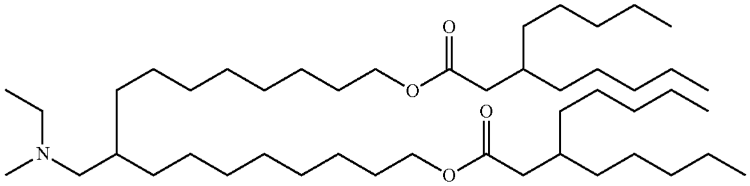

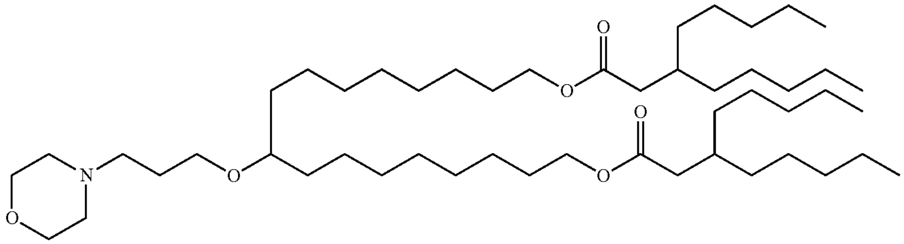

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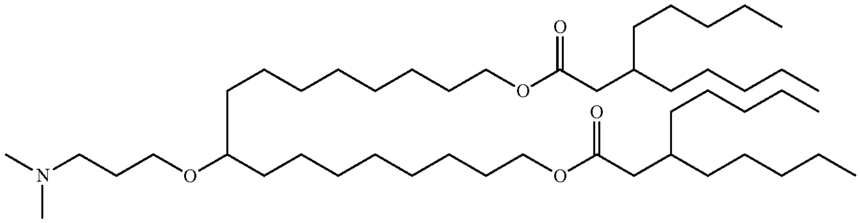

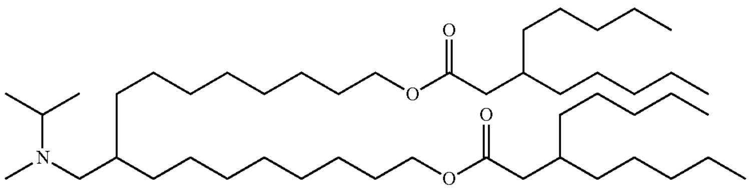

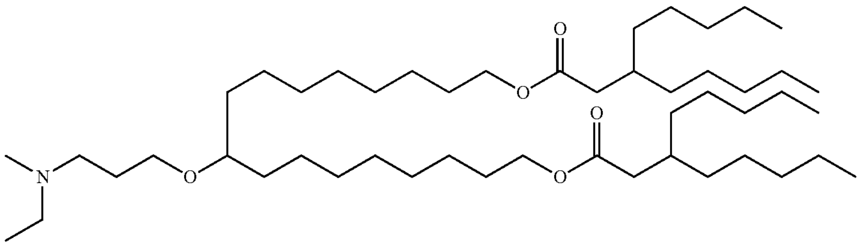

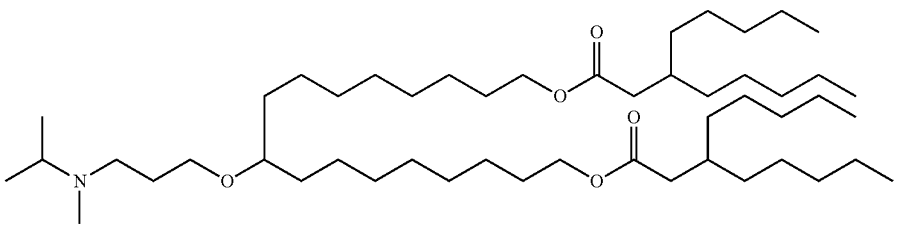

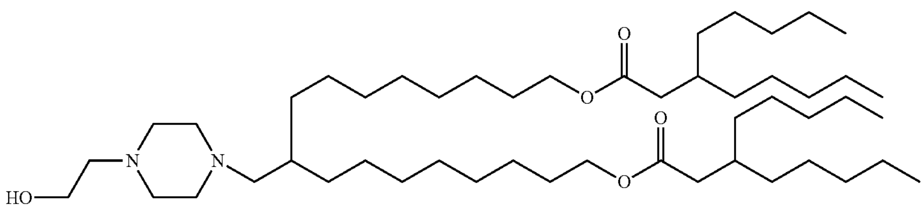

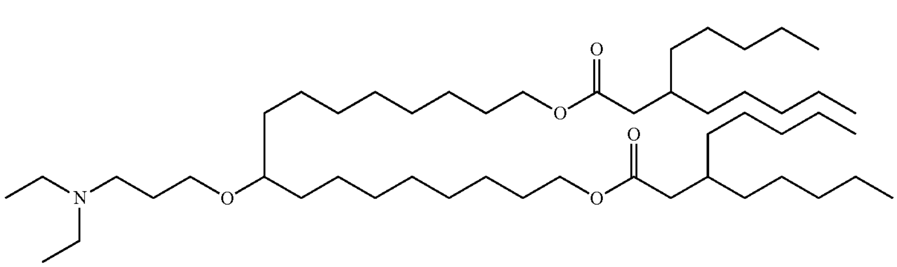

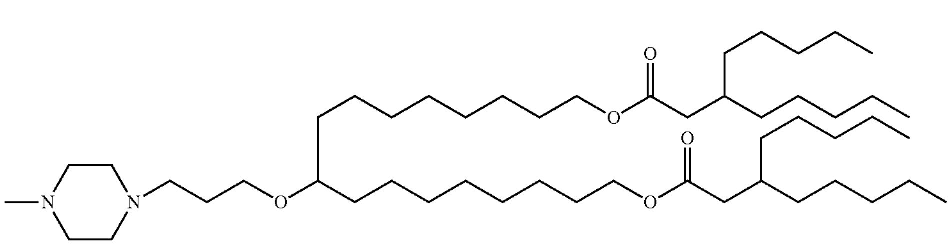

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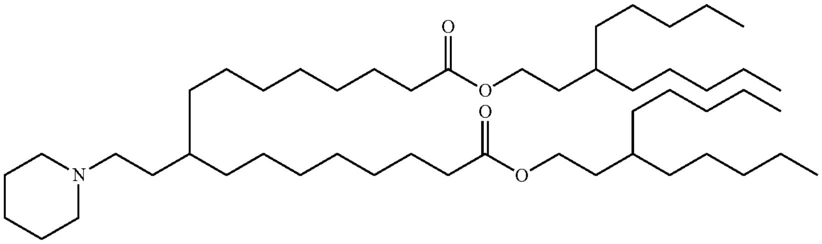

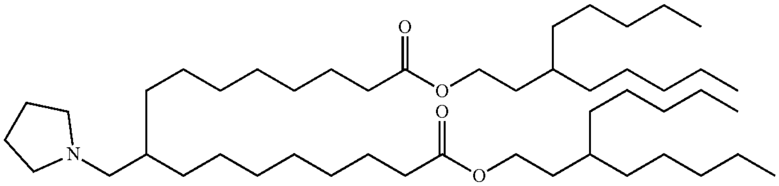

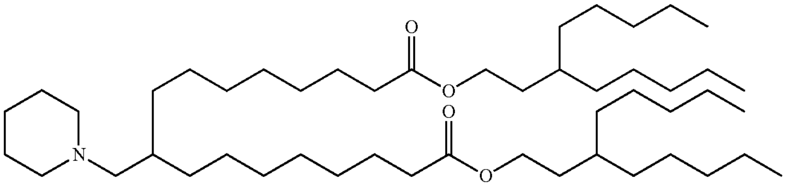

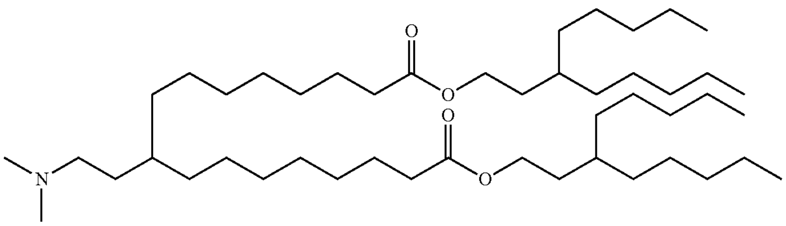

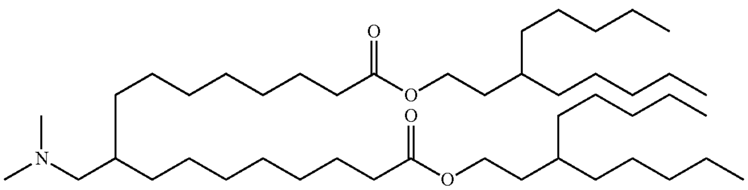

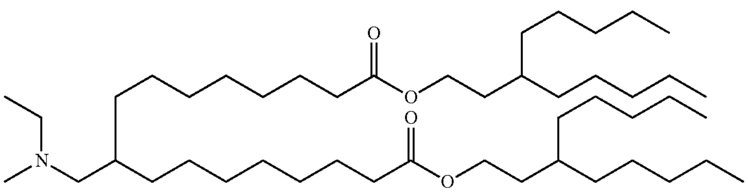

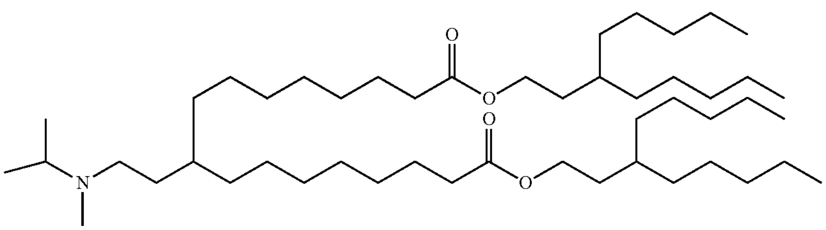

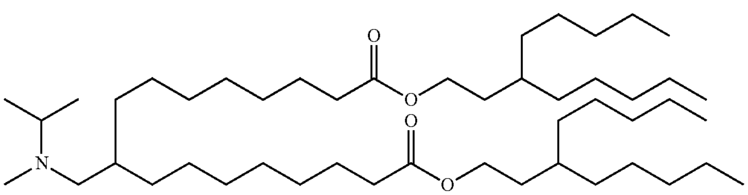

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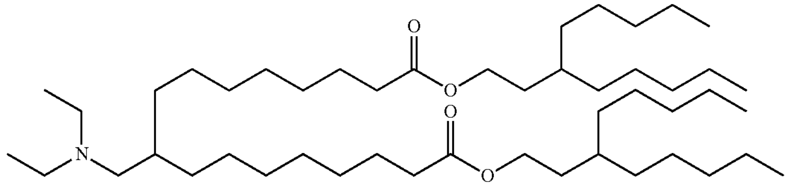

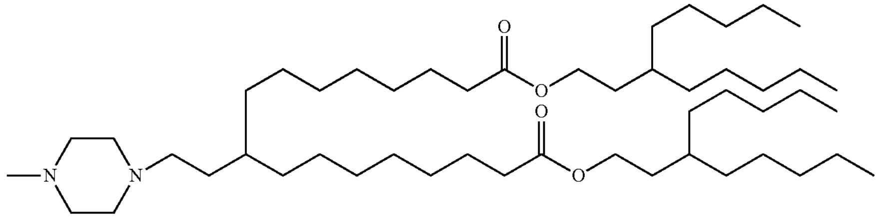

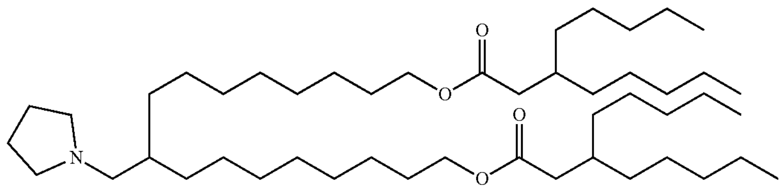

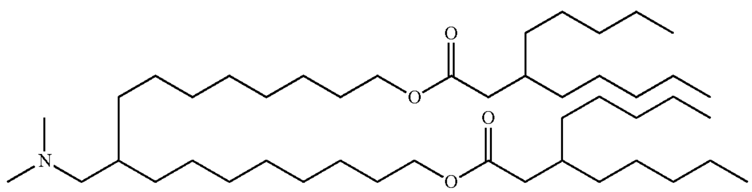

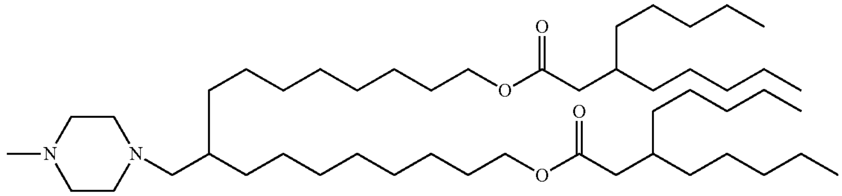

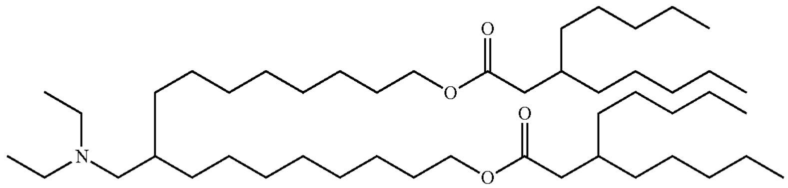

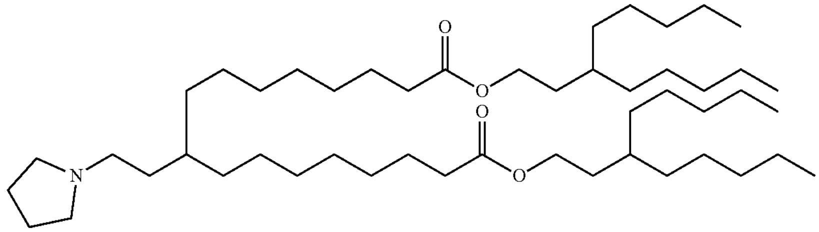

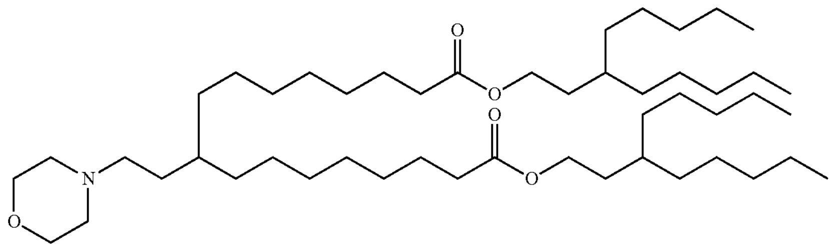

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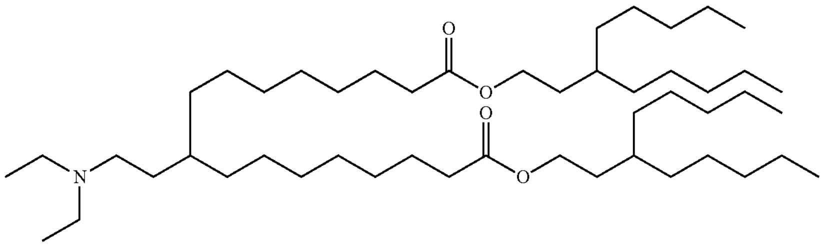

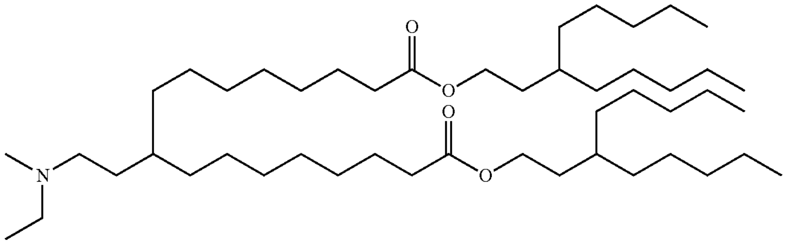

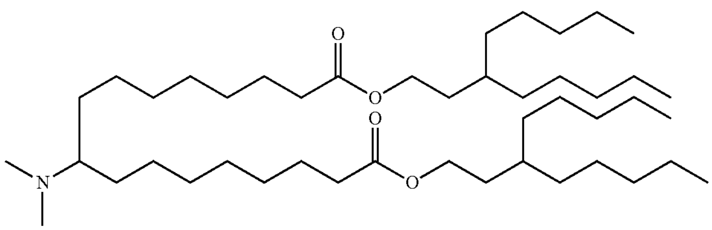

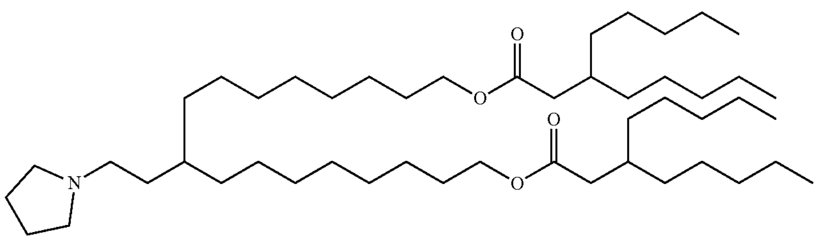

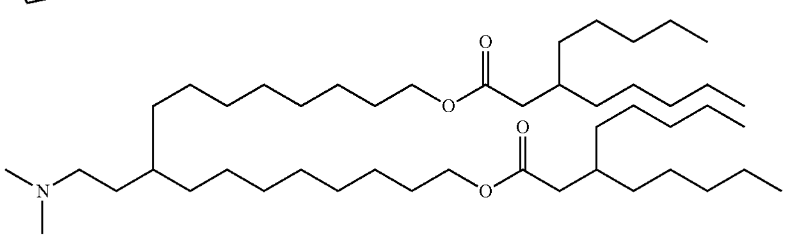

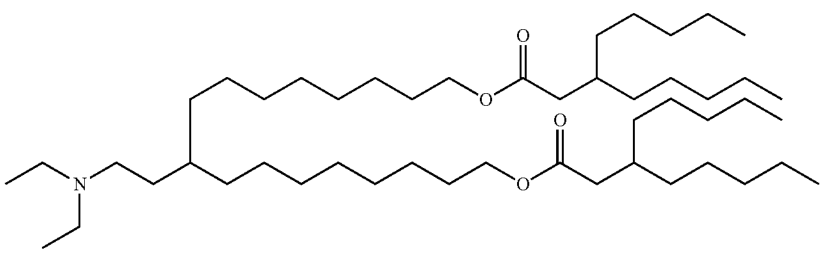

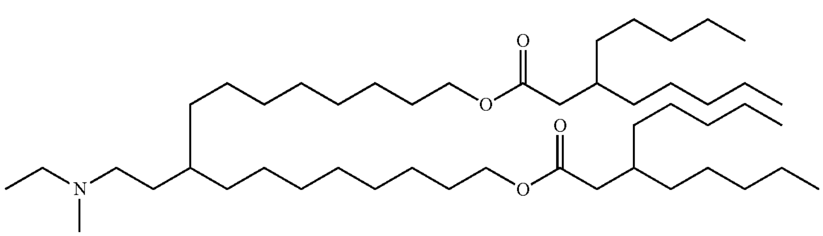

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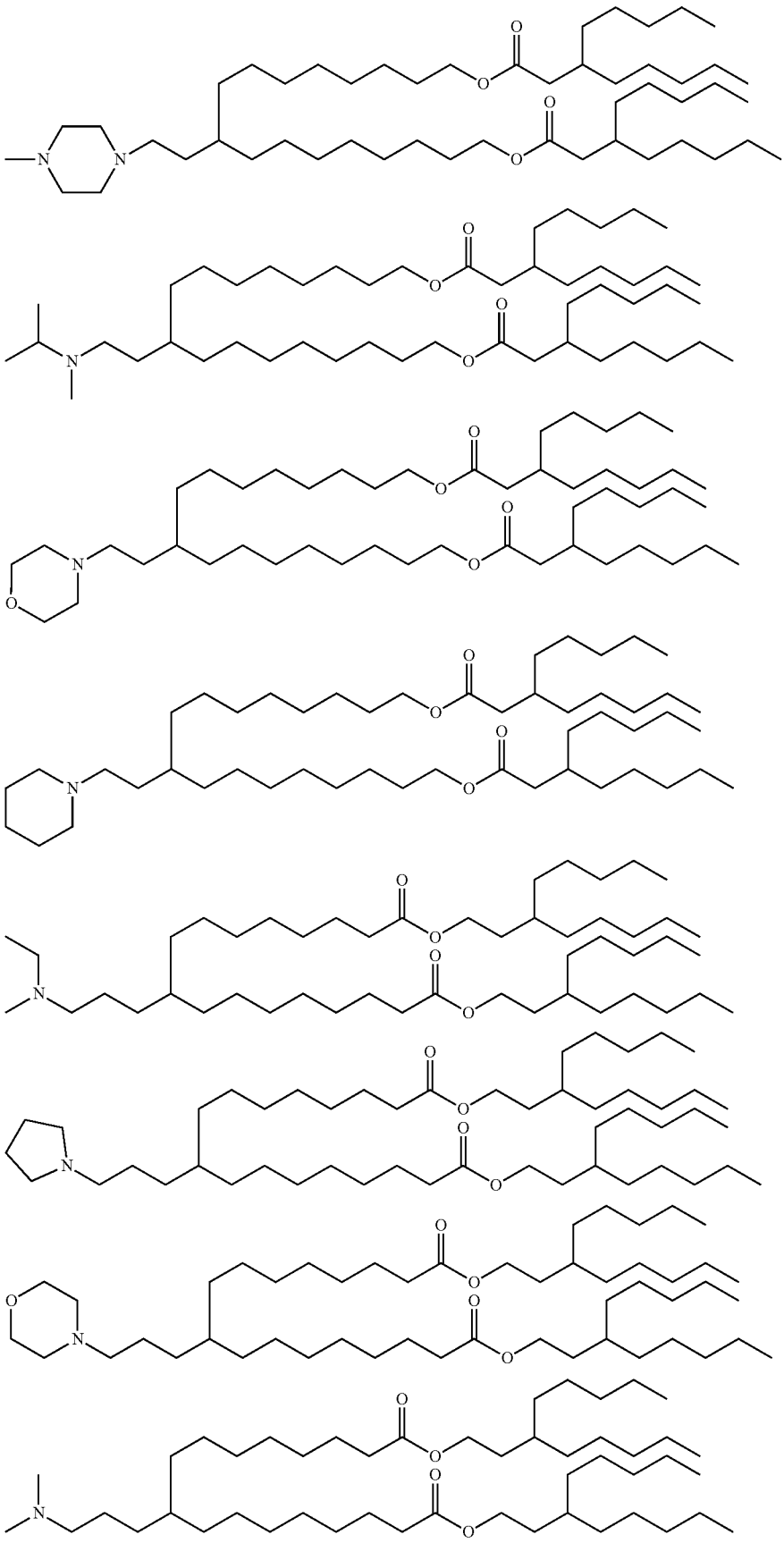

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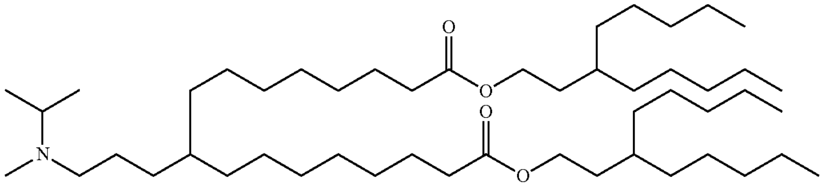

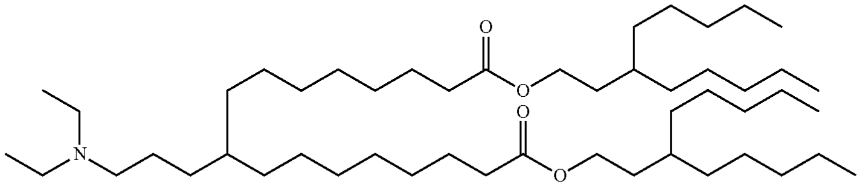

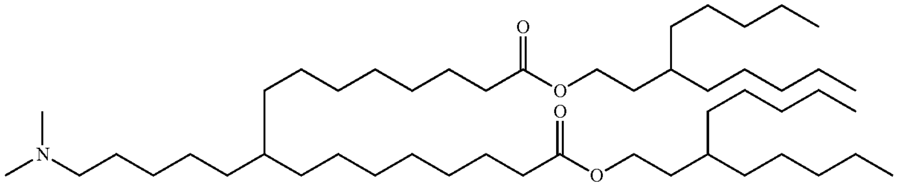

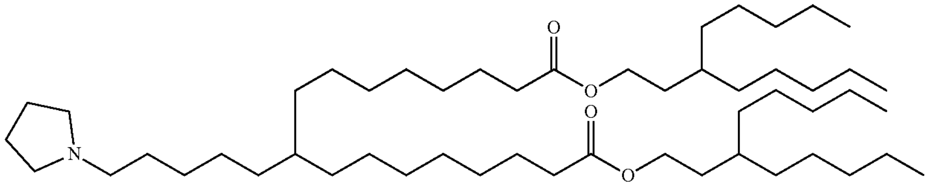

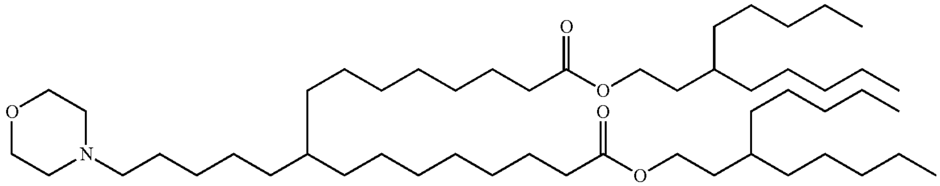

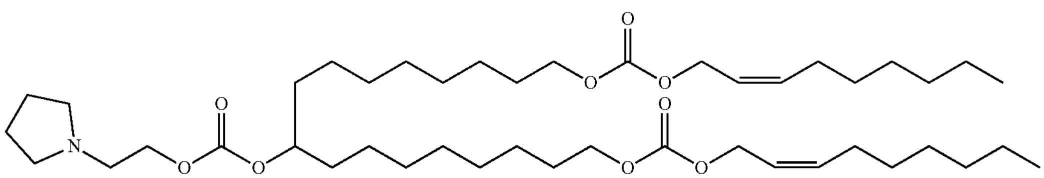

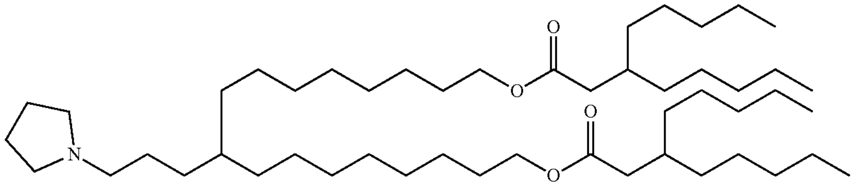

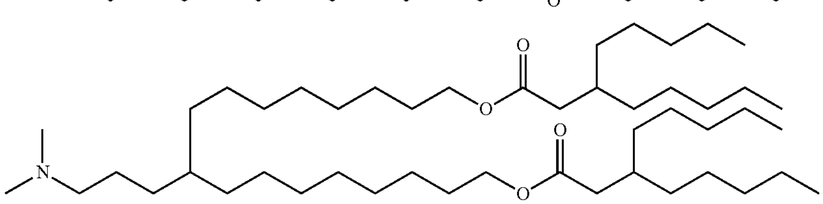

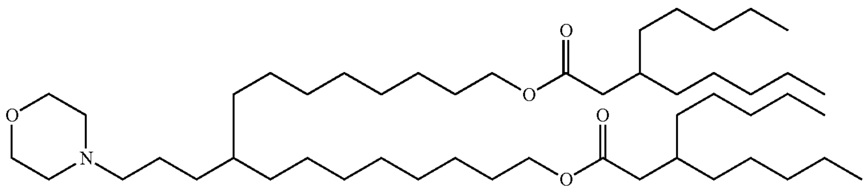

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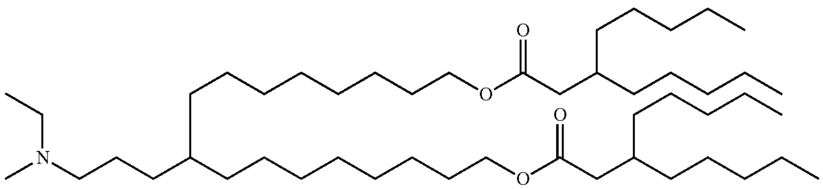

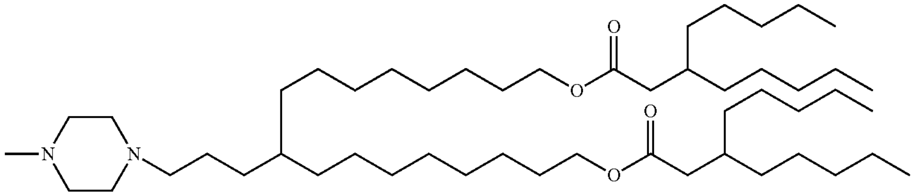

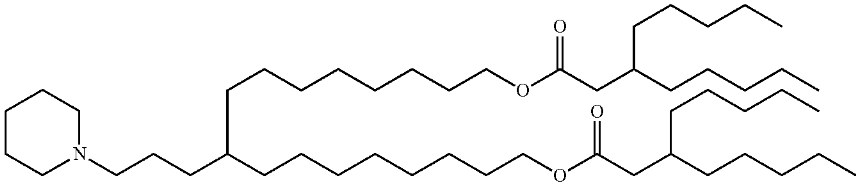

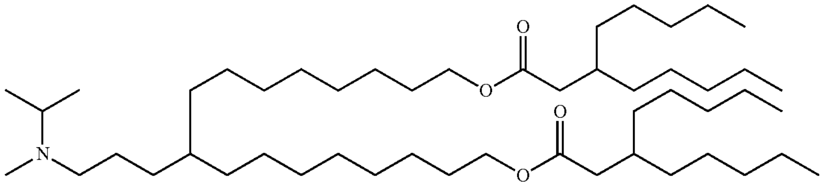

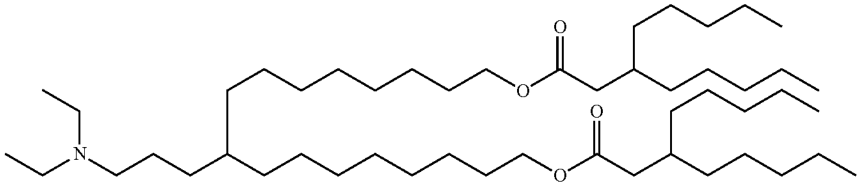

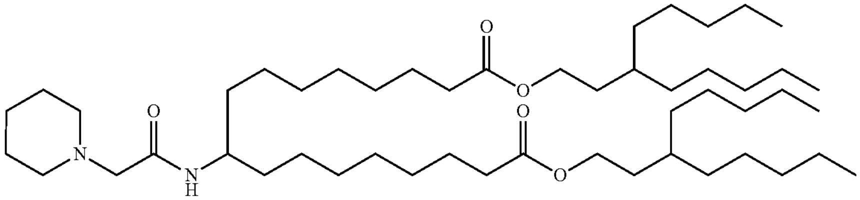

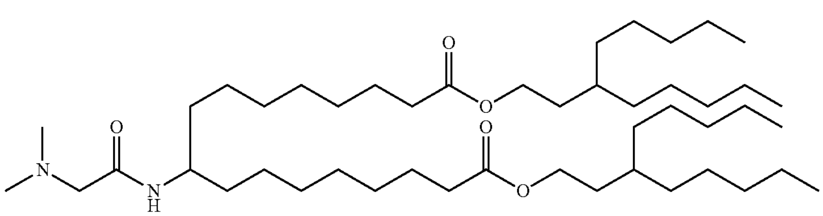

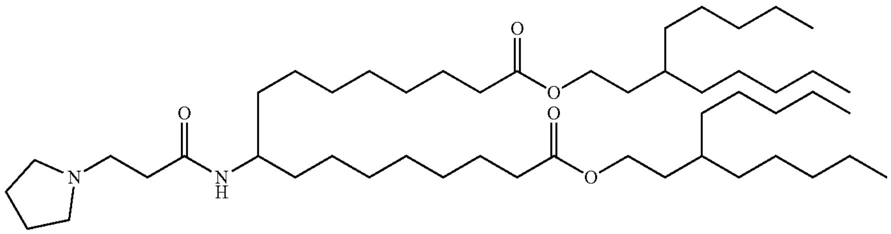

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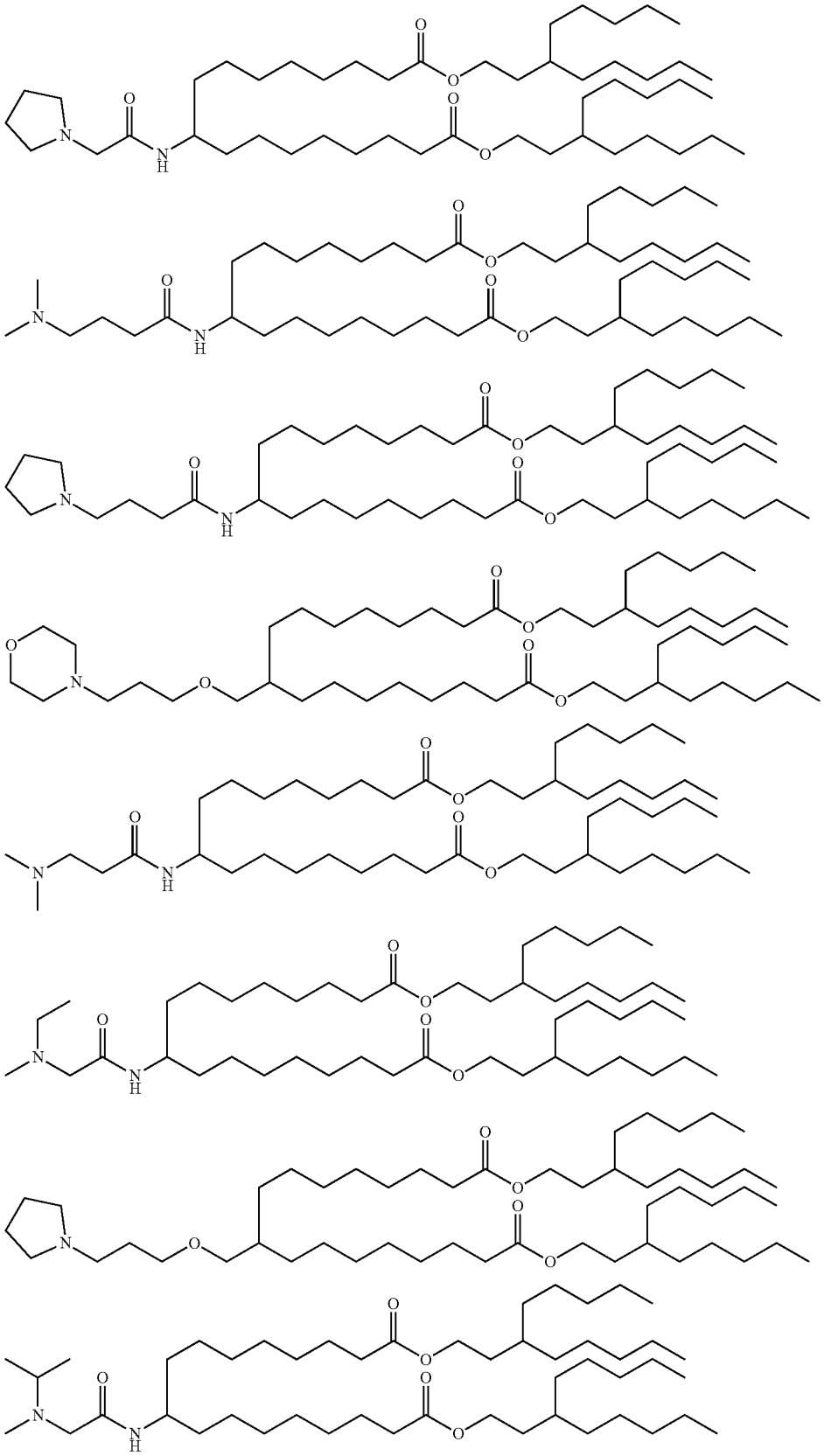

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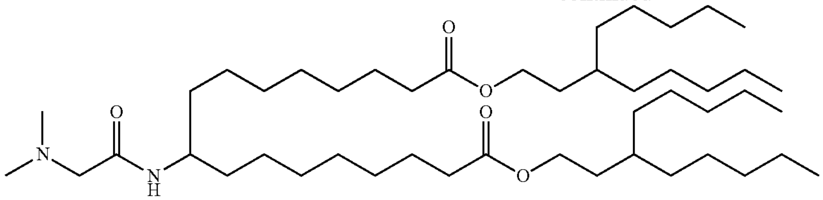

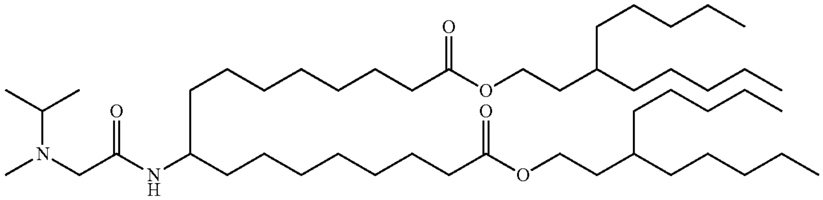

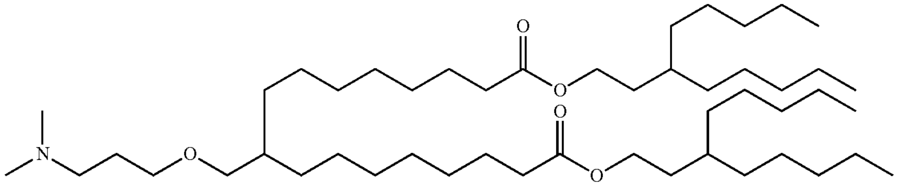

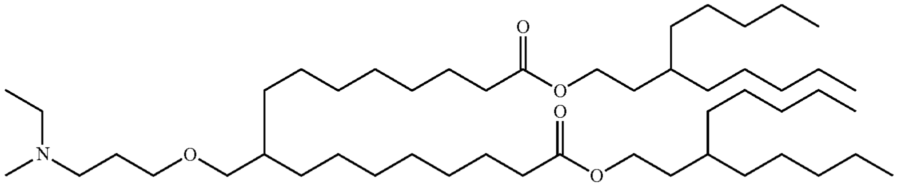

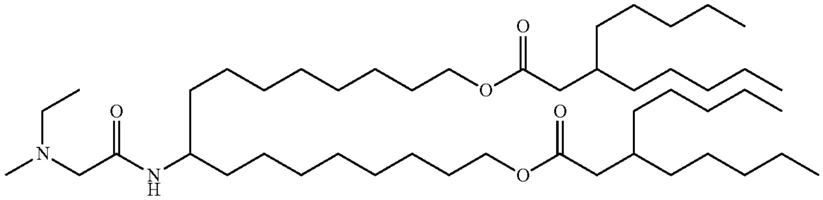

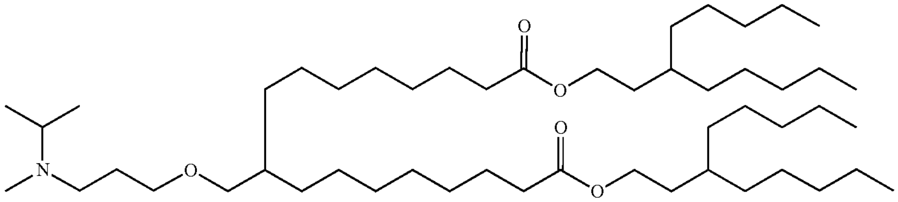

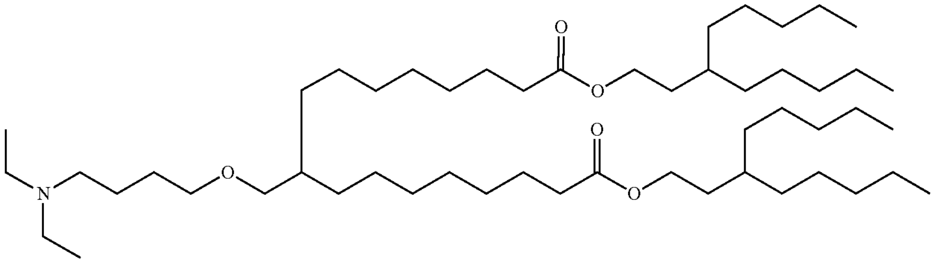

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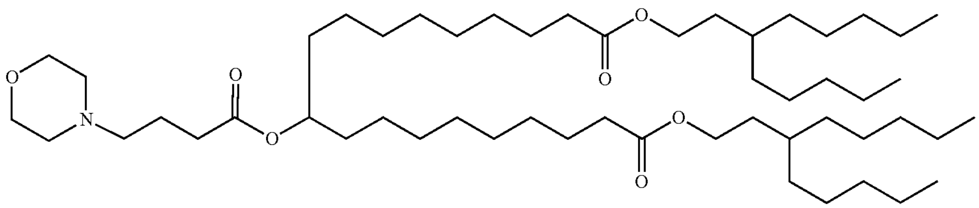

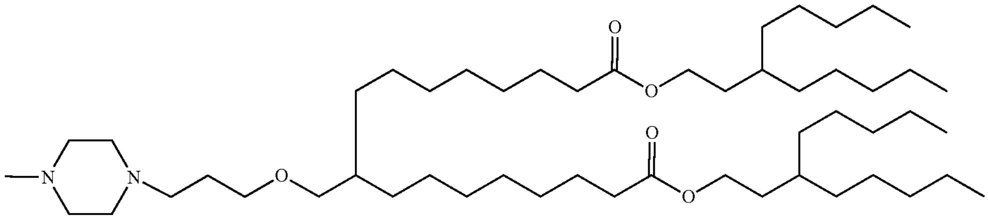

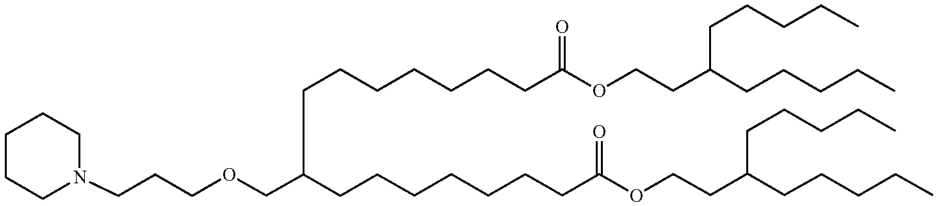

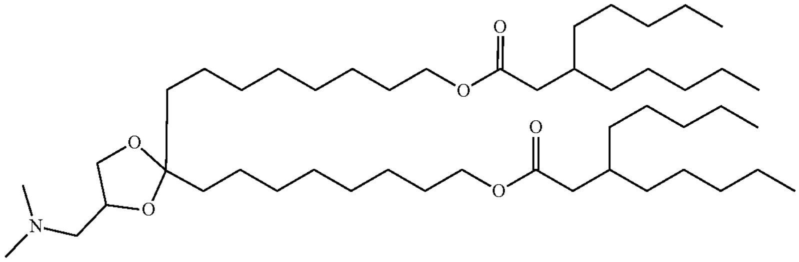

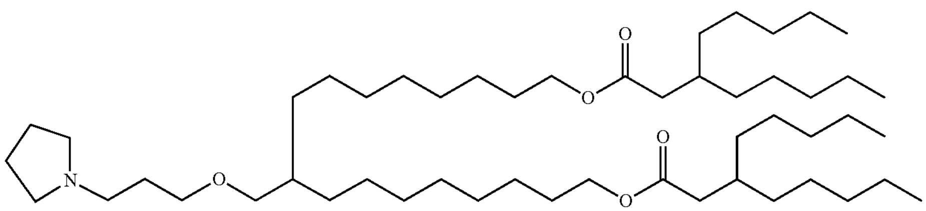

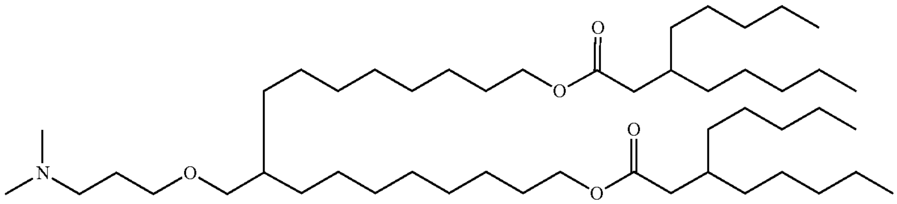

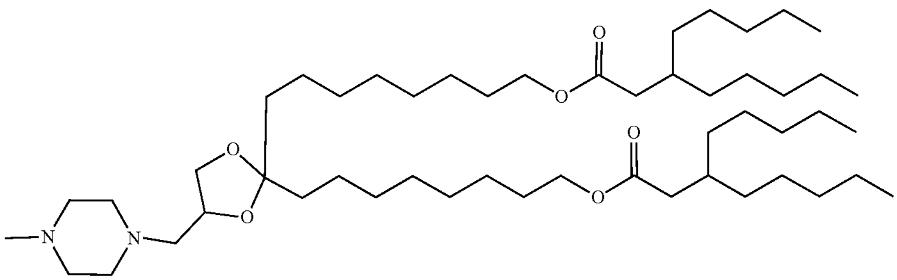

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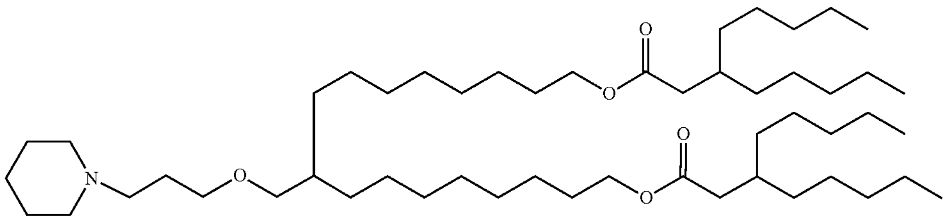

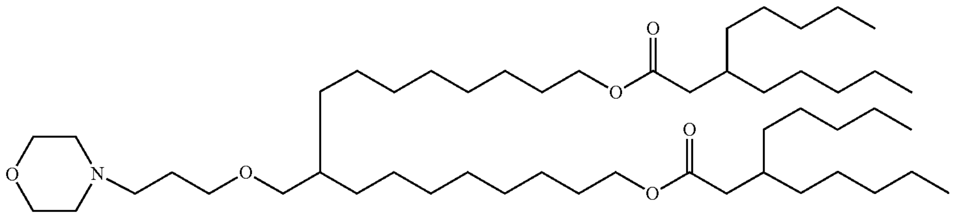

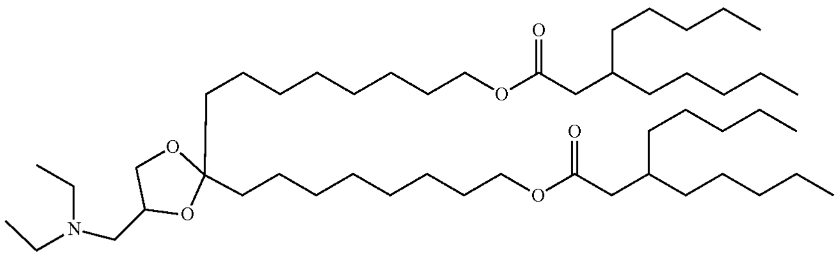

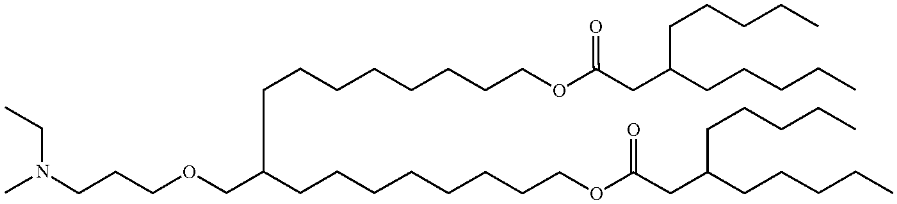

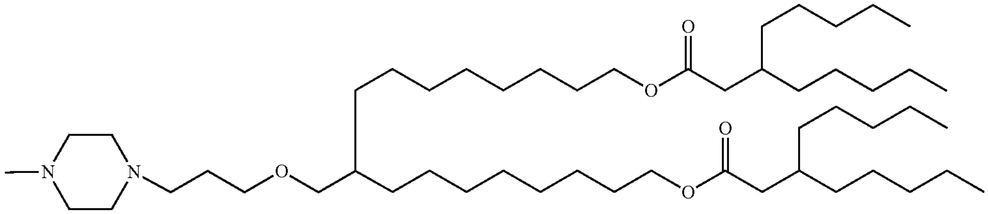

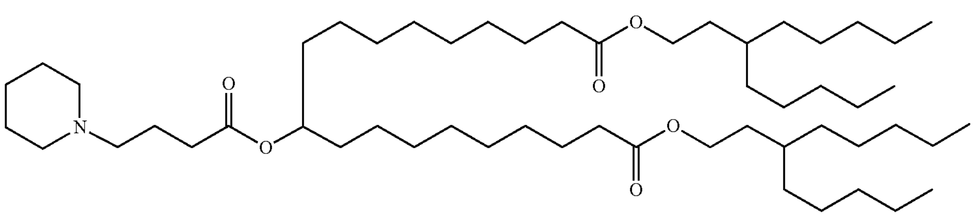

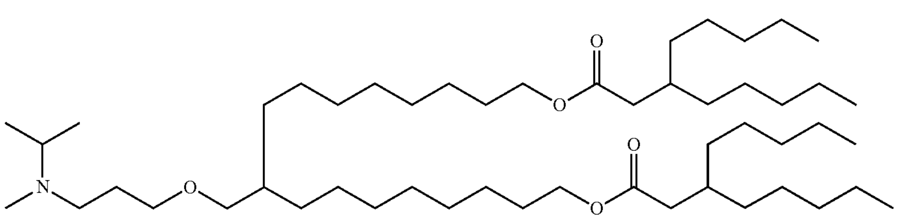

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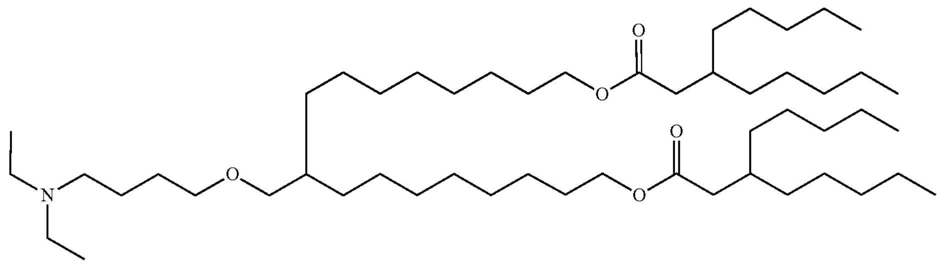

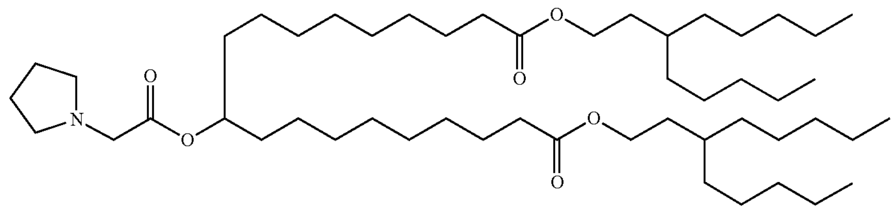

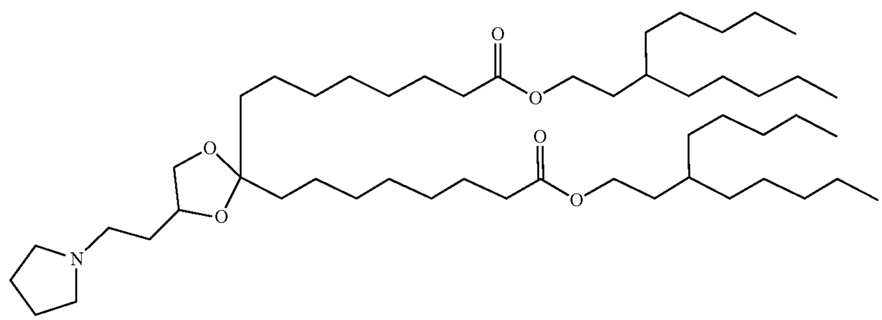

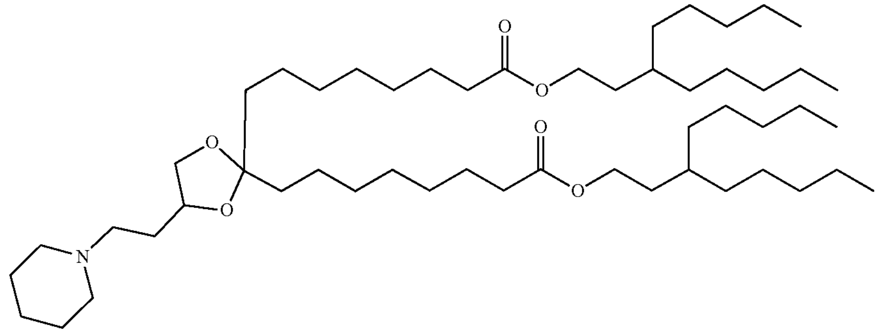

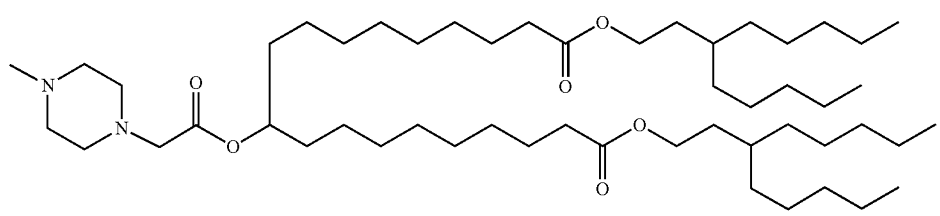

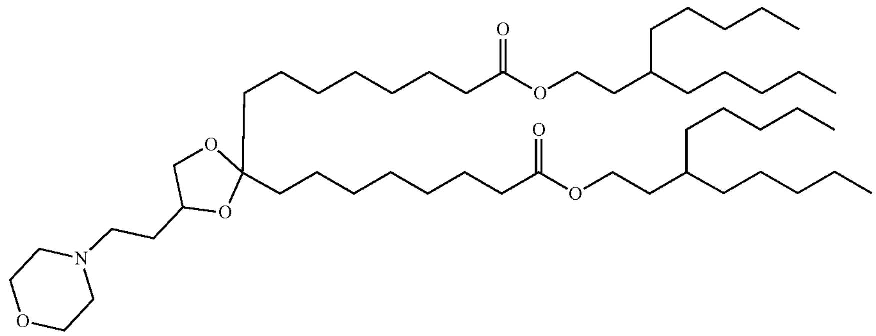

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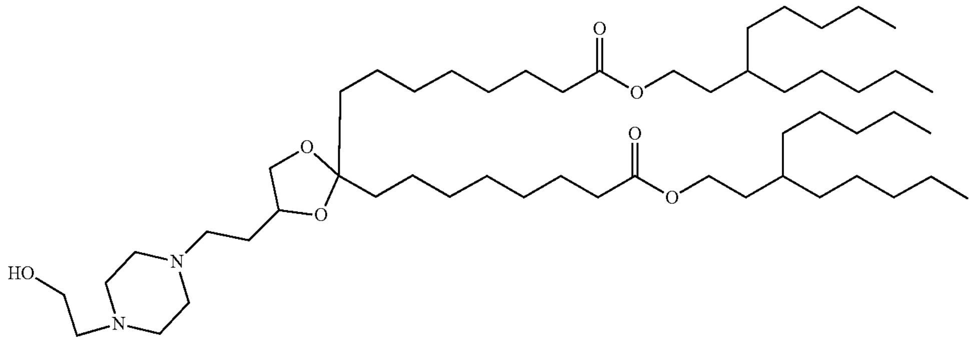

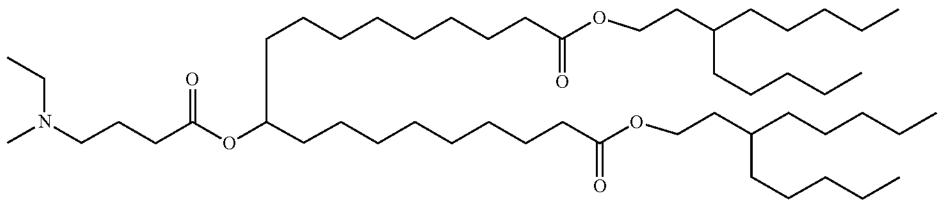

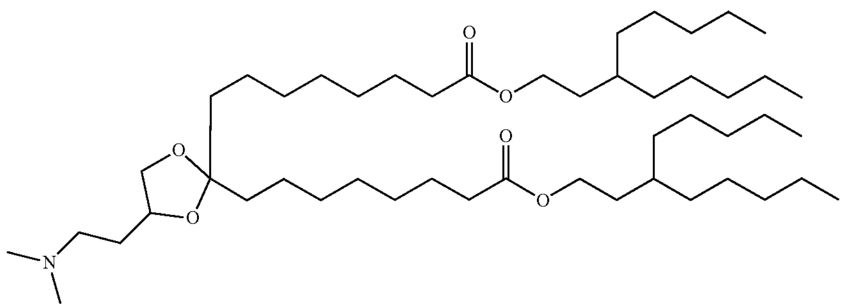

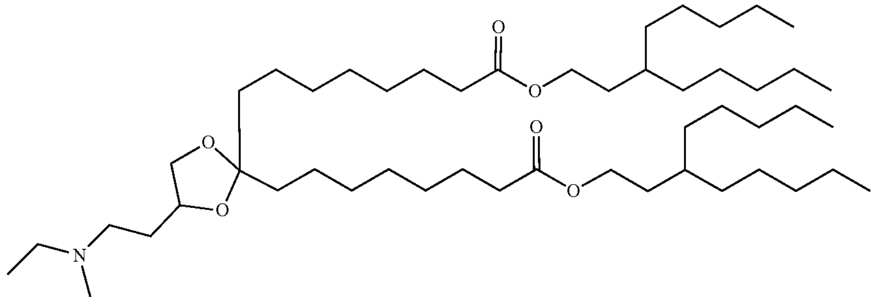

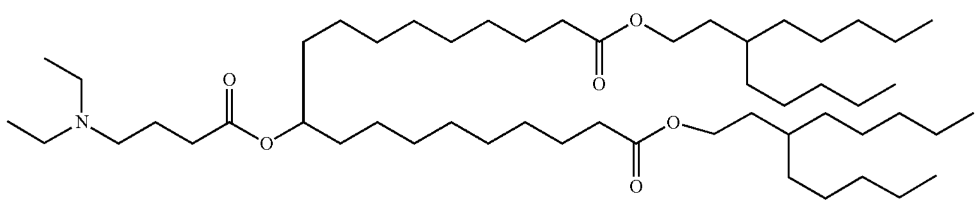

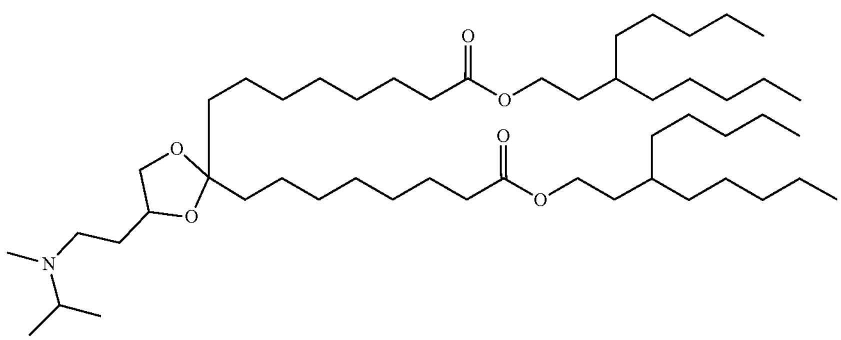

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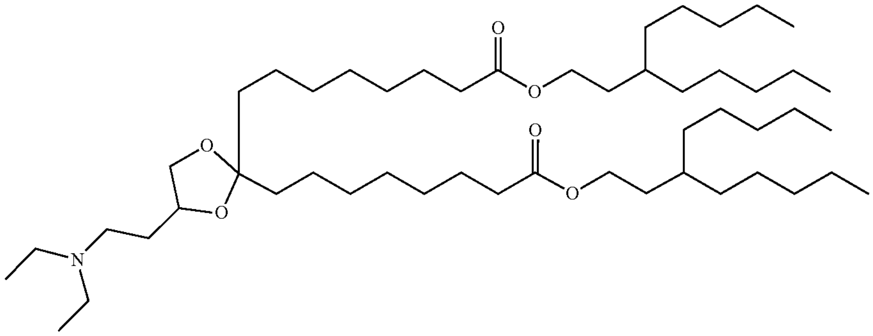

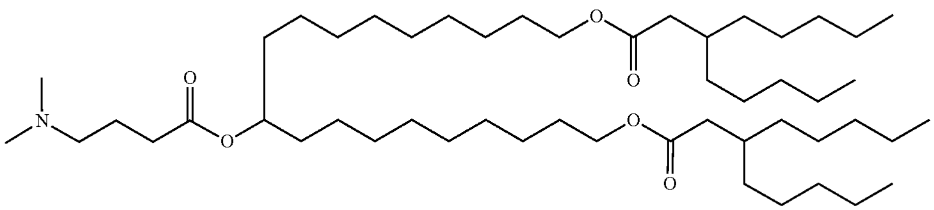

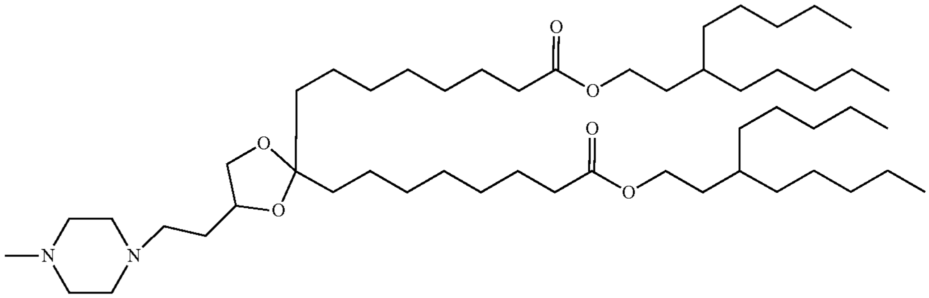

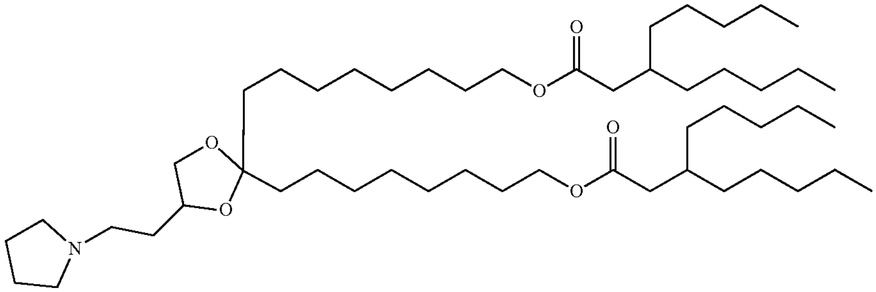

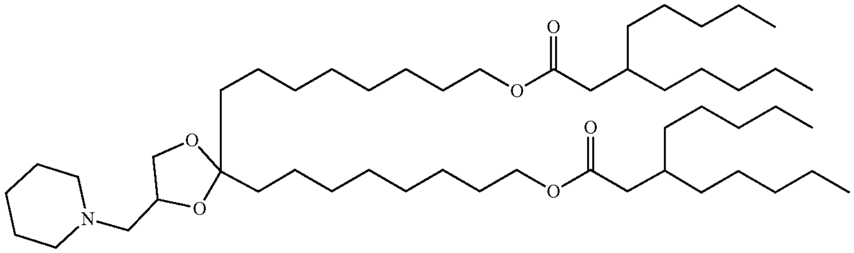

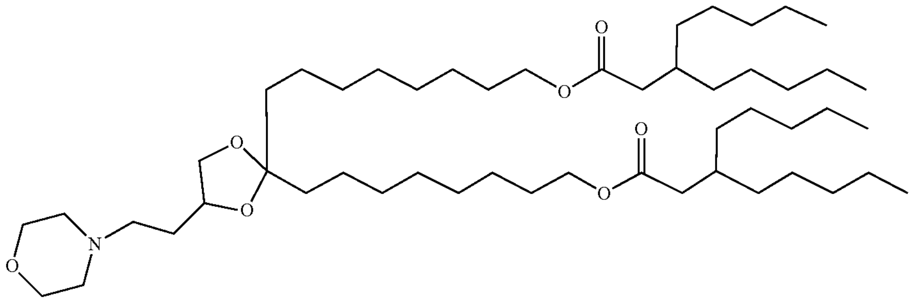

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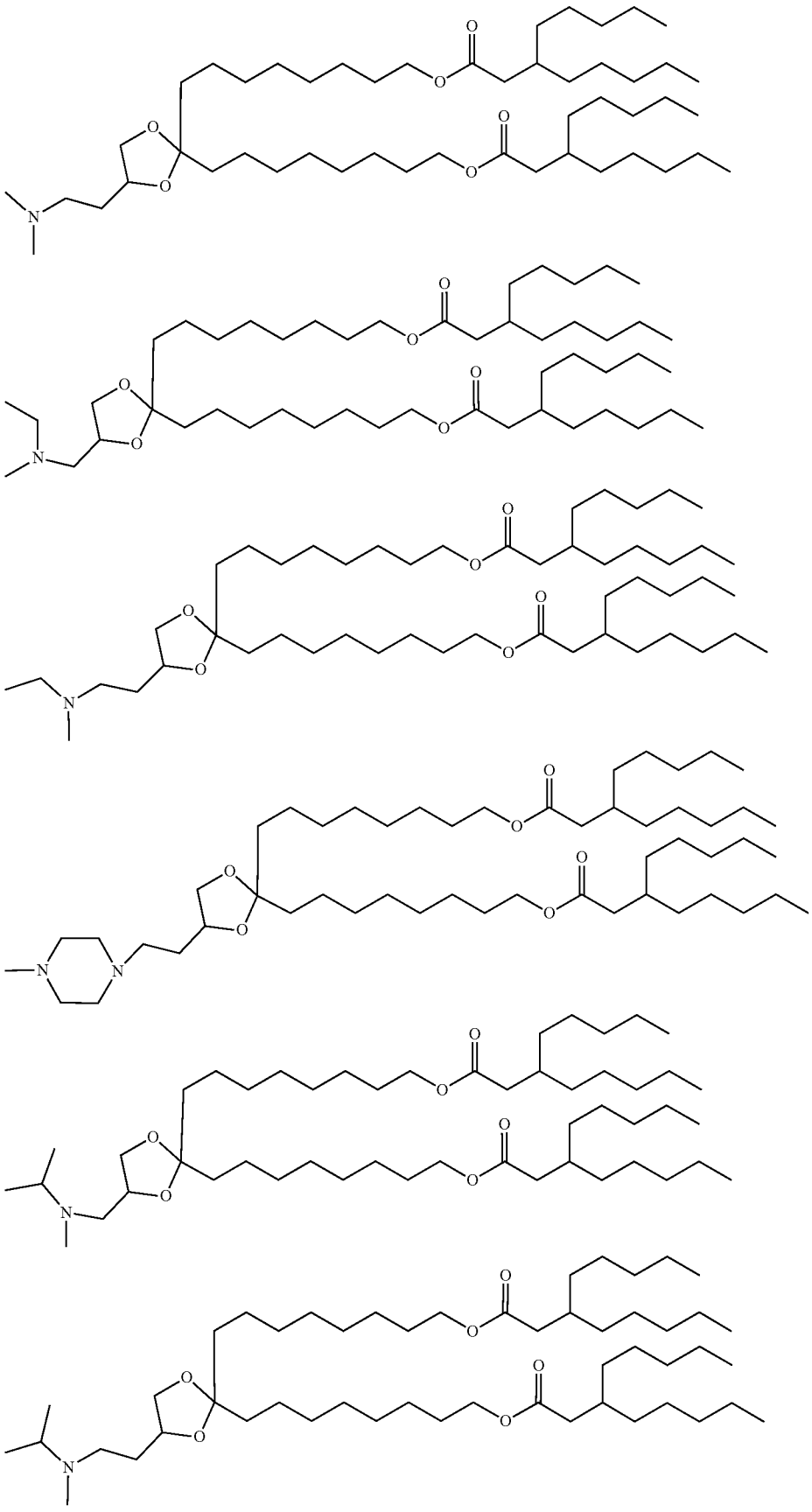

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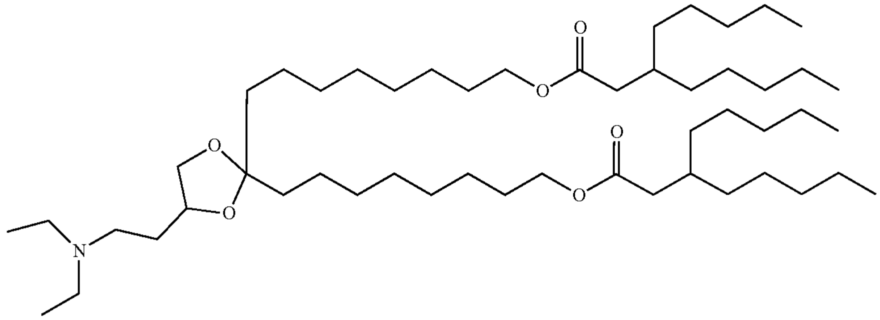

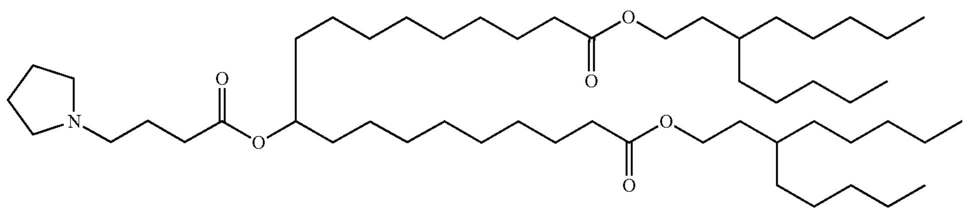

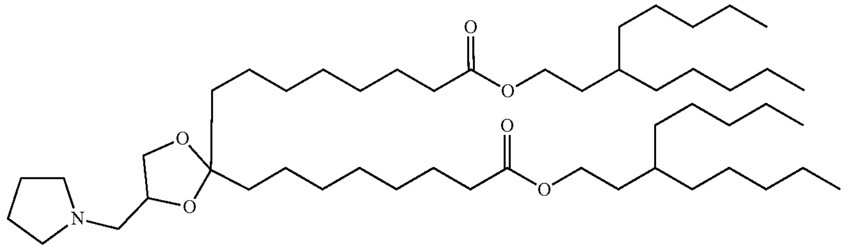

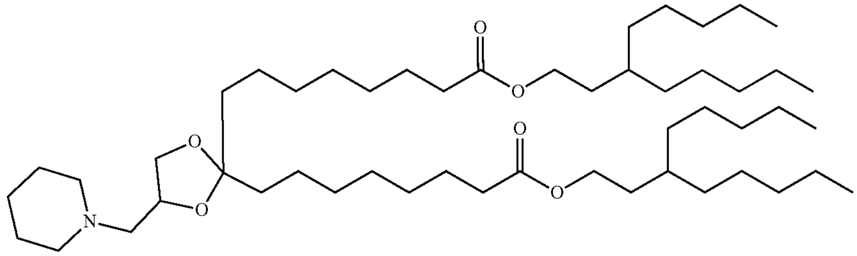

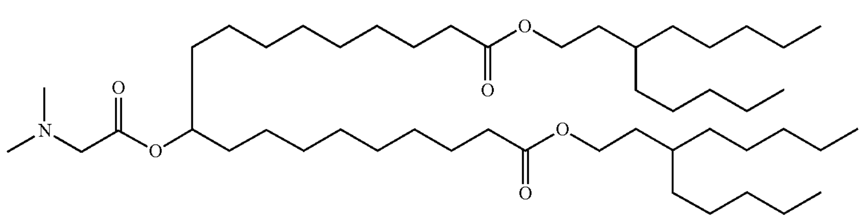

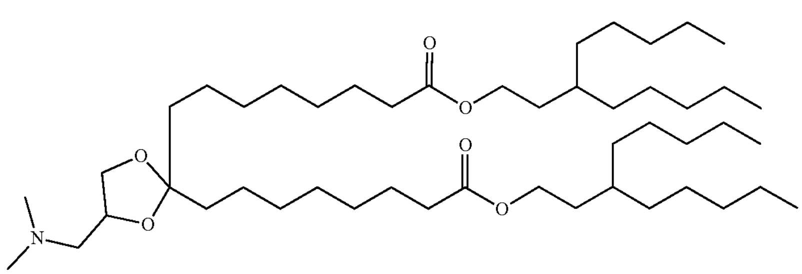

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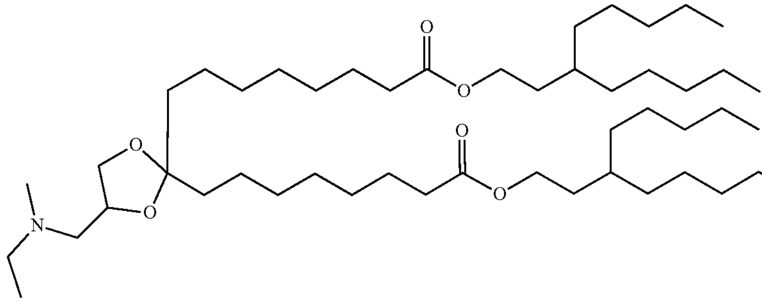

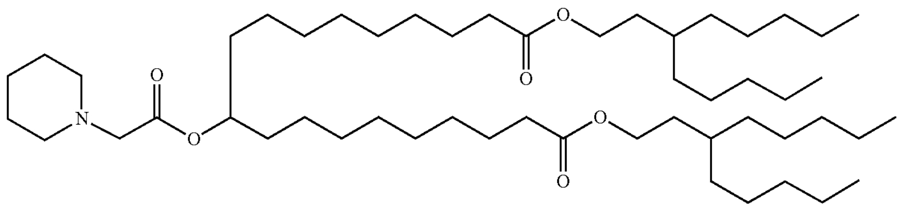

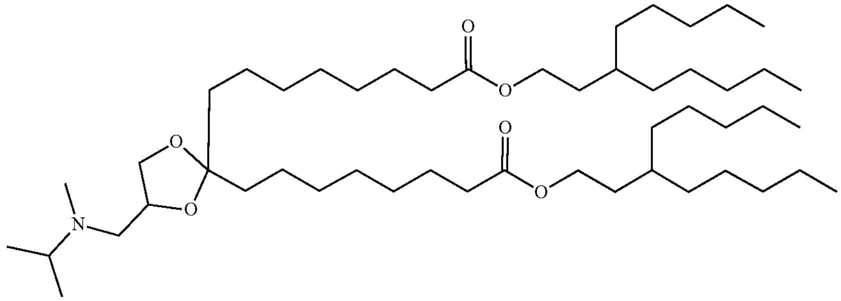

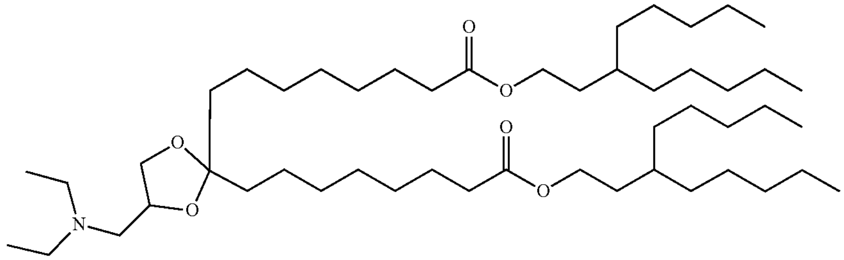

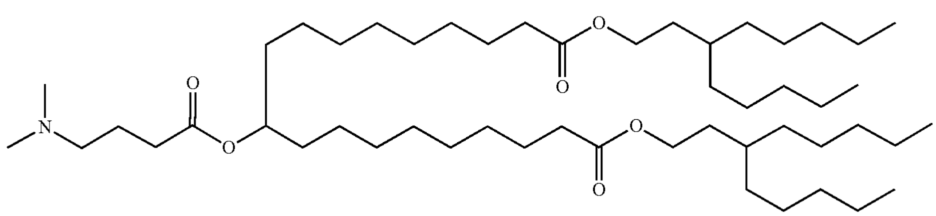

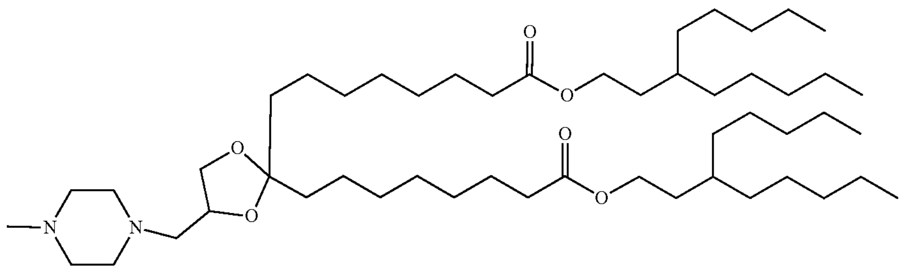

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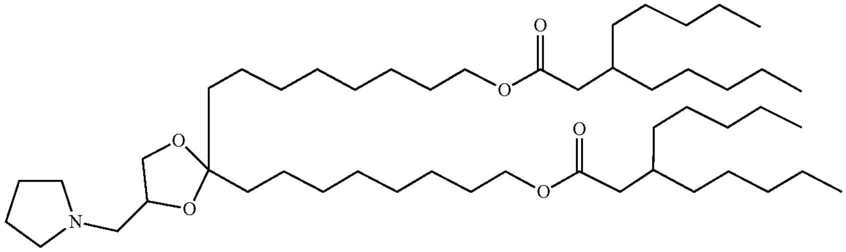

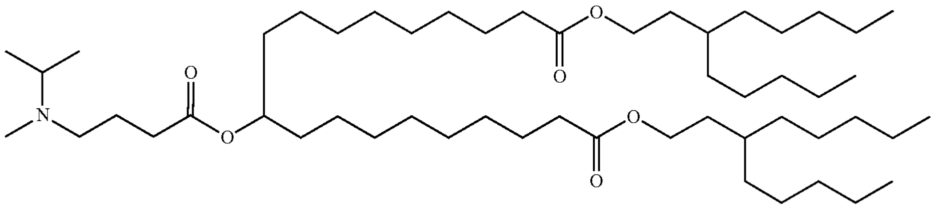

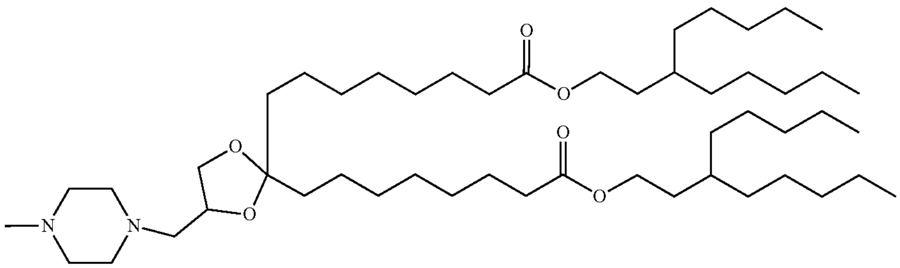

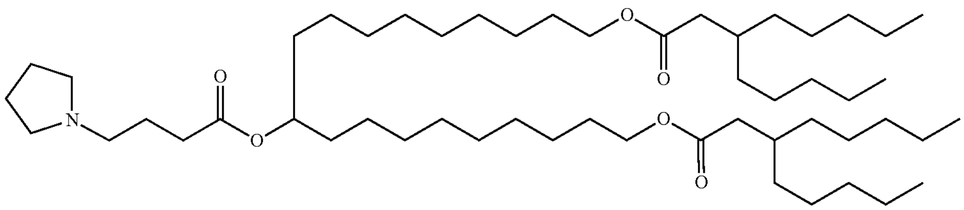

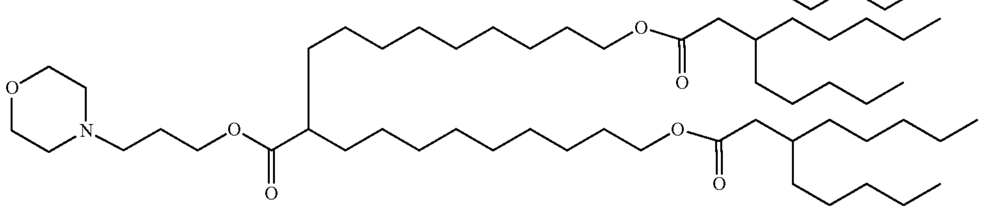

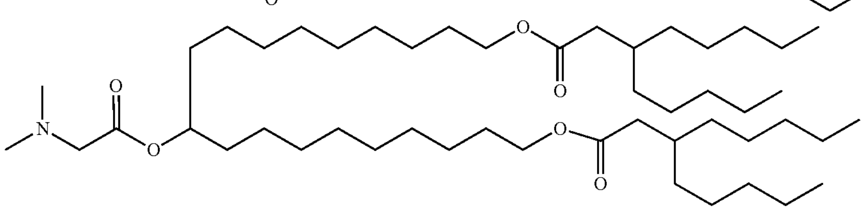

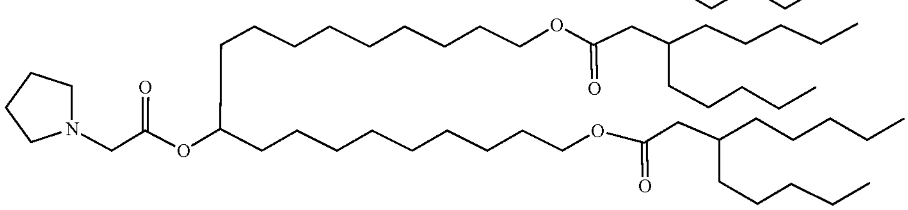

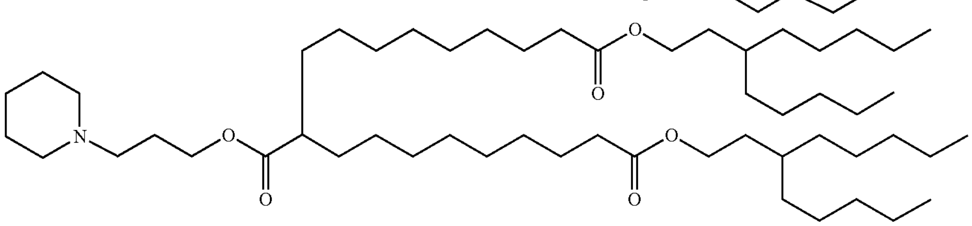

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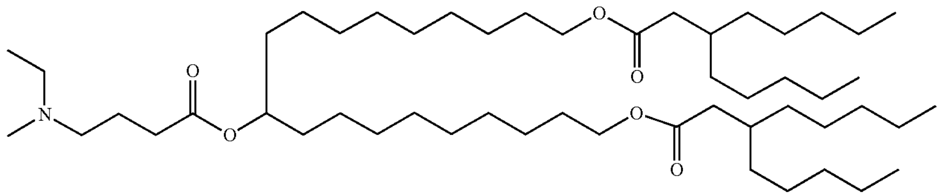

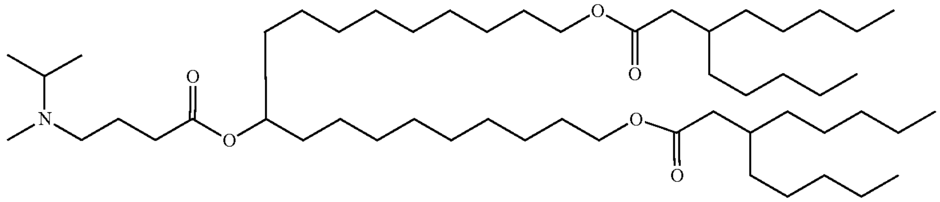

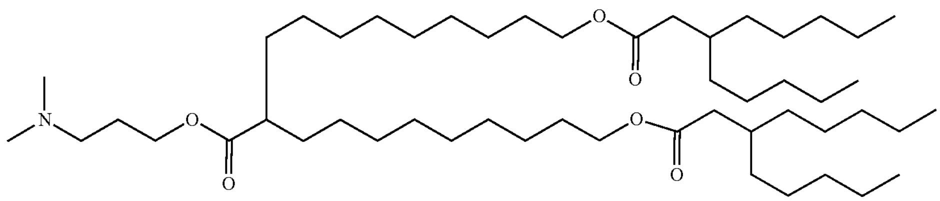

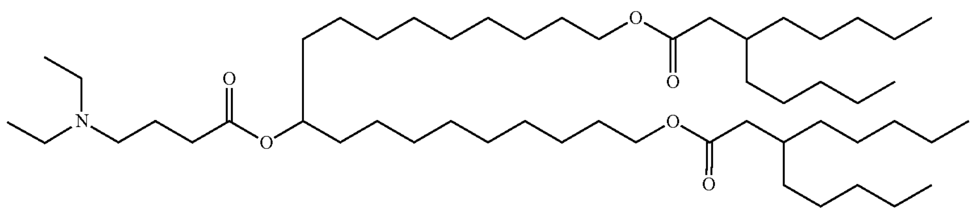

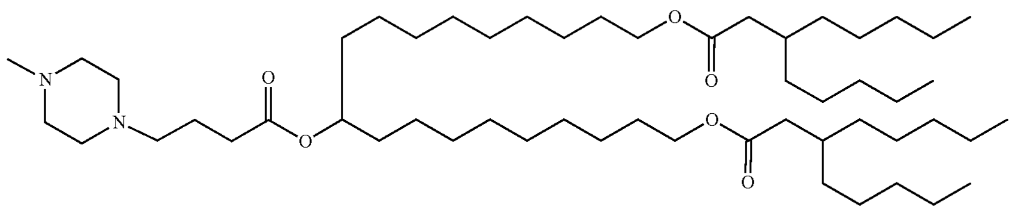

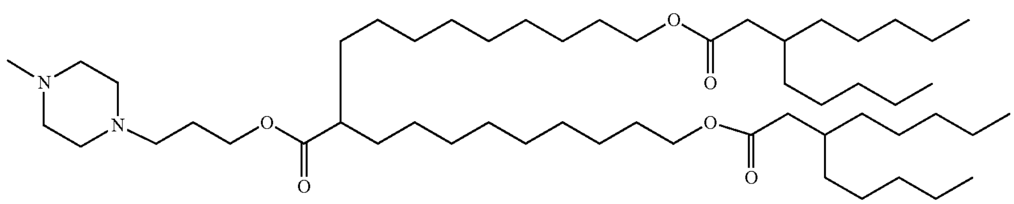

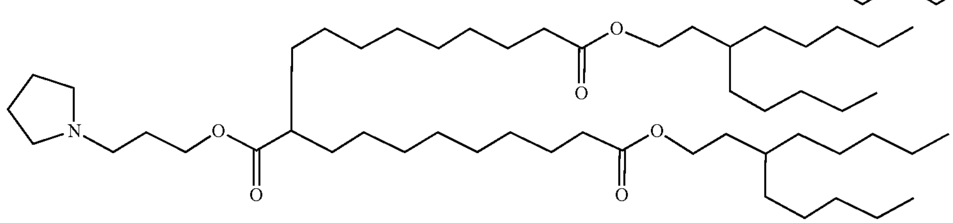

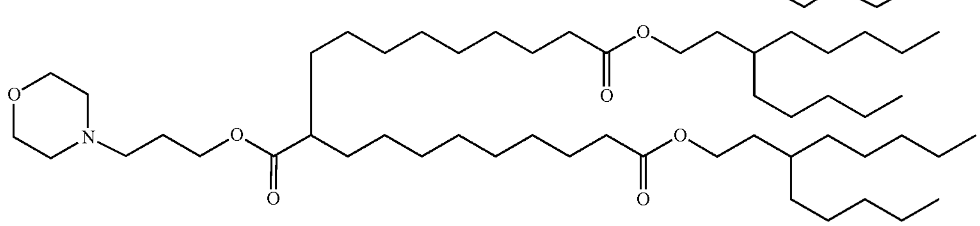

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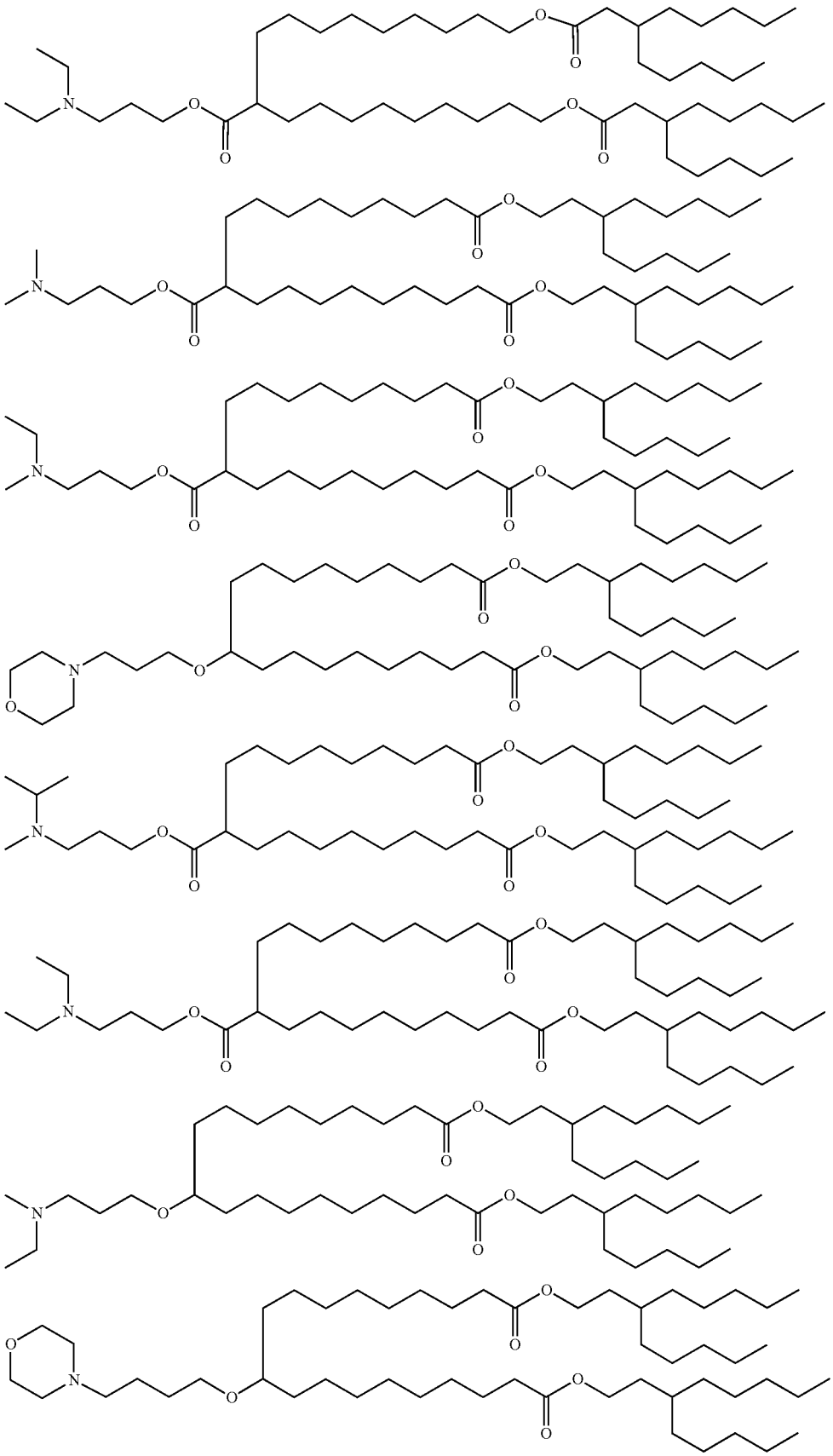

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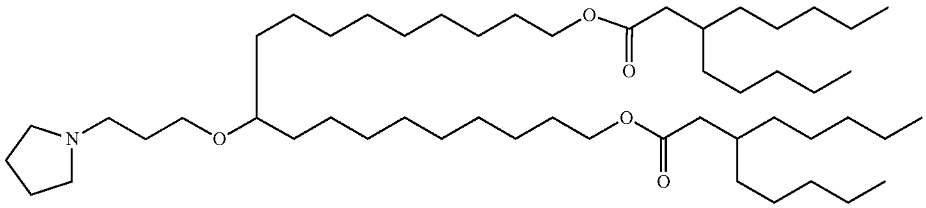

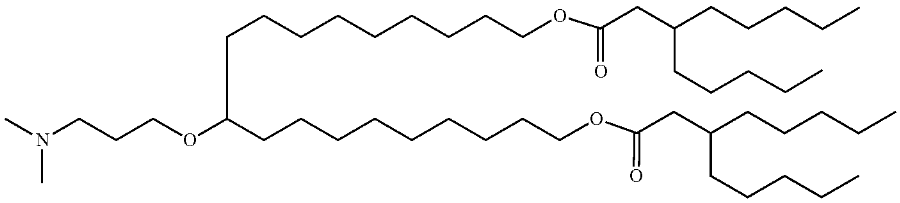

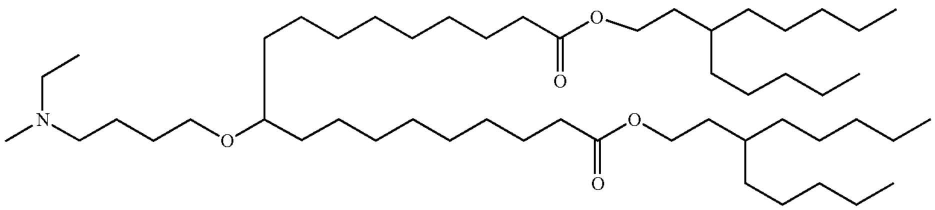

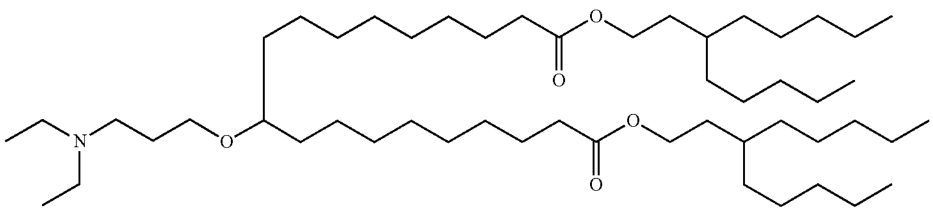

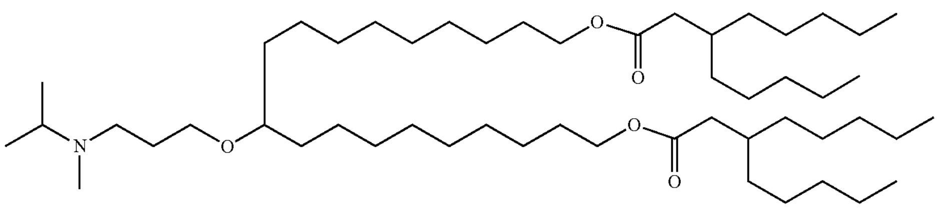

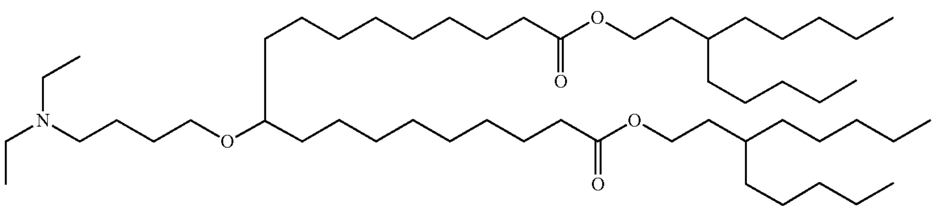

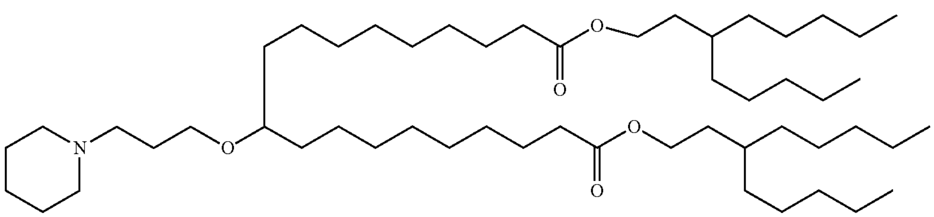

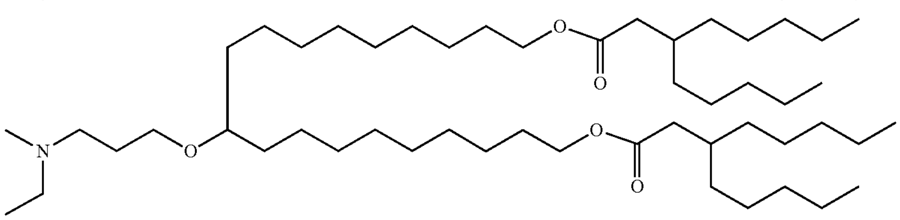

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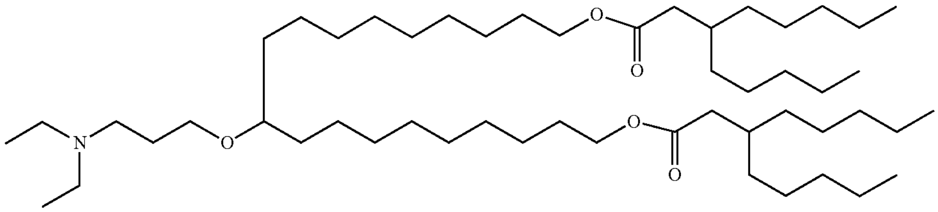

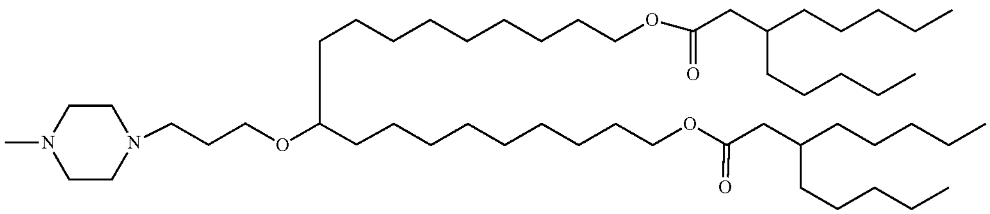

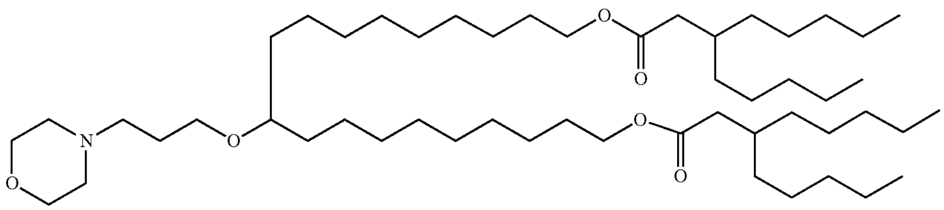

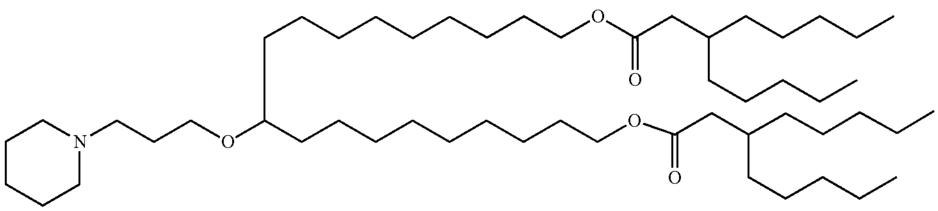

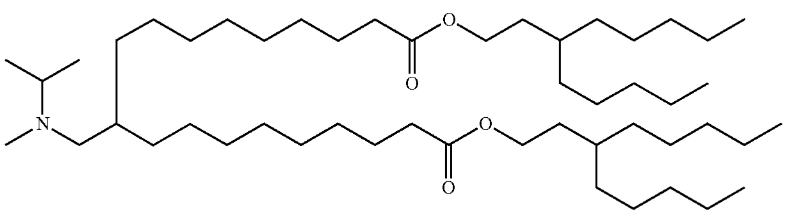

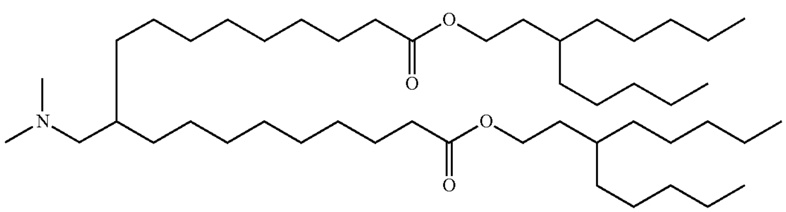

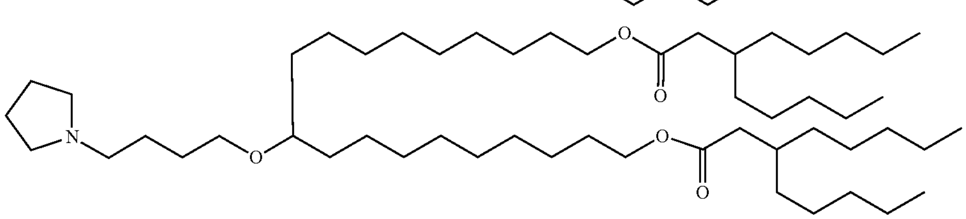

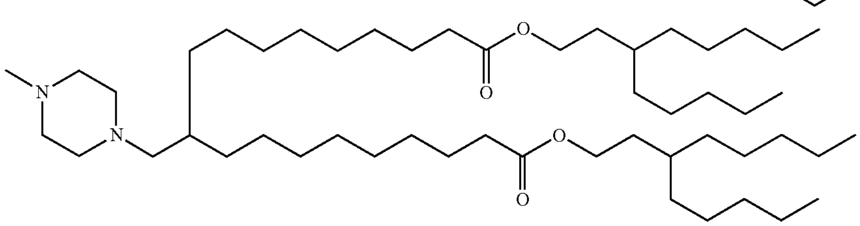

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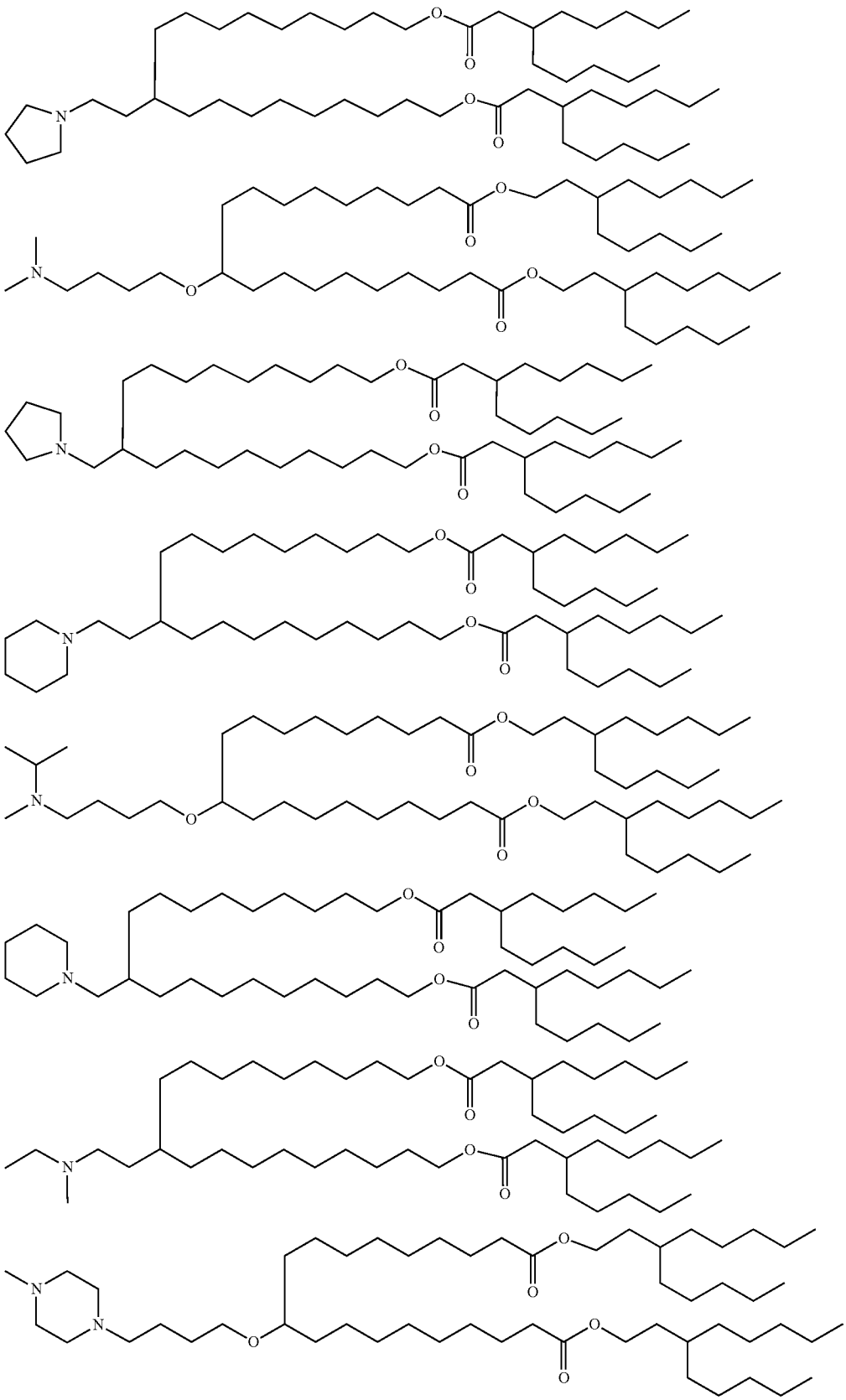

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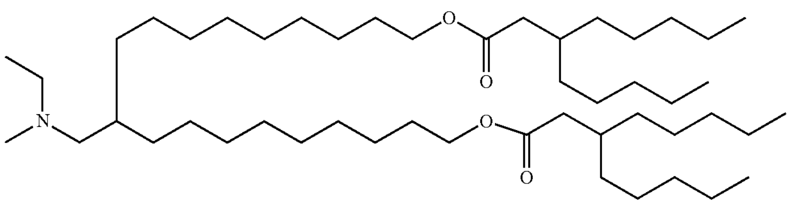

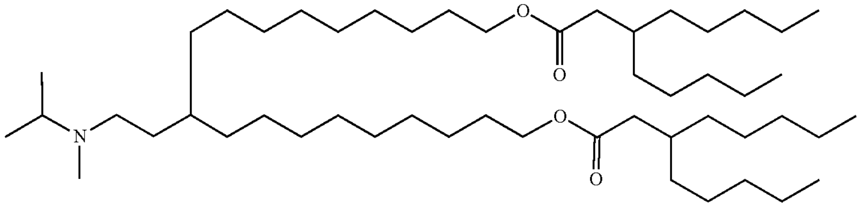

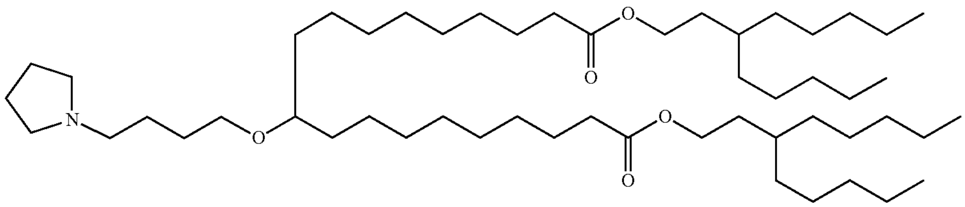

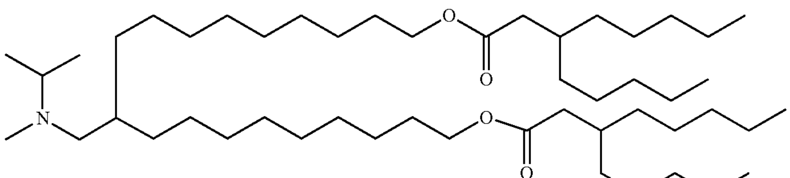

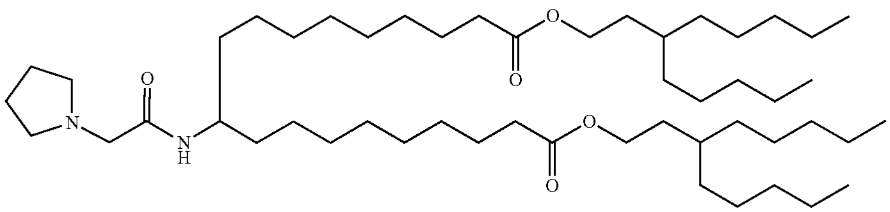

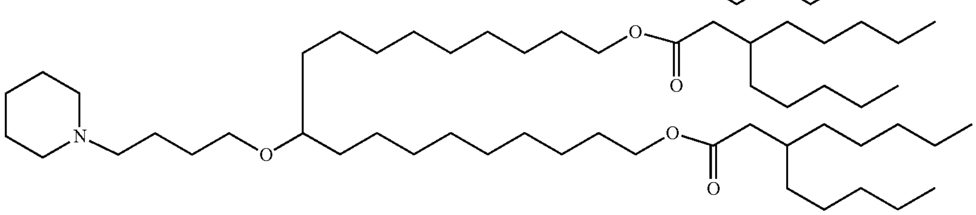

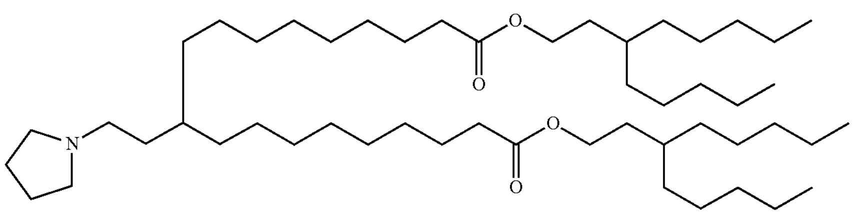

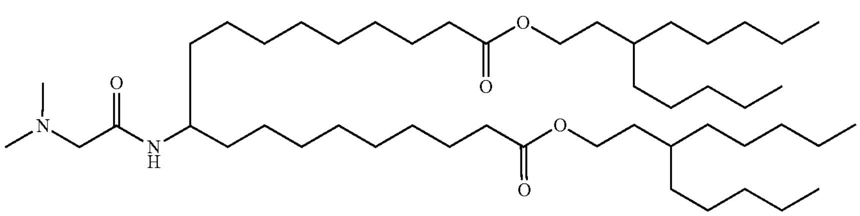

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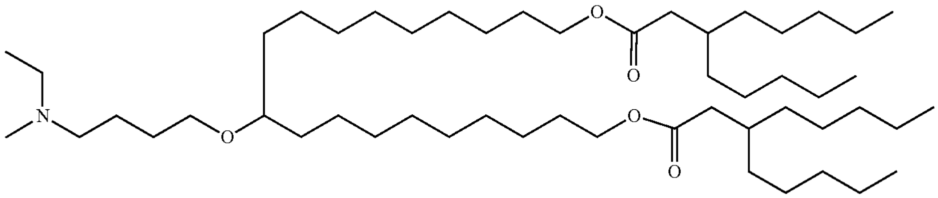

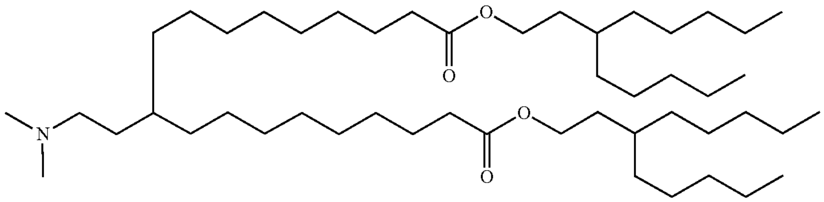

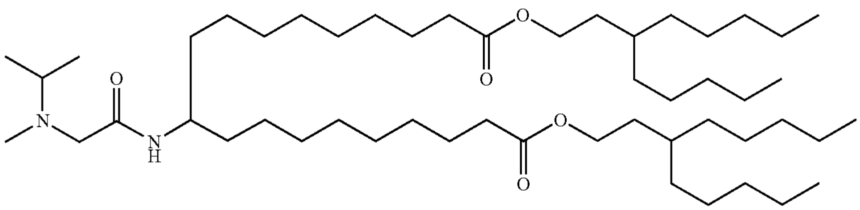

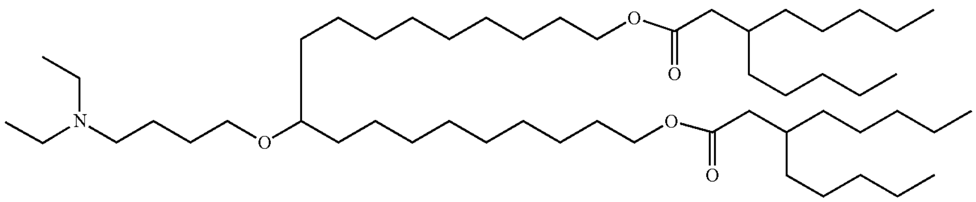

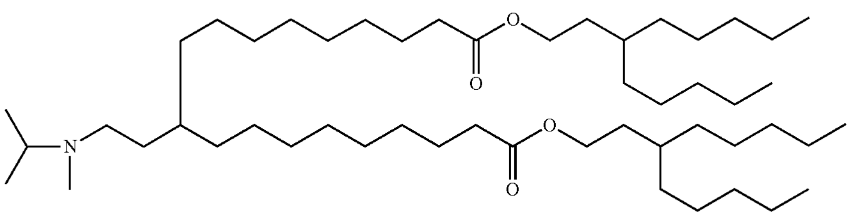

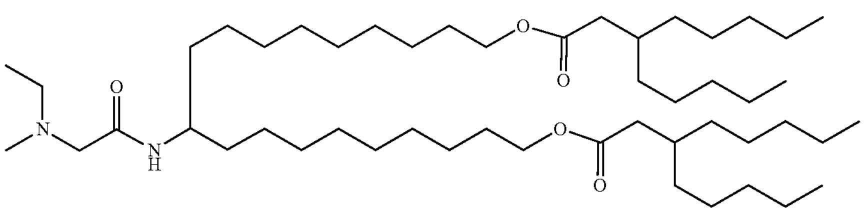

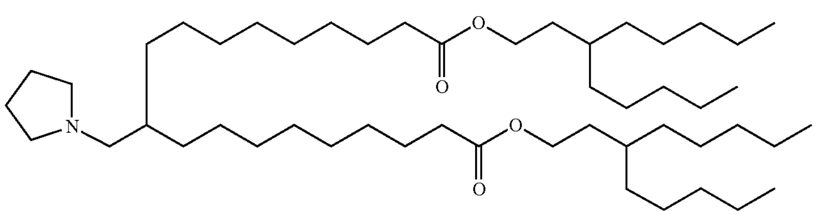

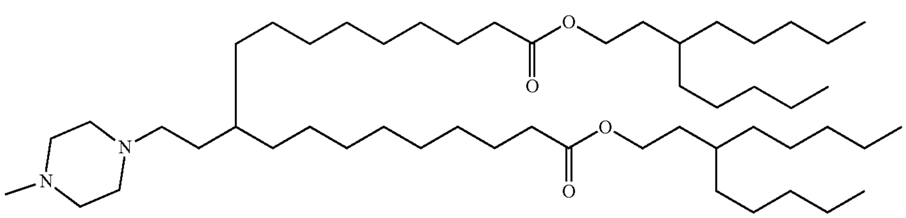

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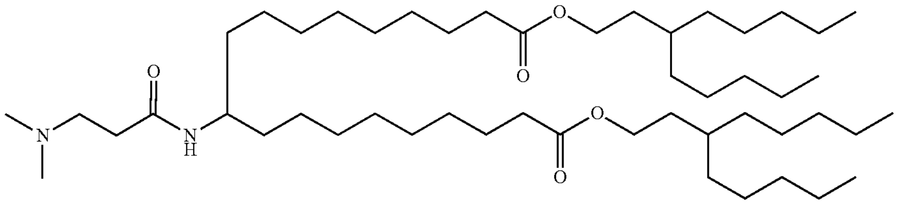

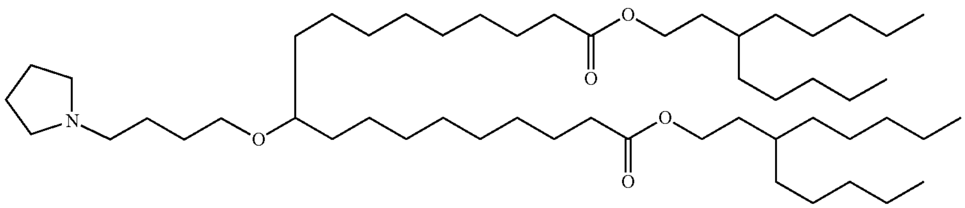

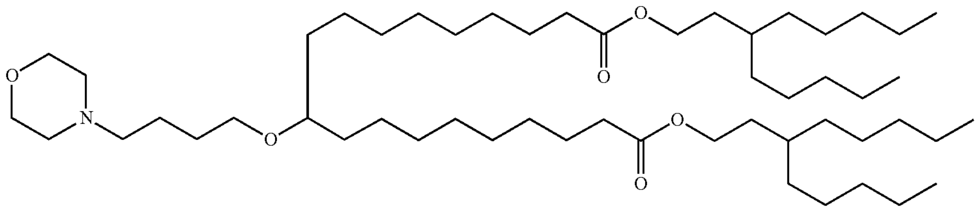

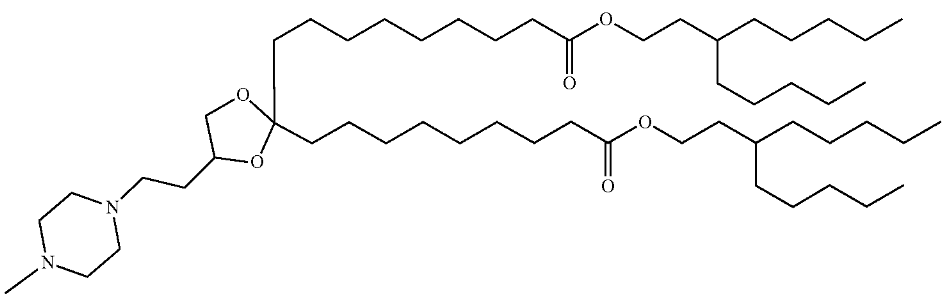

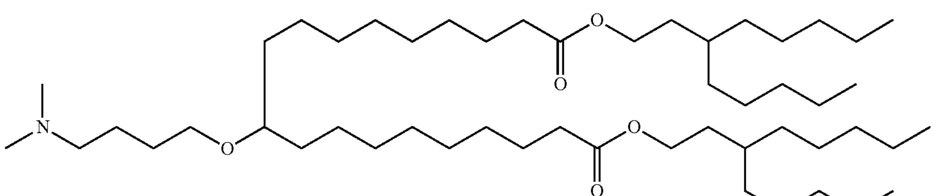

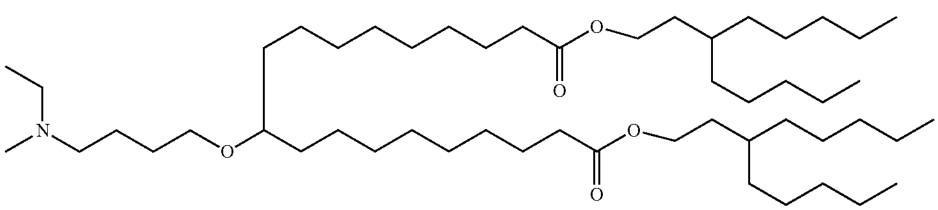

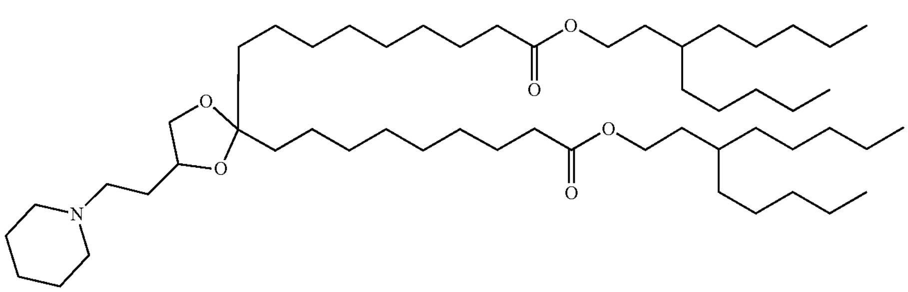

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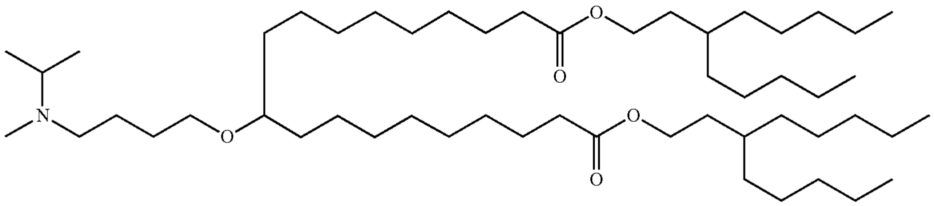

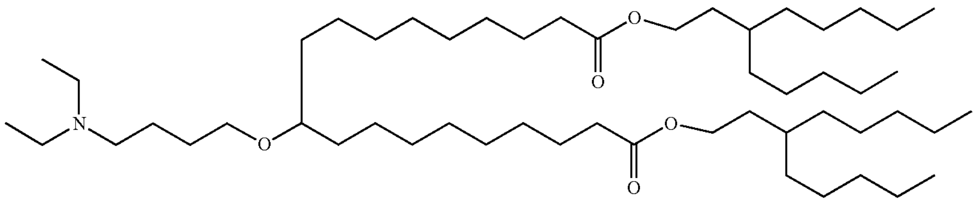

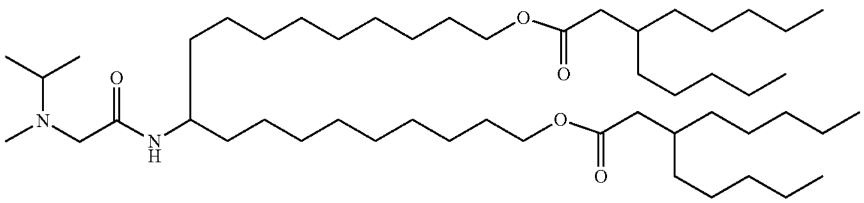

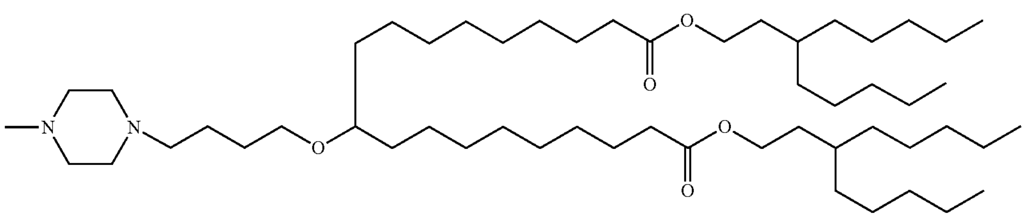

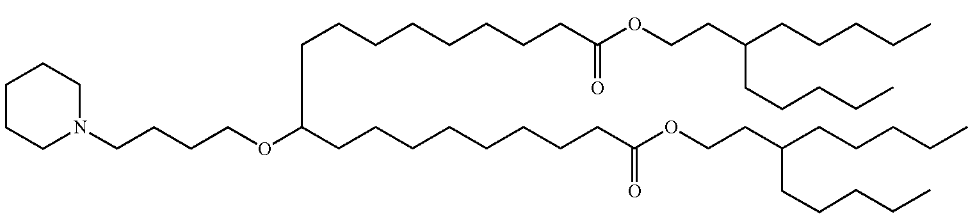

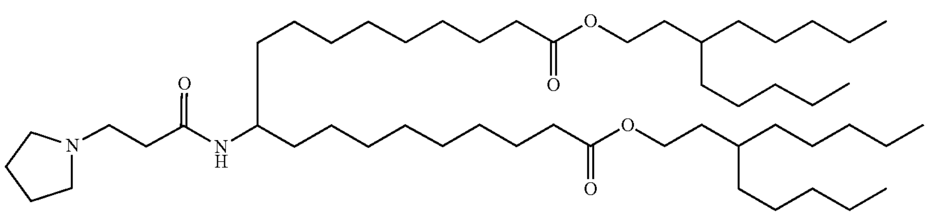

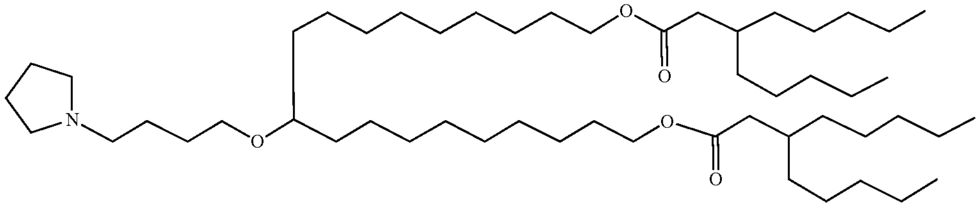

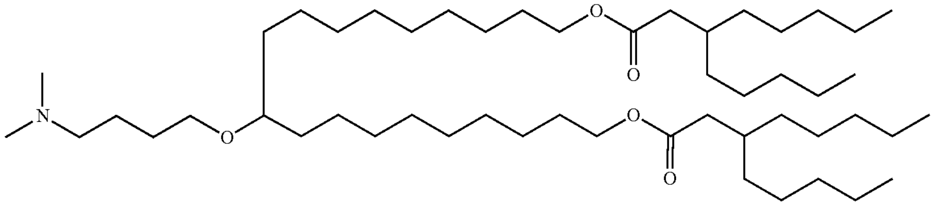

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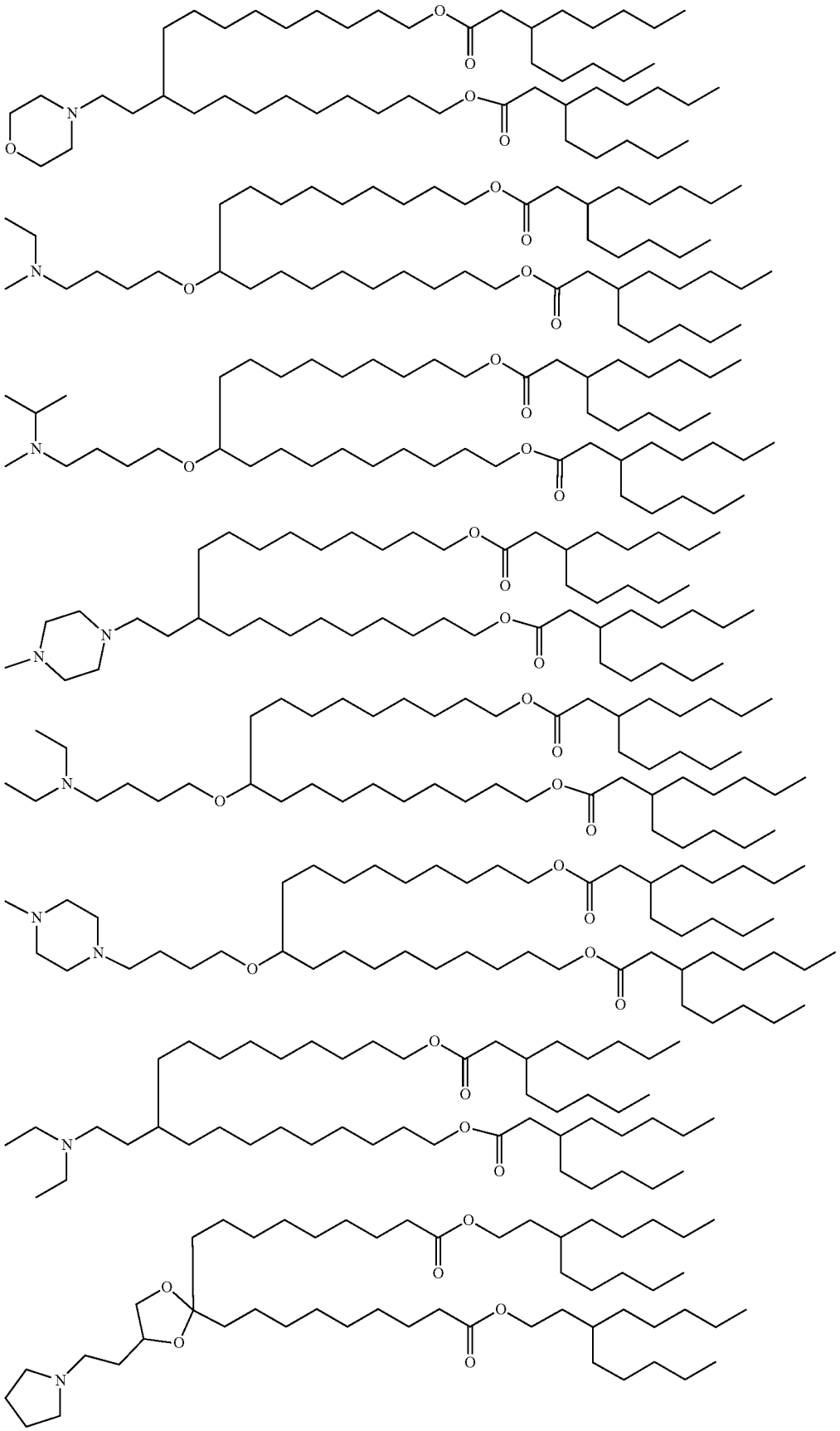

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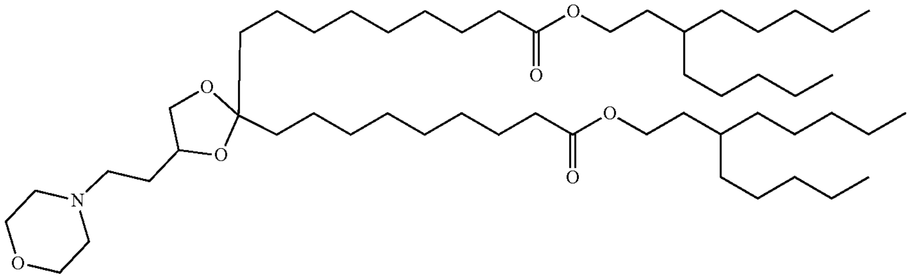

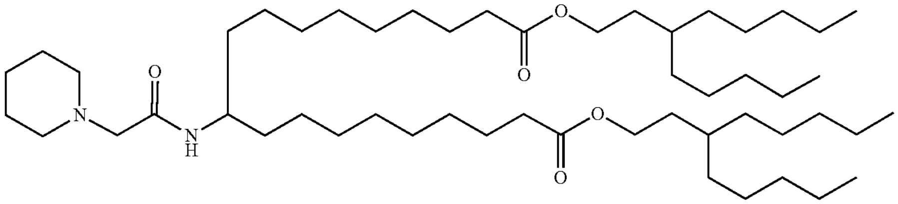

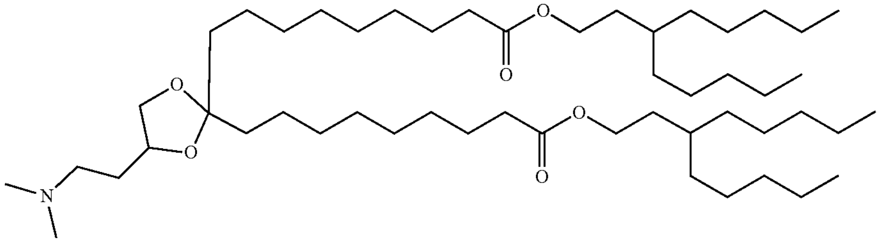

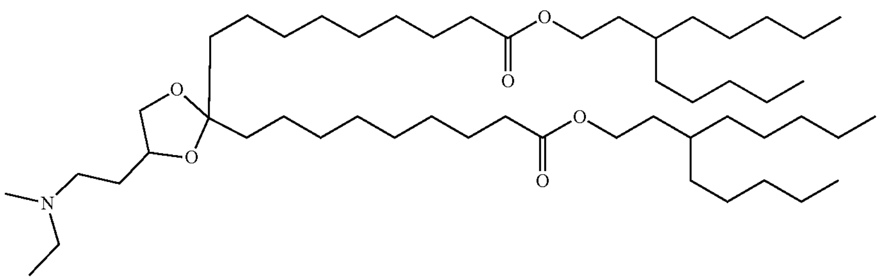

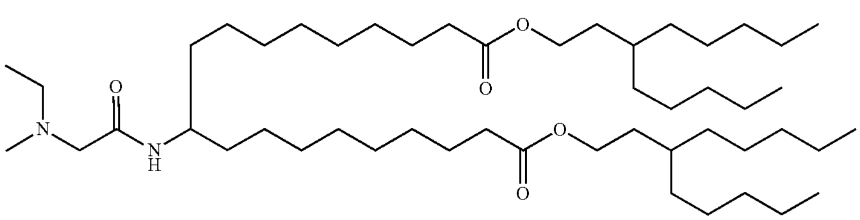

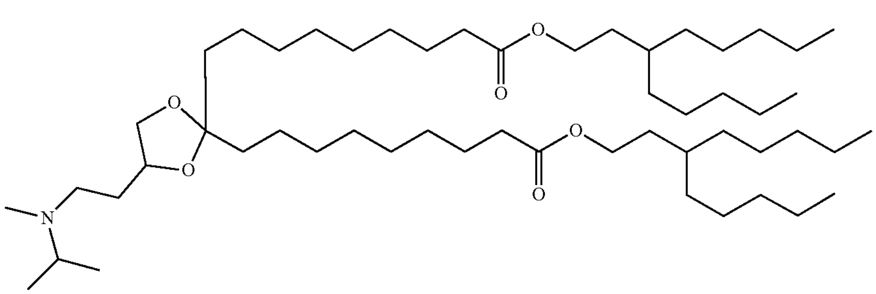

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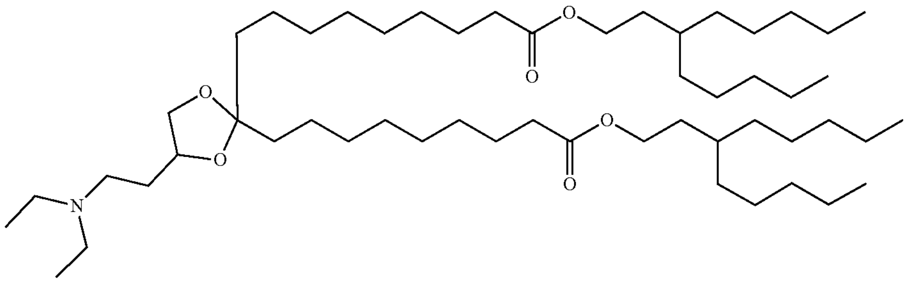

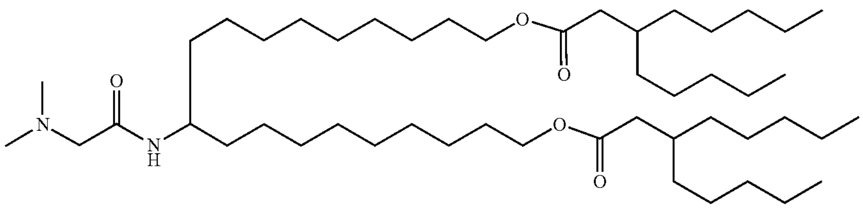

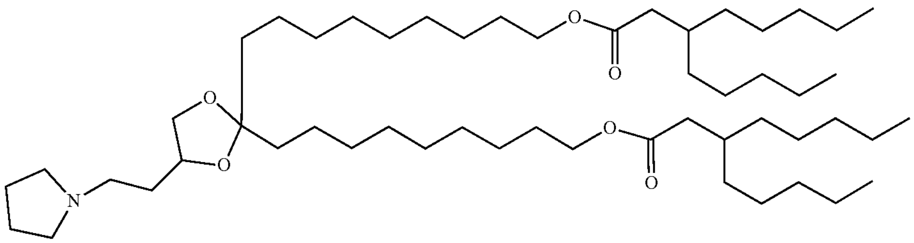

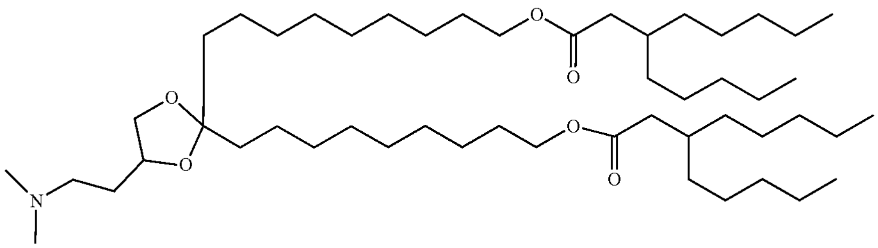

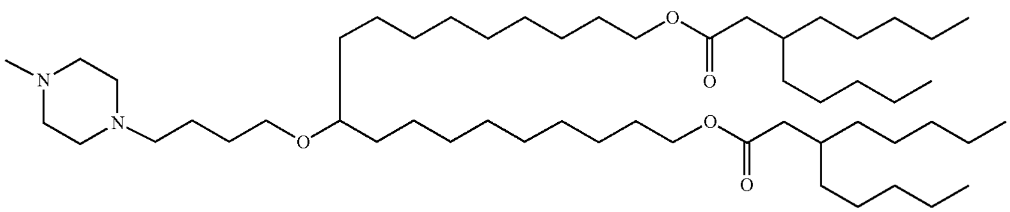

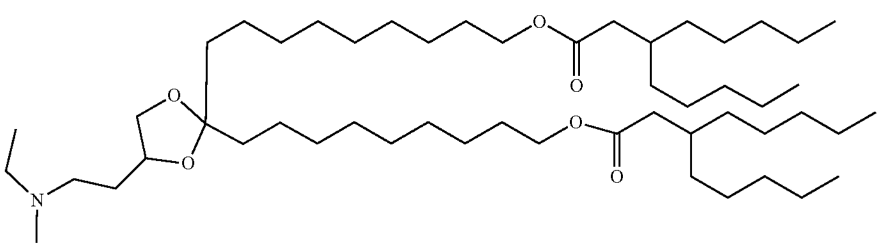

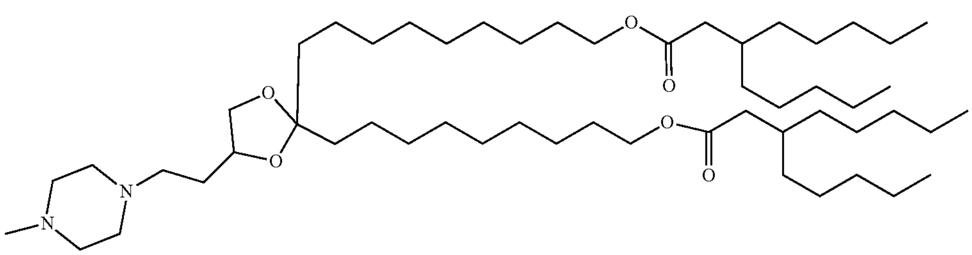

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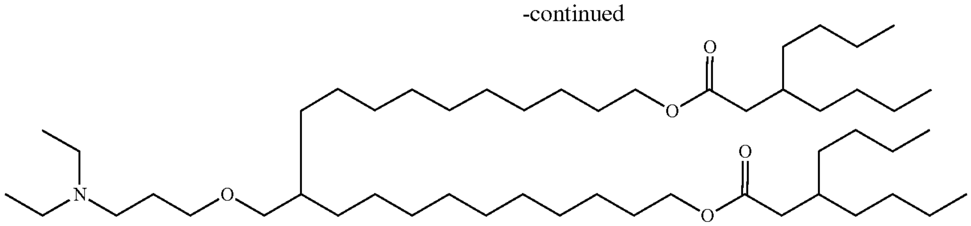

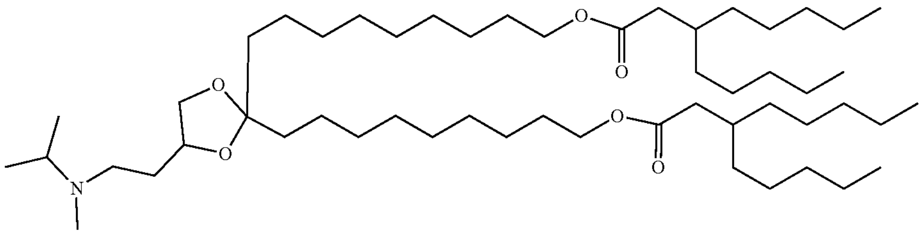

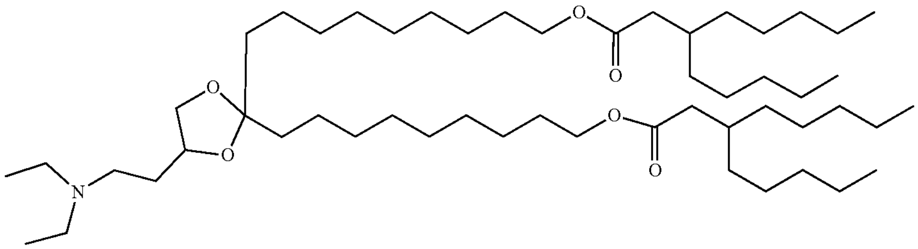

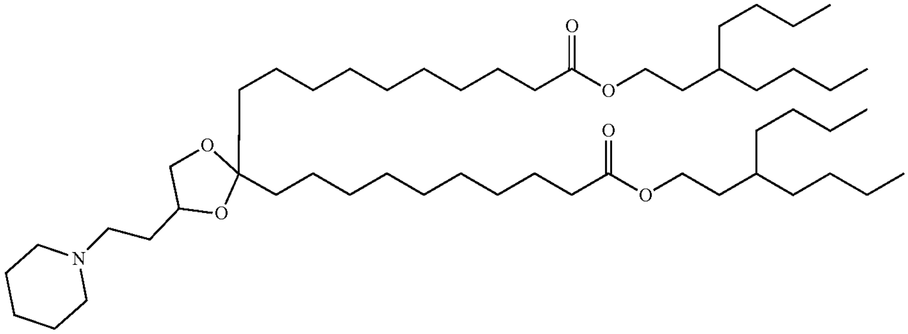

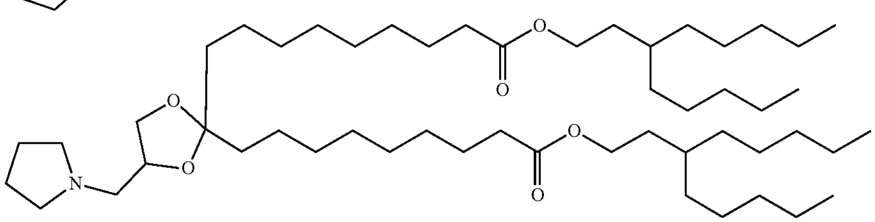

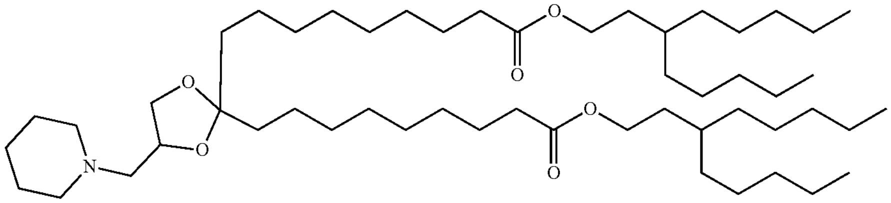

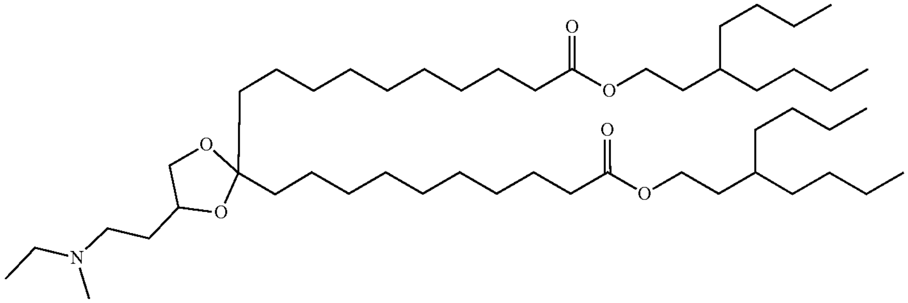

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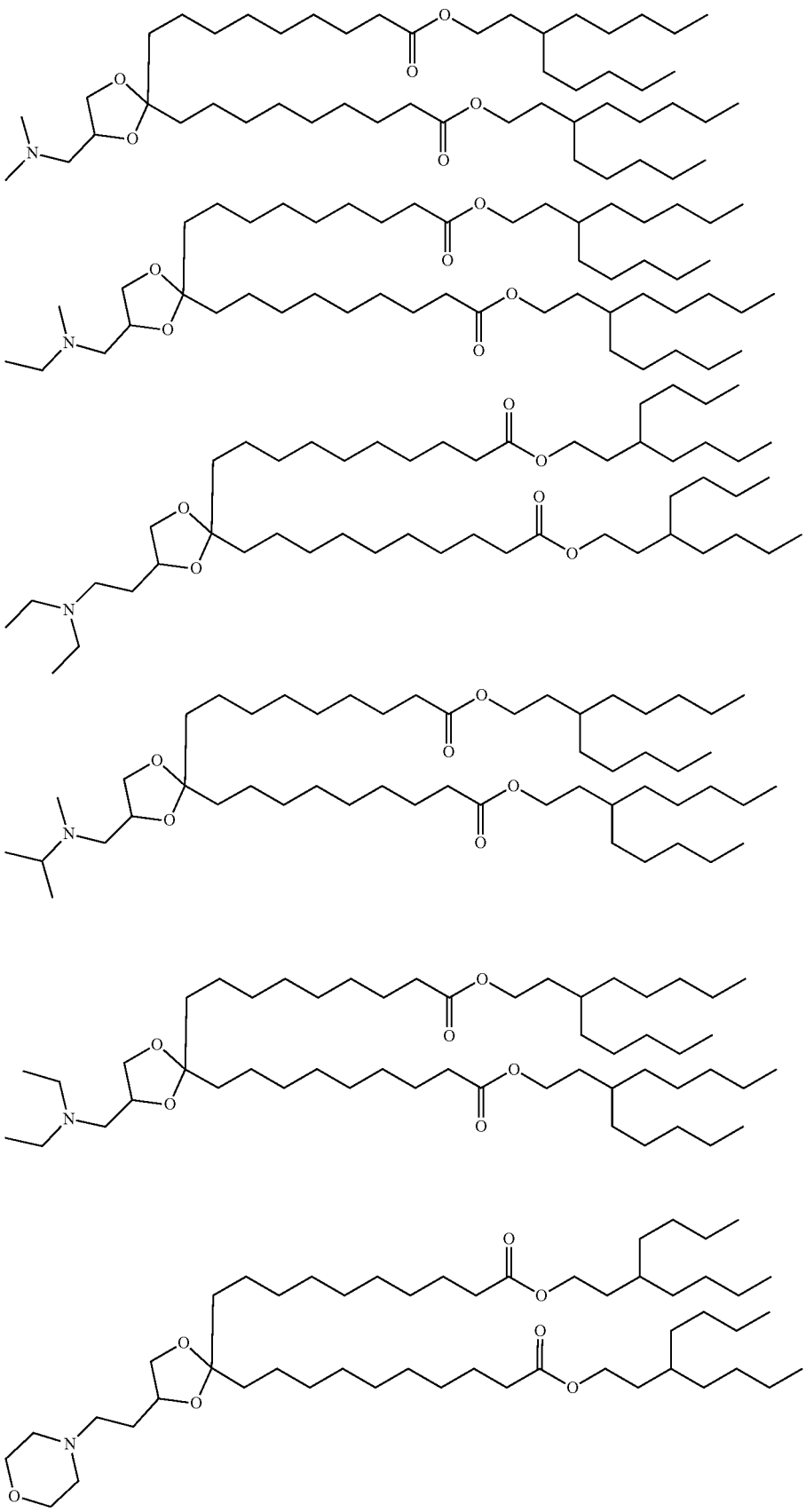

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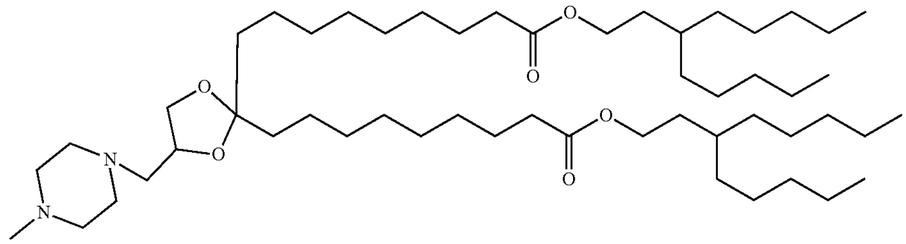

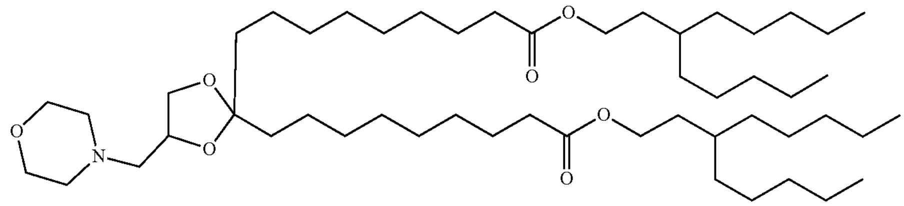

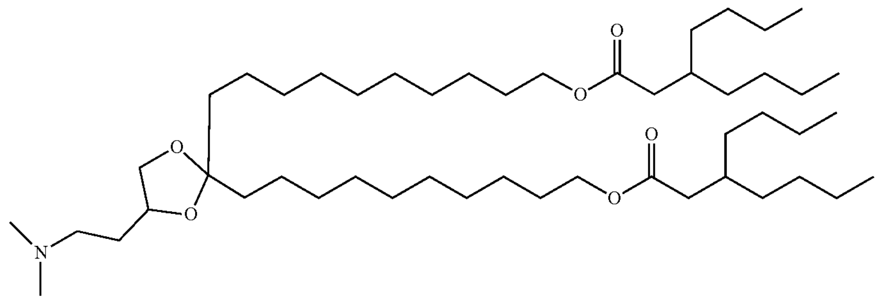

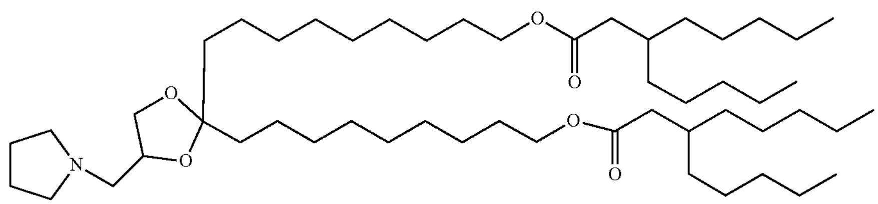

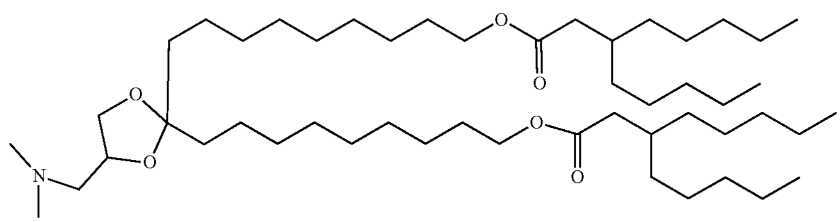

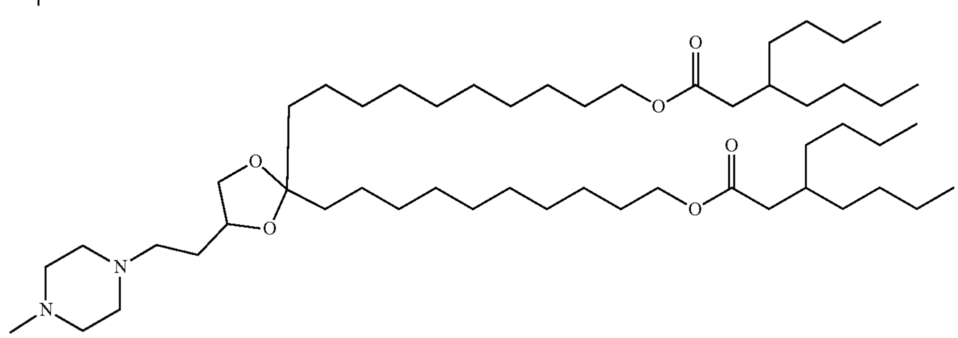

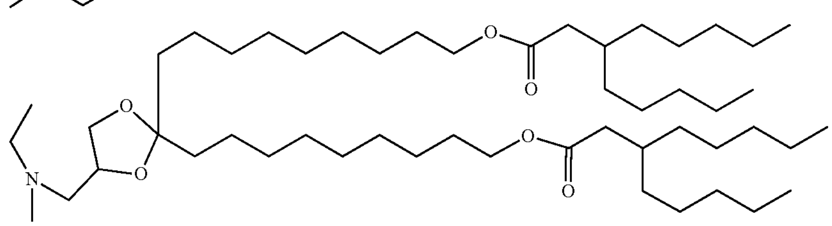

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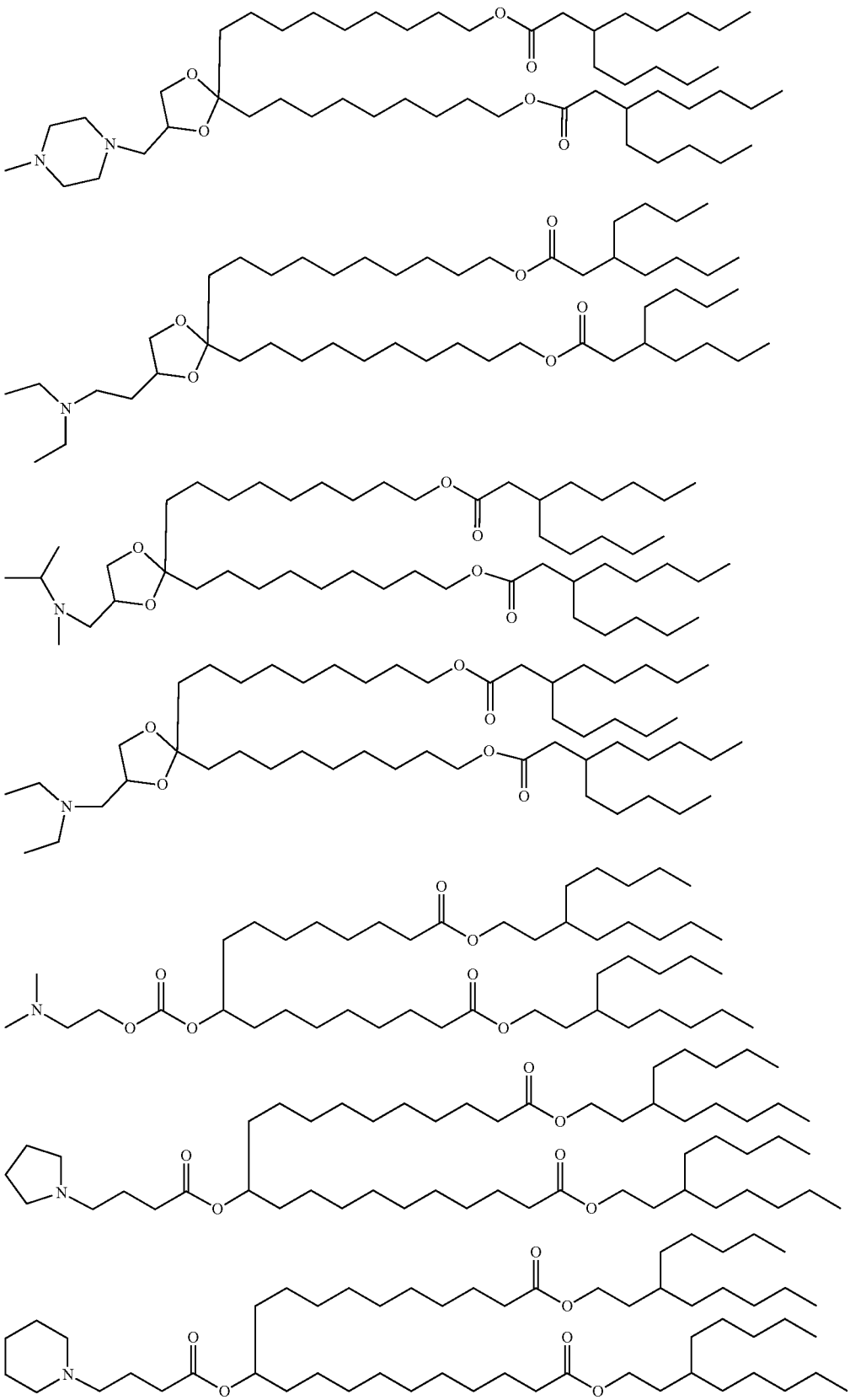

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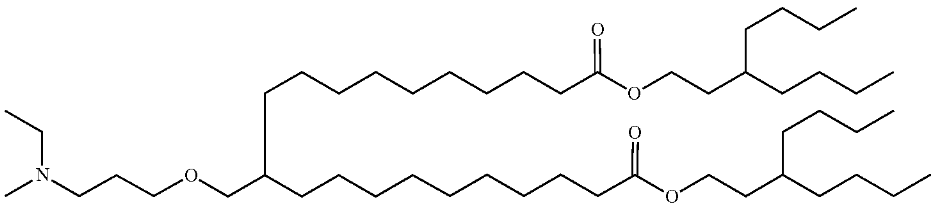

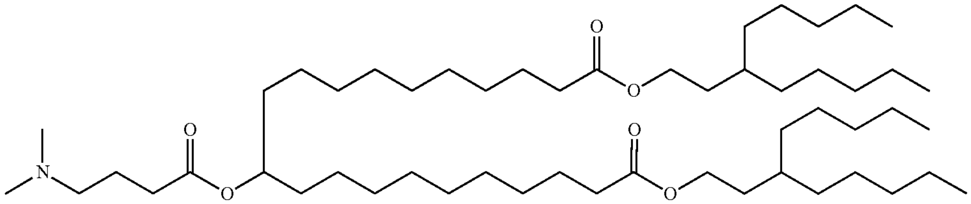

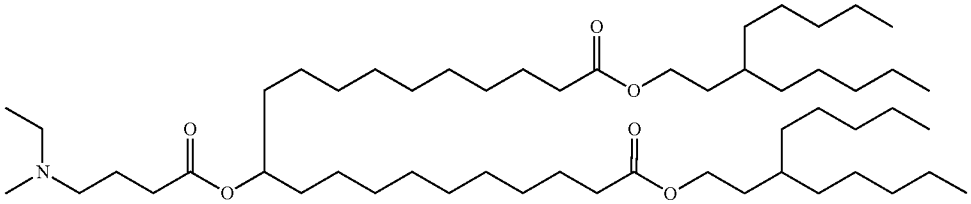

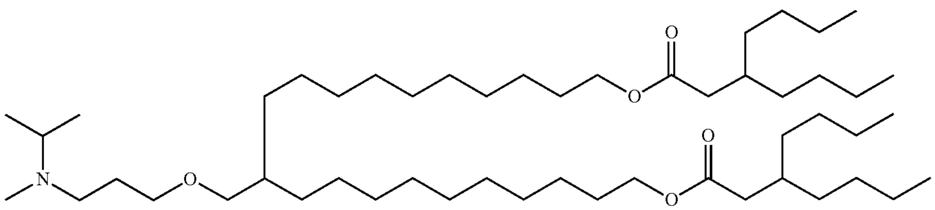

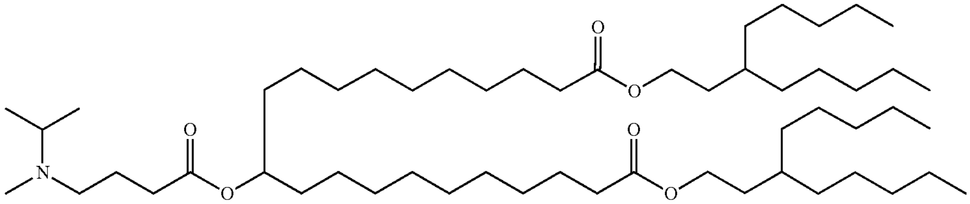

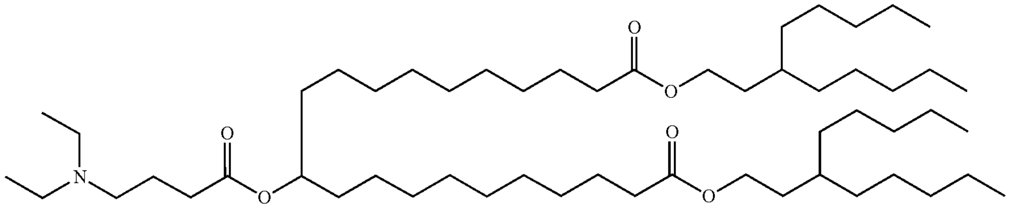

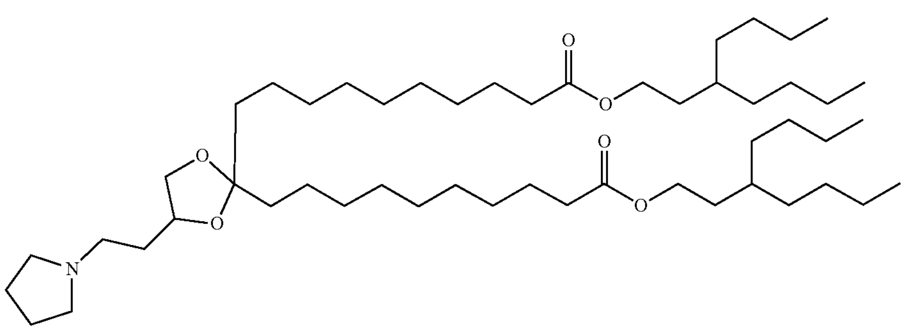

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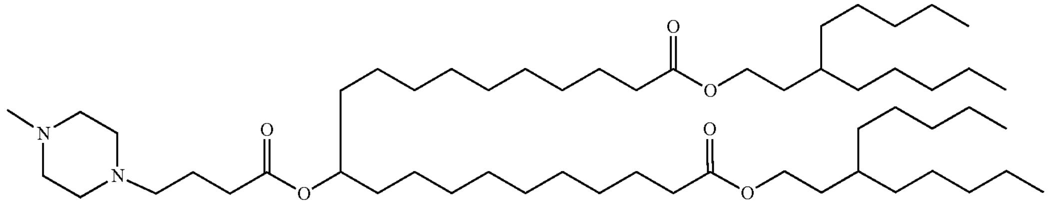

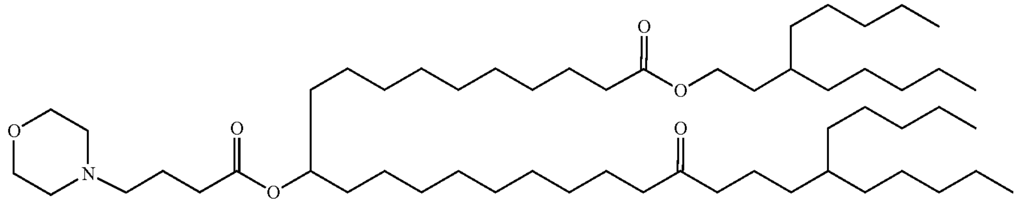

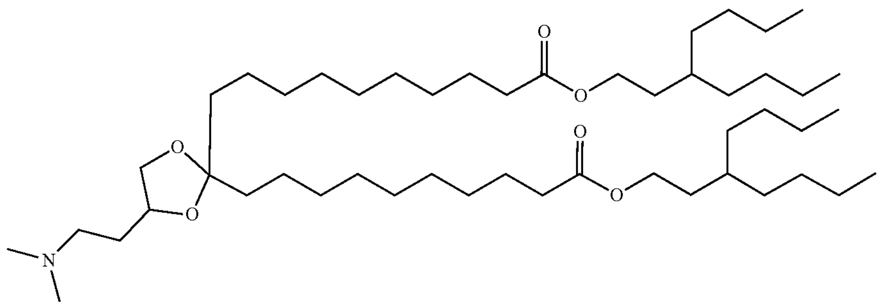

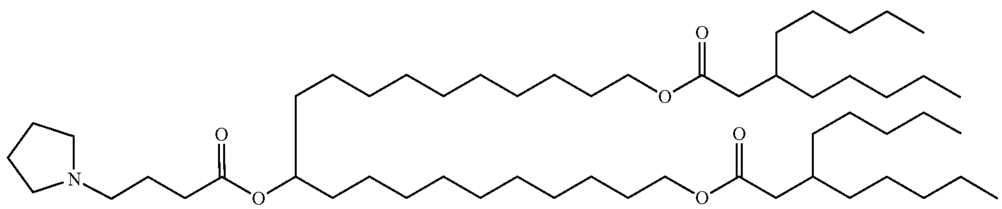

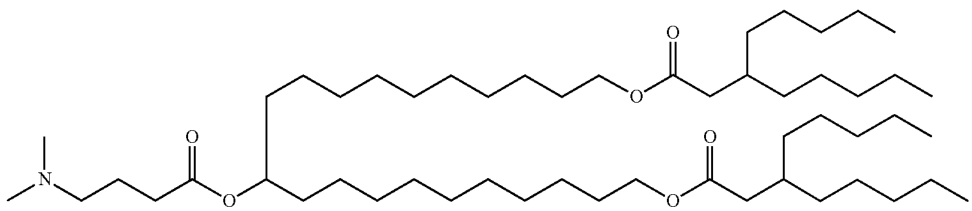

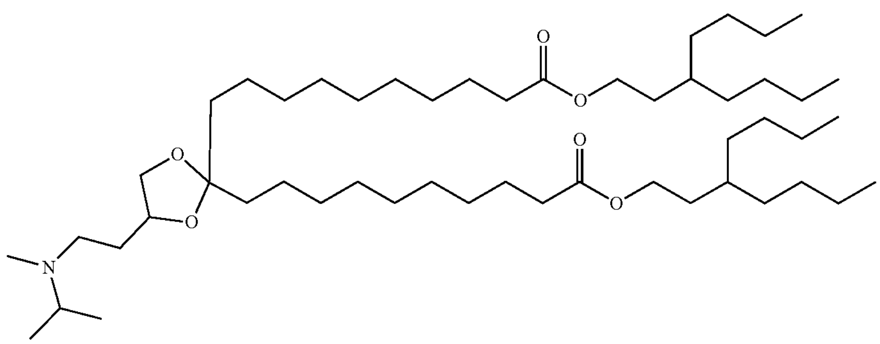

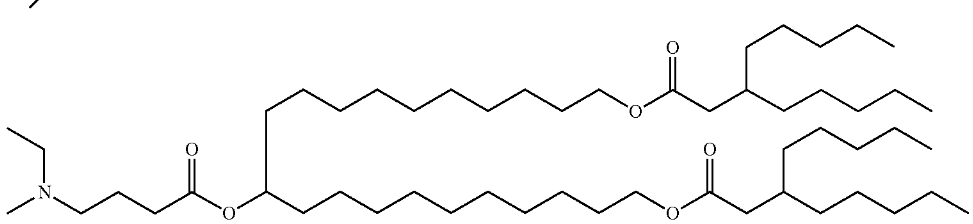

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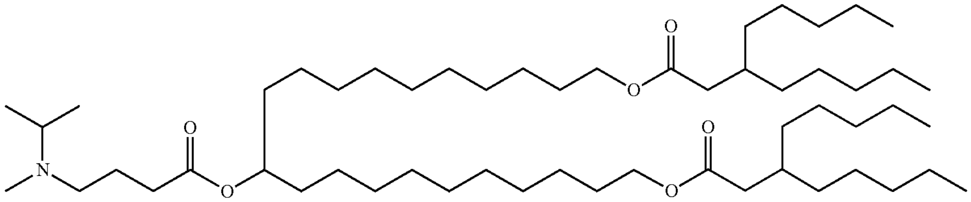

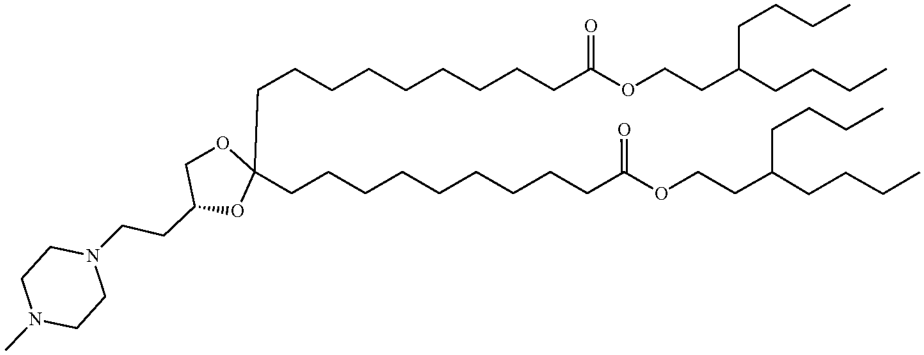

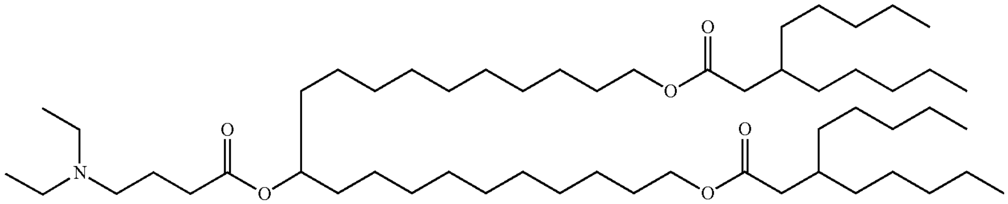

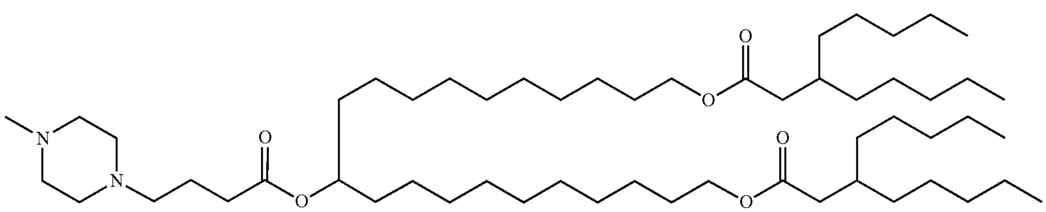

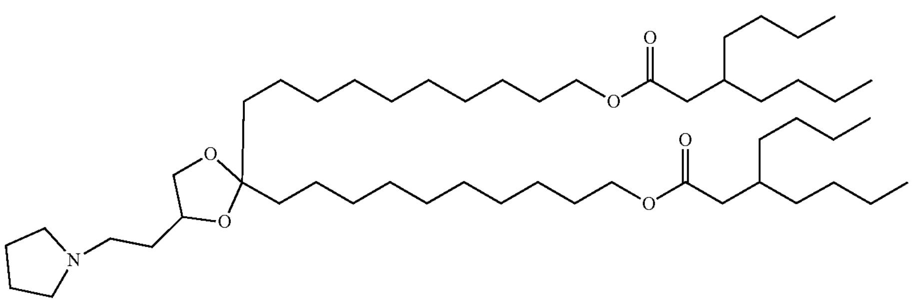

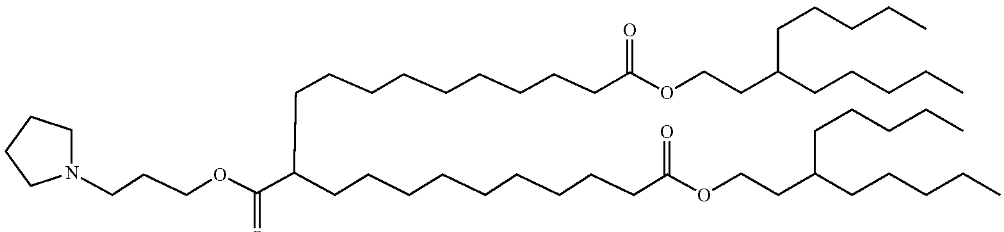

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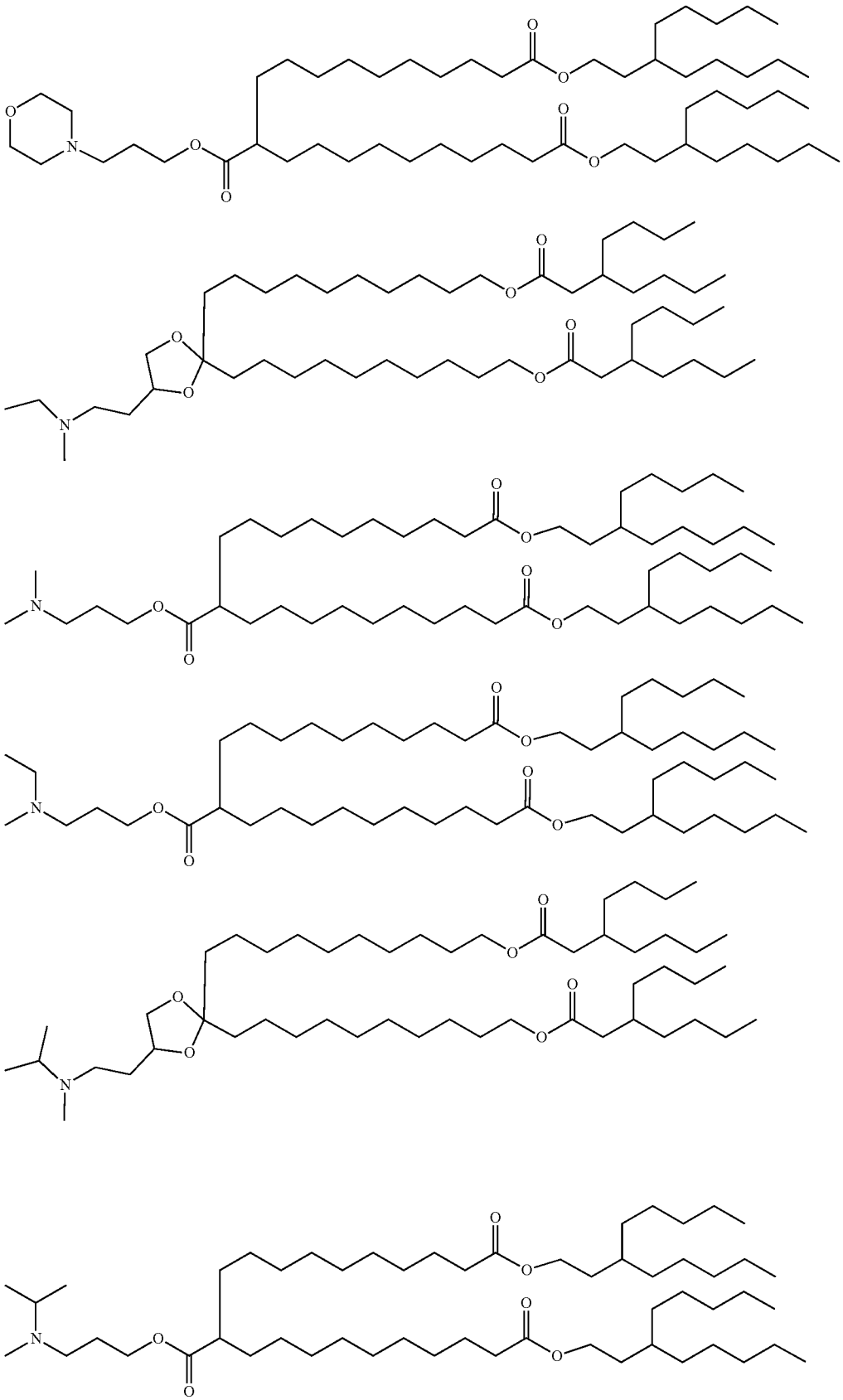

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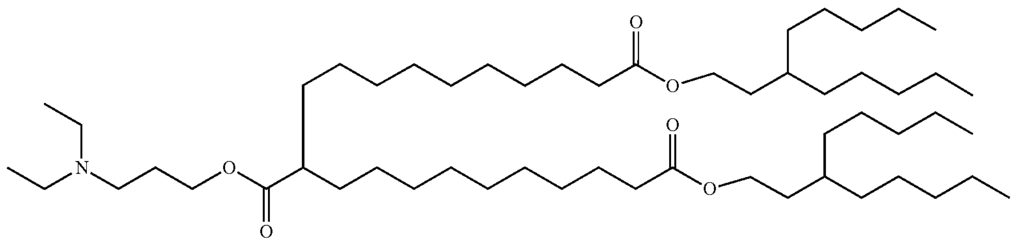

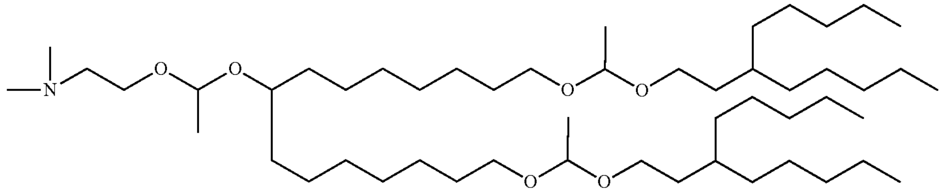

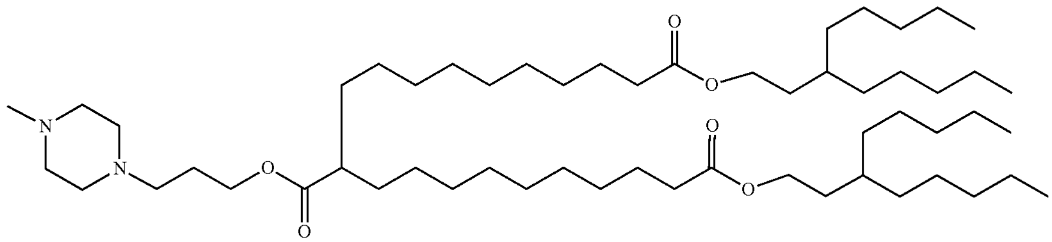

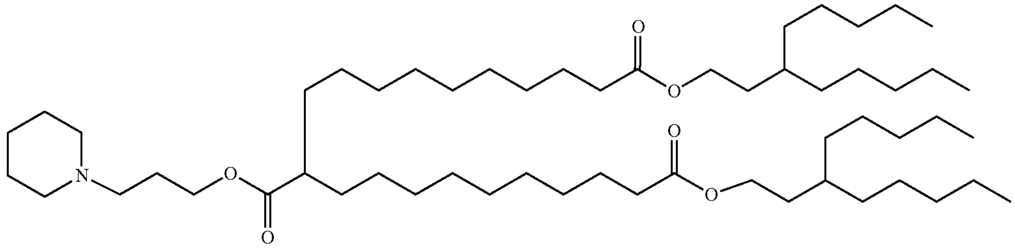

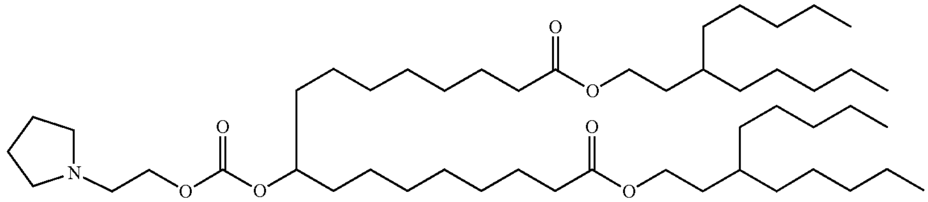

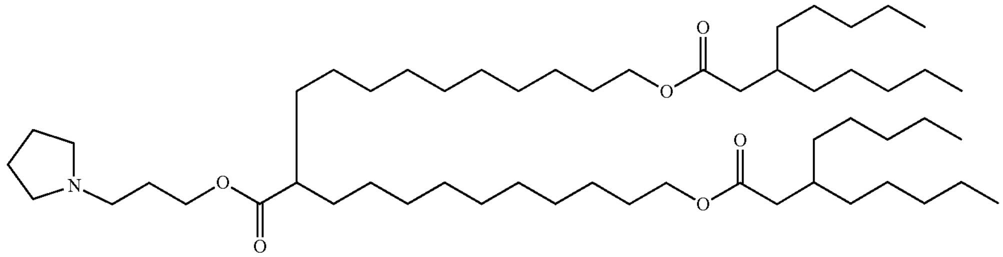

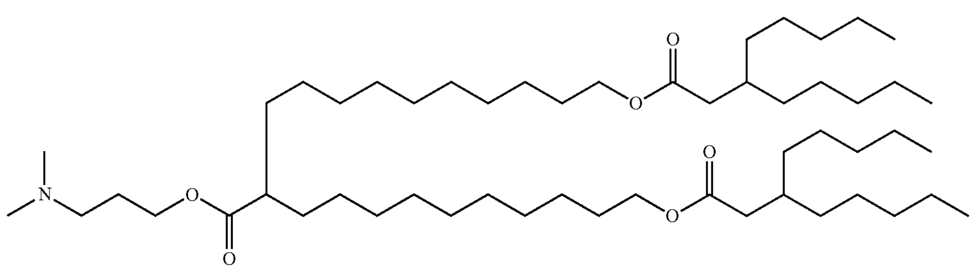

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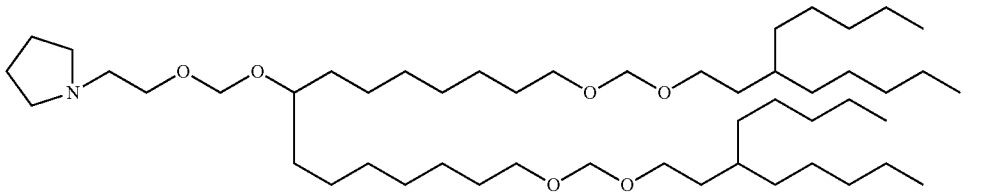

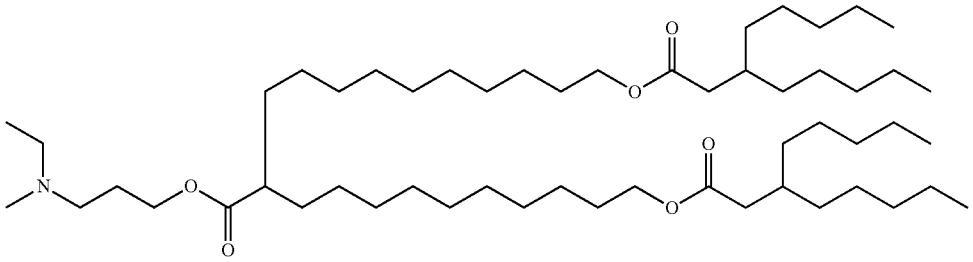

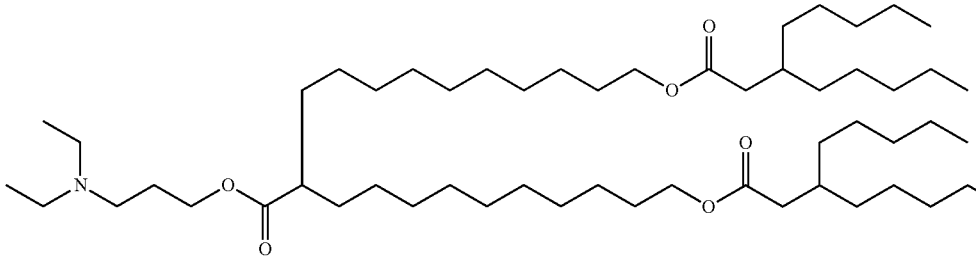

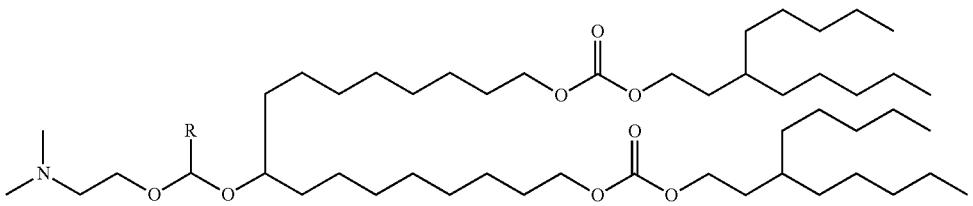

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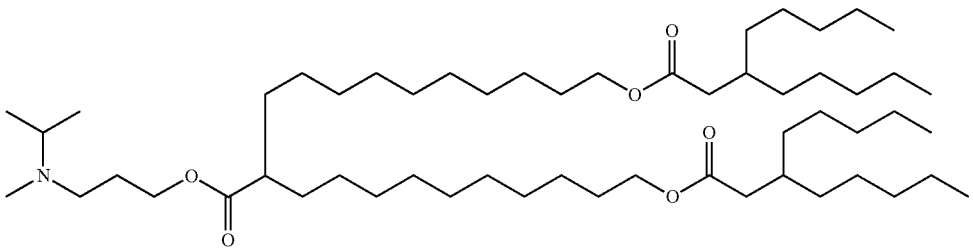

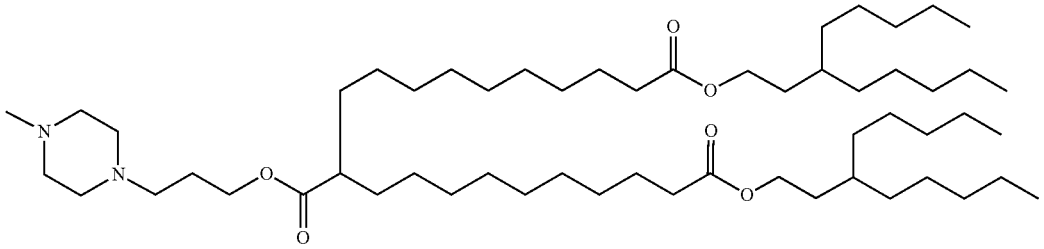

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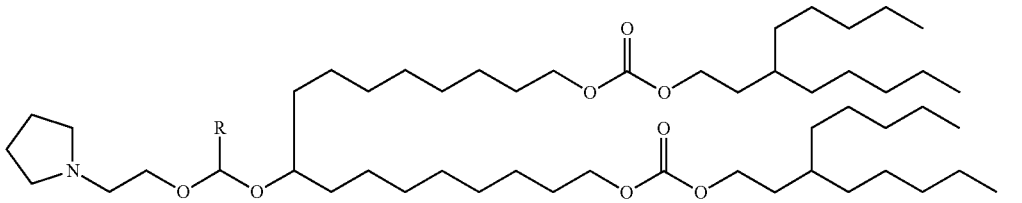

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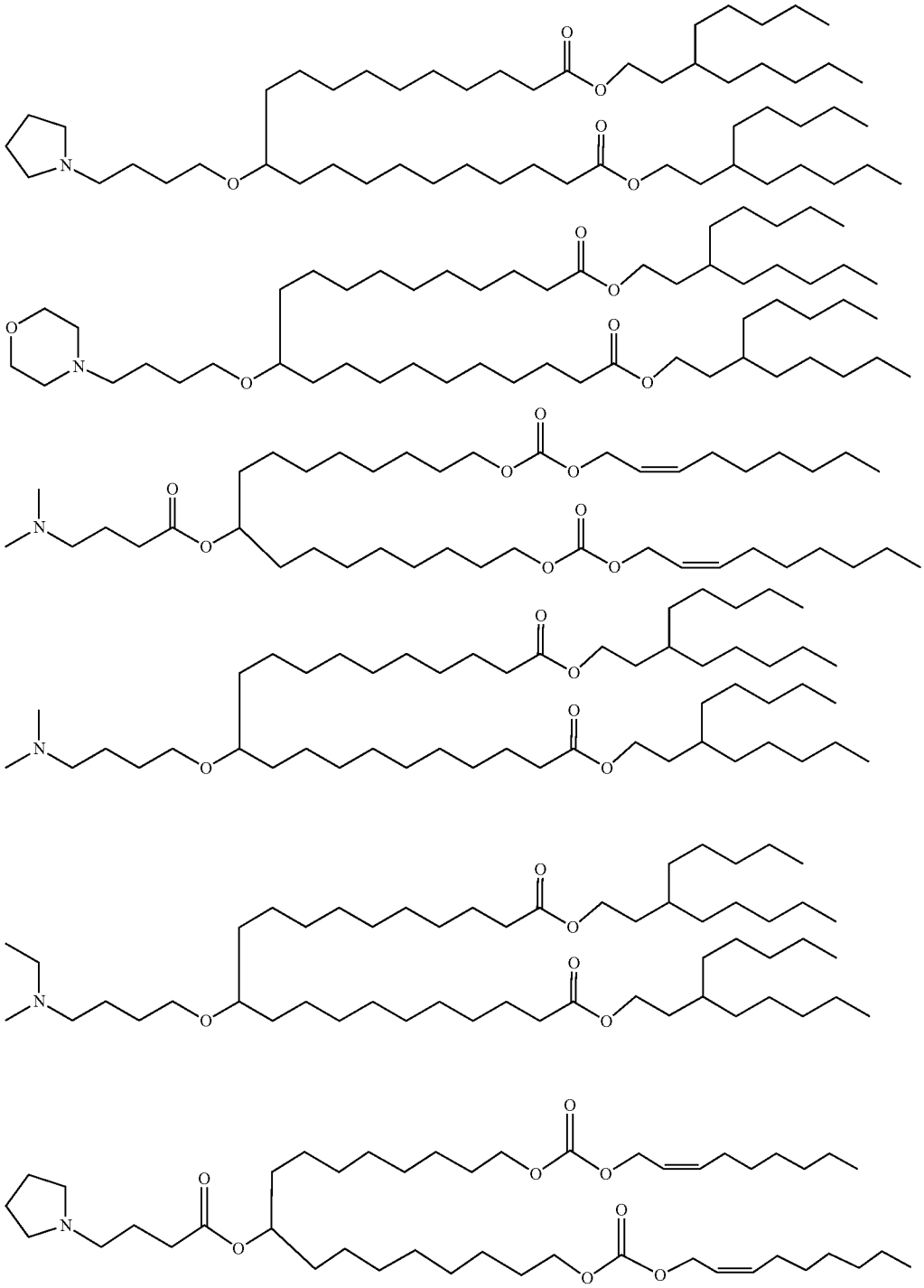

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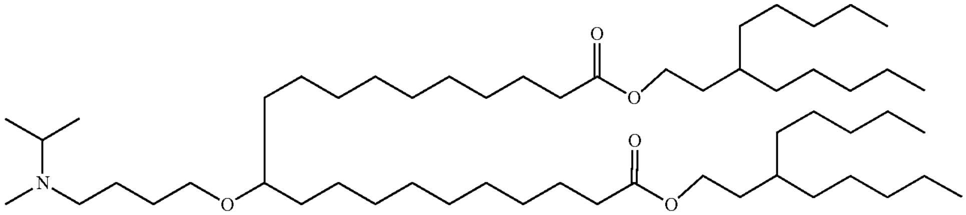

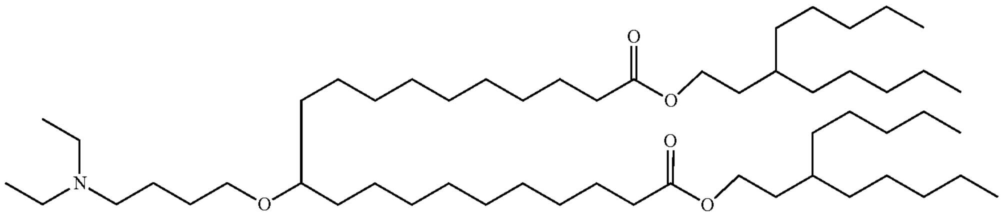

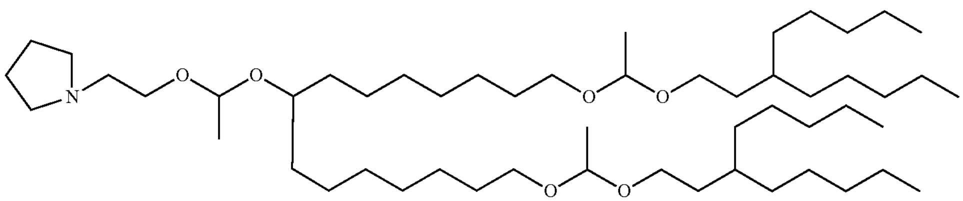

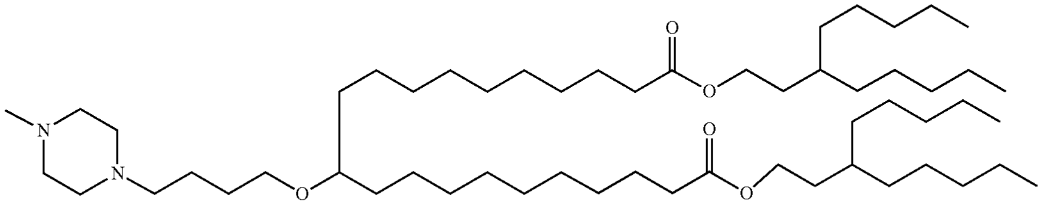

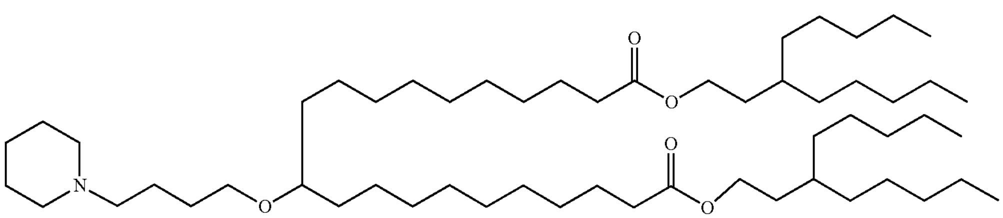

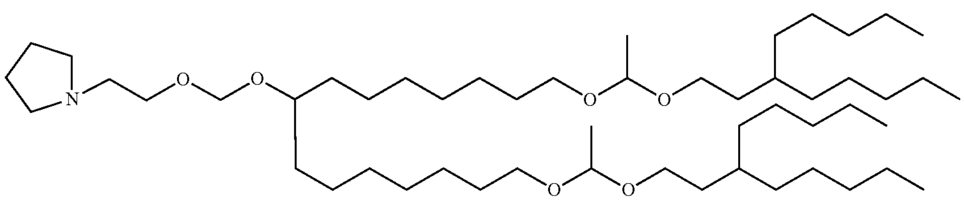

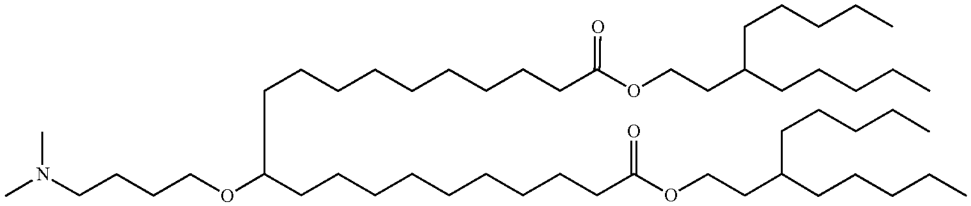

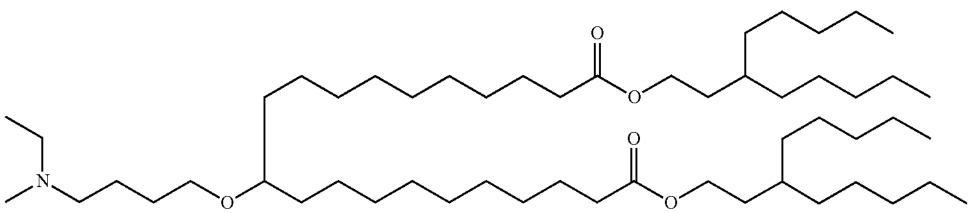

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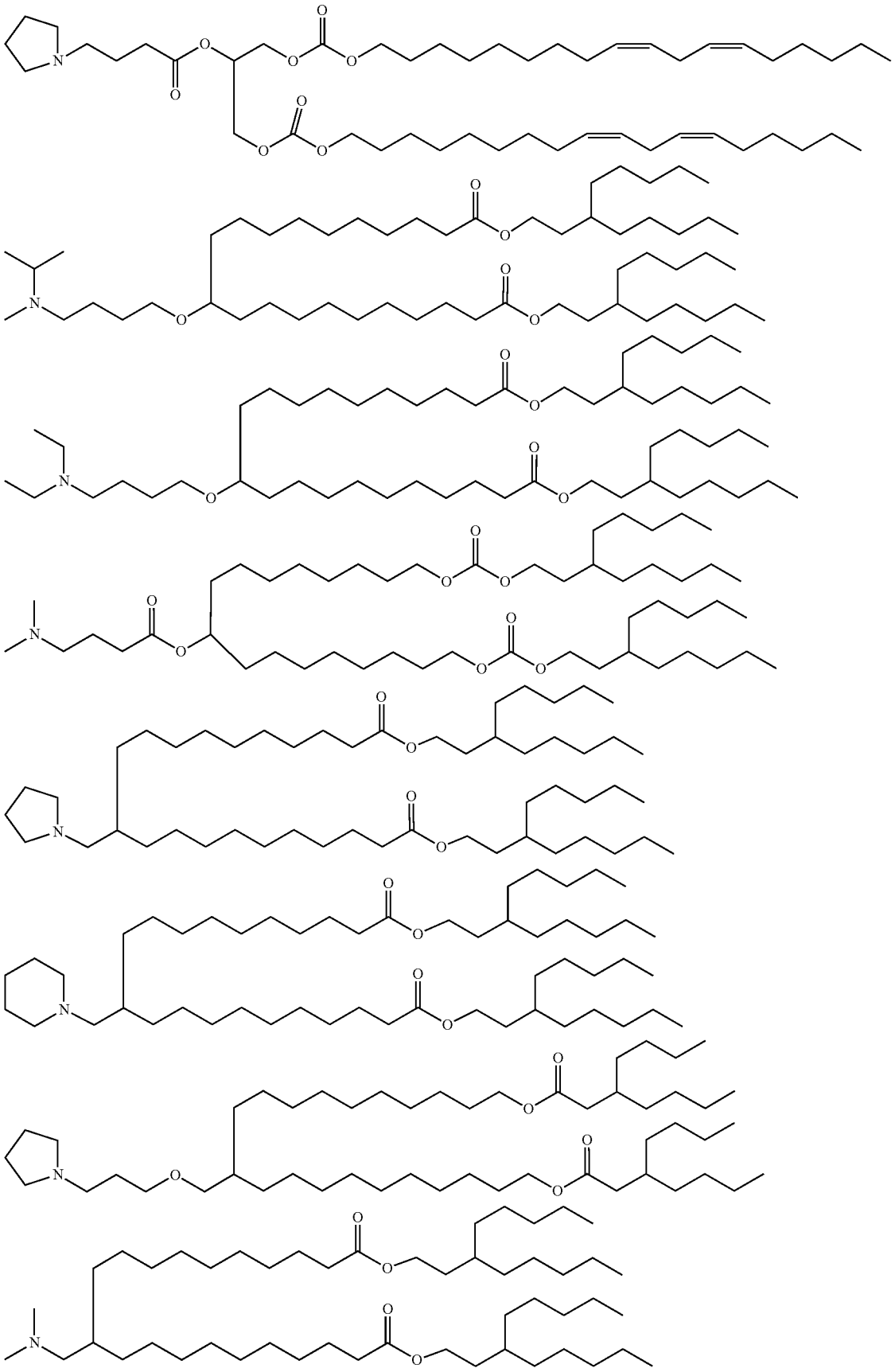

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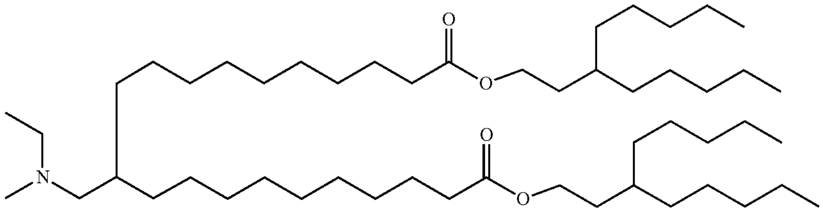

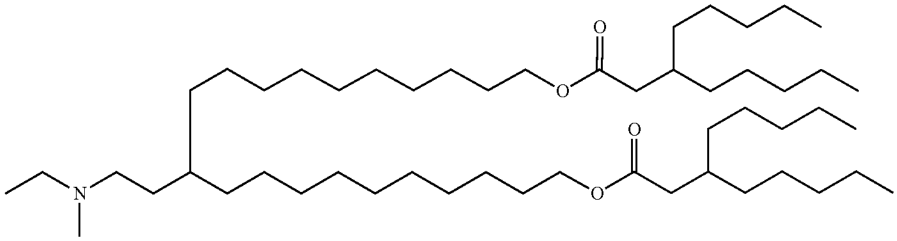

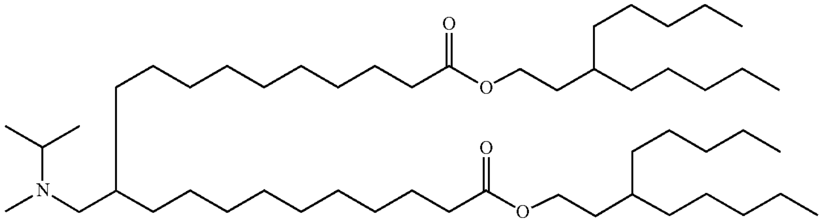

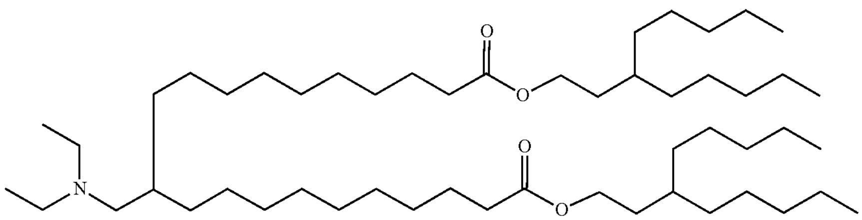

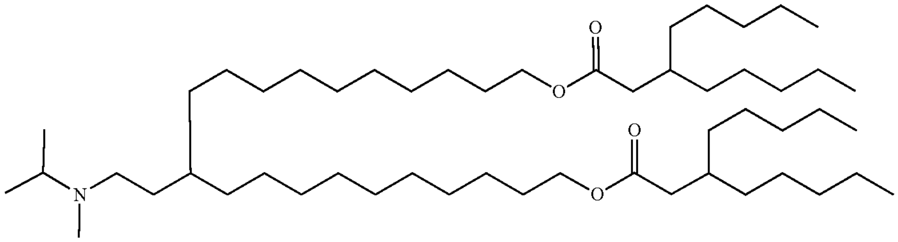

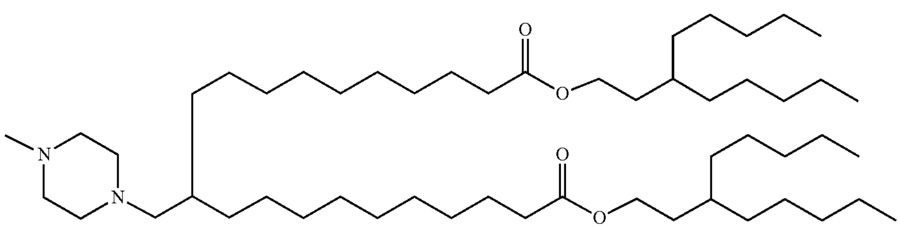

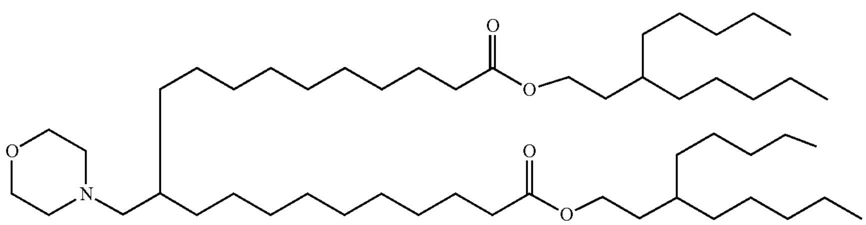

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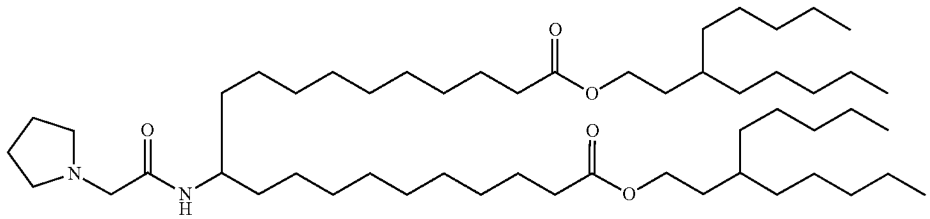

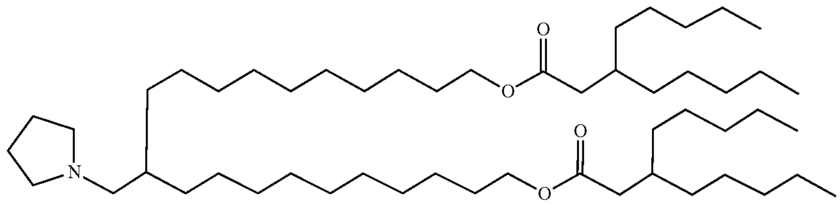

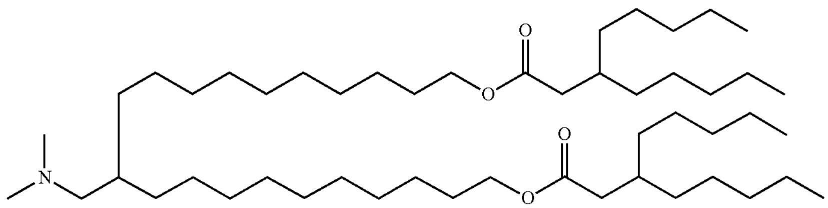

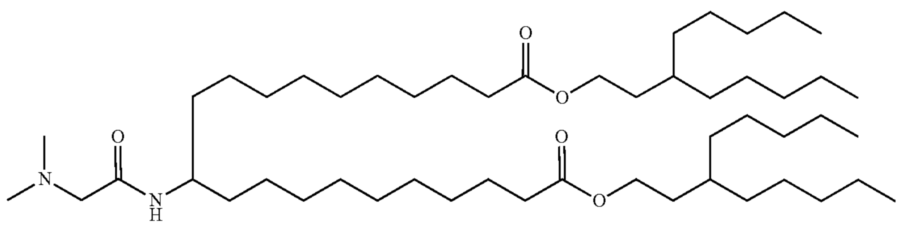

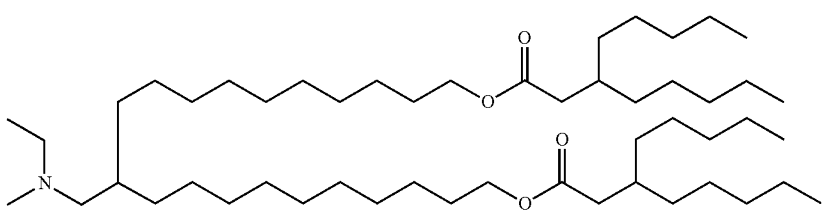

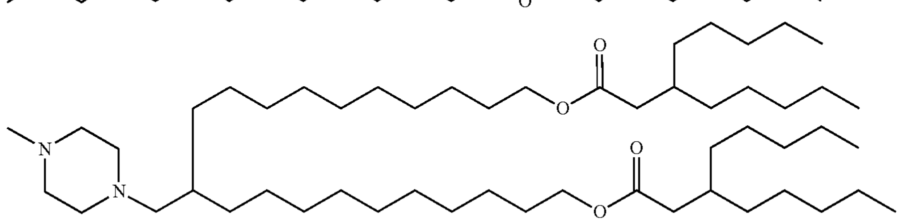

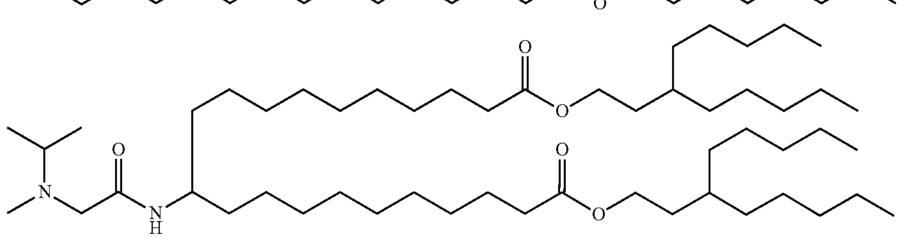

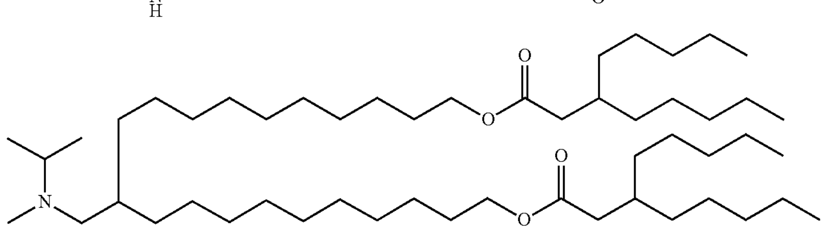

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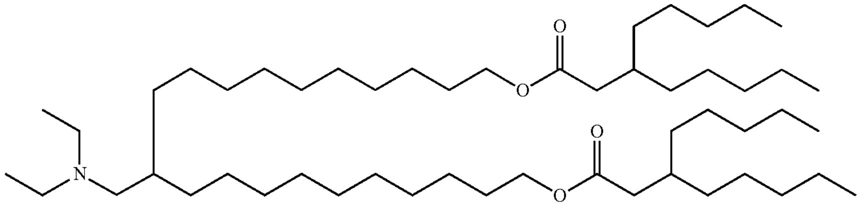

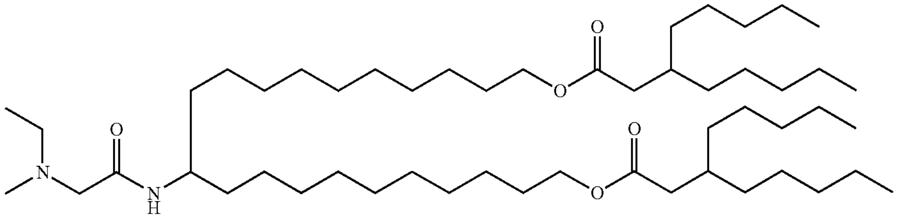

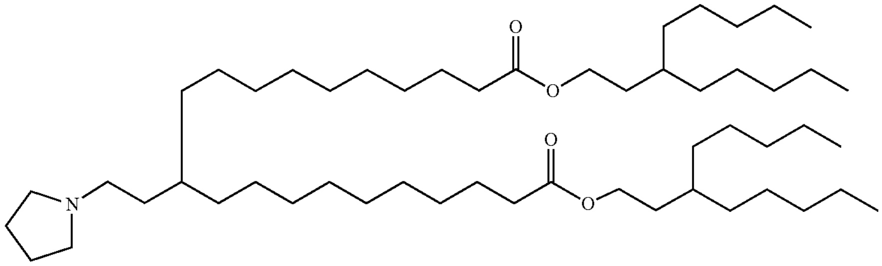

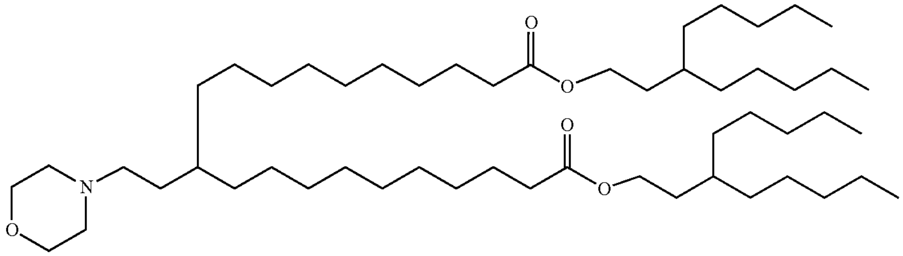

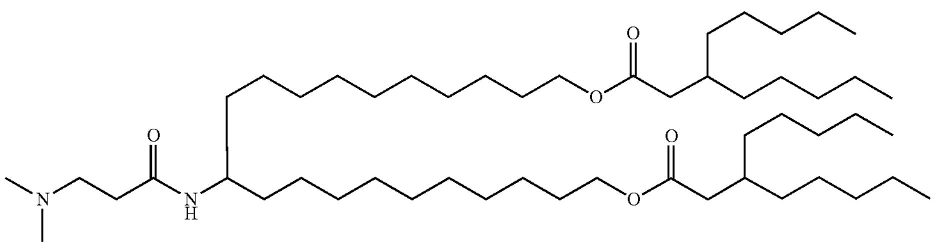

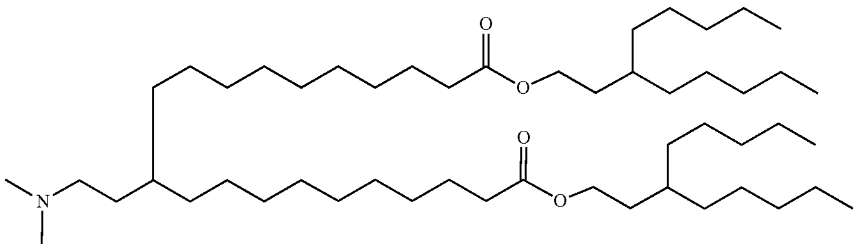

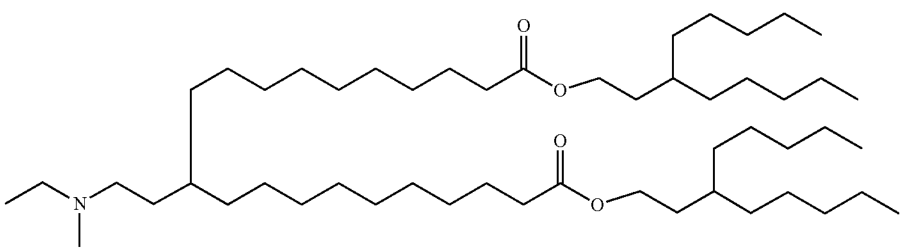

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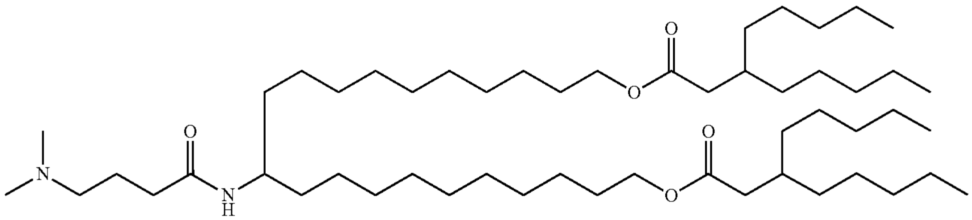

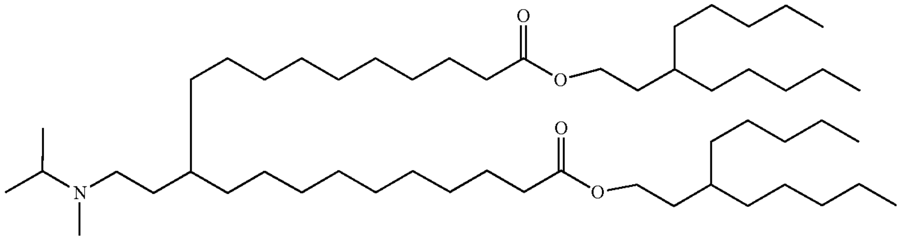

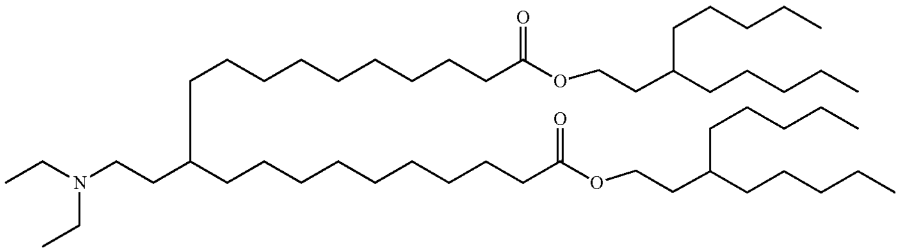

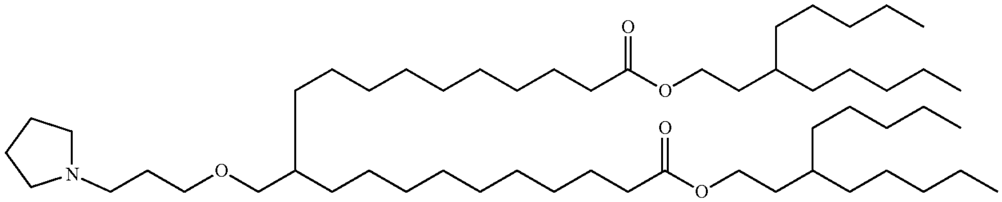

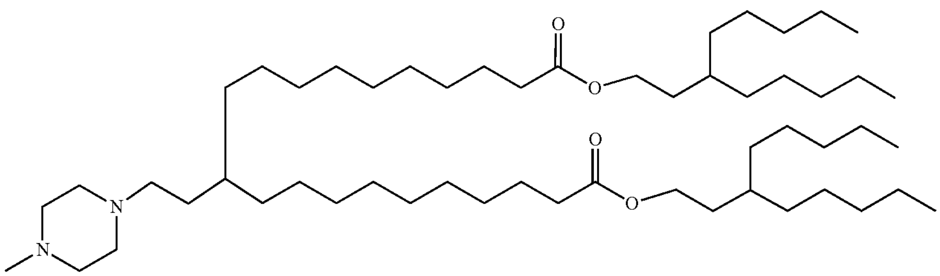

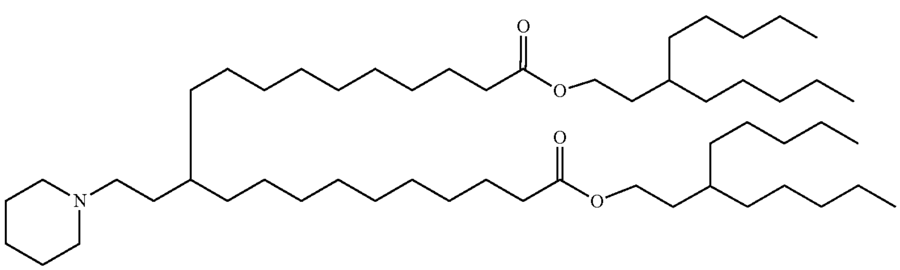

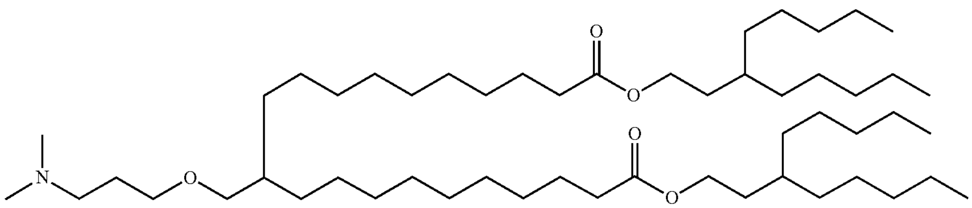

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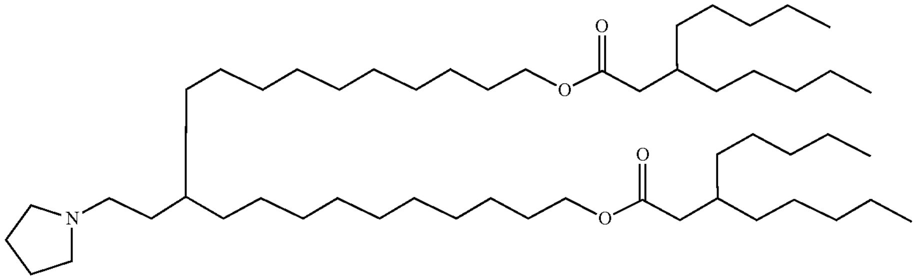

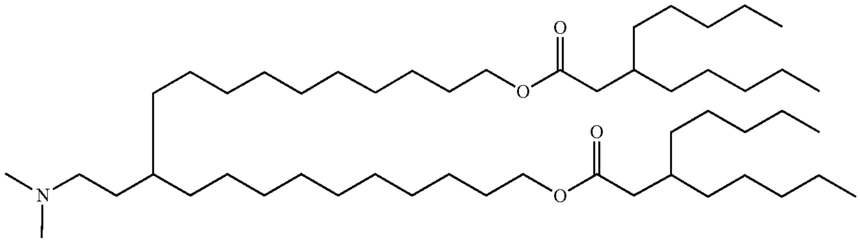

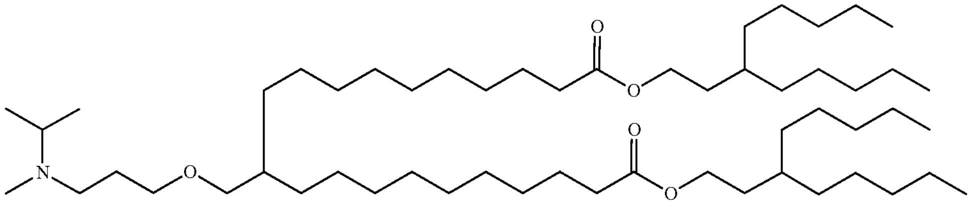

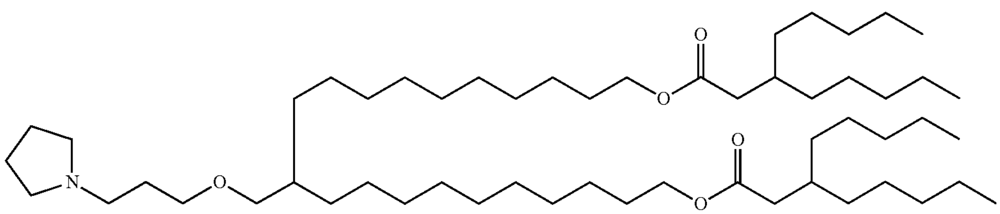

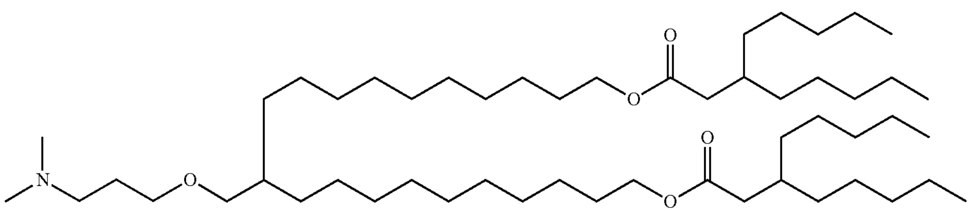

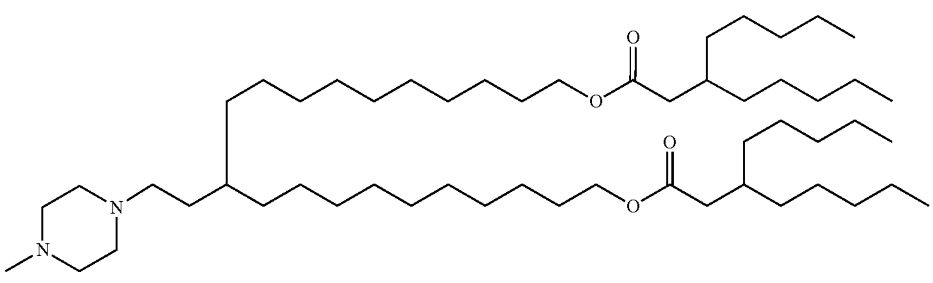

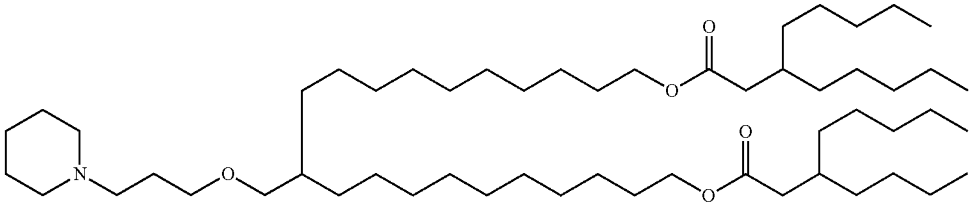

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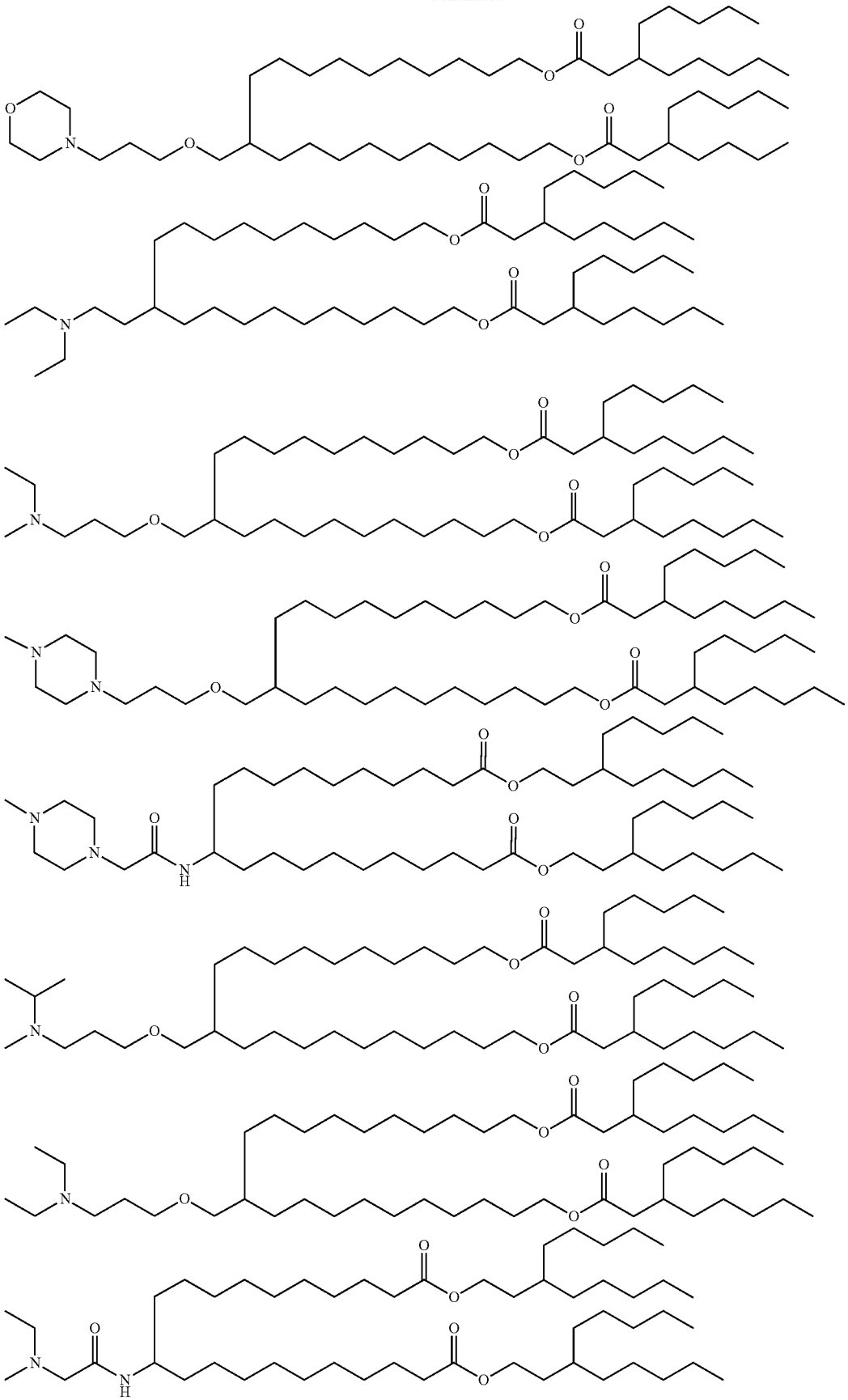

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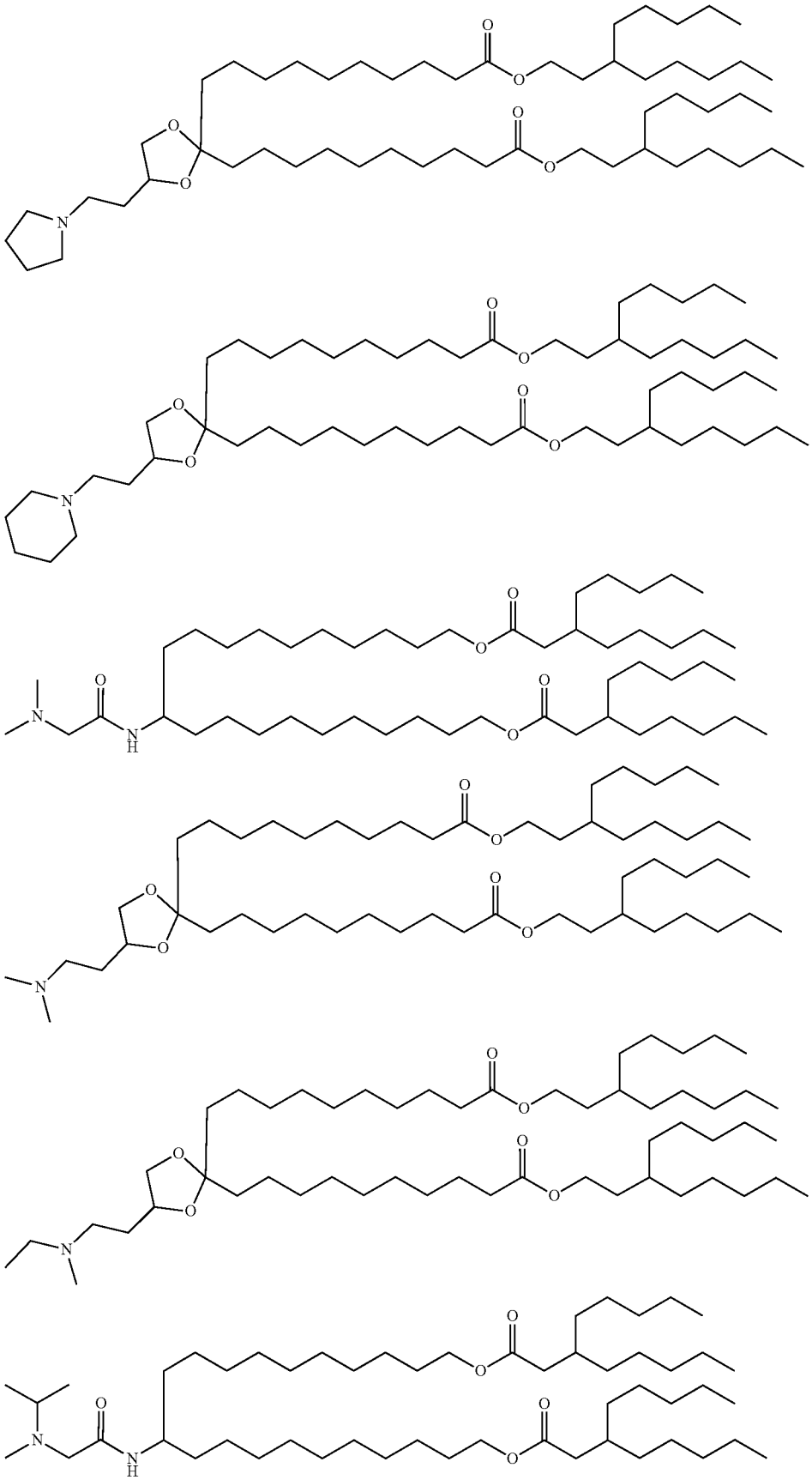

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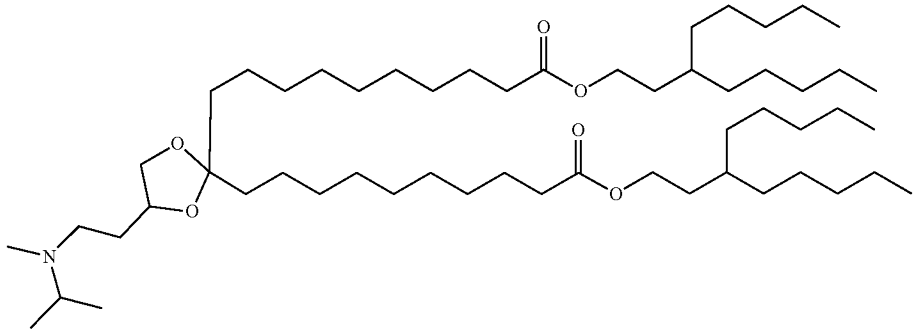

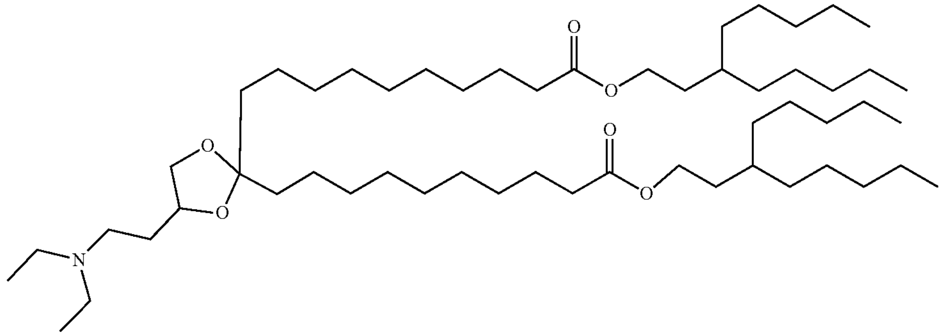

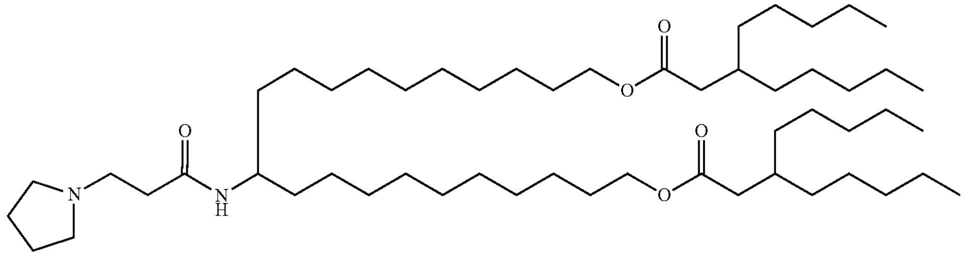

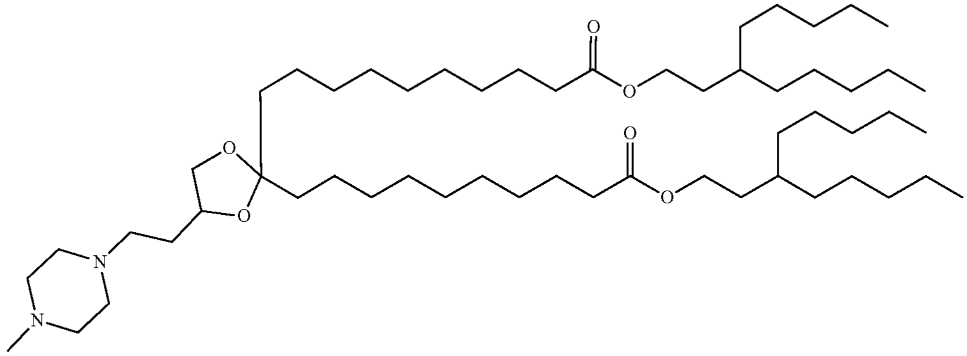

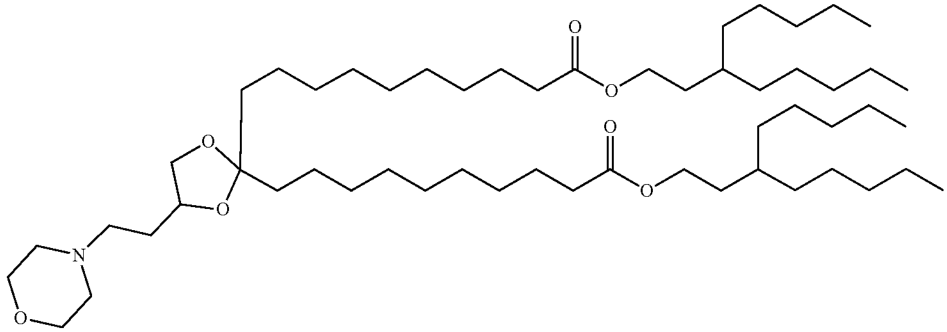

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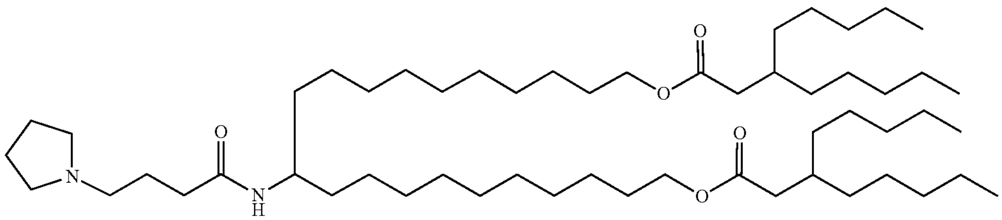

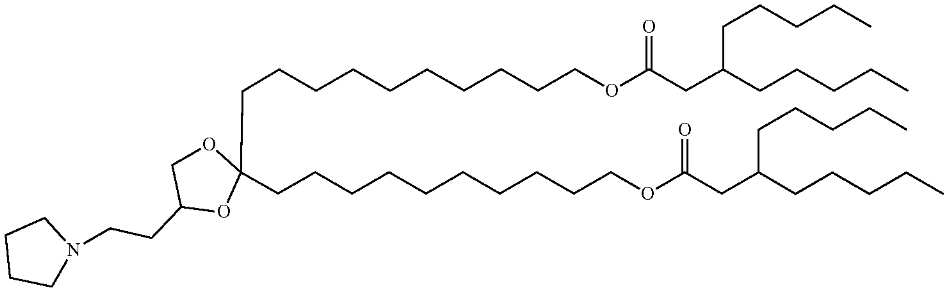

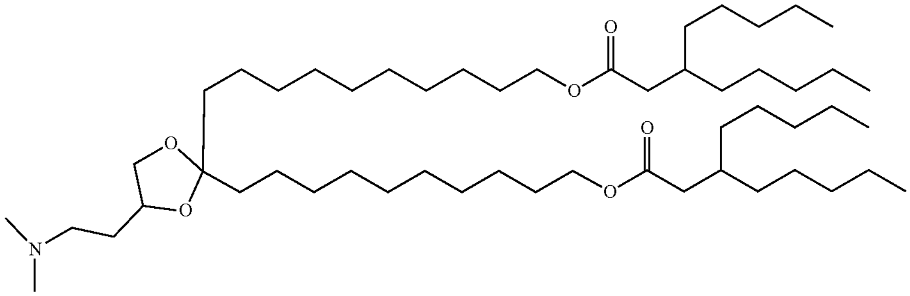

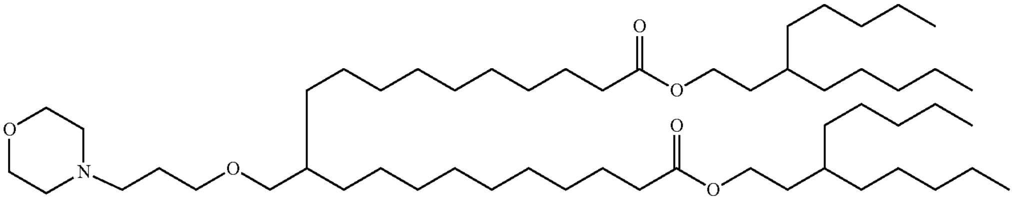

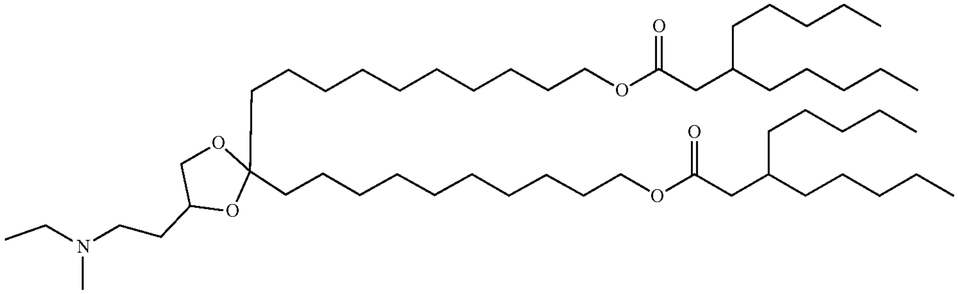

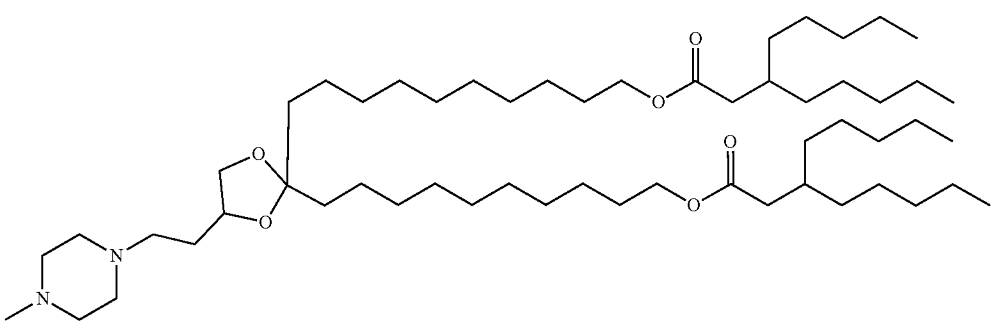

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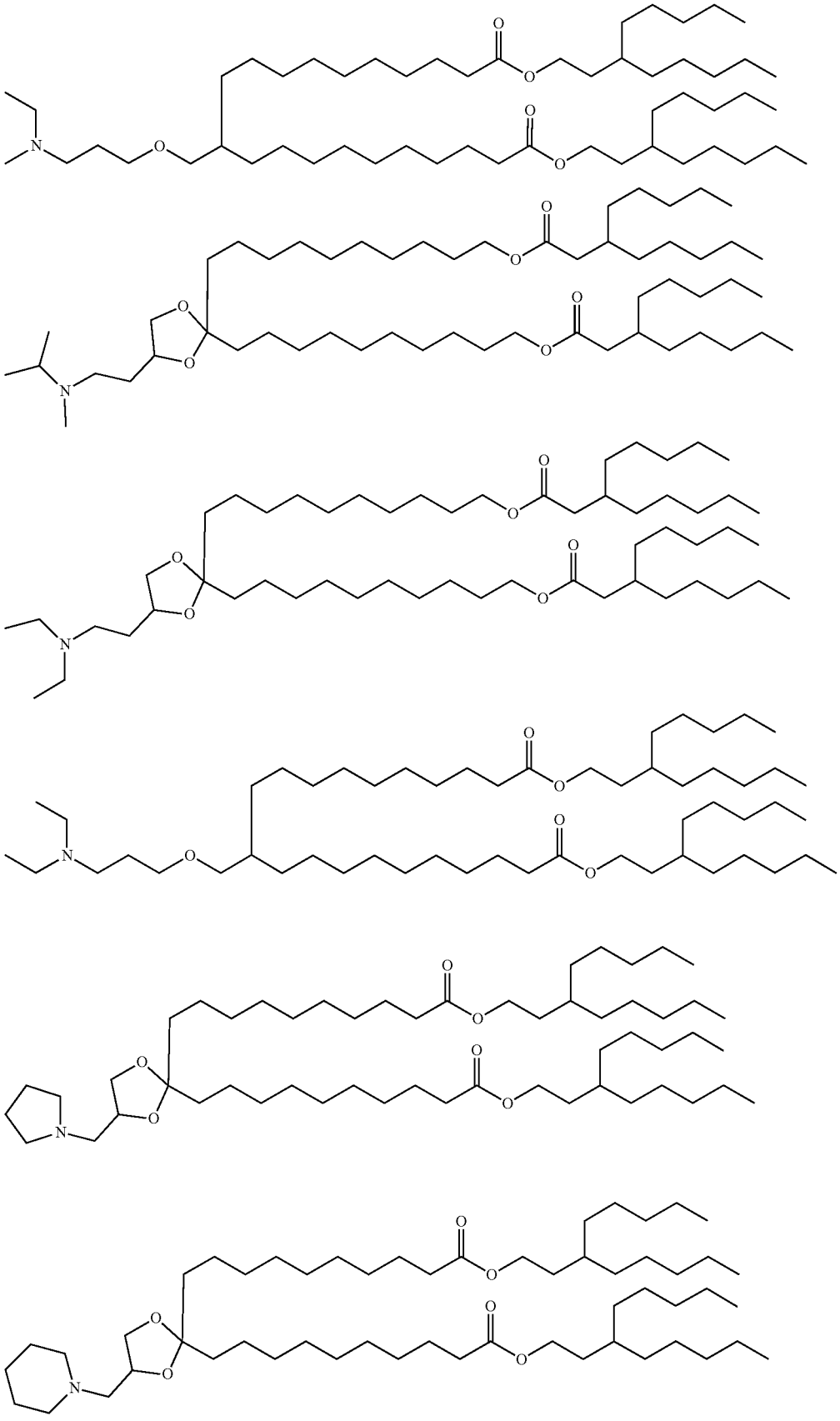

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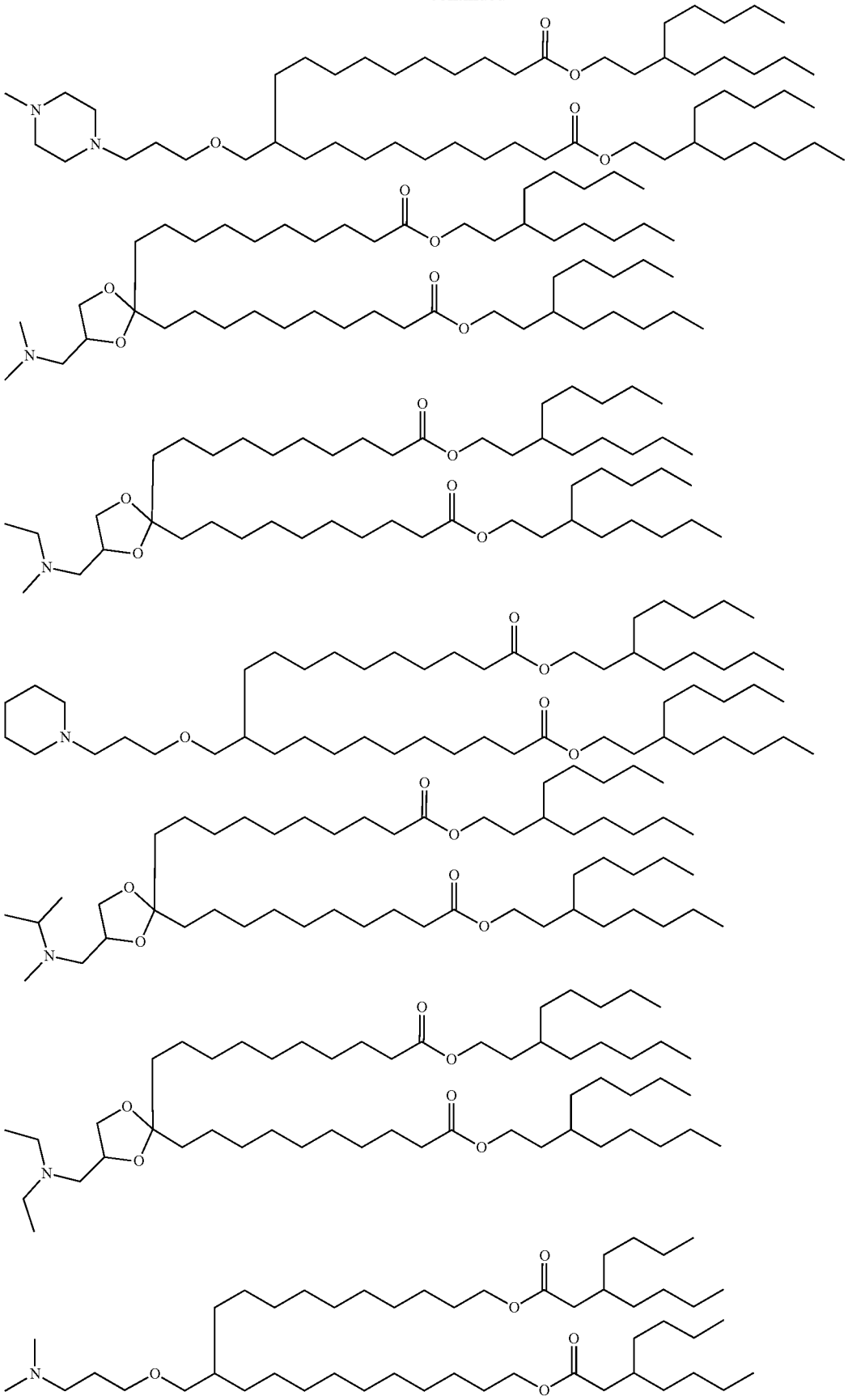

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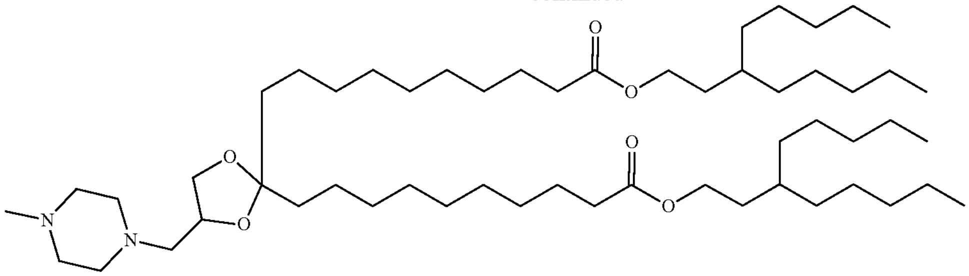

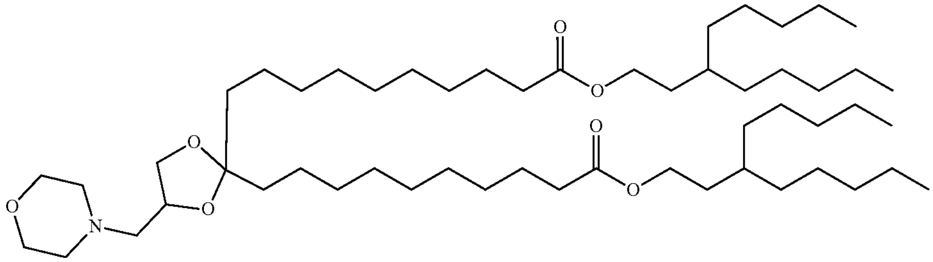

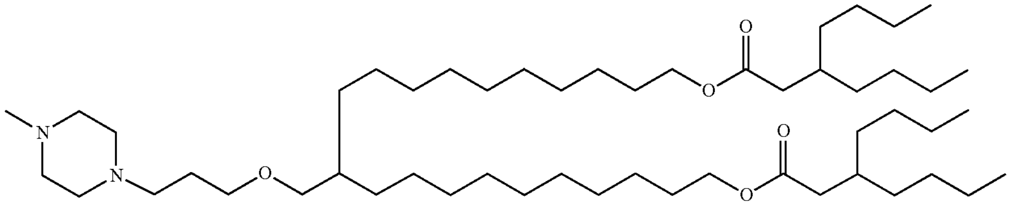

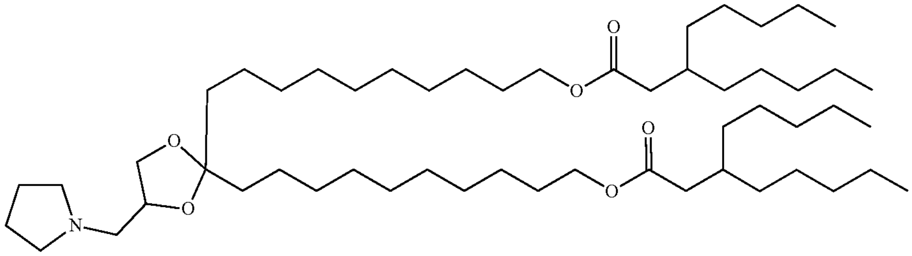

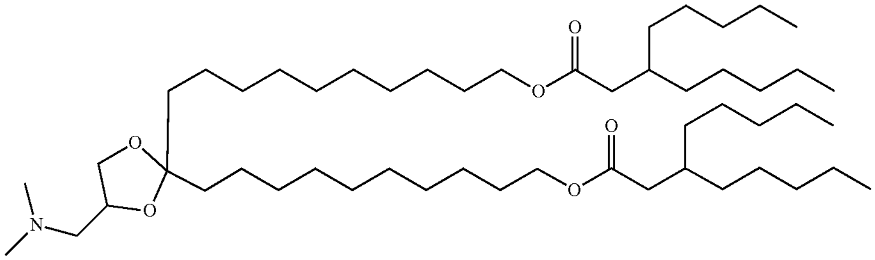

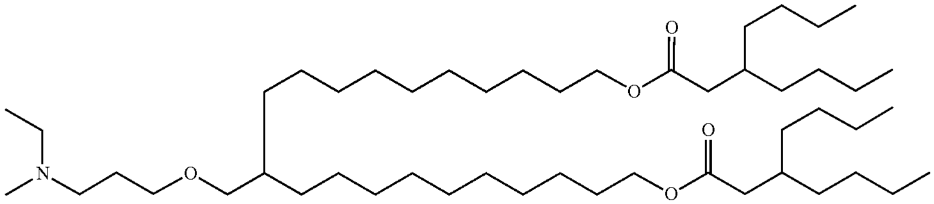

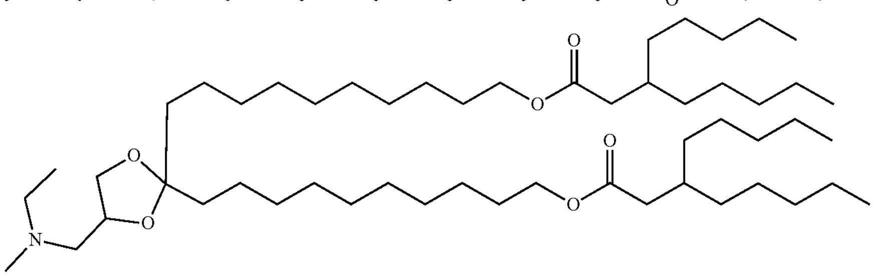

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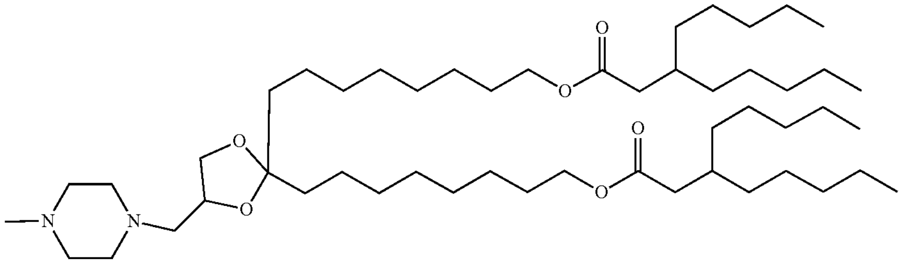

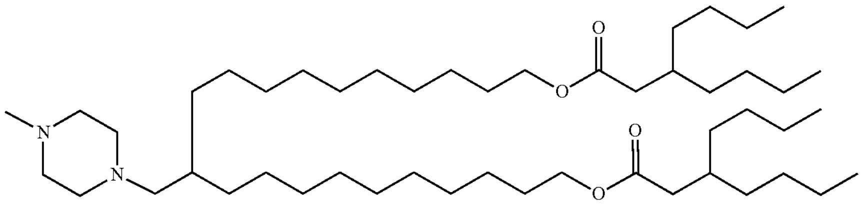

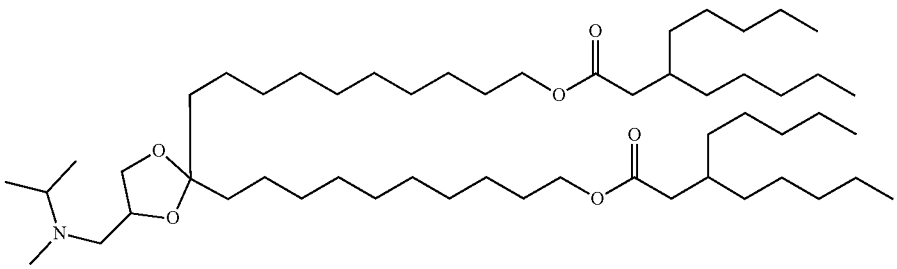

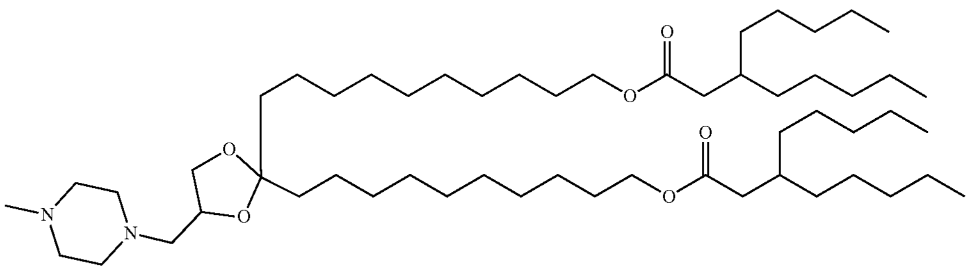

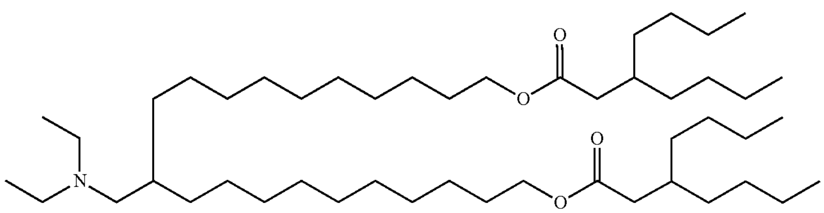

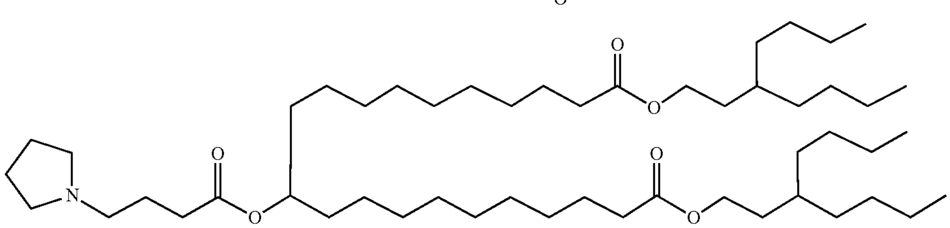

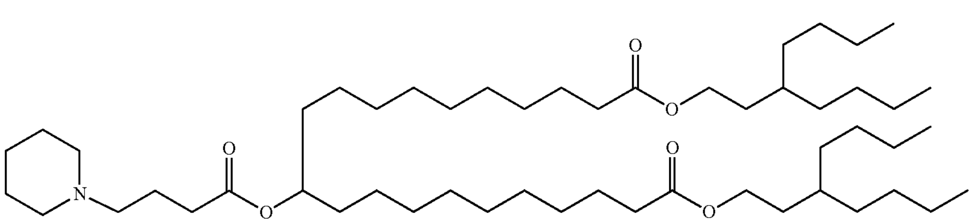

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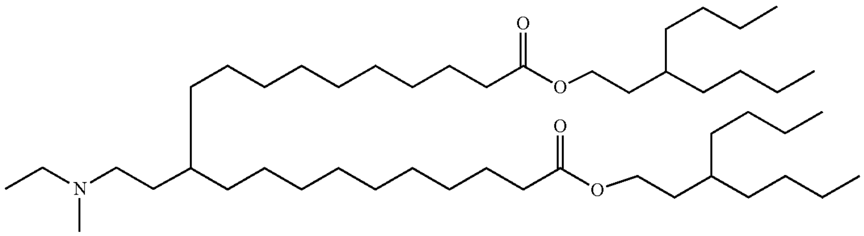

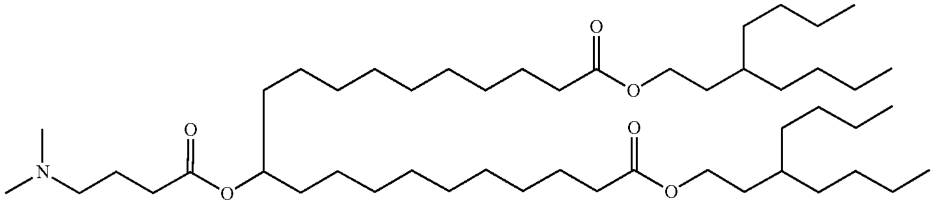

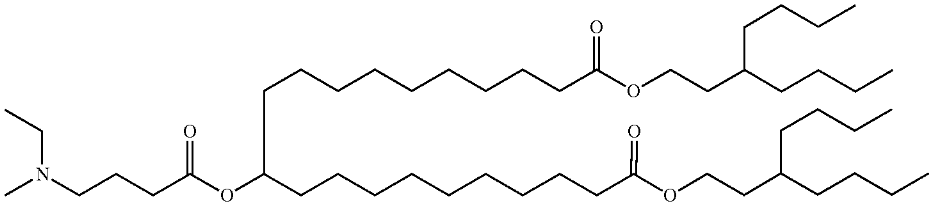

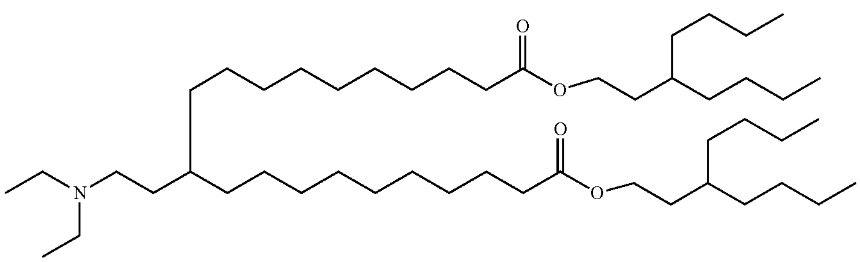

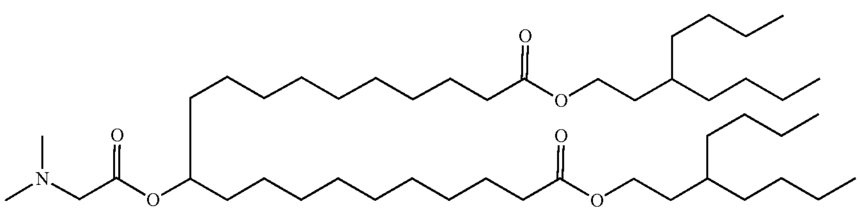

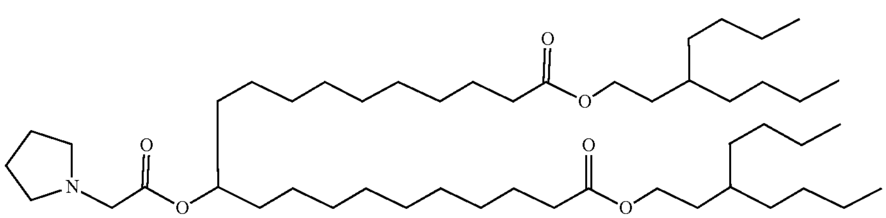

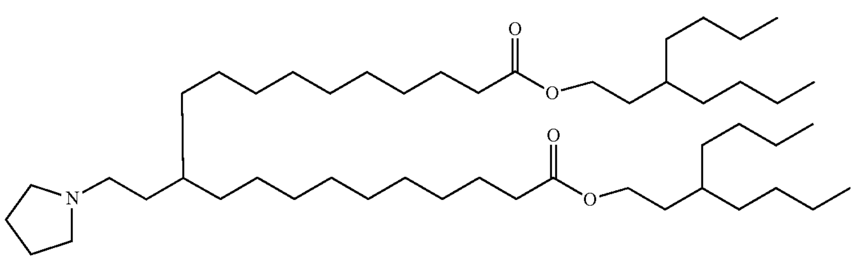

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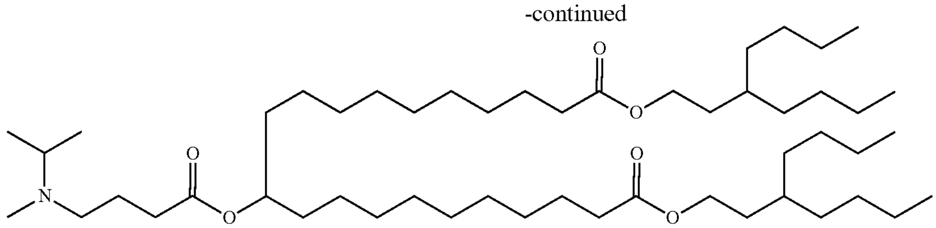

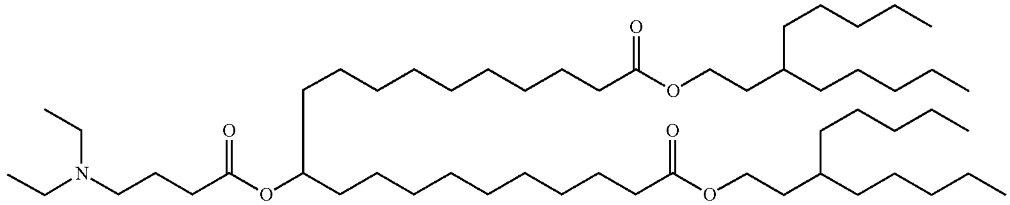

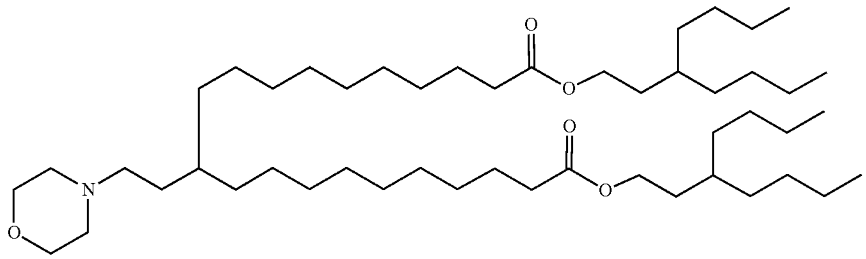

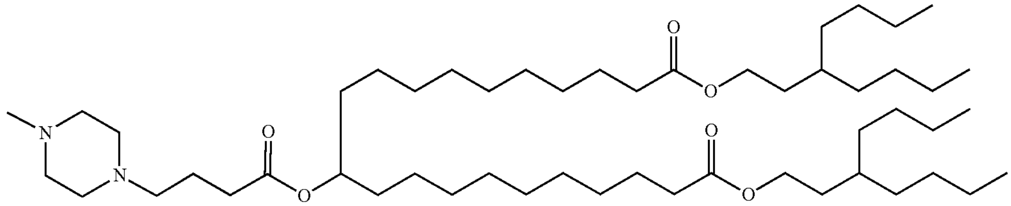

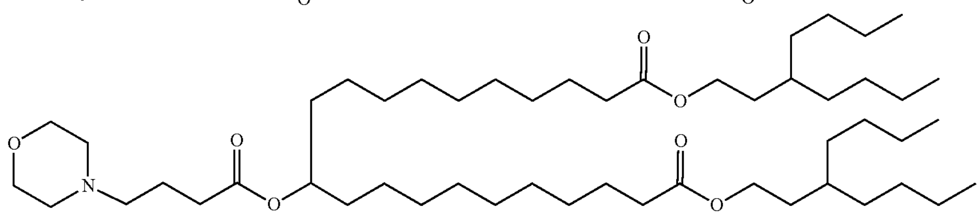

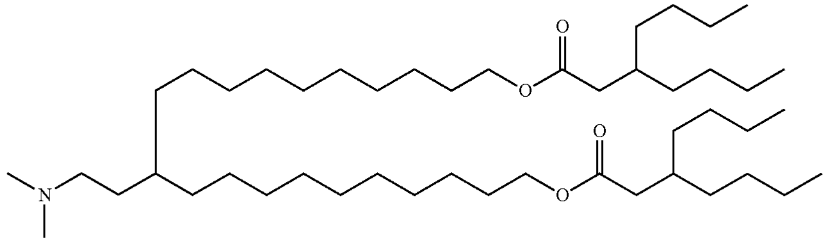

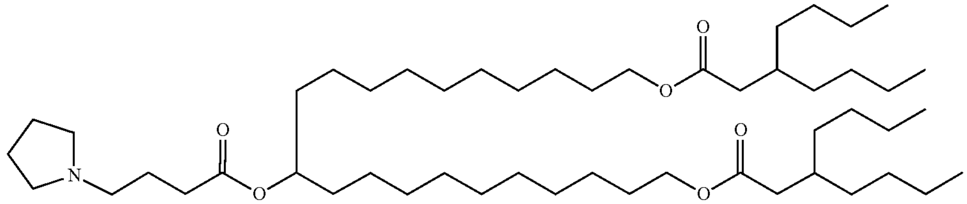

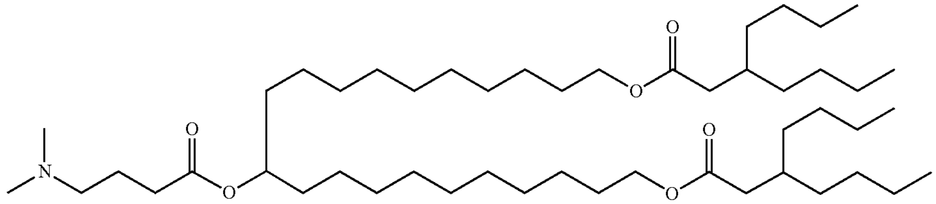

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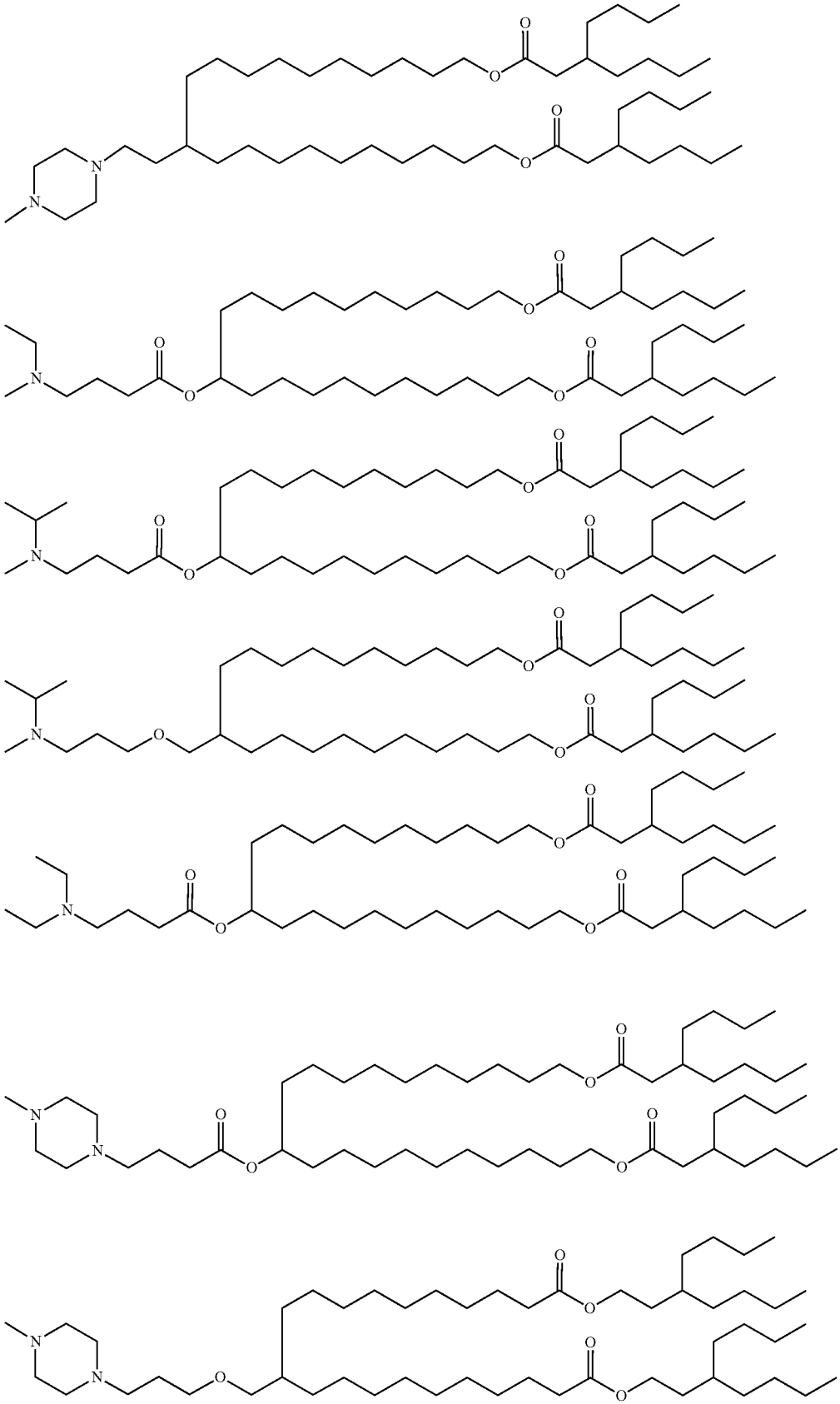

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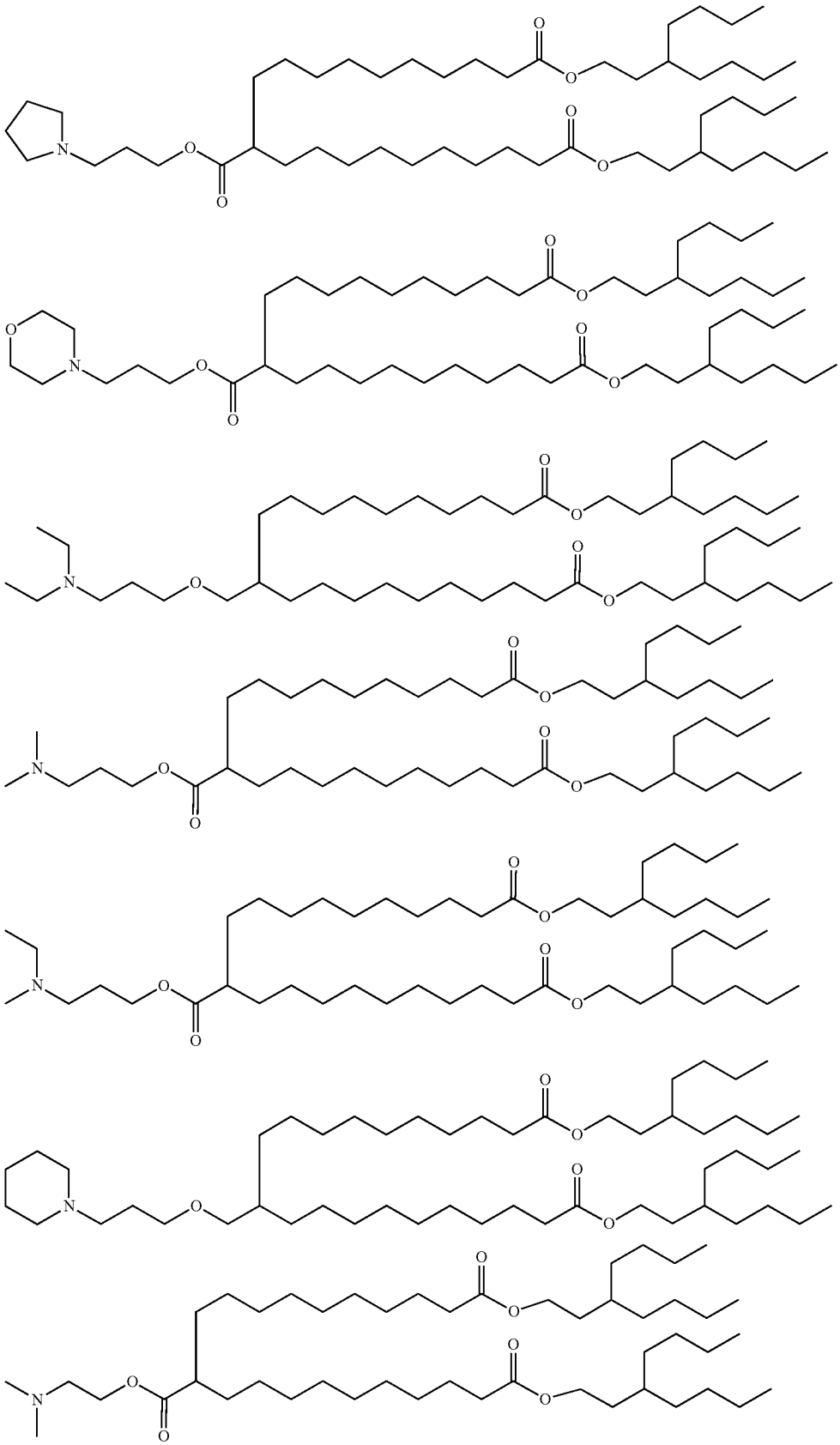

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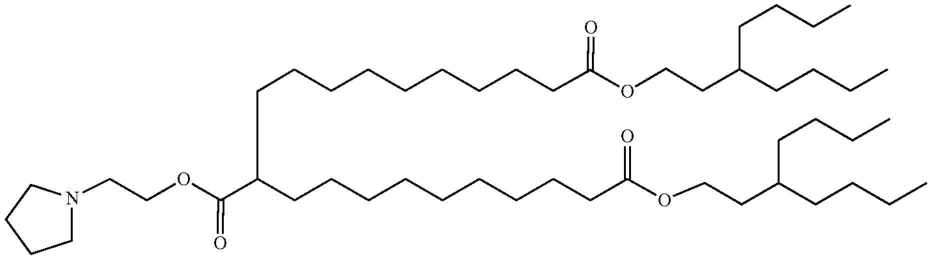

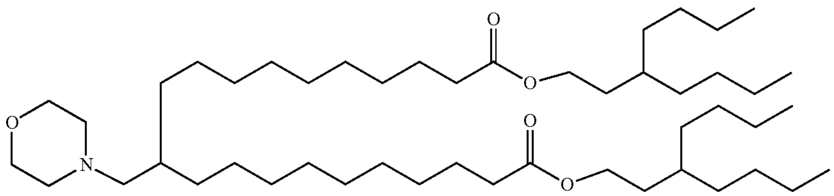

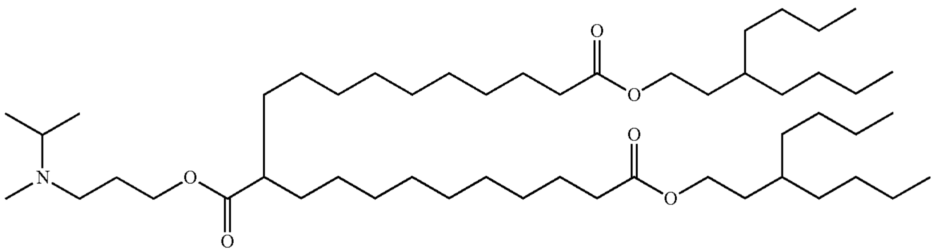

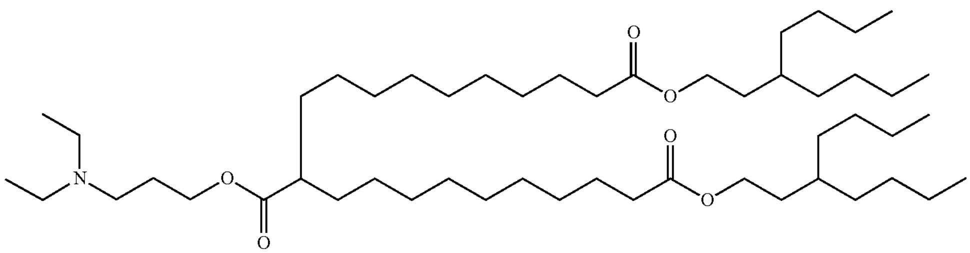

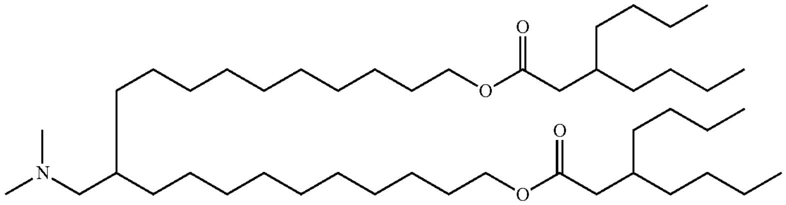

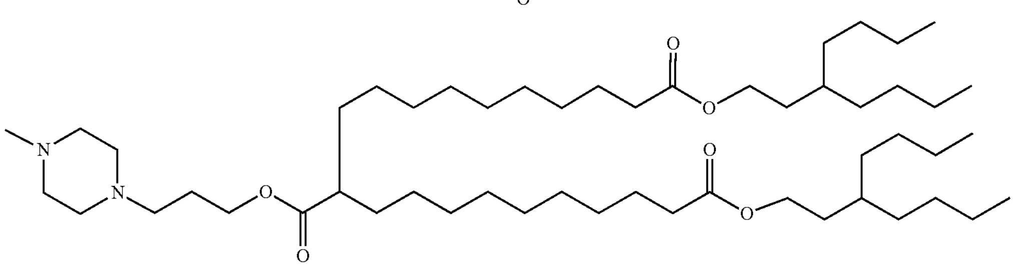

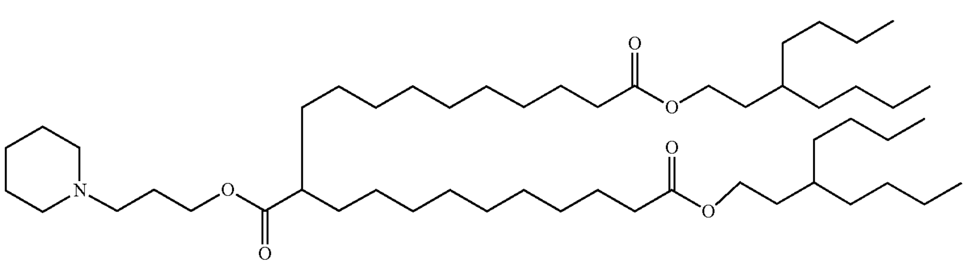

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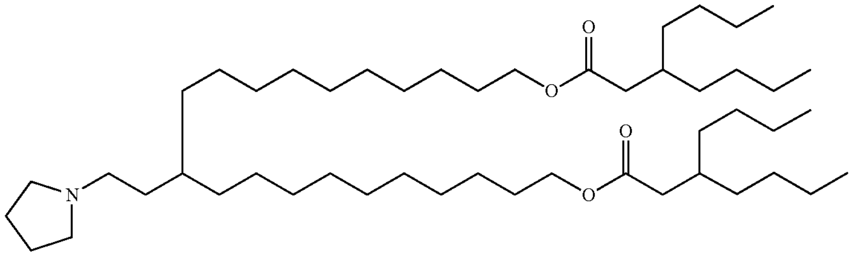

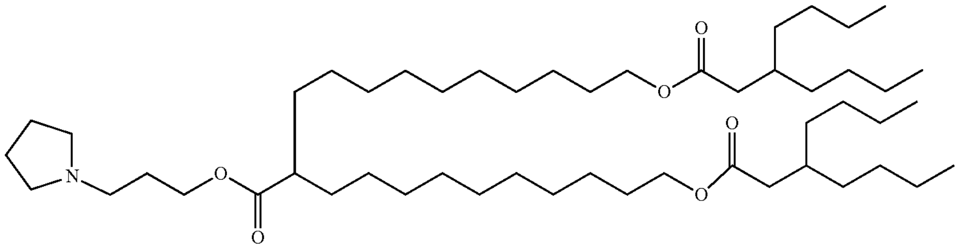

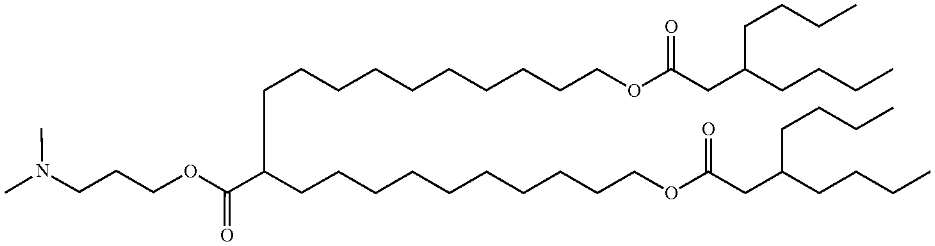

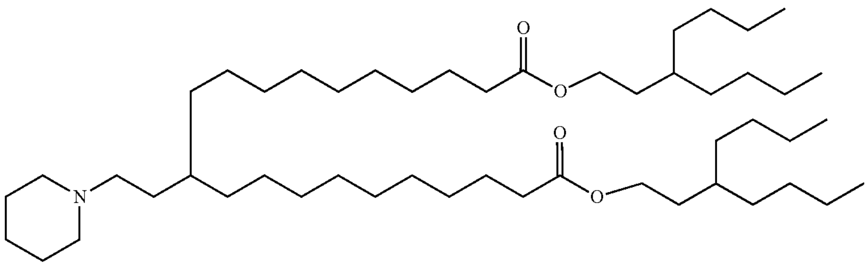

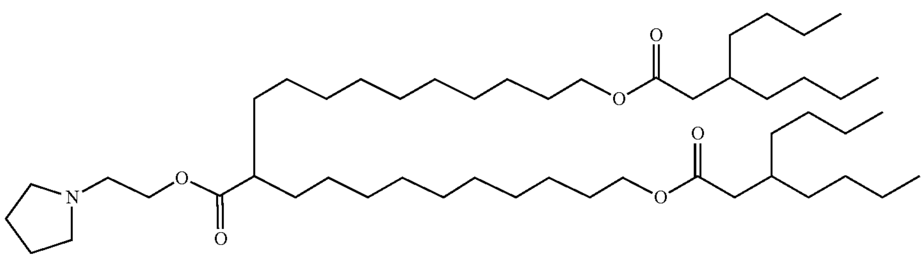

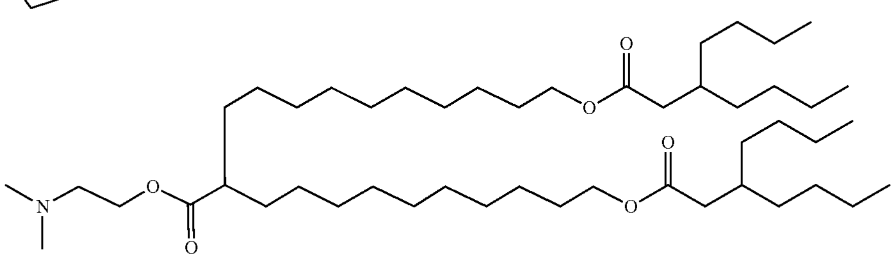

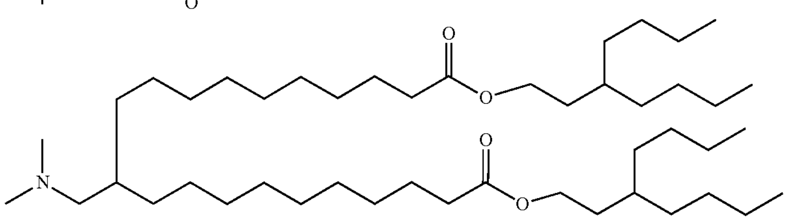

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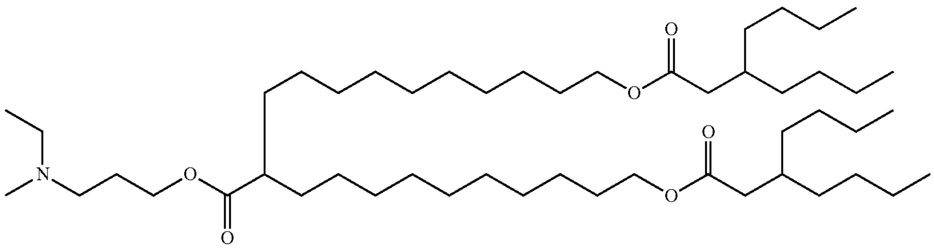

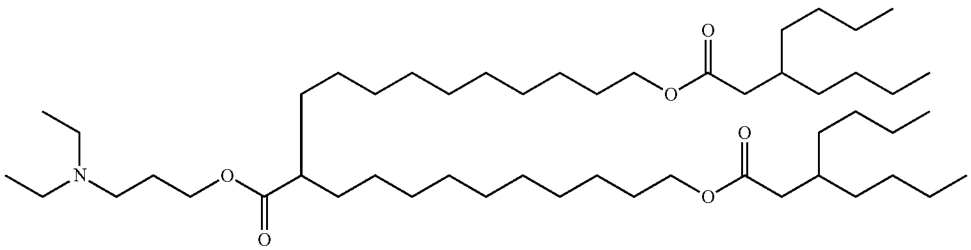

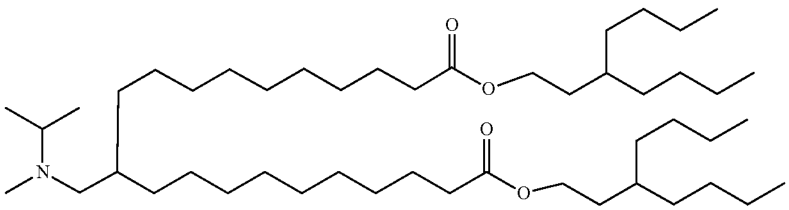

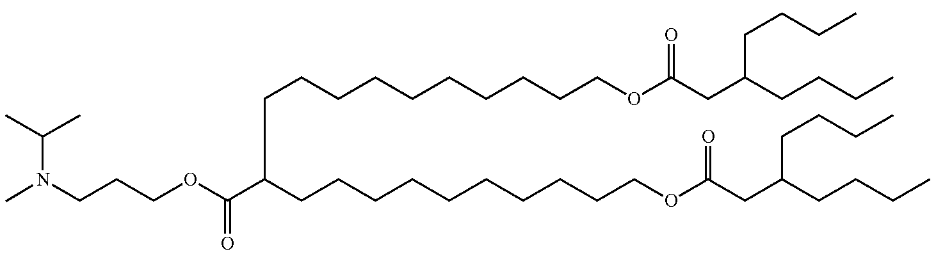

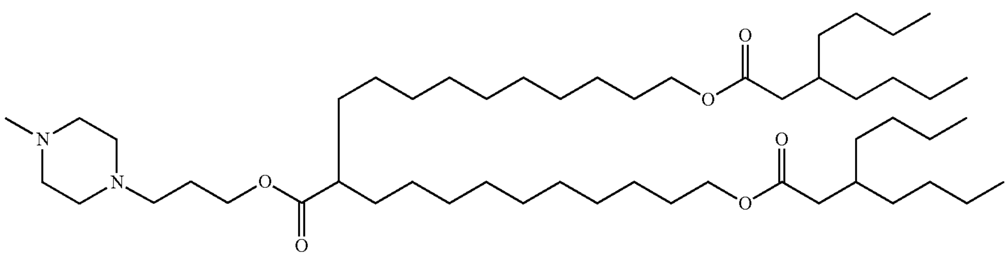

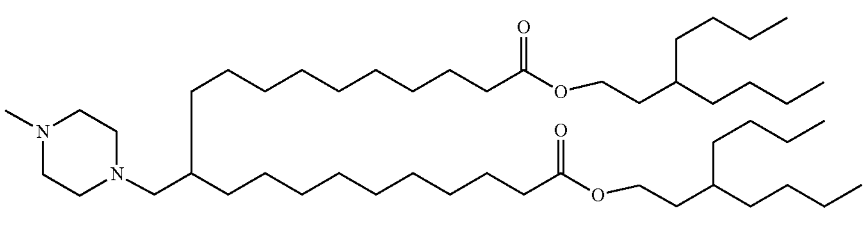

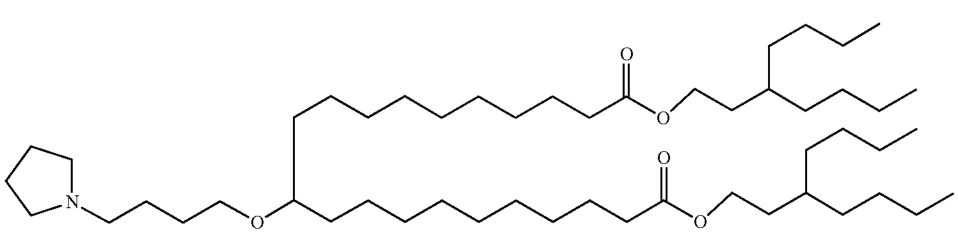

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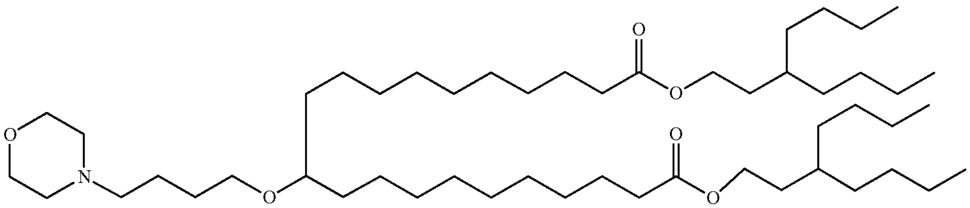

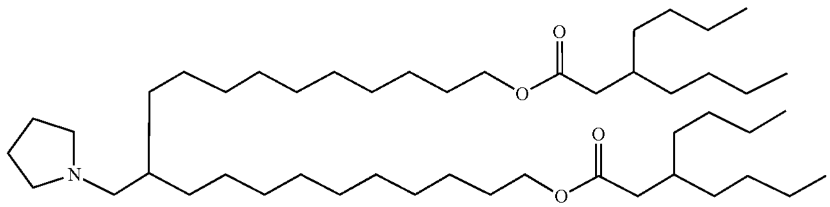

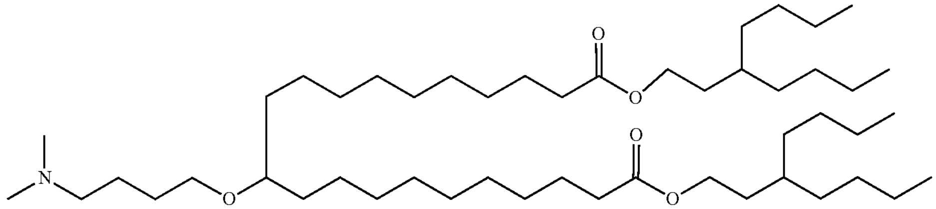

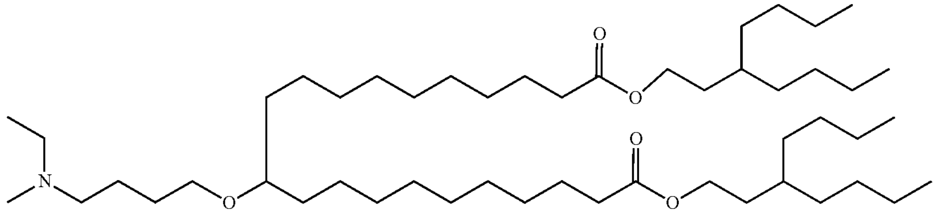

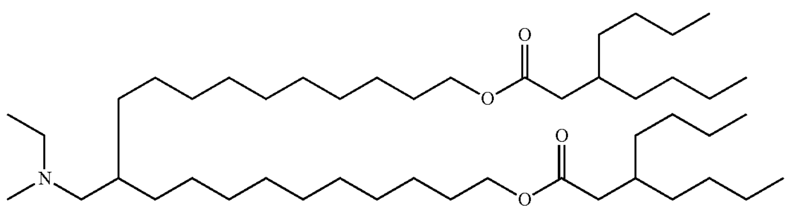

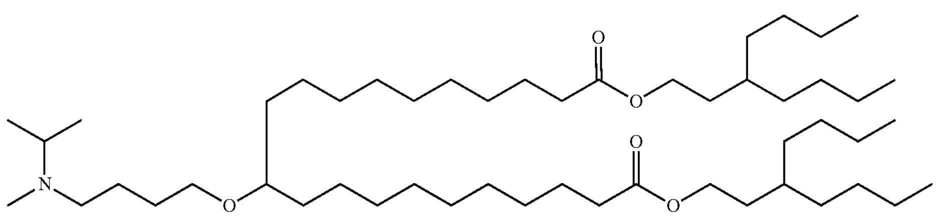

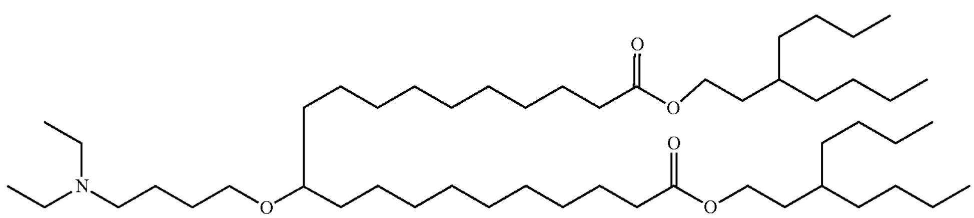

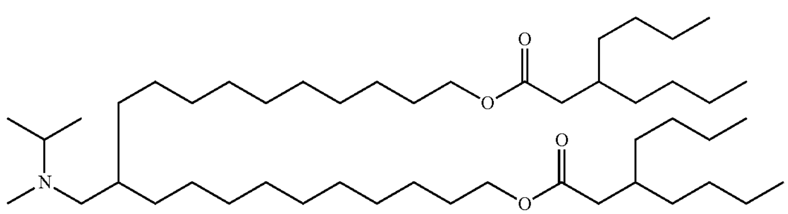

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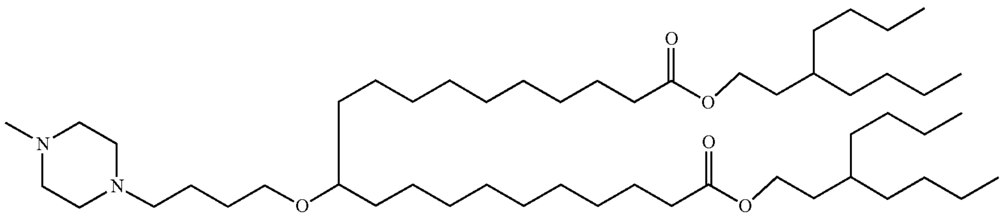

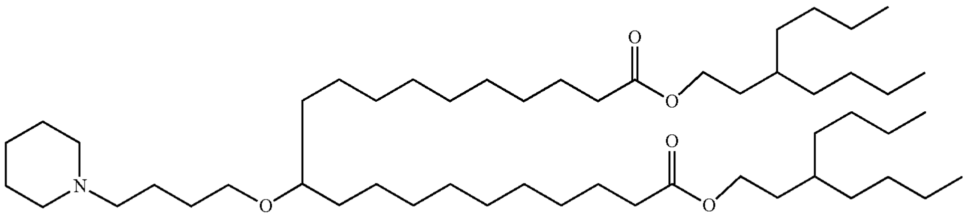

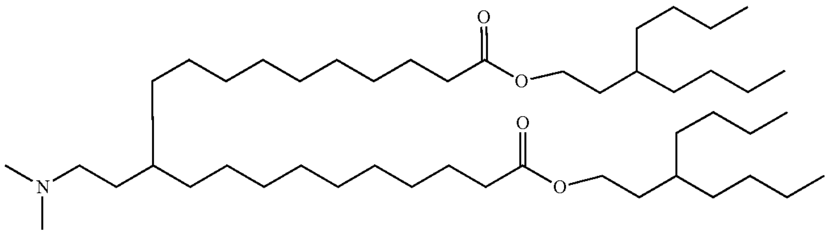

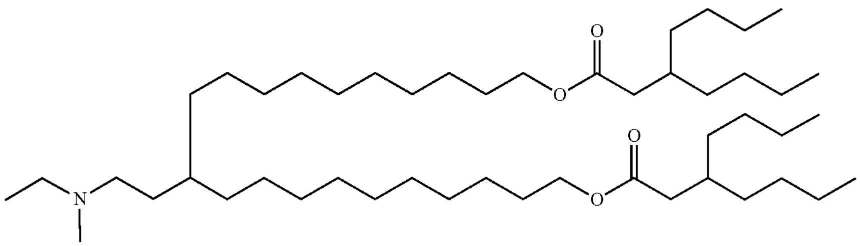

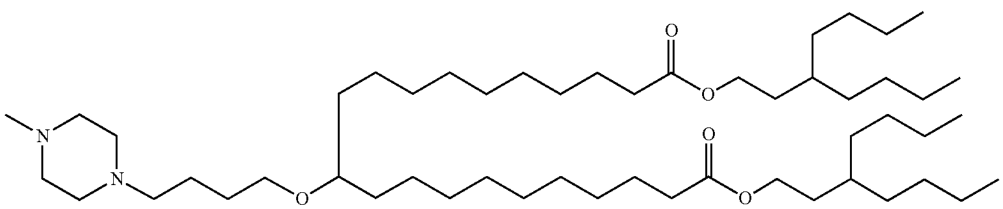

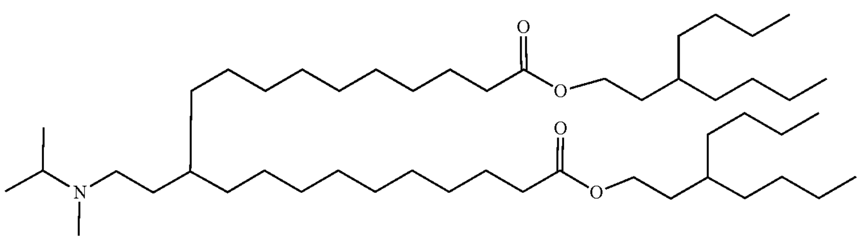

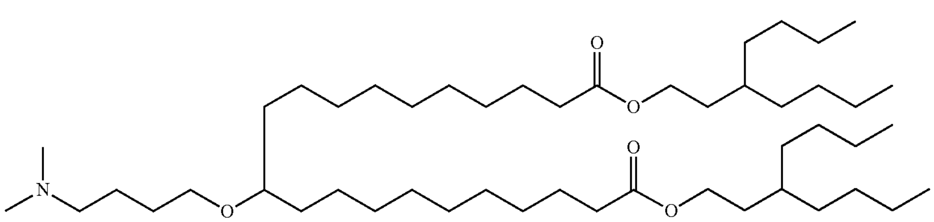

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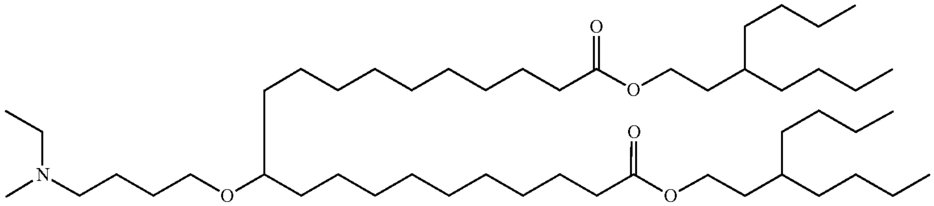

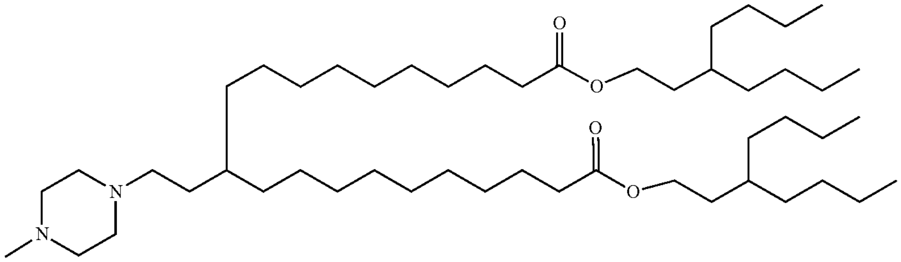

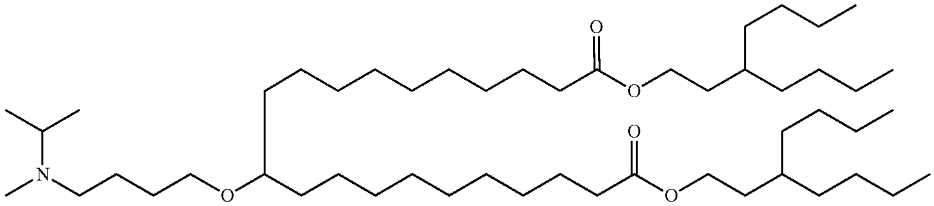

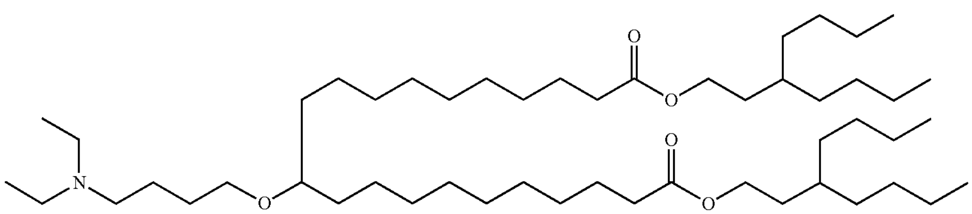

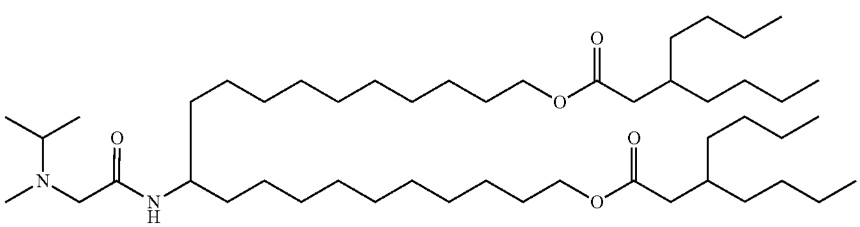

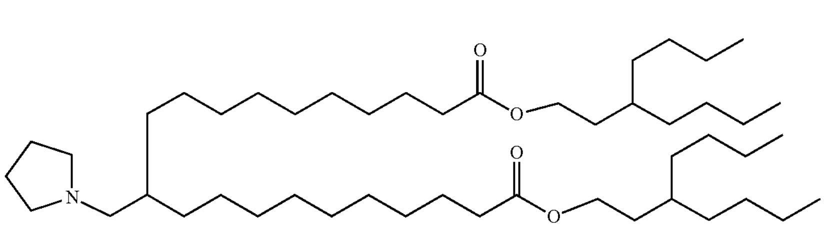

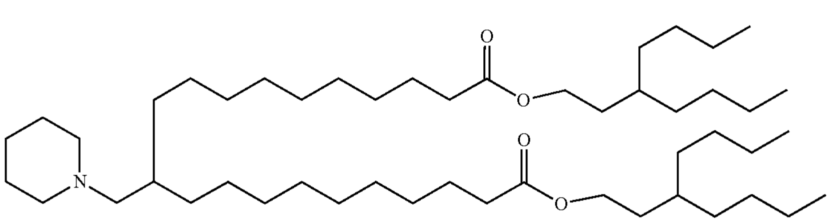

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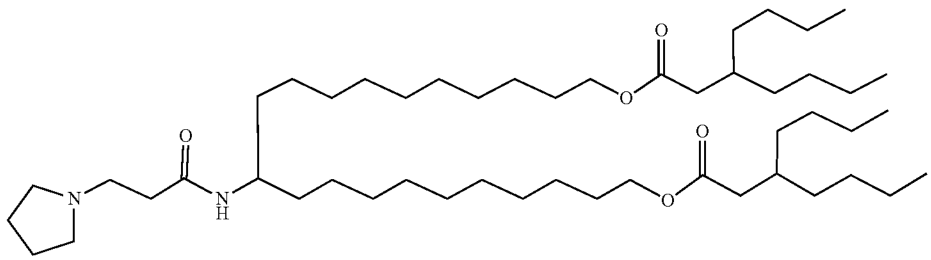

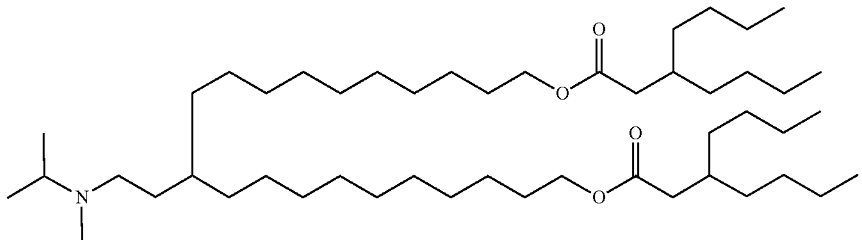

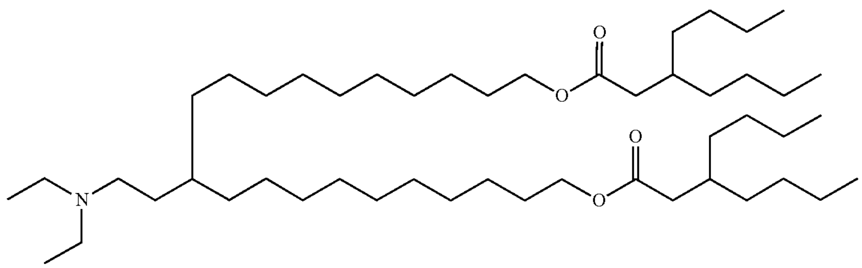

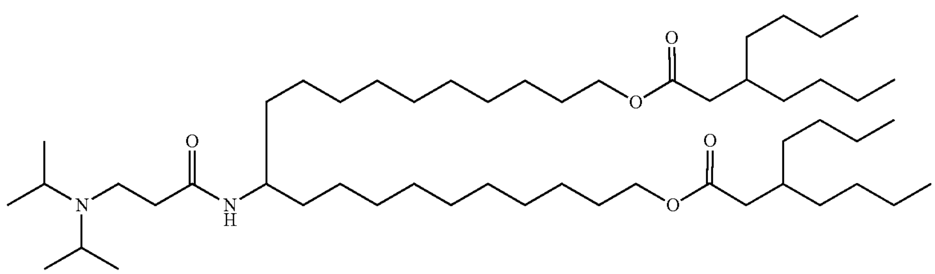

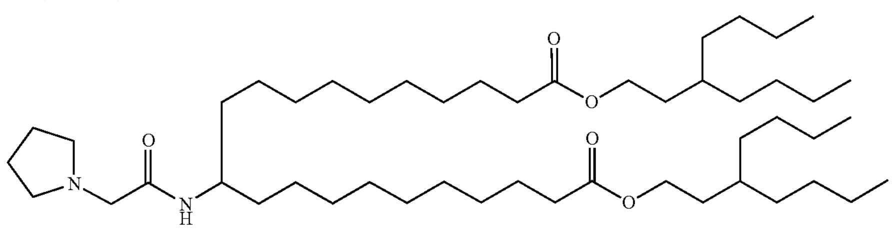

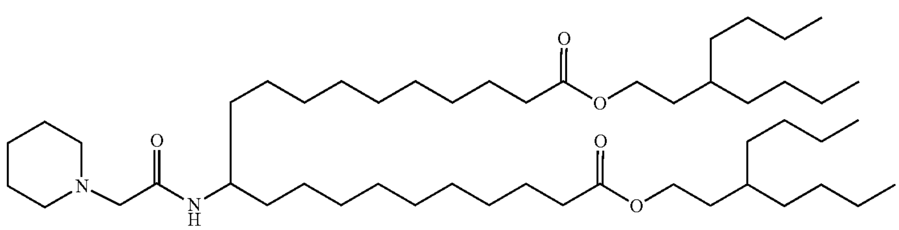

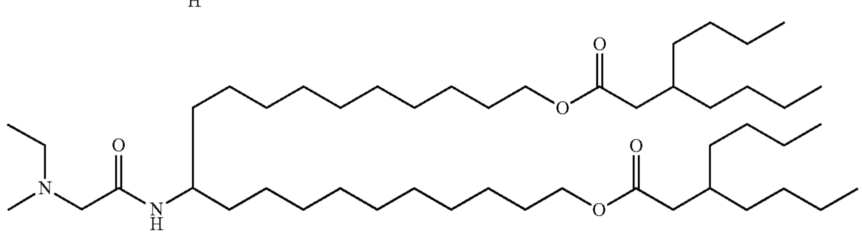

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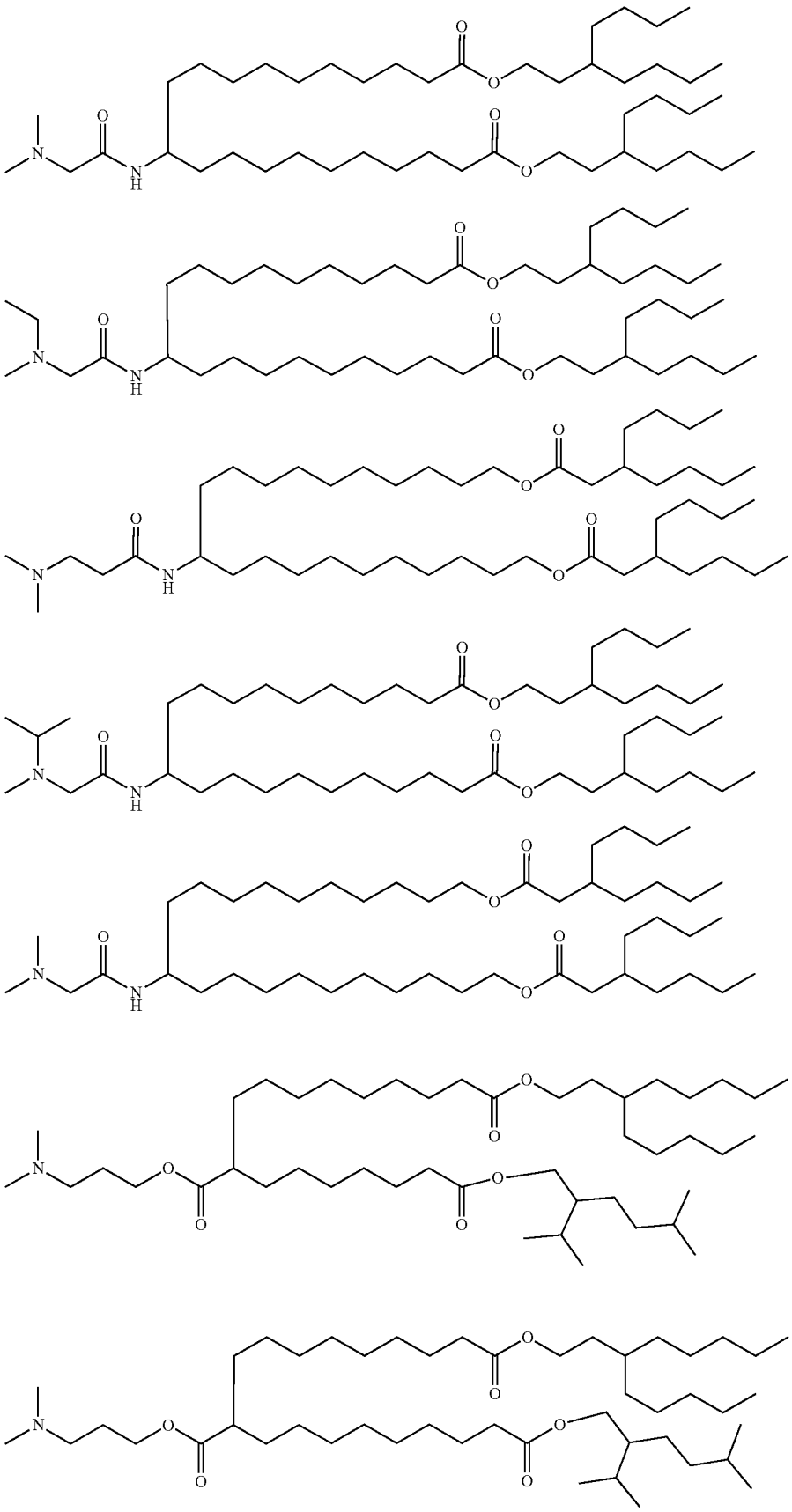

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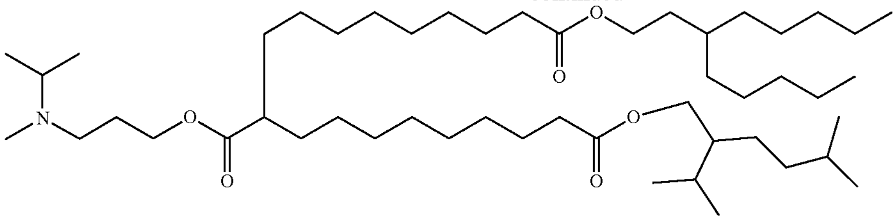
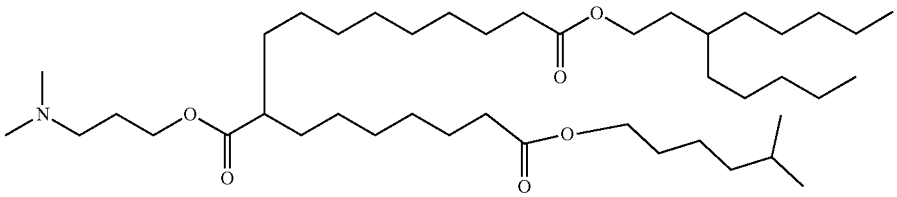
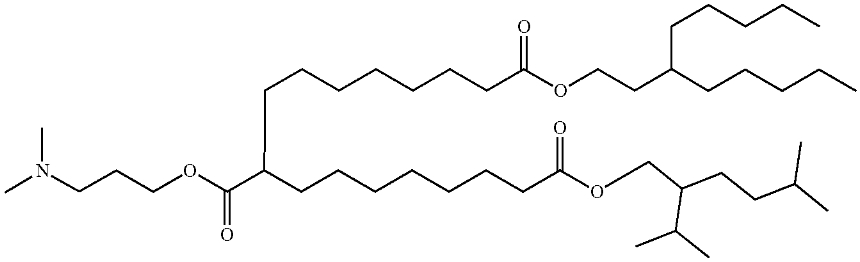
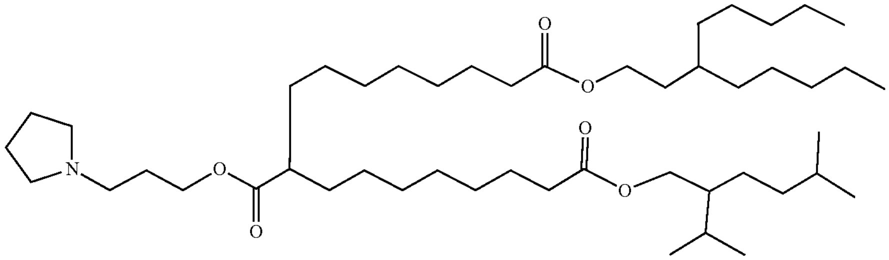
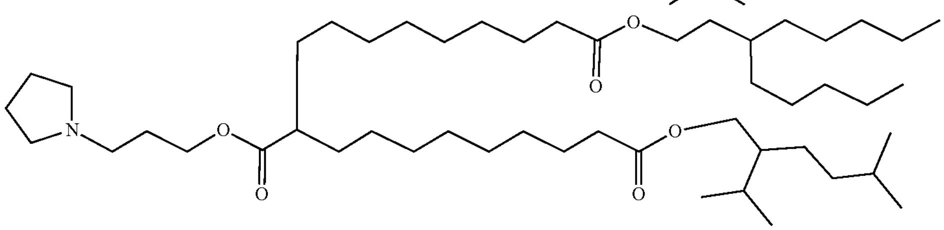




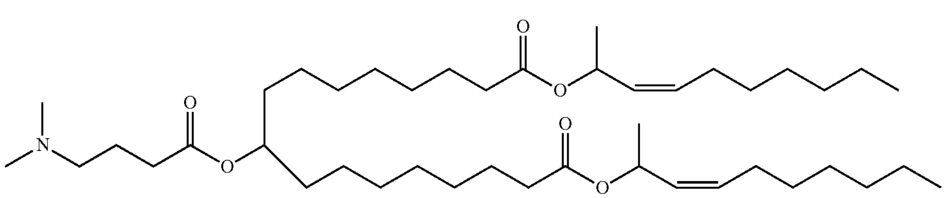






In another aspect, the present invention relates to a method of preparing a compound of Formula I-VII. Suitable exemplary synthetic methods are illustrated in Schemes 1-27 shown in the Examples section below.

In one embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof). These cationic lipids are suitable for forming nucleic acid-lipid particles.



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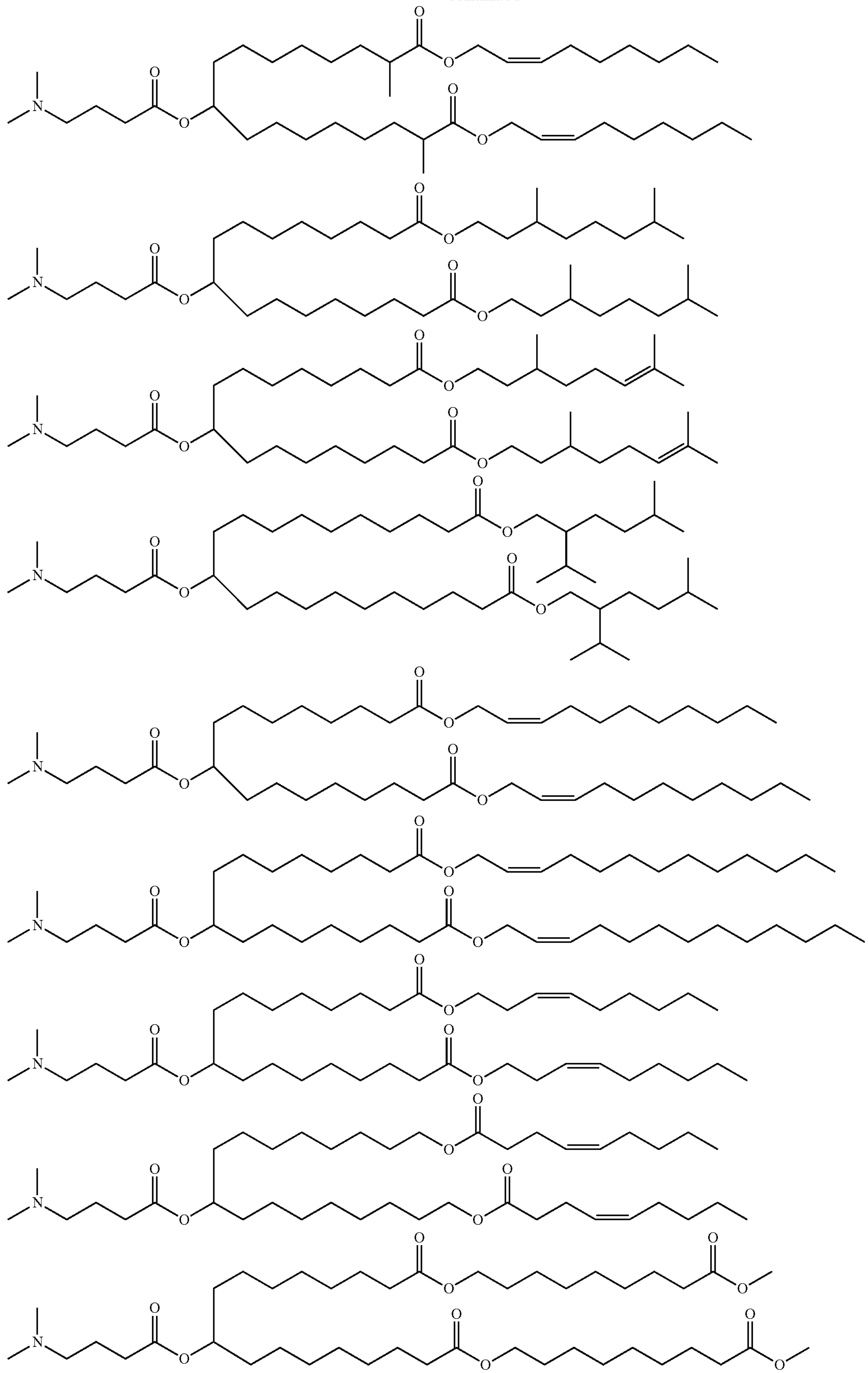

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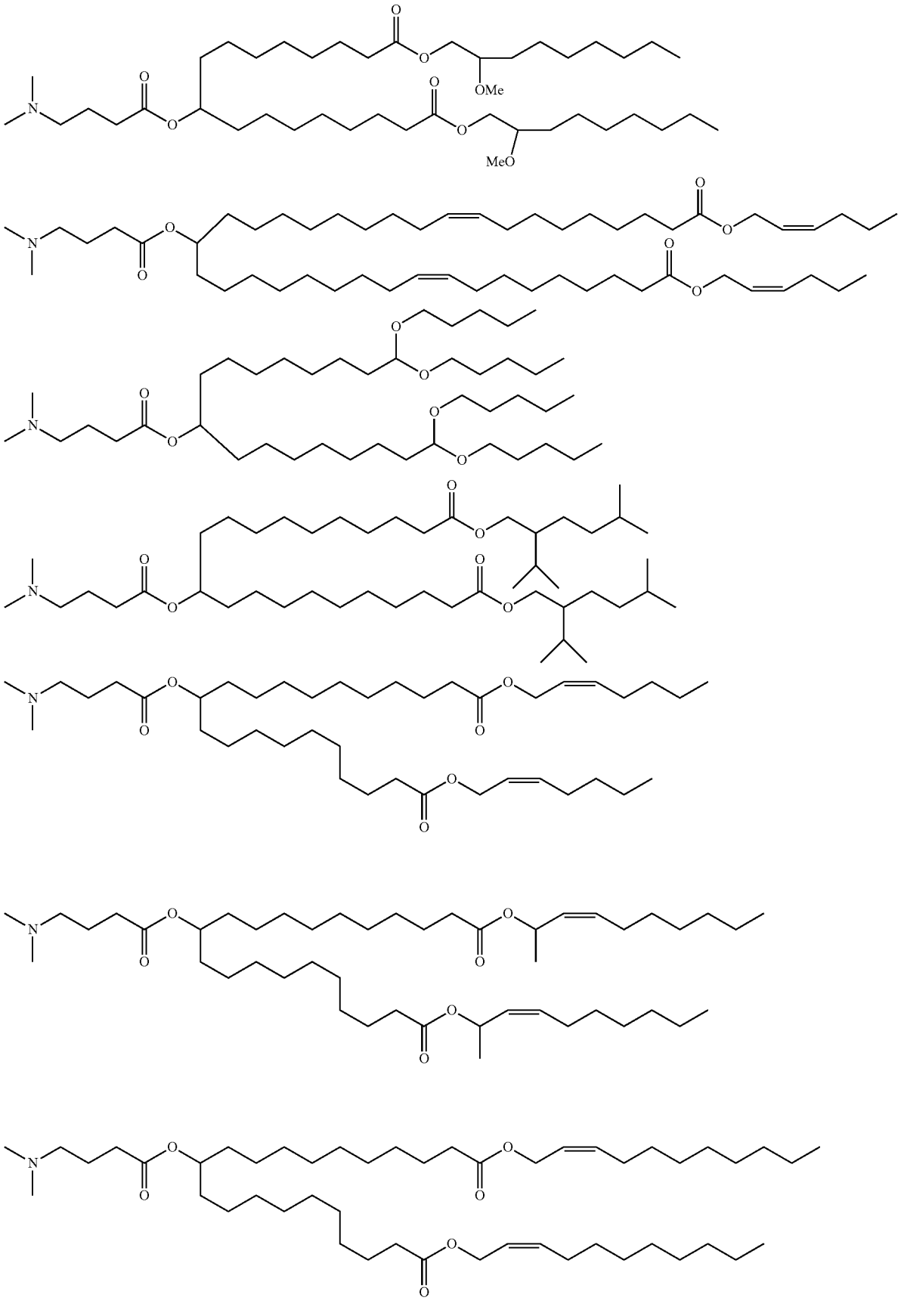

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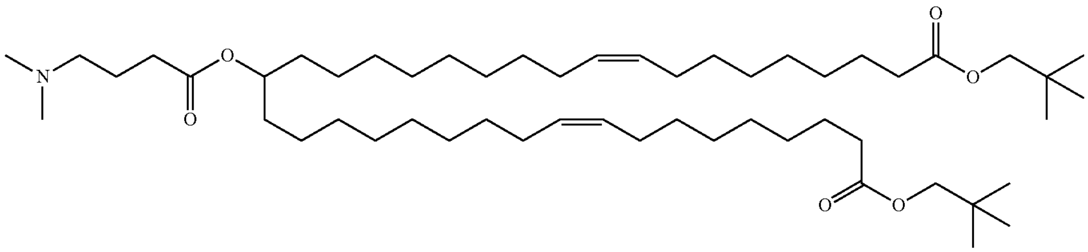

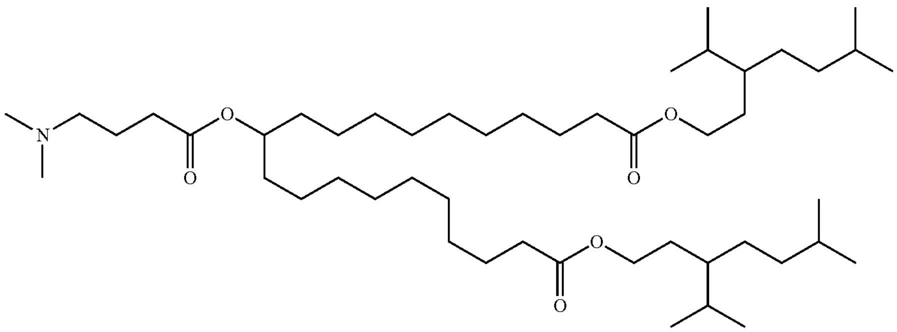

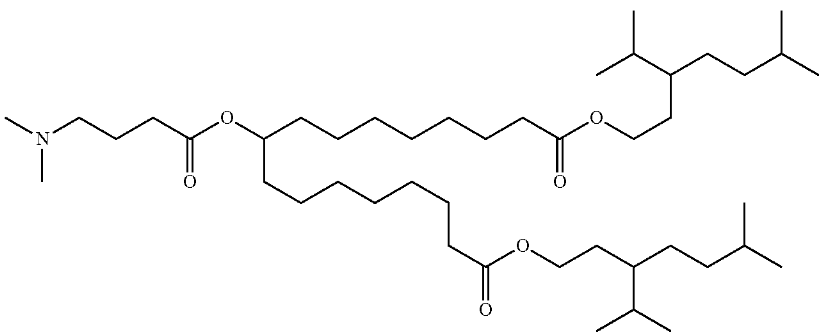

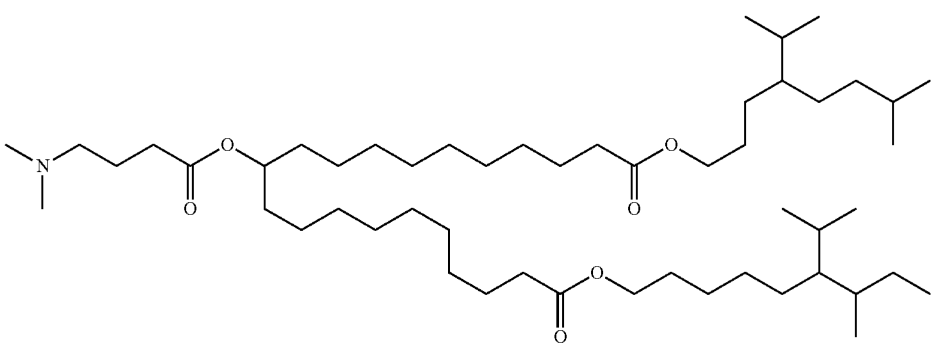

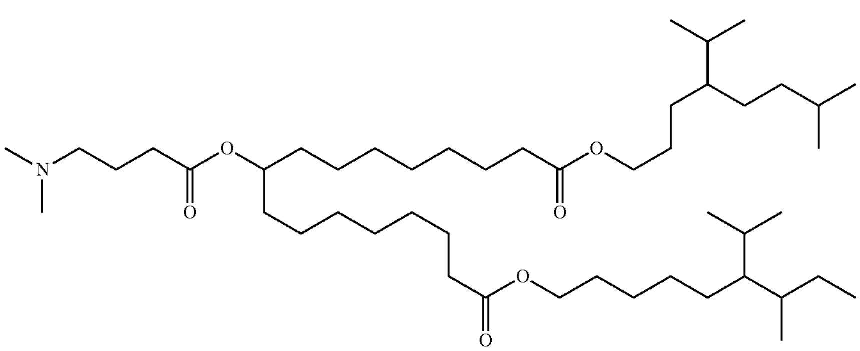

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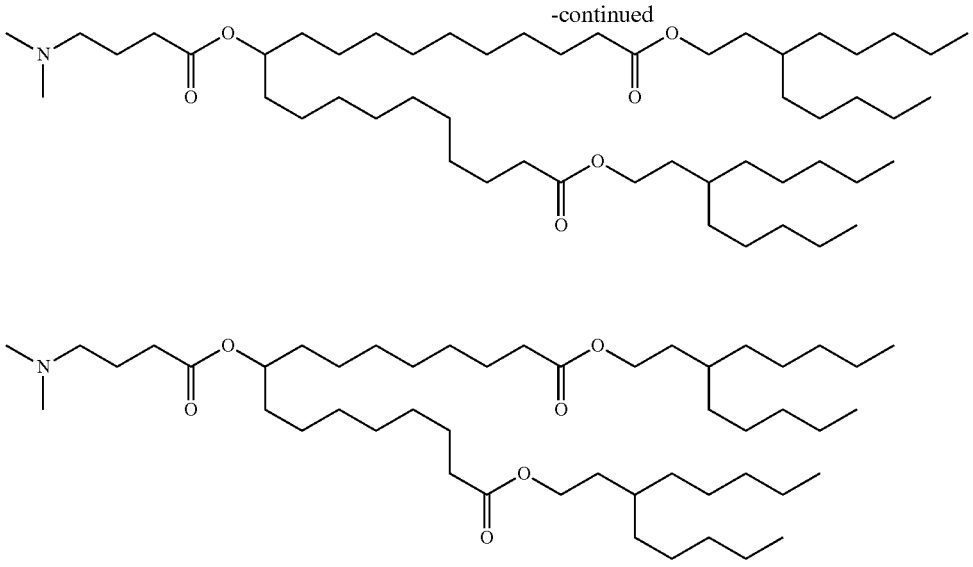
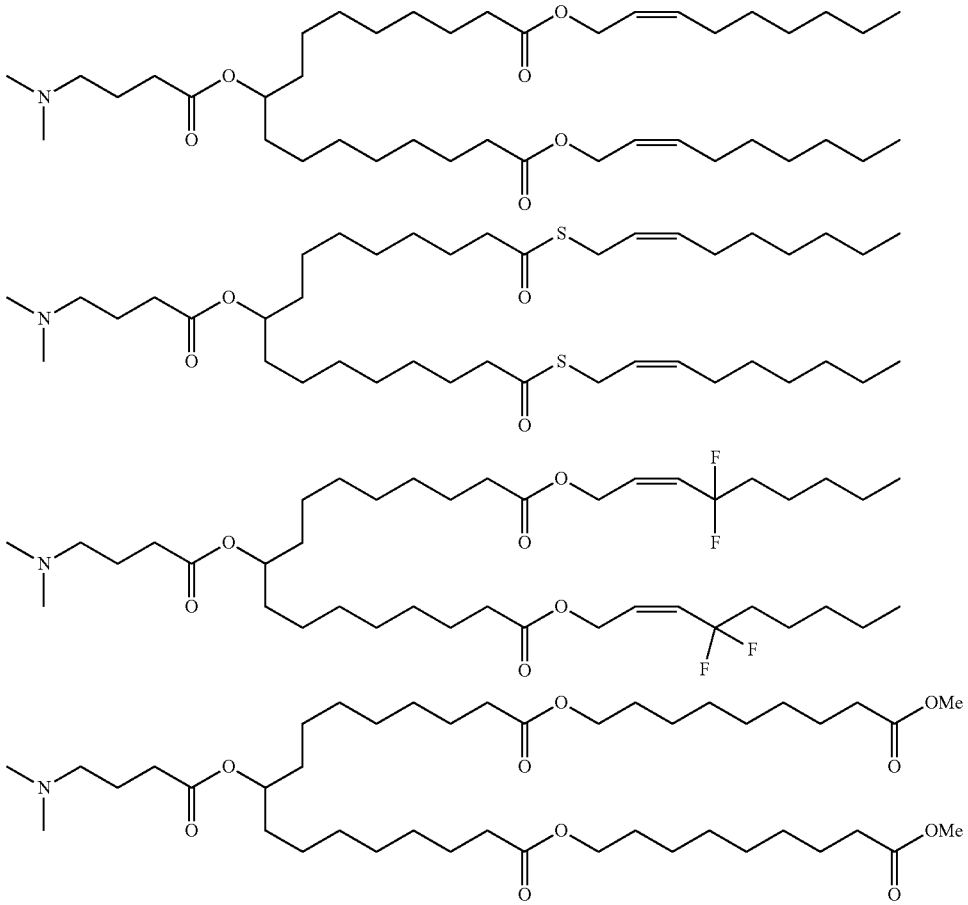


In another embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof):



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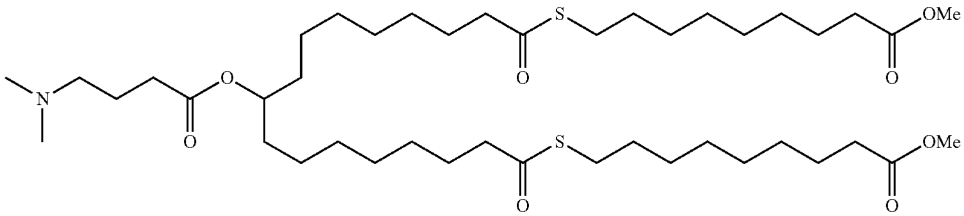

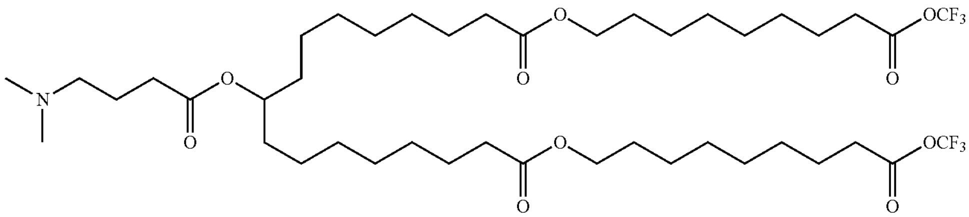

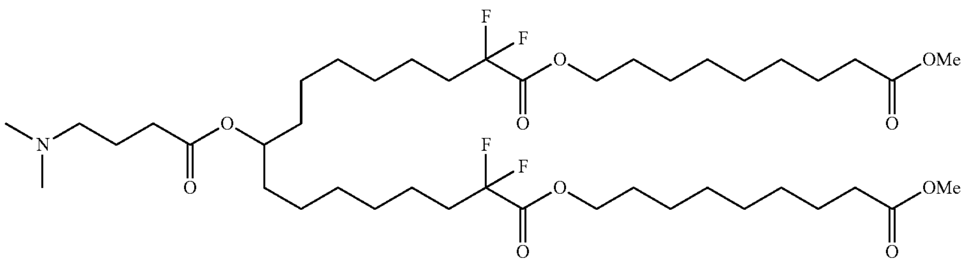

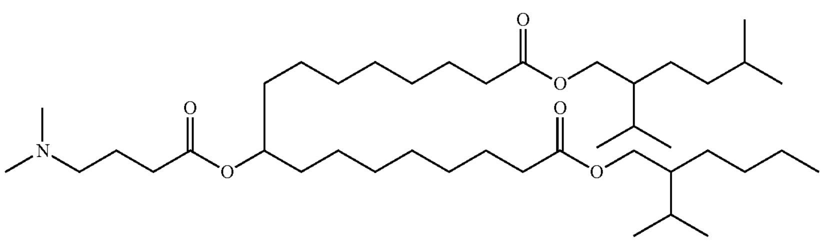

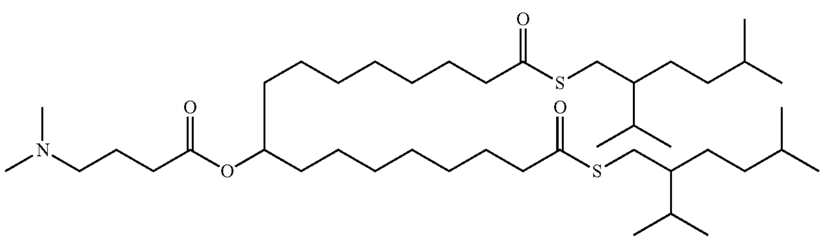

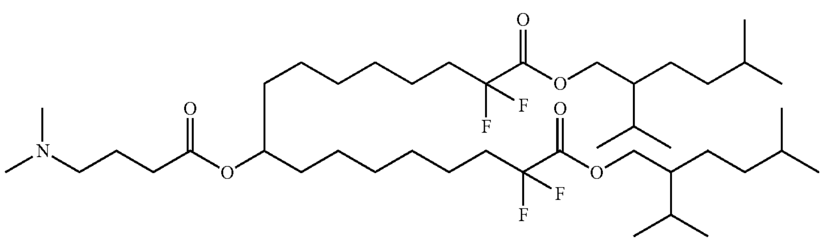

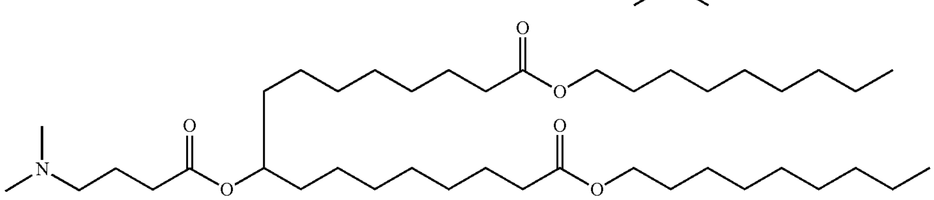

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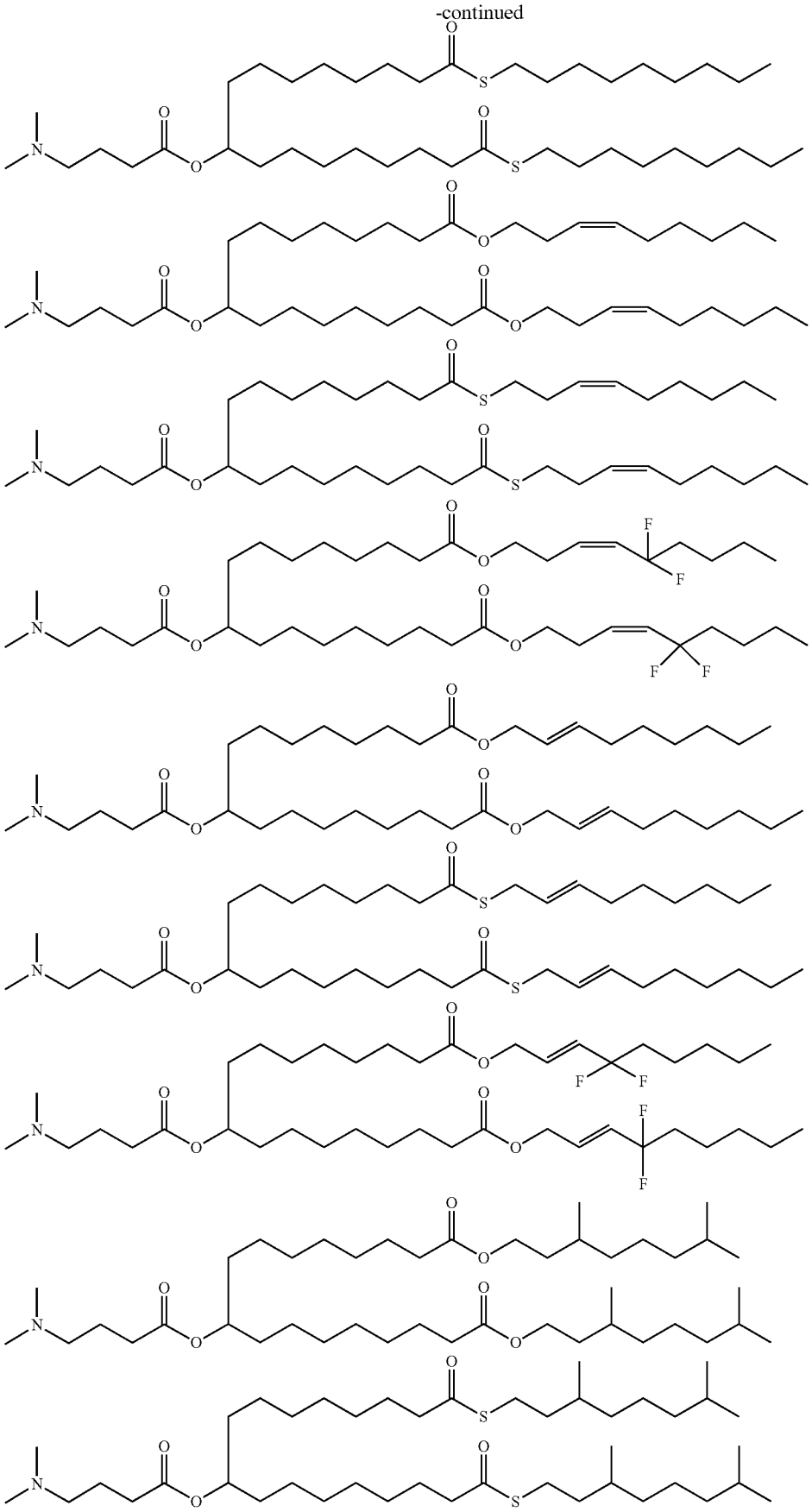

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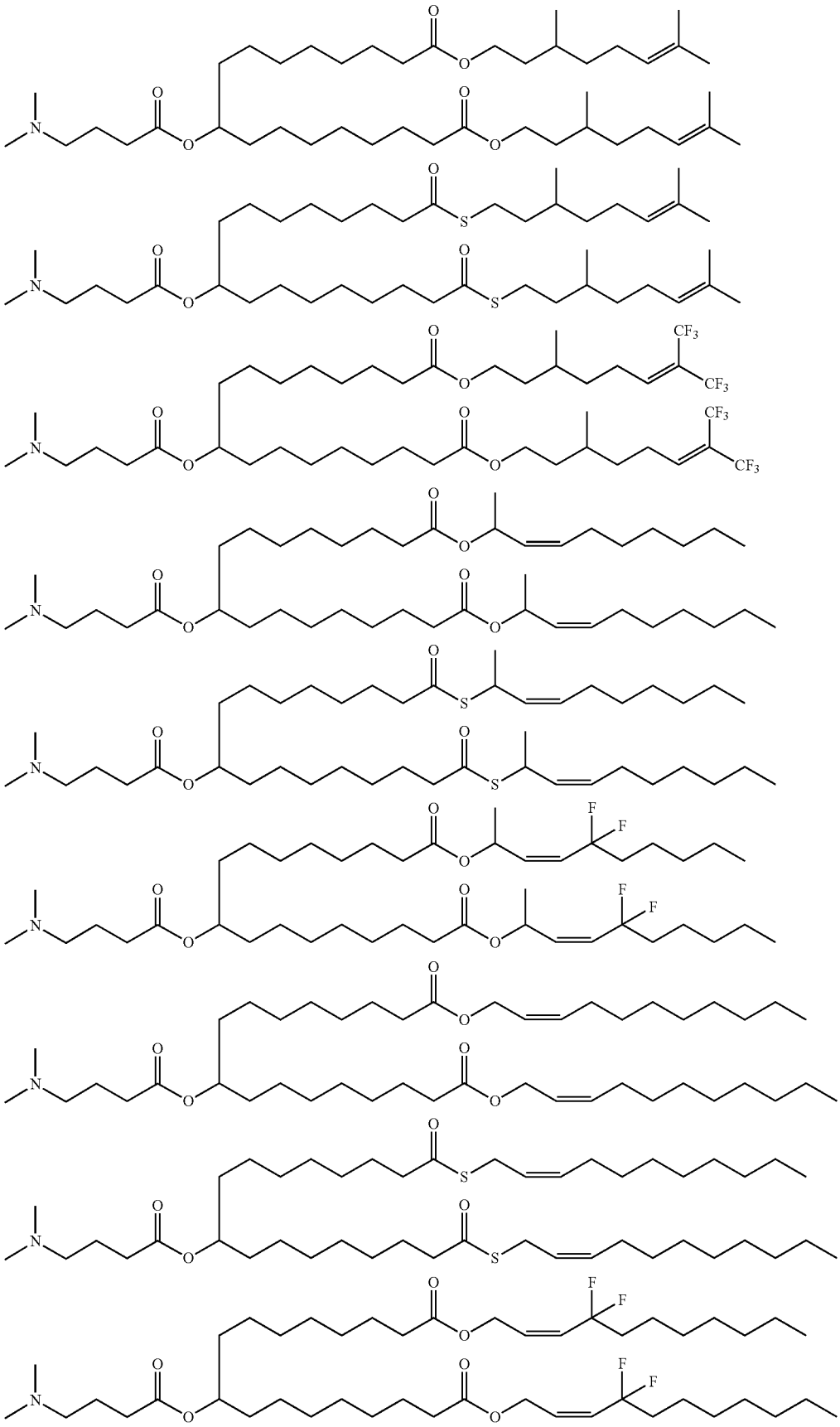

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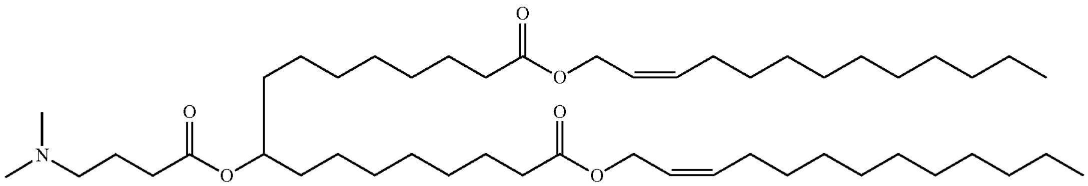

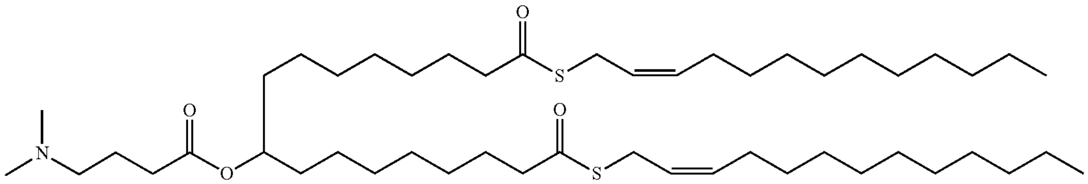

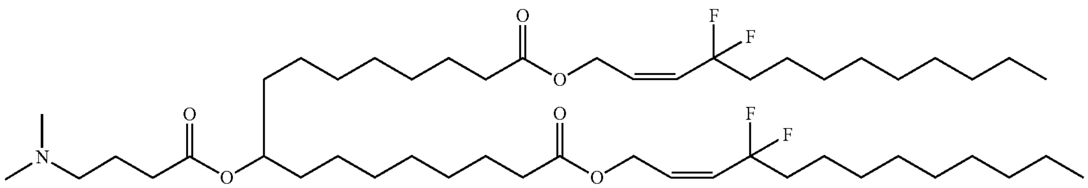

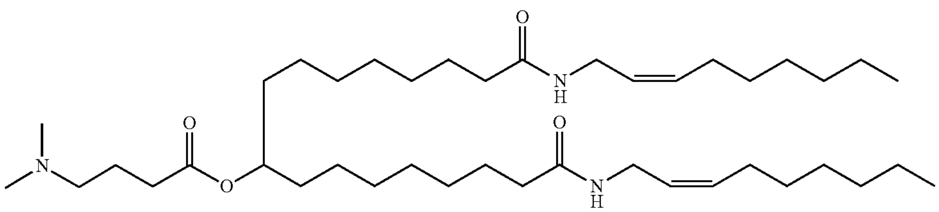

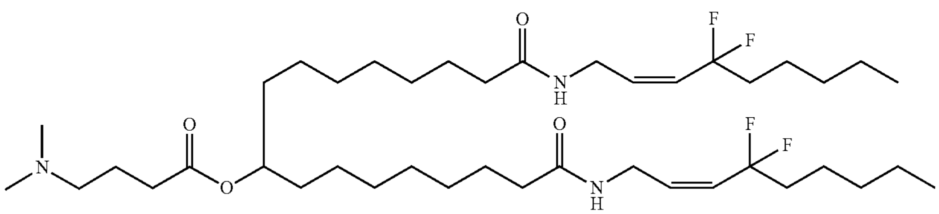

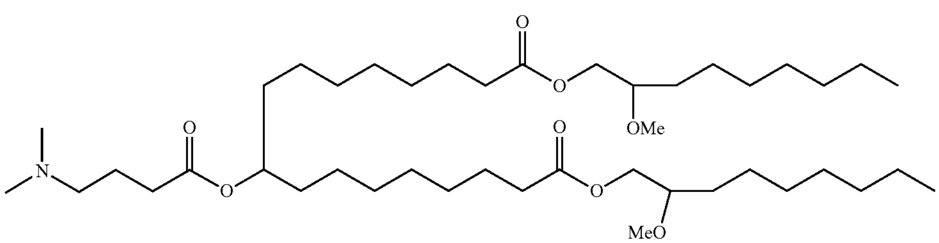

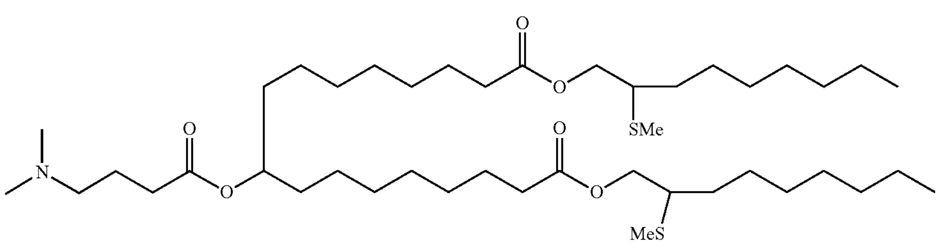

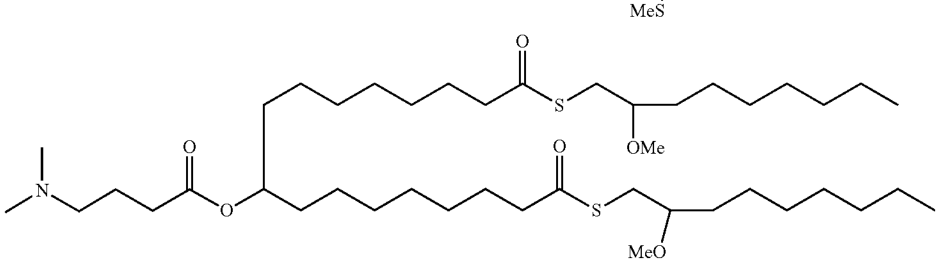

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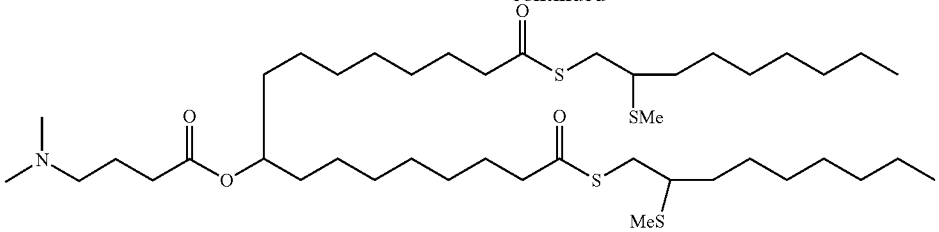
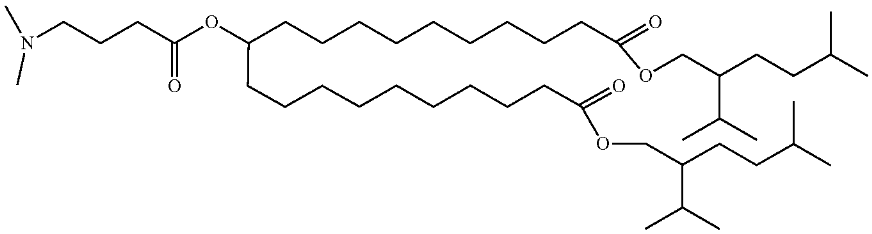
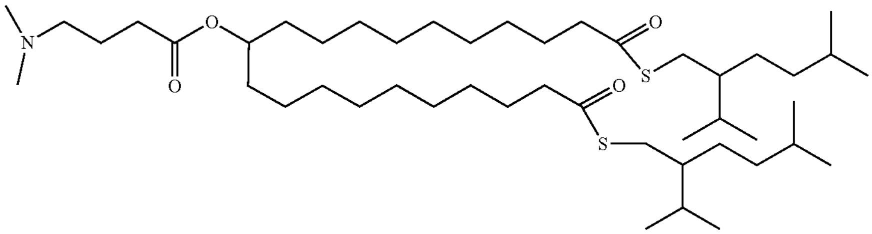
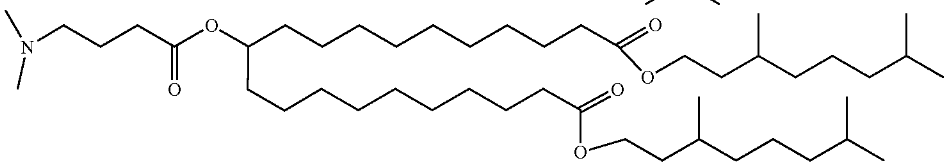
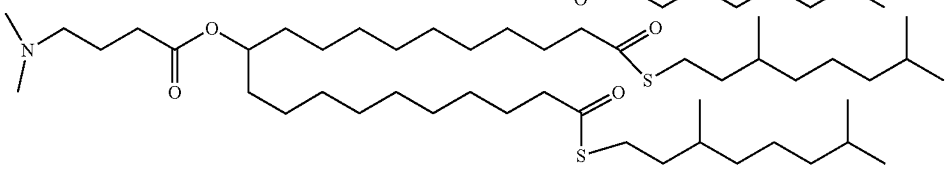
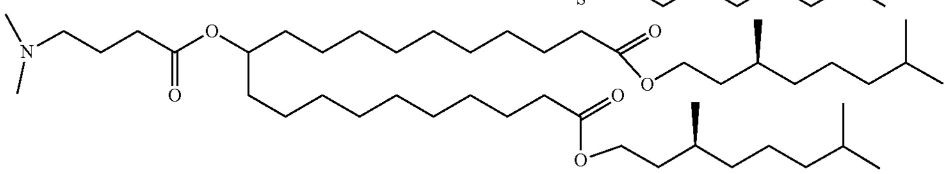


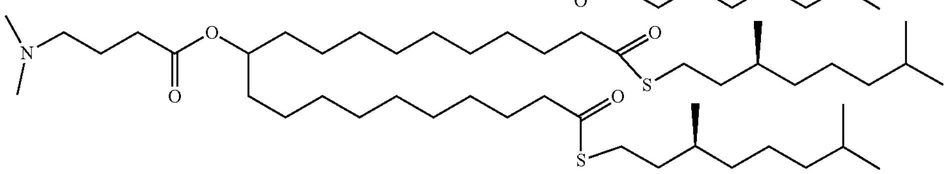




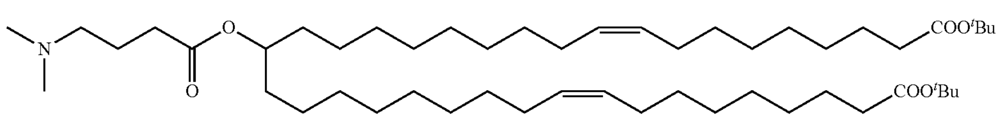








In another embodiment, the cationic lipid of the present invention is selected from the following compounds, and salts thereof (including pharmaceutically acceptable salts thereof):



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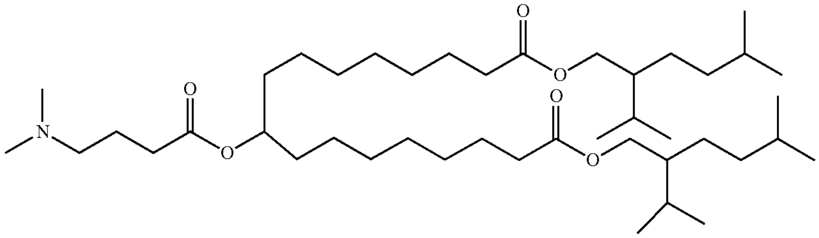
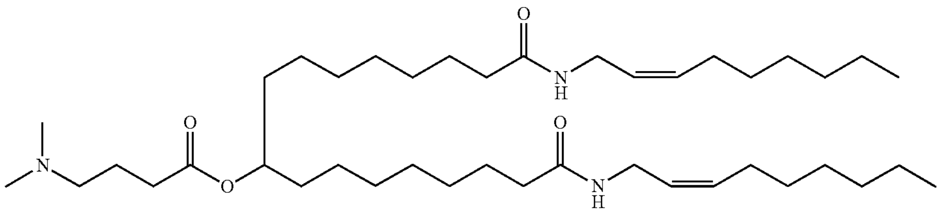
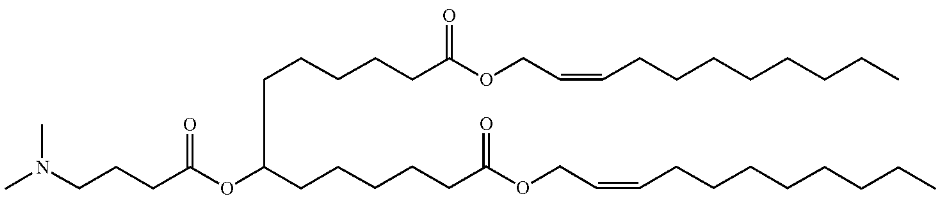
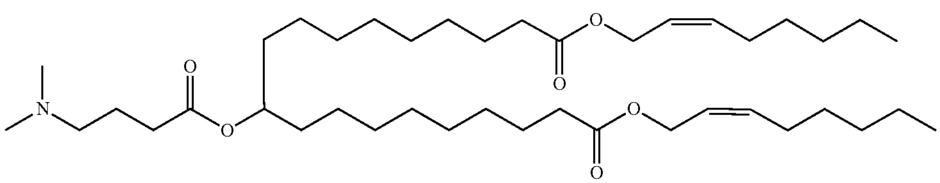
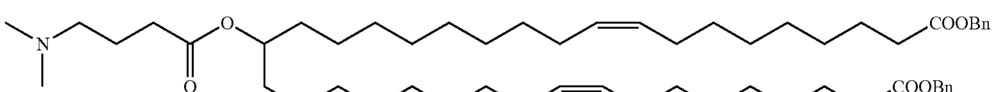
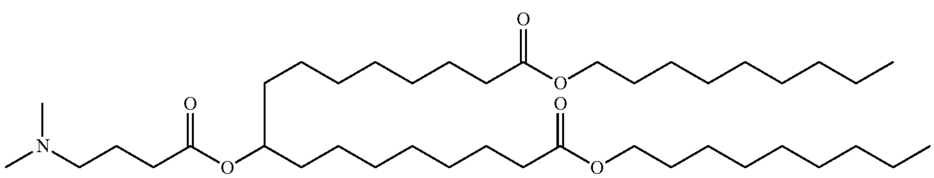


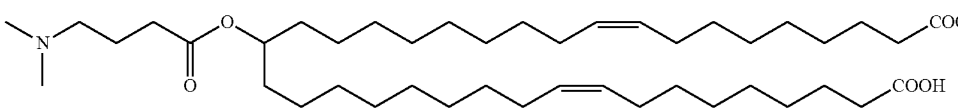


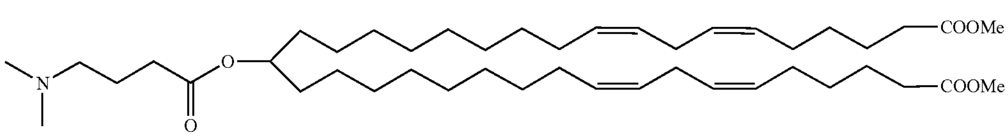


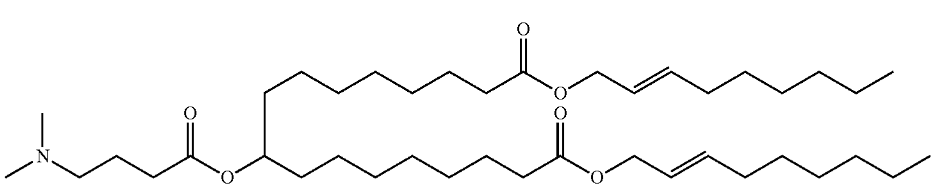




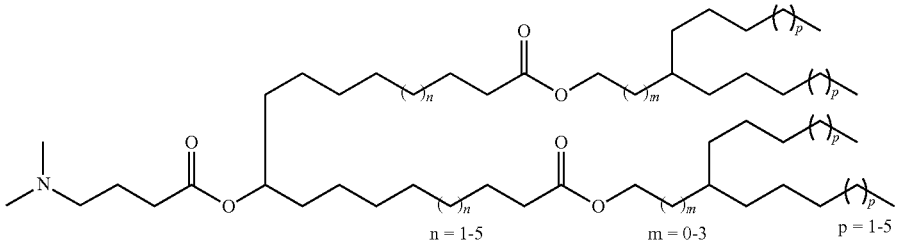


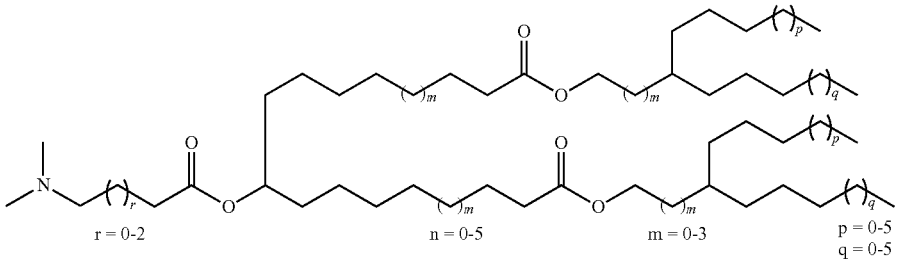


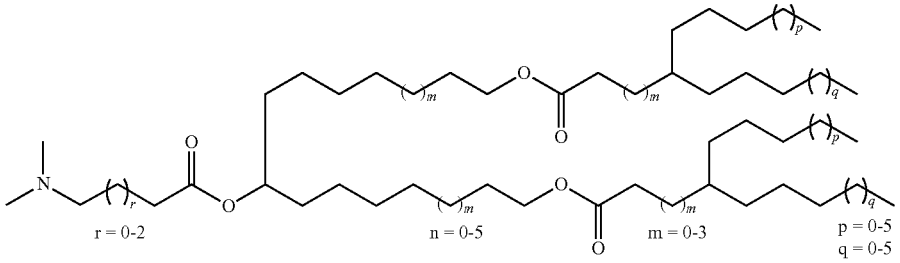


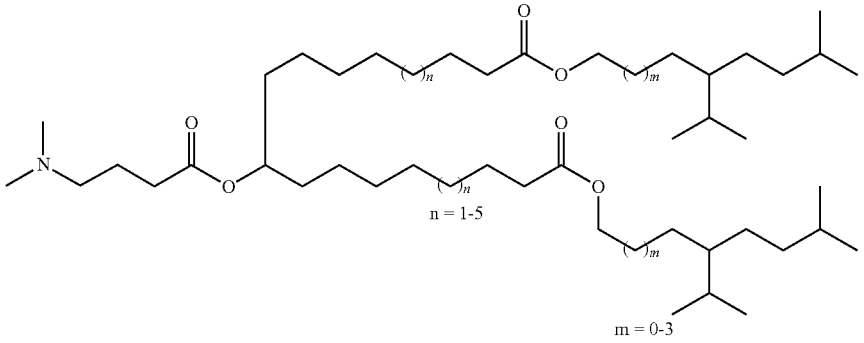


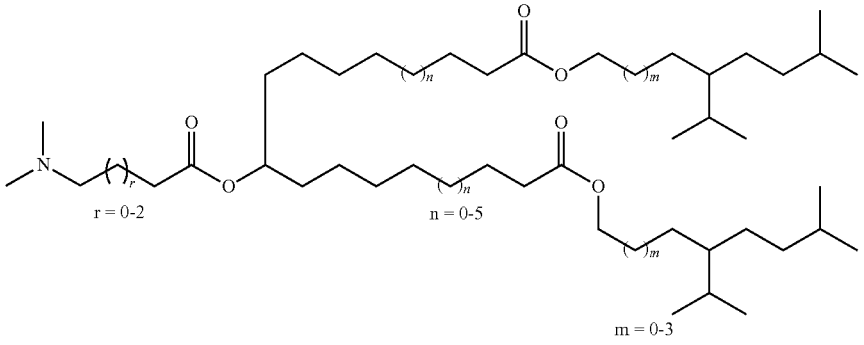
Additional representative cationic lipids include, but are not limited to:











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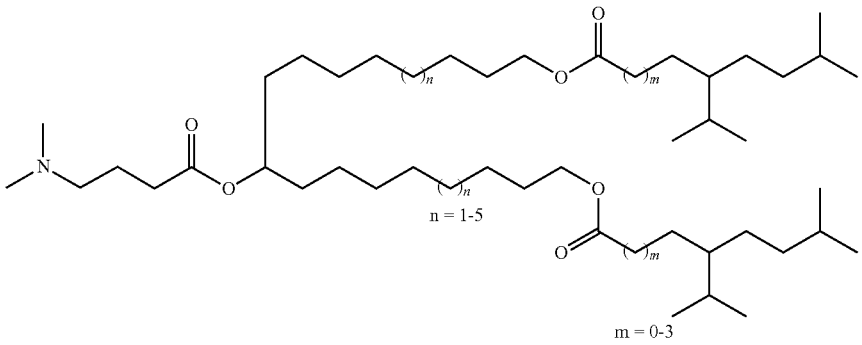
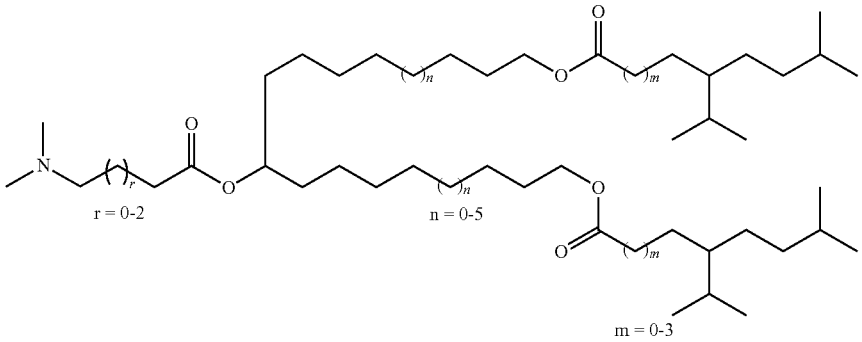
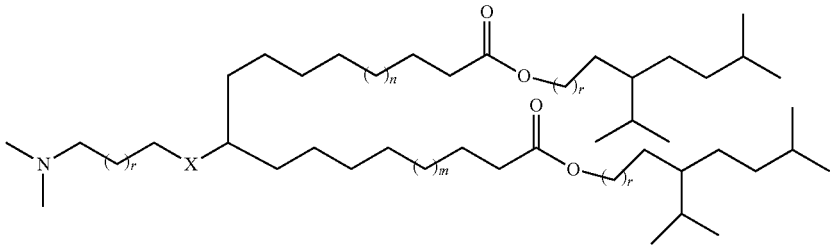
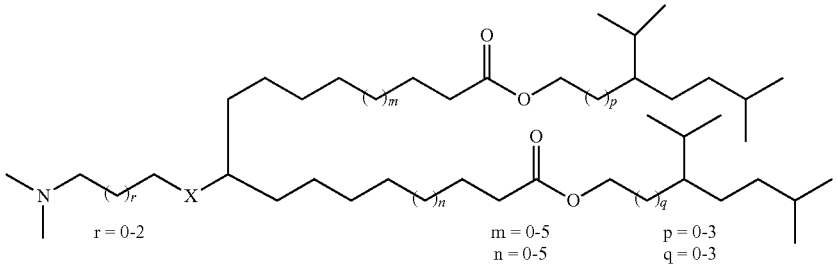
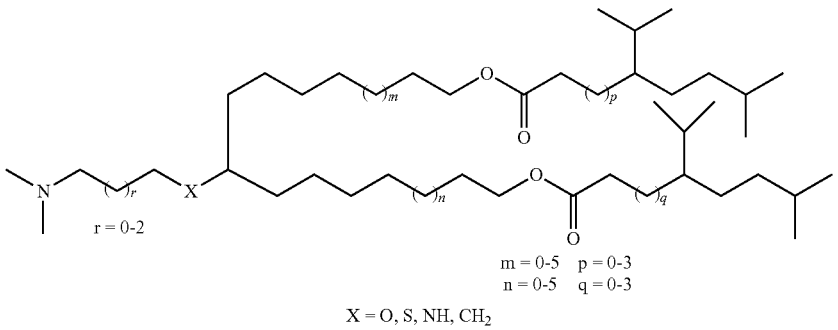

n = 1-5
m = 0-3


r = 0-2
n = 0-5
m = 0-3


X = O, S, NH, CH2
r = 0-2, N = 1-5, and m = 1-5


r = 0-2
m = 0-5
n = 0-5
p = 0-3
q = 0-3
X = O, S, NH, CH2


r = 0-2
m = 0-5 p = 0-3
n = 0-5 q = 0-3
X = O, S, NH, CH2

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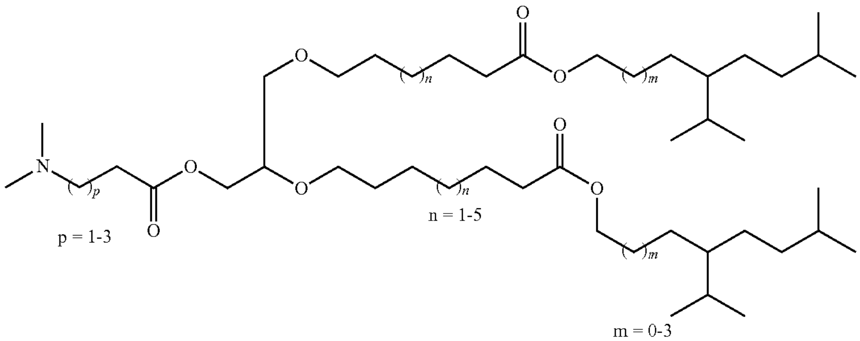
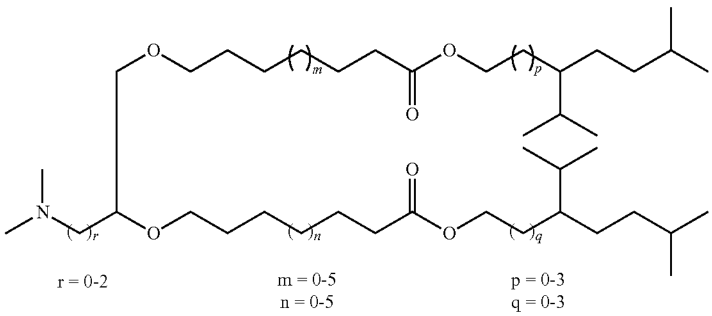
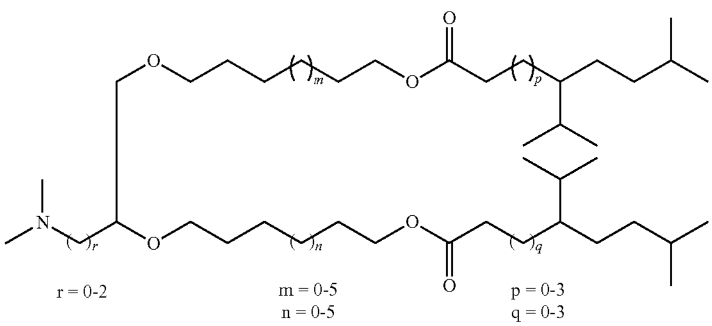
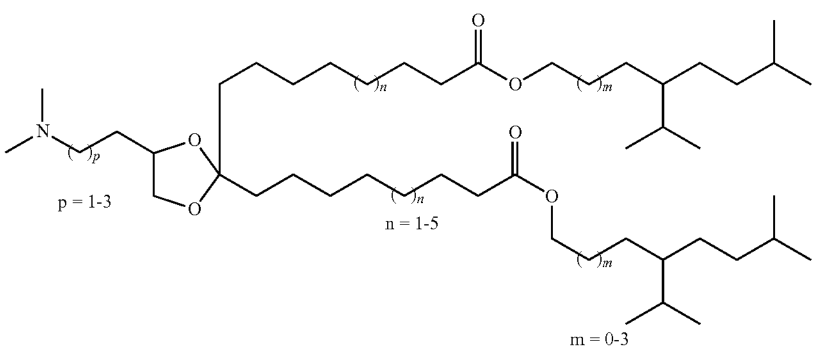
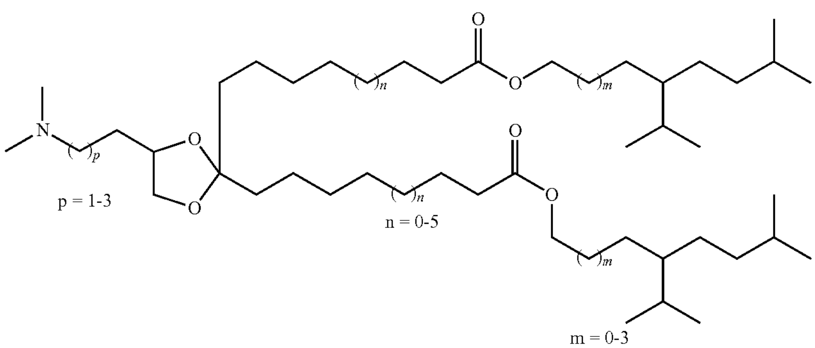

p = 1-3
n = 1-5
m = 0-3


r = 0-2
m = 0-5
n = 0-5
p = 0-3
q = 0-3


r = 0-2
m = 0-5
n = 0-5
p = 0-3
q = 0-3


p = 1-3
n = 1-5
m = 0-3


p = 1-3
n = 0-5
m = 0-3

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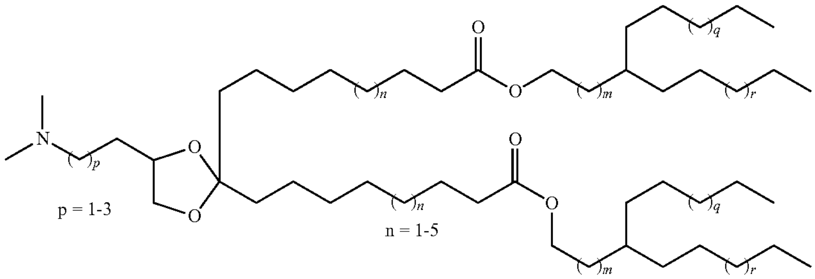
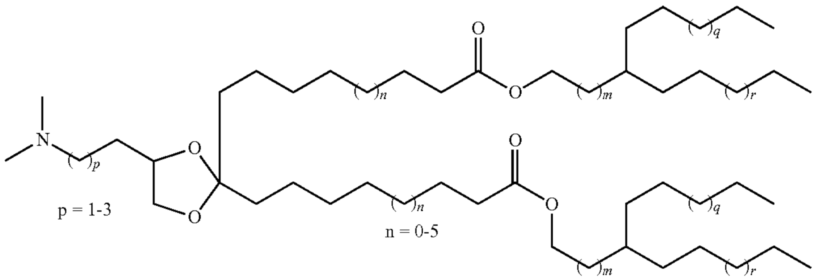


p = 1-3

n = 1-5

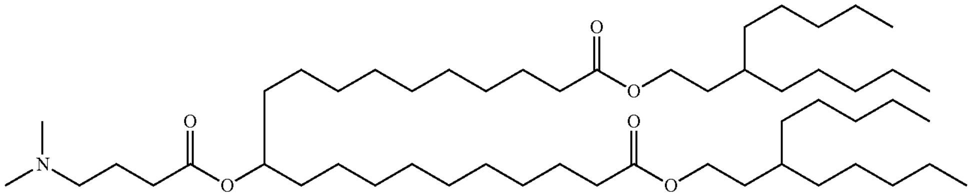
m = 0-3; q = 0-4; r = 0-4

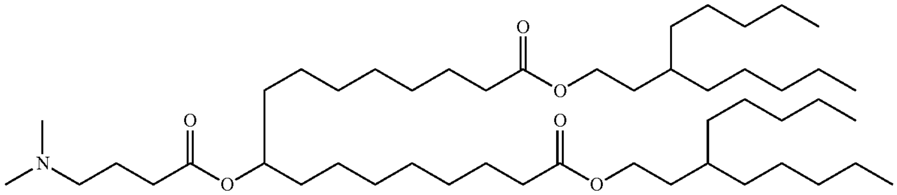


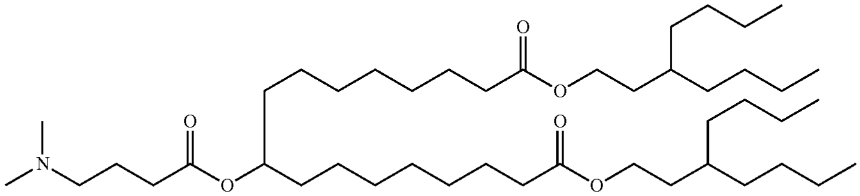
p = 1-3

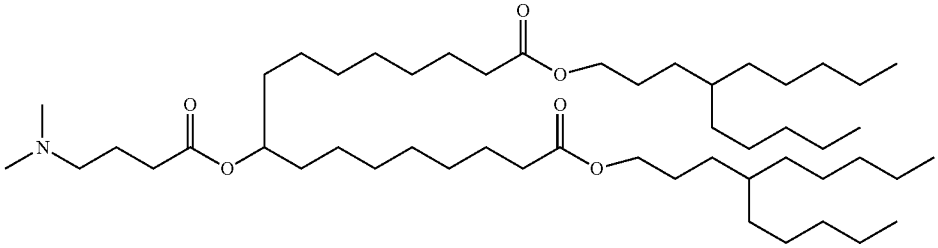
n = 0-5

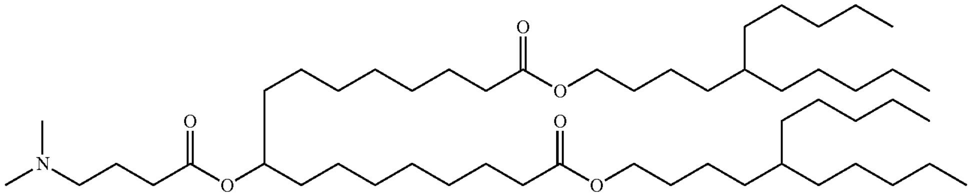
m = 0-3; q = 0-4; r = 0-4











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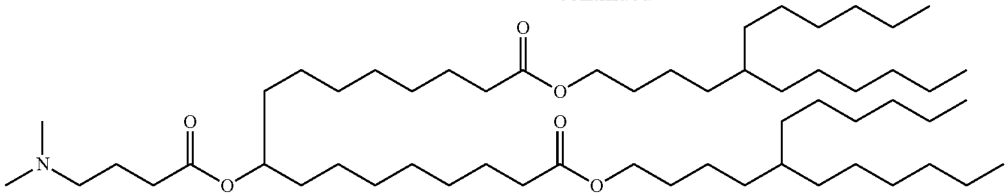

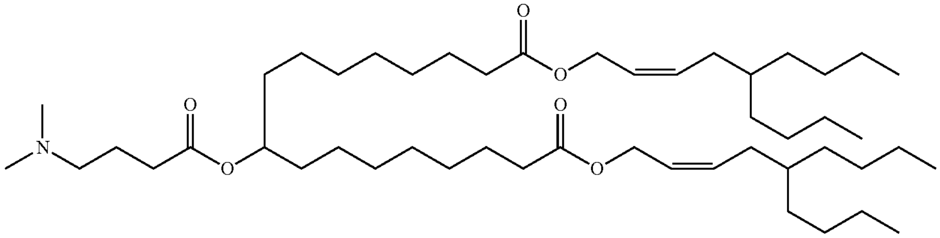

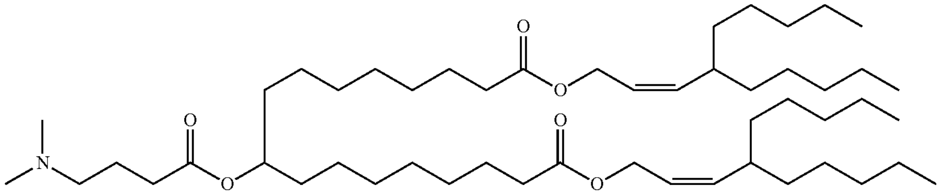

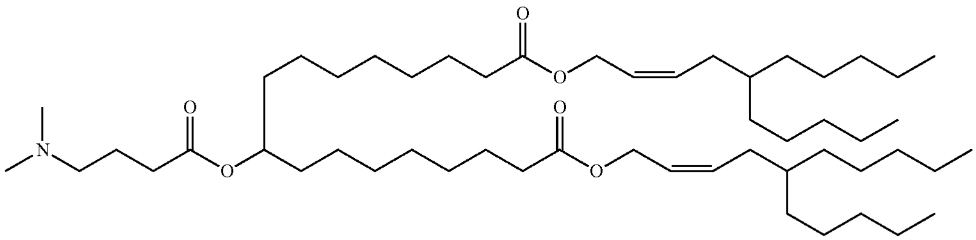

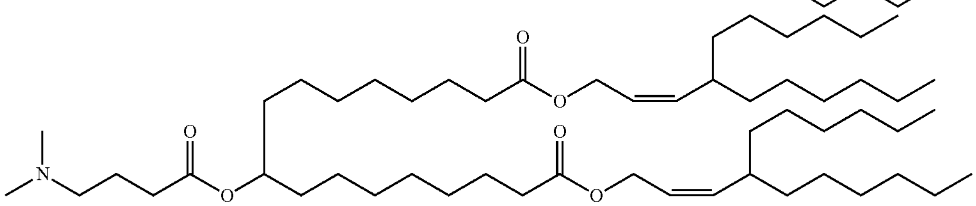

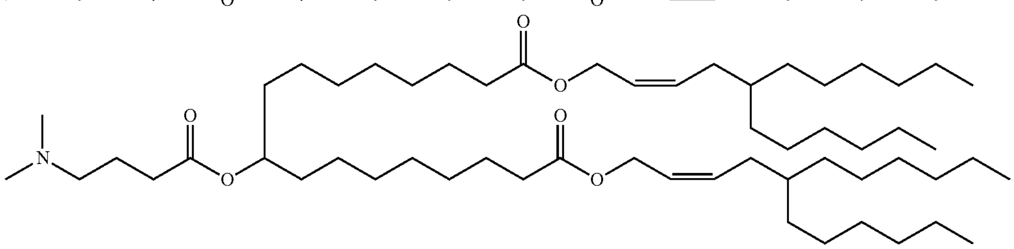

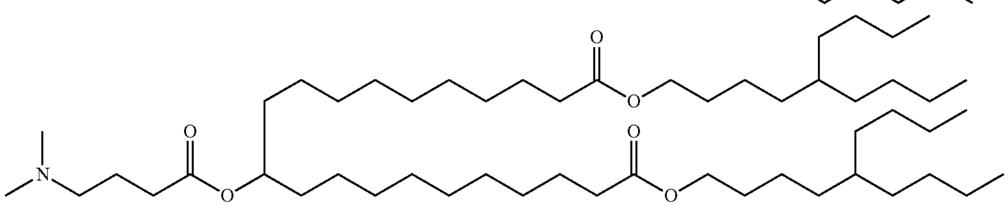

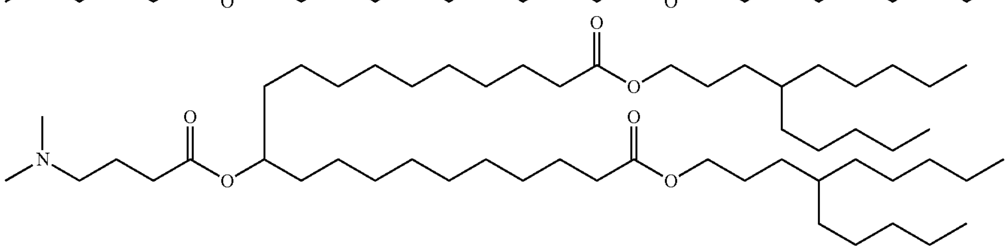

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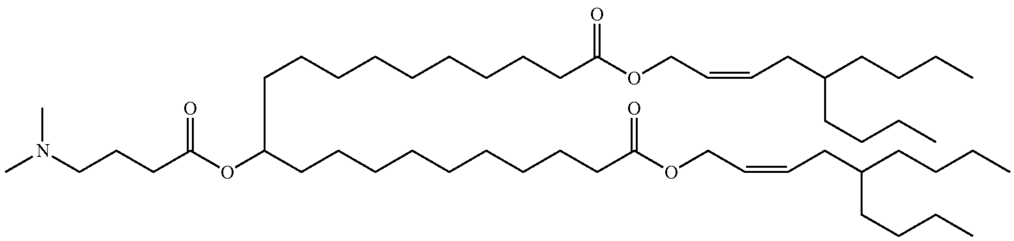
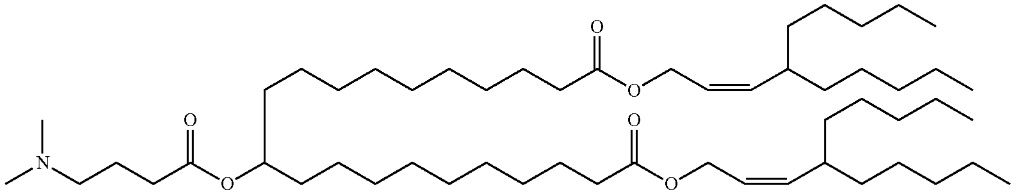
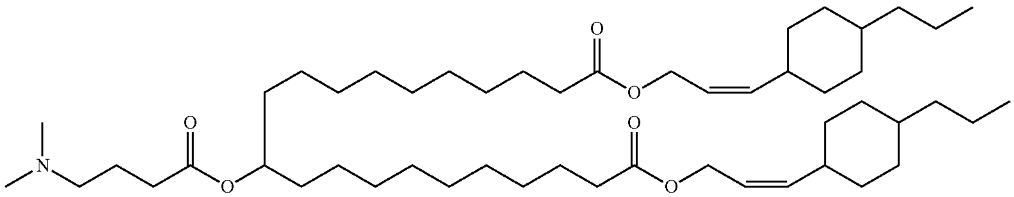
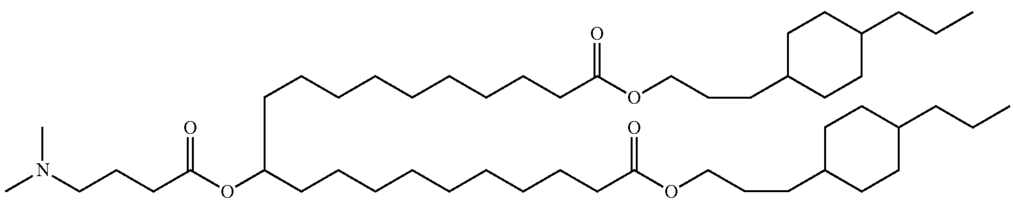
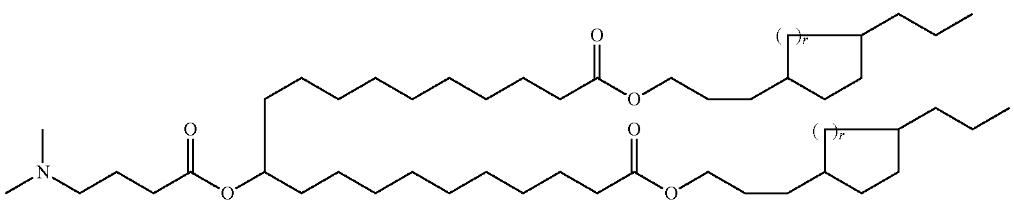


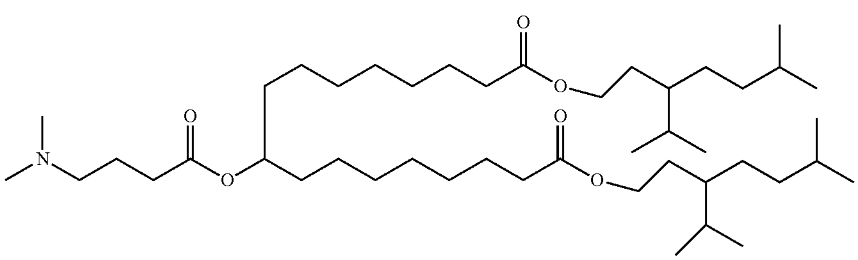








r = 0, 1, or 2



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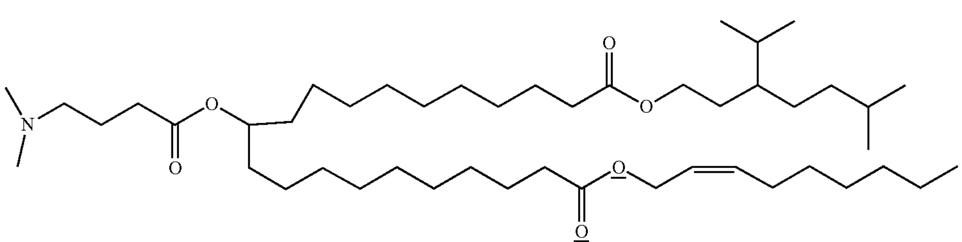

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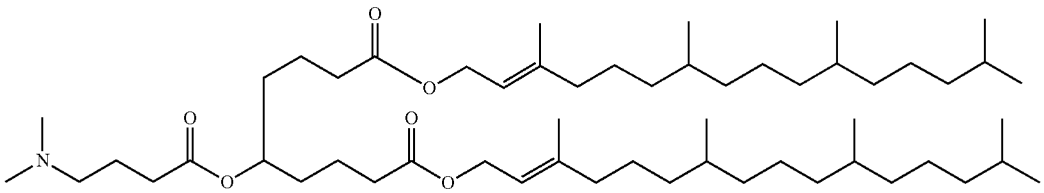
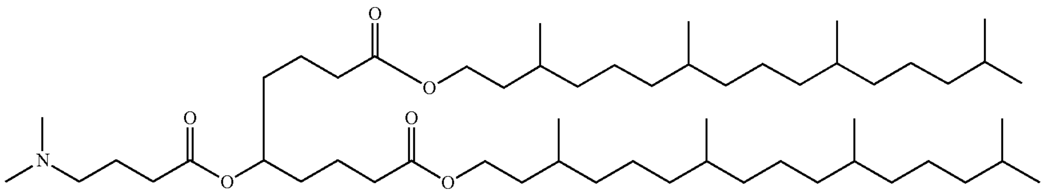
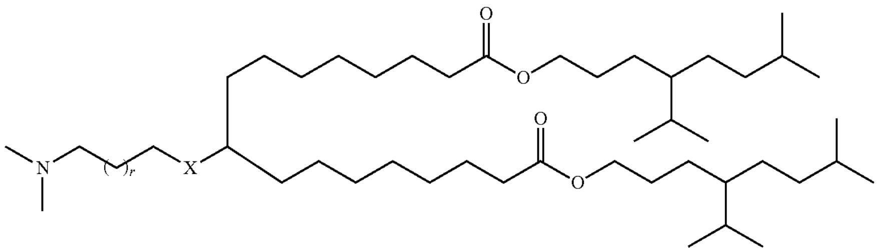
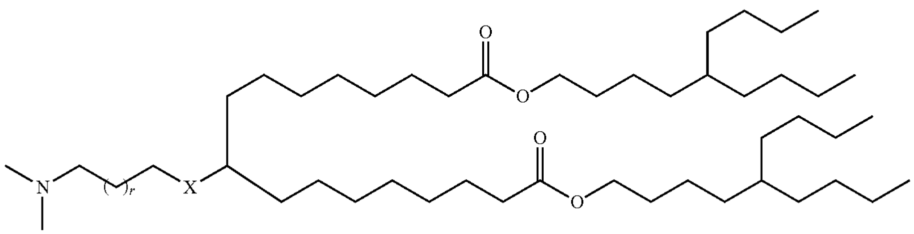


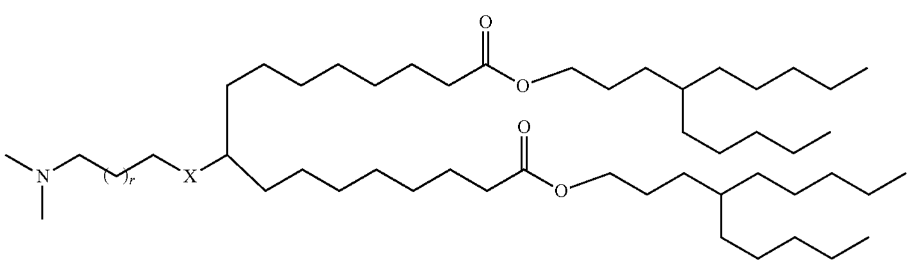




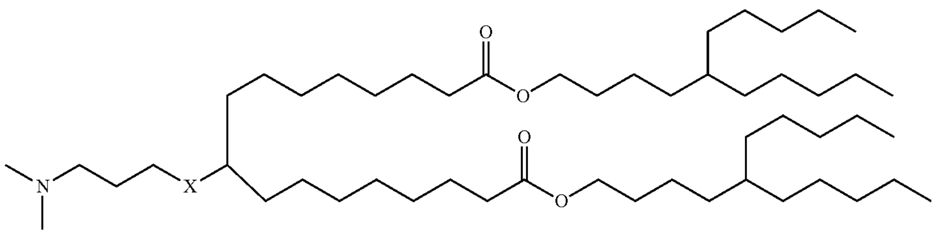
X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2

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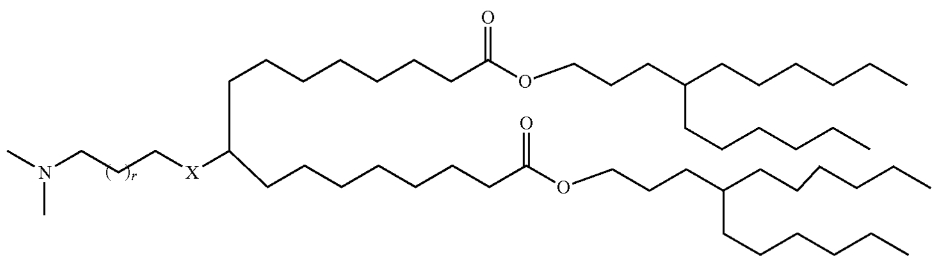
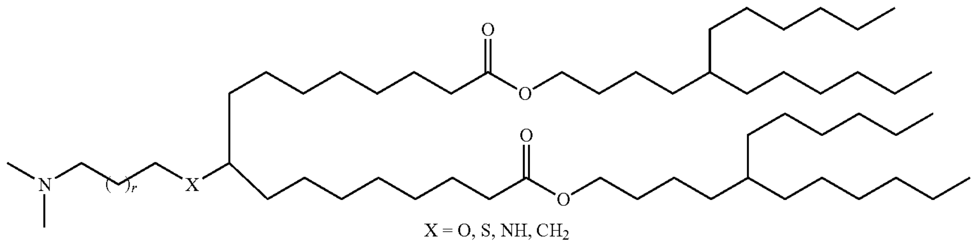
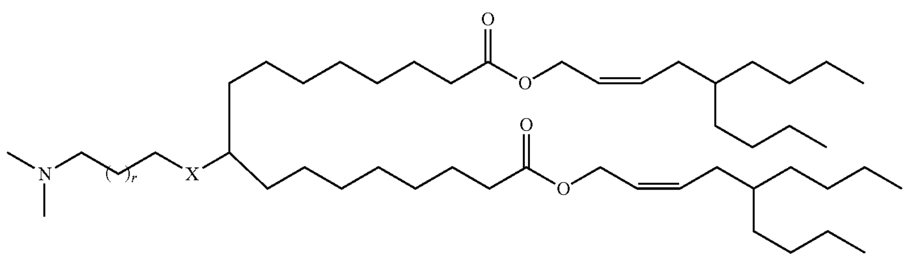


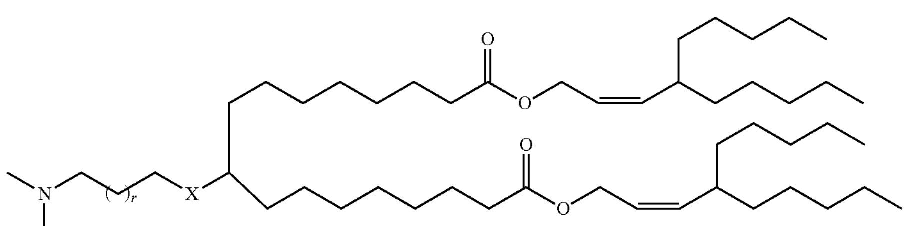
X = O, S, NR, CH_2
r = 0, 1, or 2



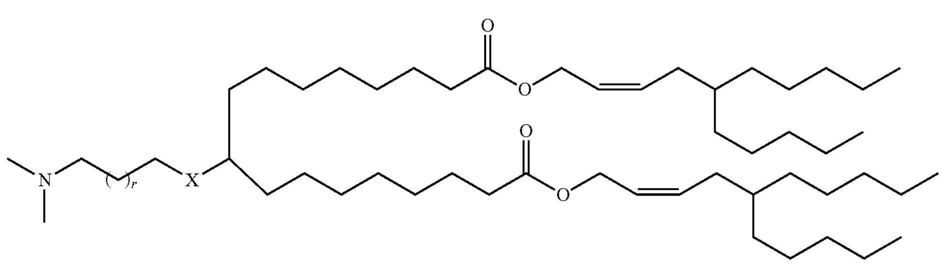
X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2

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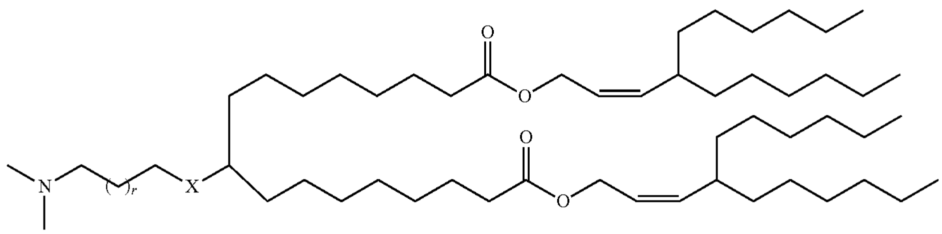
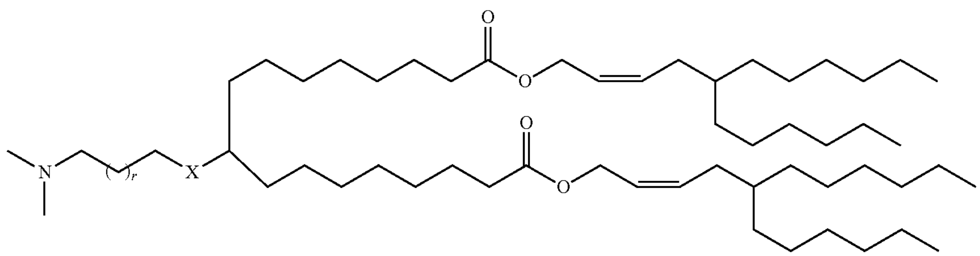
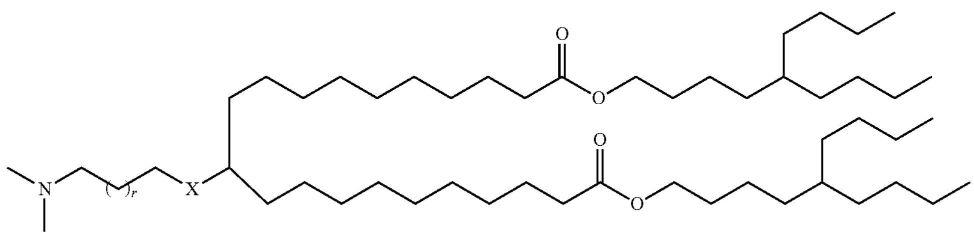


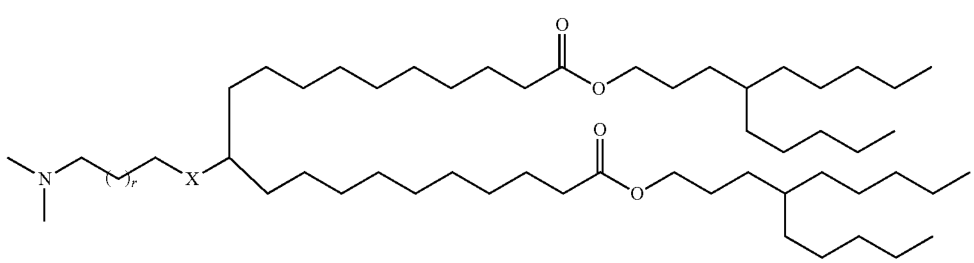
X = O, S, NH, CH_2
r = 0, 1, or 2



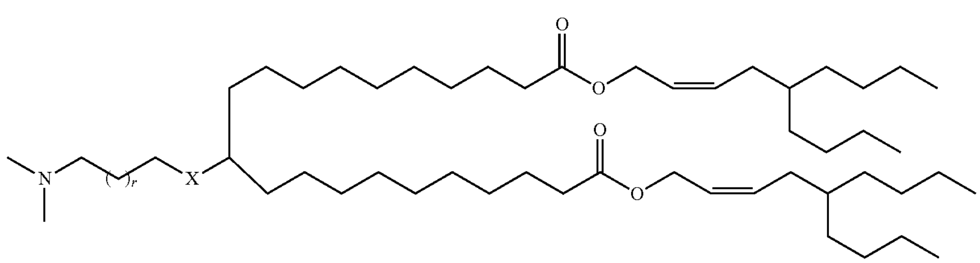
X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2



X = O, S, NH, CH_2
r = 0, 1, or 2

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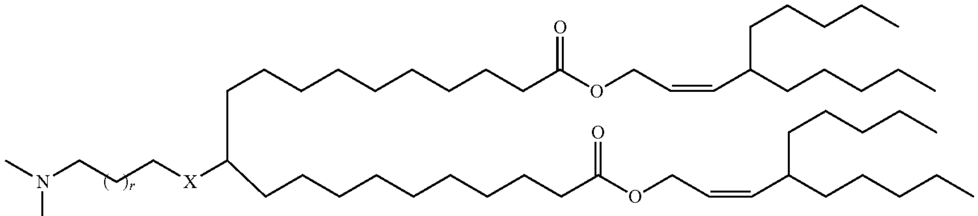
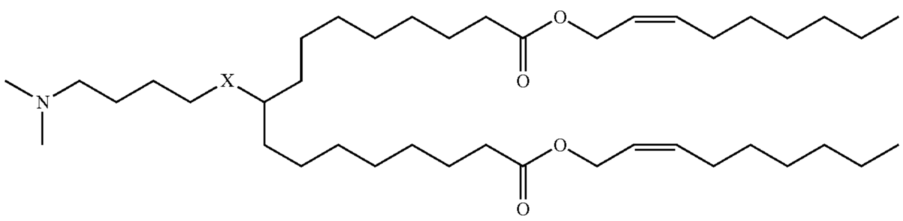


X = O, S, NH, CH_2
r = 0, 1, or 2



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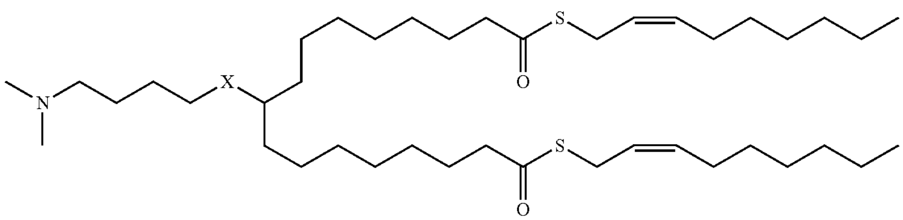
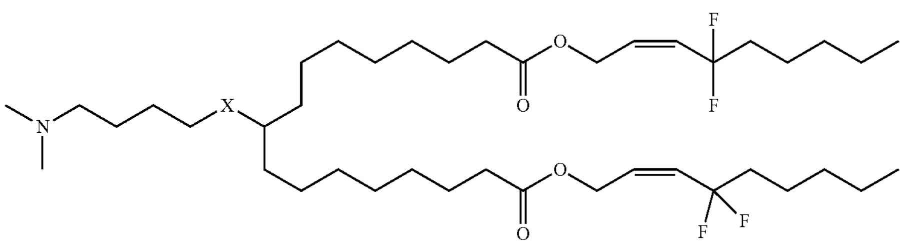


X = O, S, NH, CH_2



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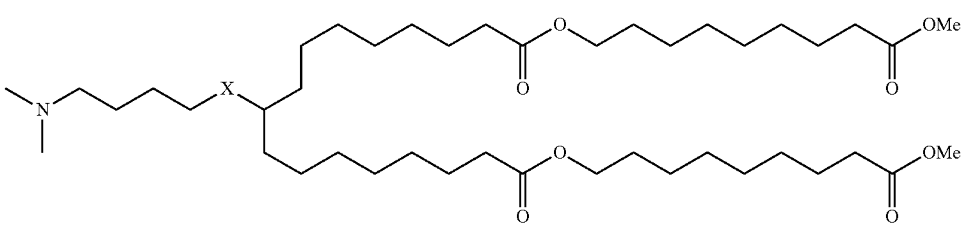

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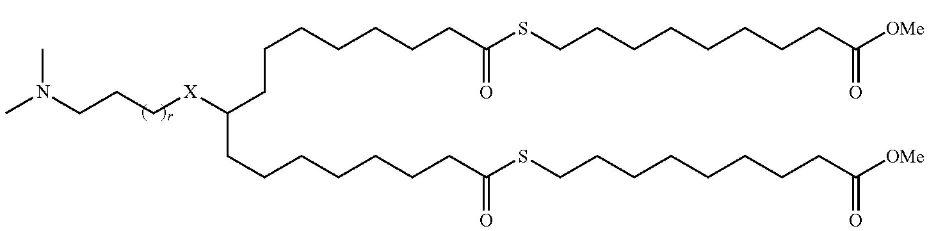

X = O, S, NH, CH_2
r = 0, 1, or 2

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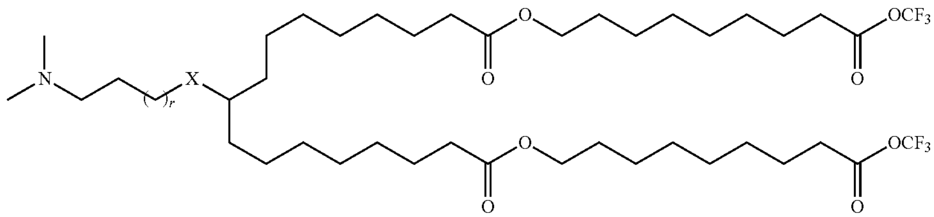
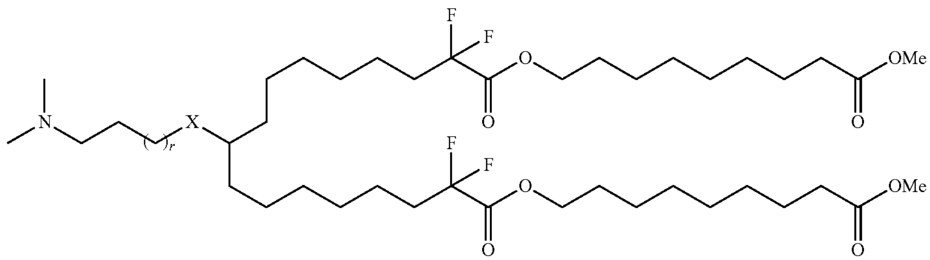
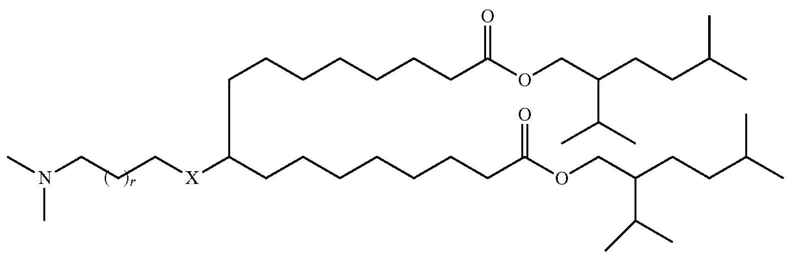


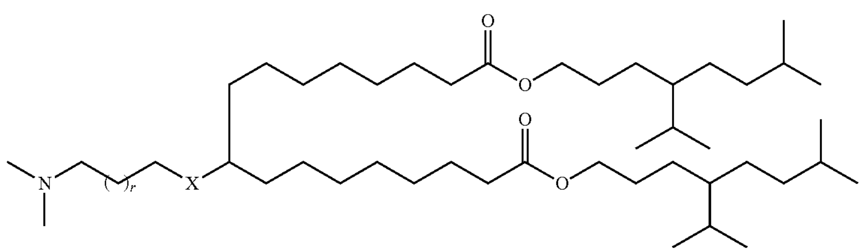
X = O, S, NR, CH_2
r = 0, 1, or 2



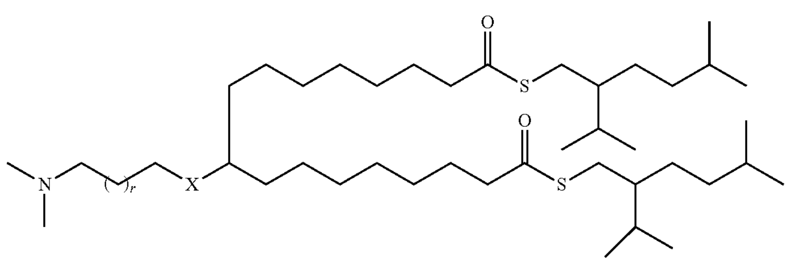
X = O, S, NR, CH_2
r = 0, 1, or 2



X = O, S, NR, CH_2
r = 0, 1, or 2



X = O, S, NR, CH_2
r = 0, 1, or 2



X = O, S, NR, CH_2
r = 0, 1, or 2

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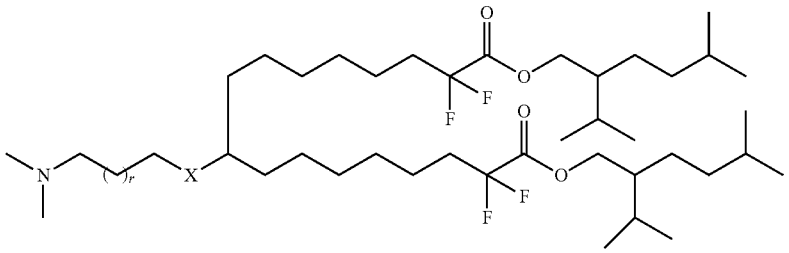
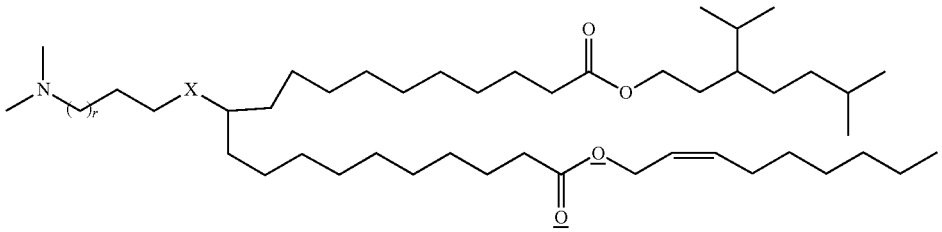
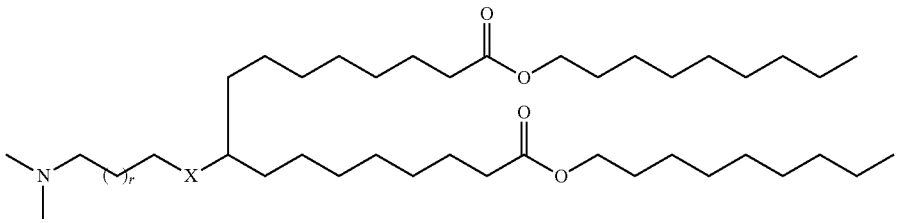


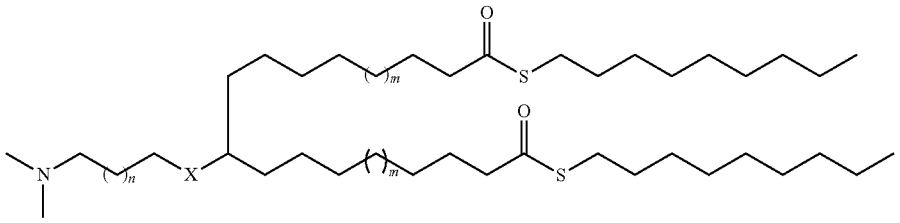
r = 0, 1, or 2



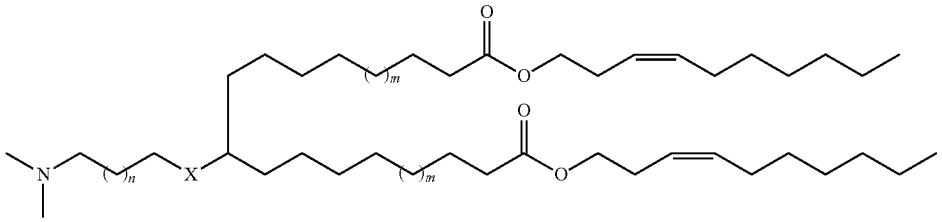
X = O, S, NR, CH_2
r = 0, 1, or 2



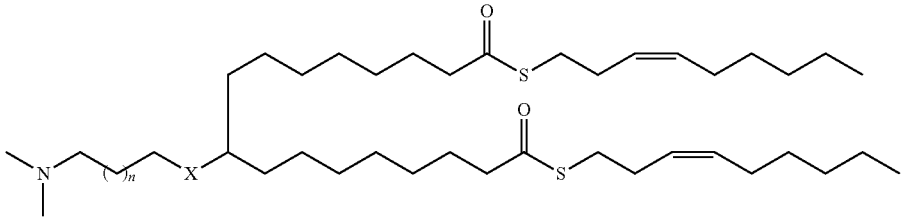
X = O, S, NR, CH_2
r = 0, 1, or 2



m = 0-5, n = 0, 1, or 2



X = O, S, NR, CH_2
m = 0-5, n = 0, 1, or 2



n = 0, 1, or 2

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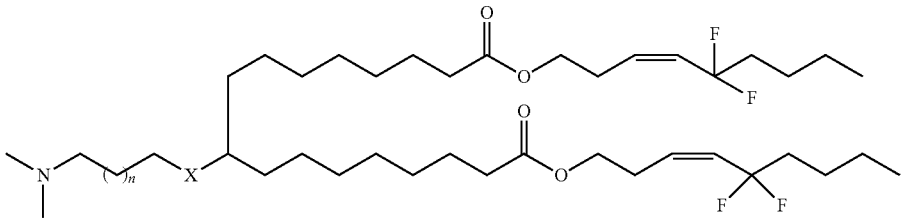
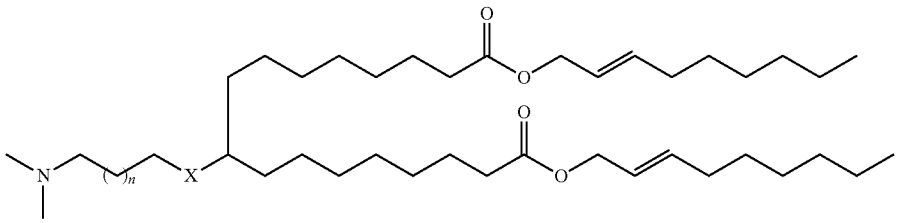
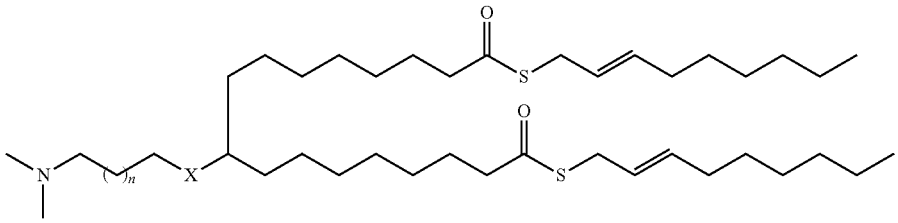


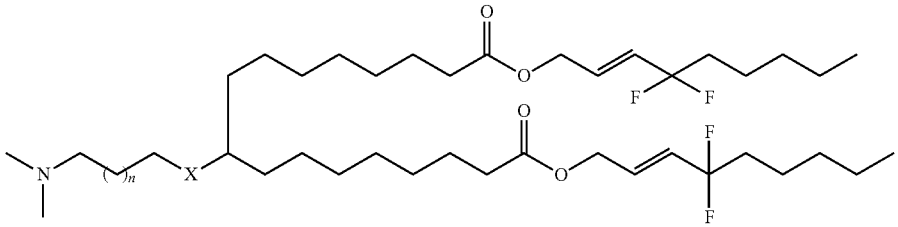
n = 0, 1, or 2



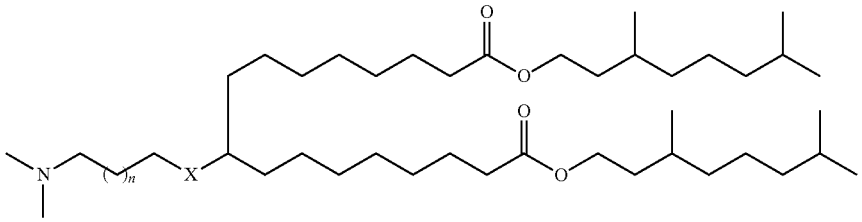
n = 0, 1, or 2



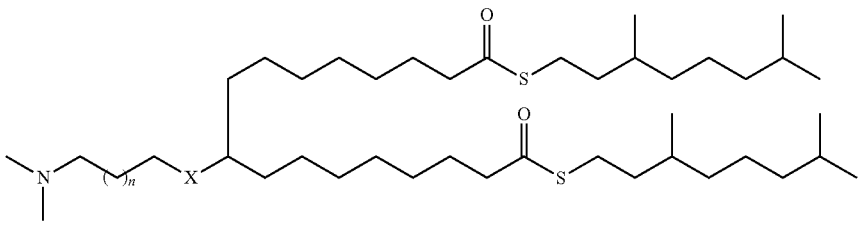
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2

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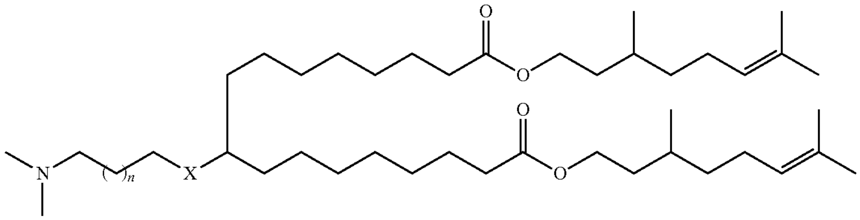
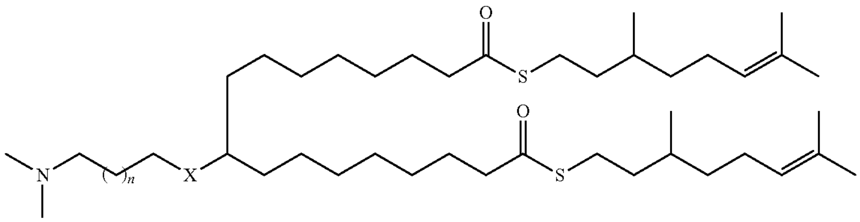
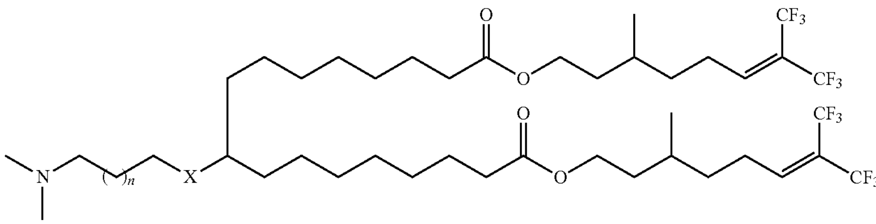


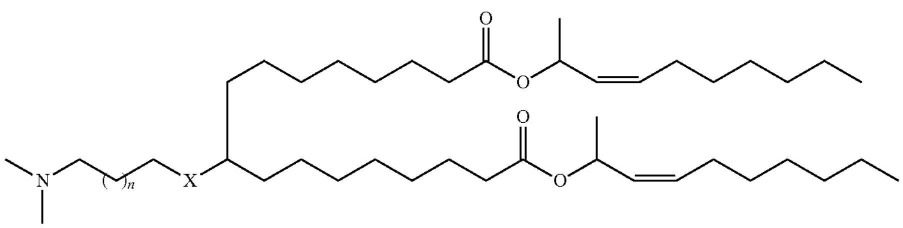
n = 0, 1, or 2



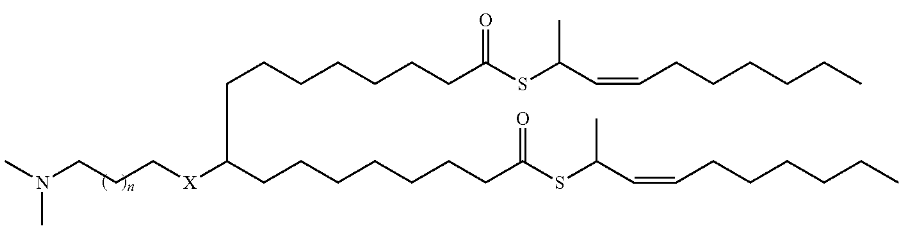
n = 0, 1, or 2



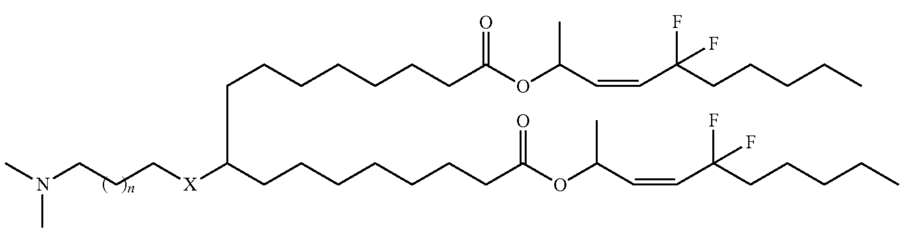
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2

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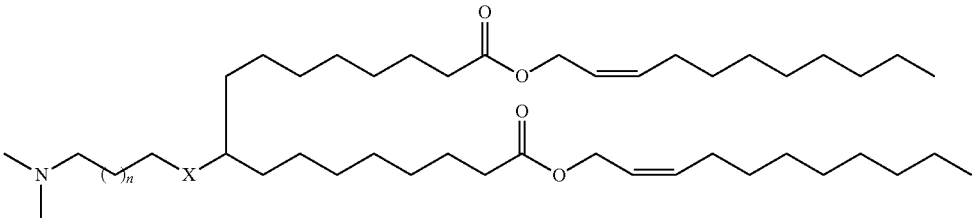
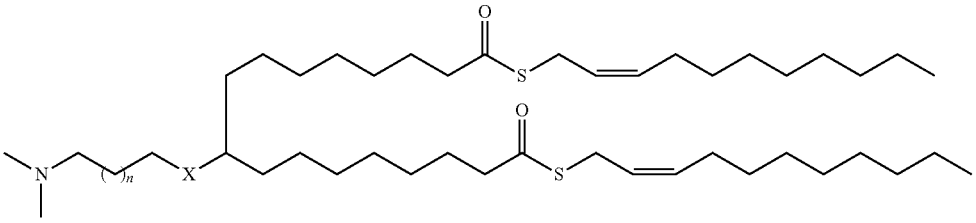
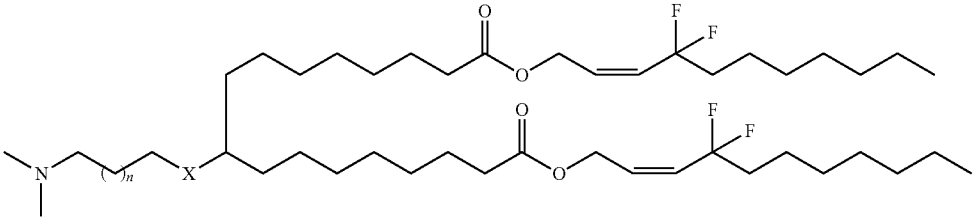


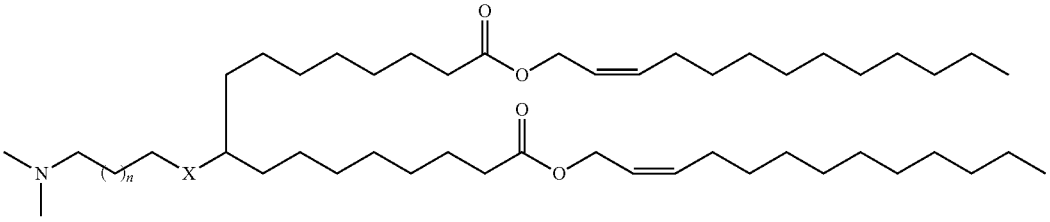
n = 0, 1, or 2



n = 0, 1, or 2



n = 0, 1, or 2



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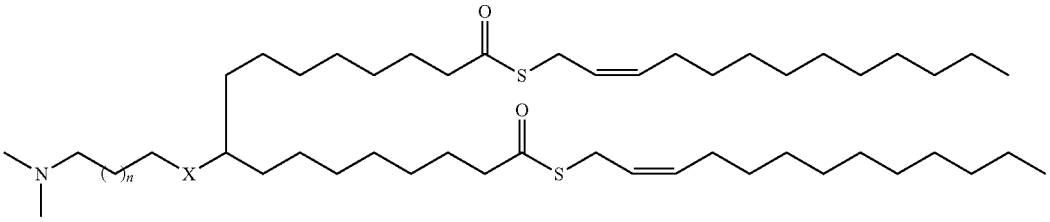
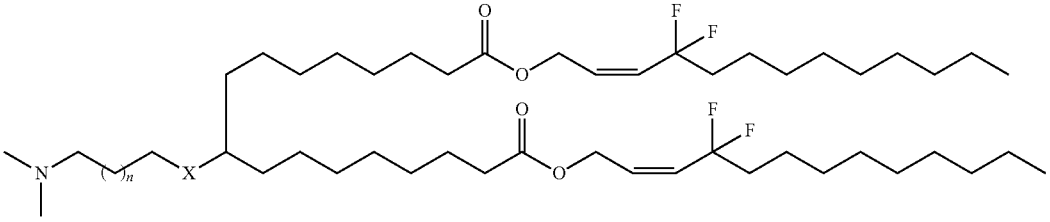


n = 0, 1, or 2



n = 0, 1, or 2

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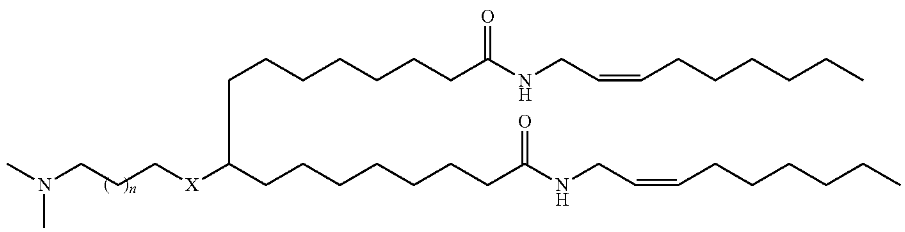
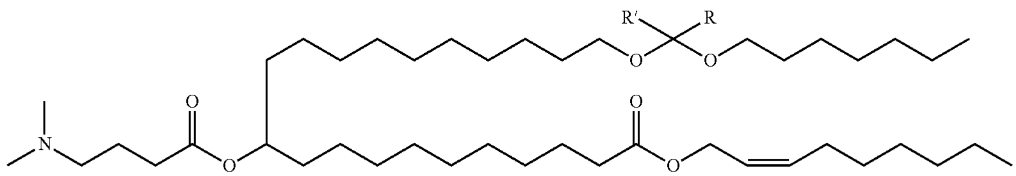
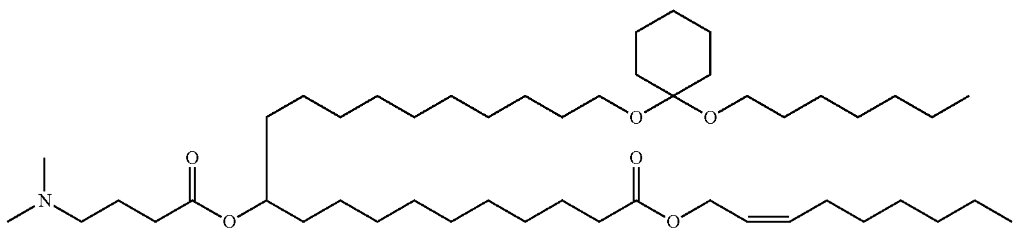
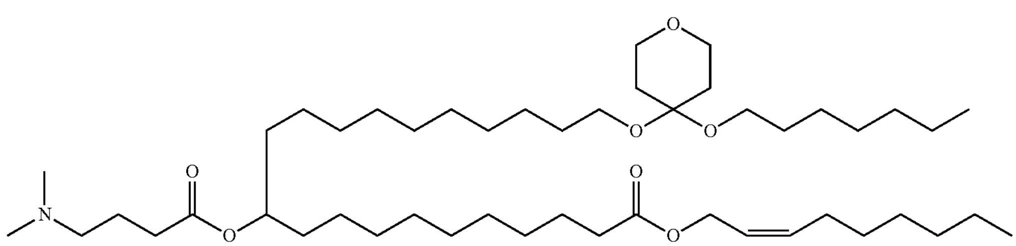


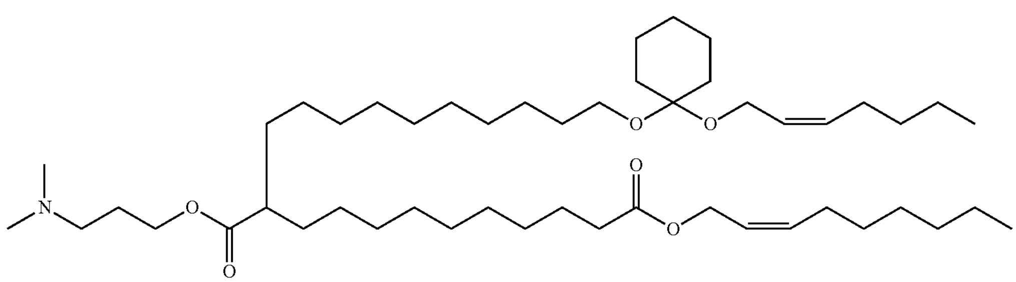
n = 0, 1, or 2

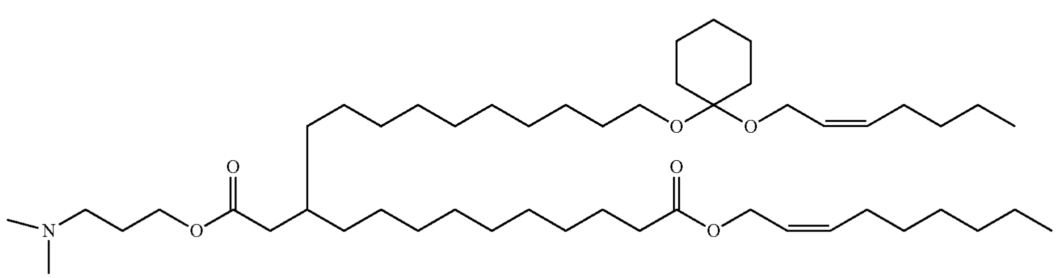


R, R′ = CH_3, Cyclopentyl etc









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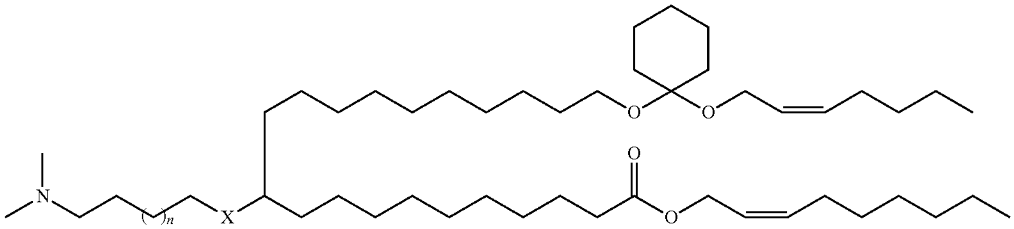
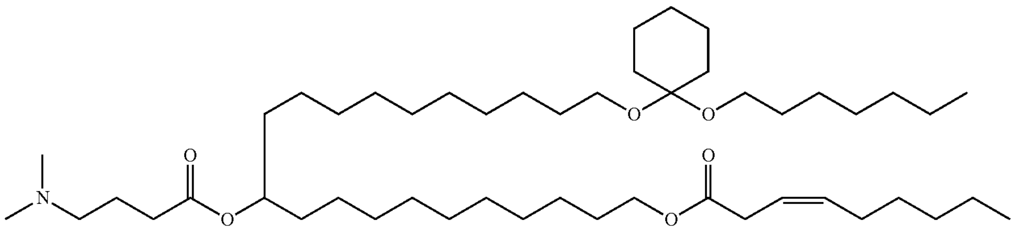
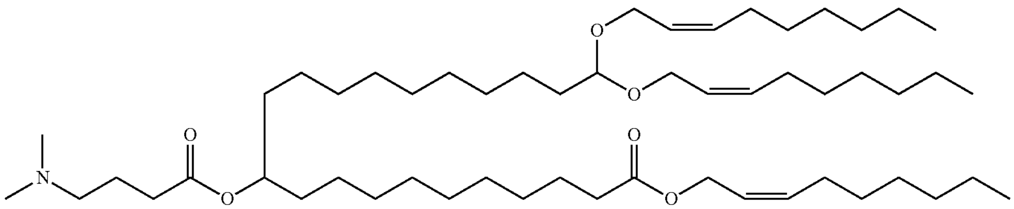
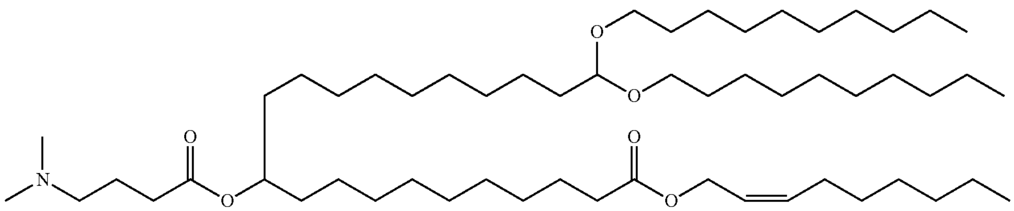
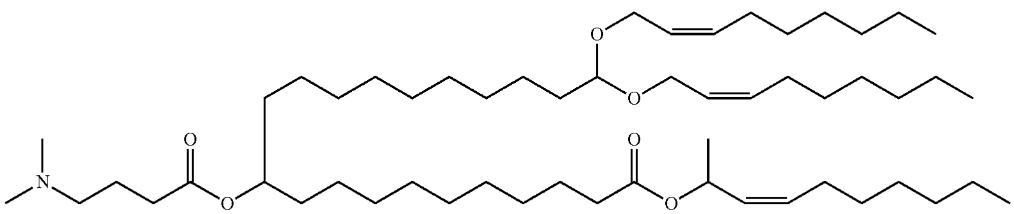


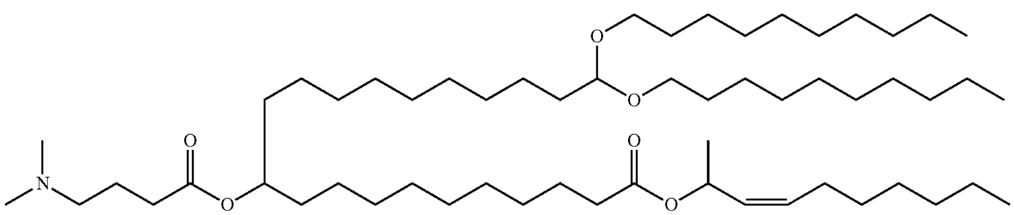
X = O, S, NR, CH_2, COO, NHCOO, OCONH
n = 0, 1, or 2

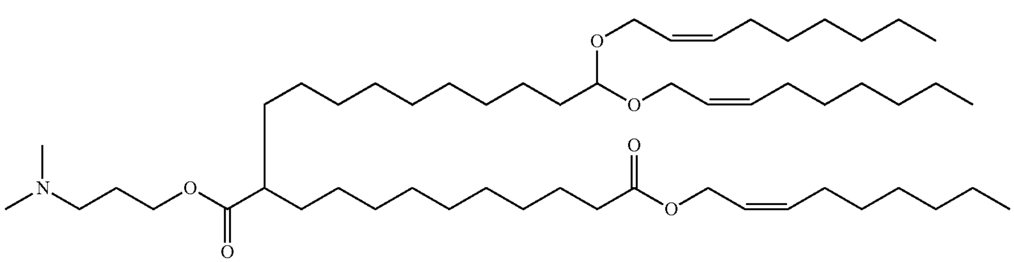












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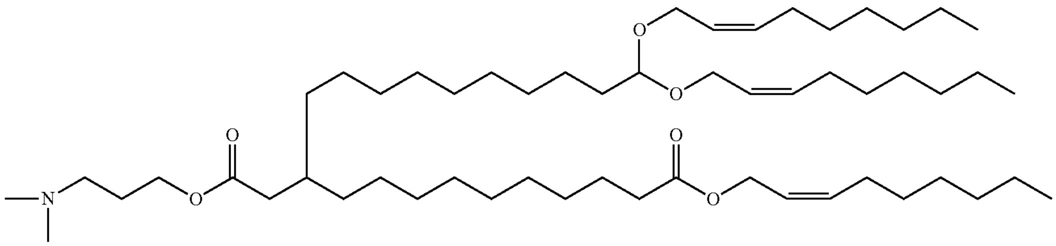
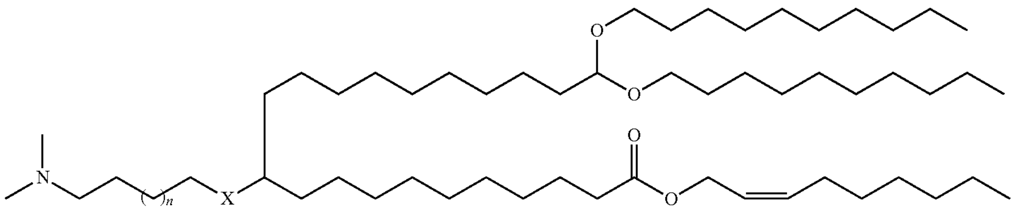
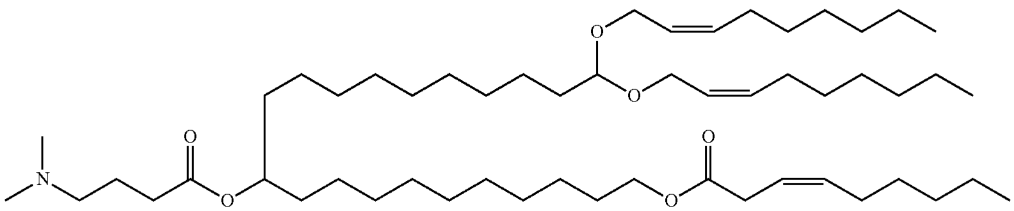
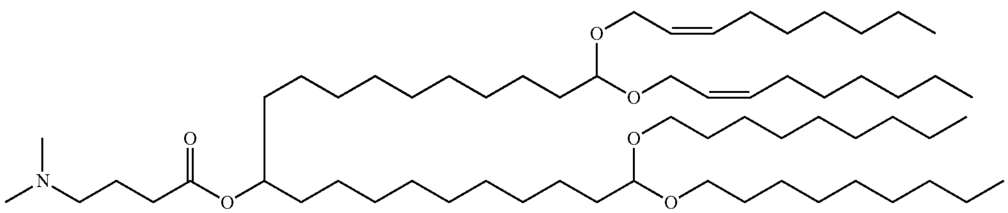
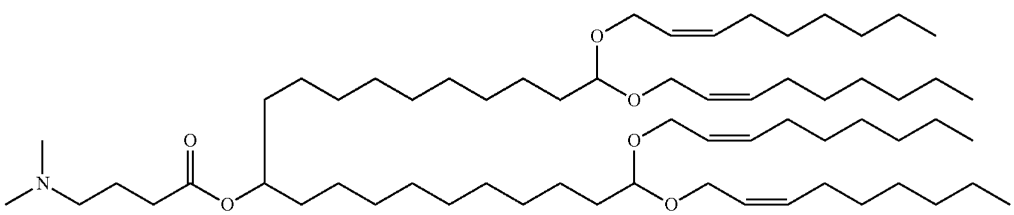


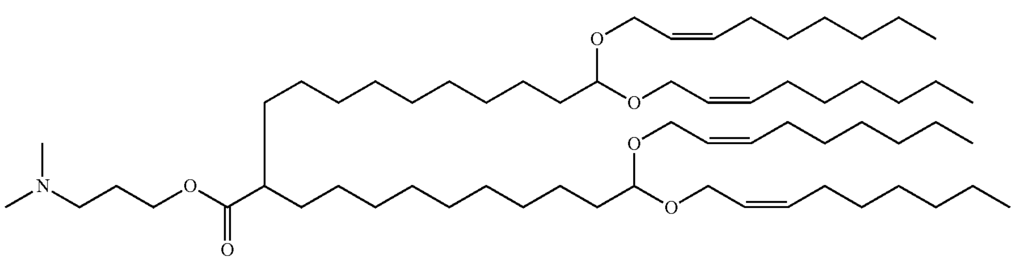


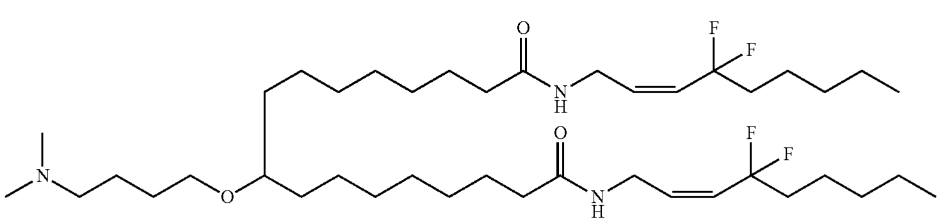
X = O, S, NR, CH_2, COO, NHCOO, OCONH
n = 0, 1, or 2











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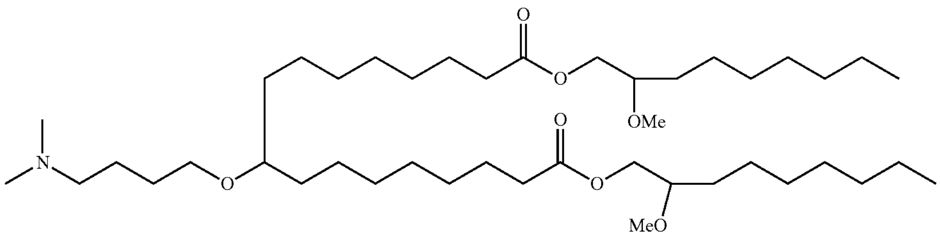
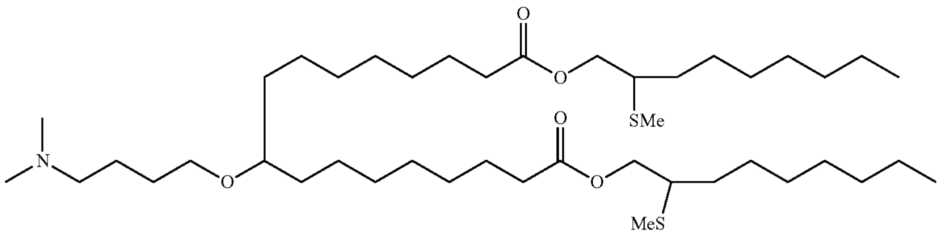
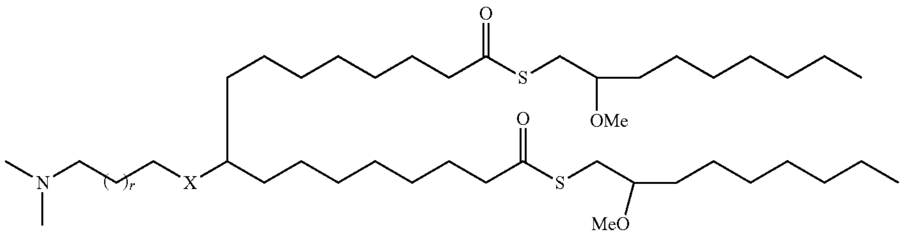
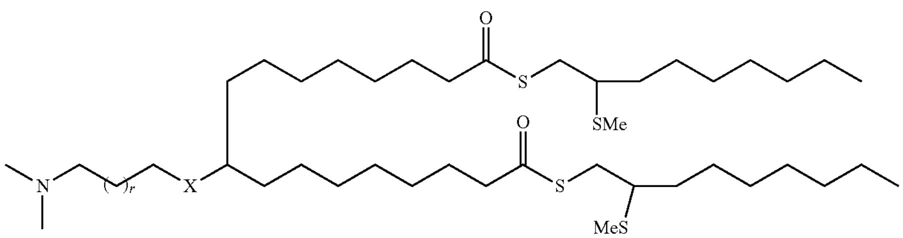


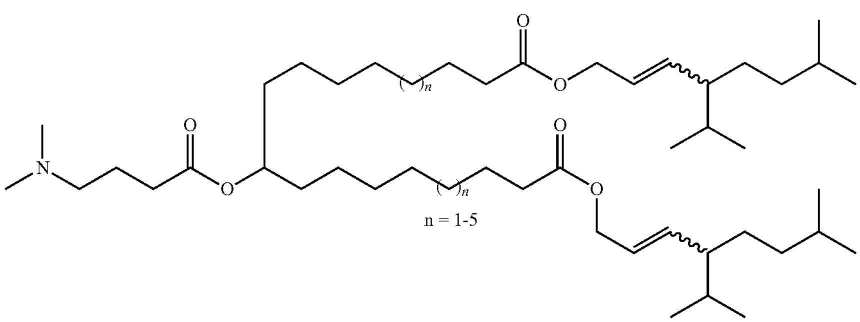


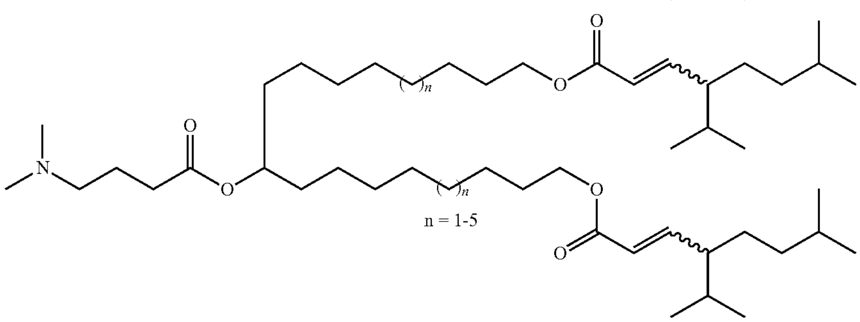


X = O, S, NR, CH_2
r = 0, 1, or 2



X = O, S, NR, CH_2
r = 0, 1, or 2





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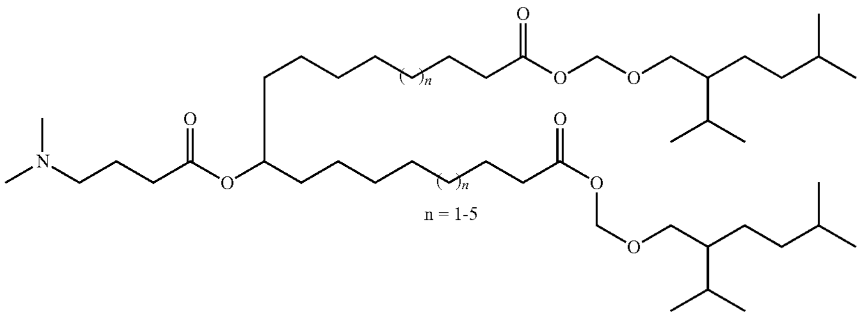
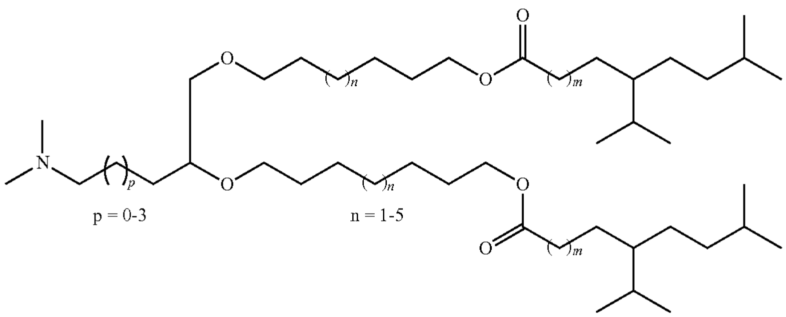
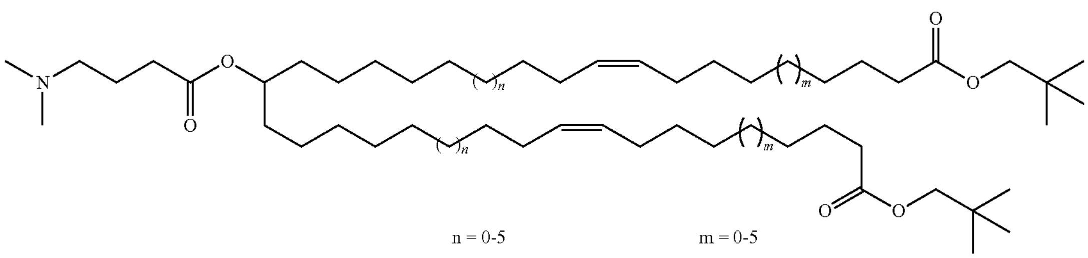


n = 1-5

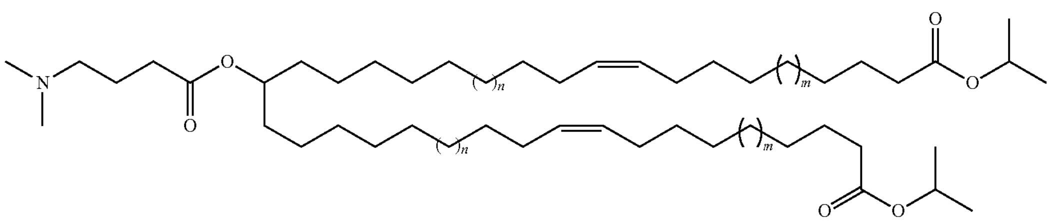


p = 0-3 n = 1-5

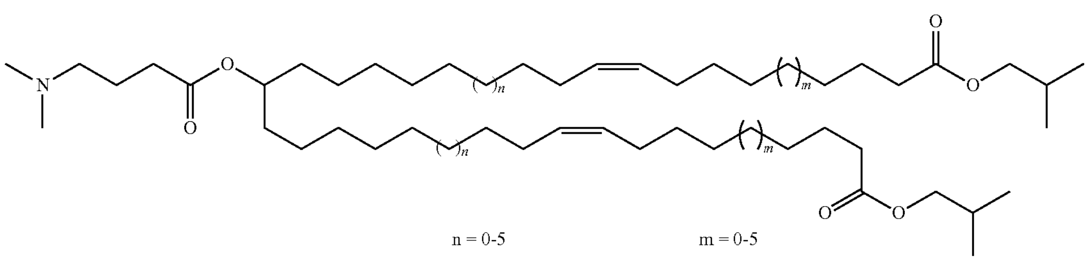
m = 0-3



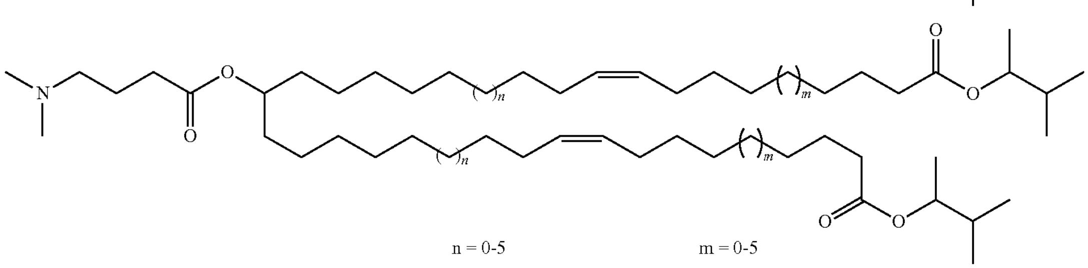
n = 0-5 m = 0-5



n = 0-5 m = 0-5

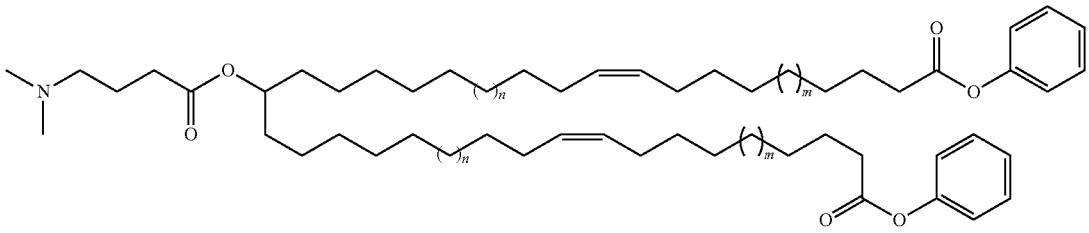


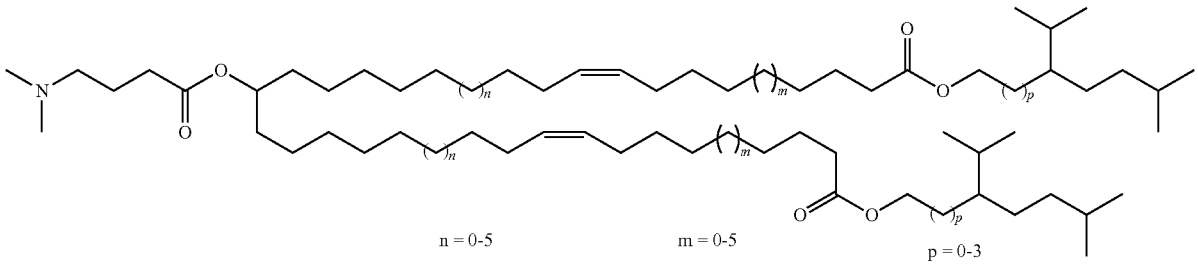
n = 0-5 m = 0-5

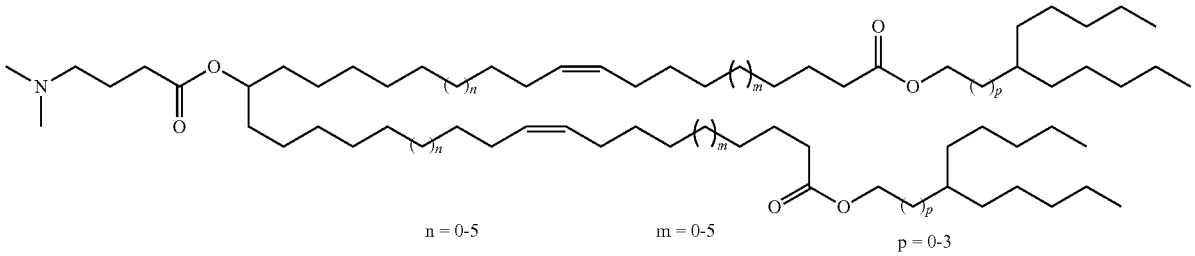


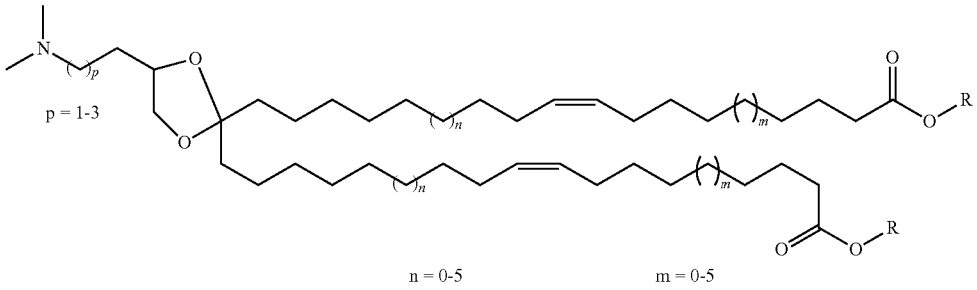
n = 0-5 m = 0-5

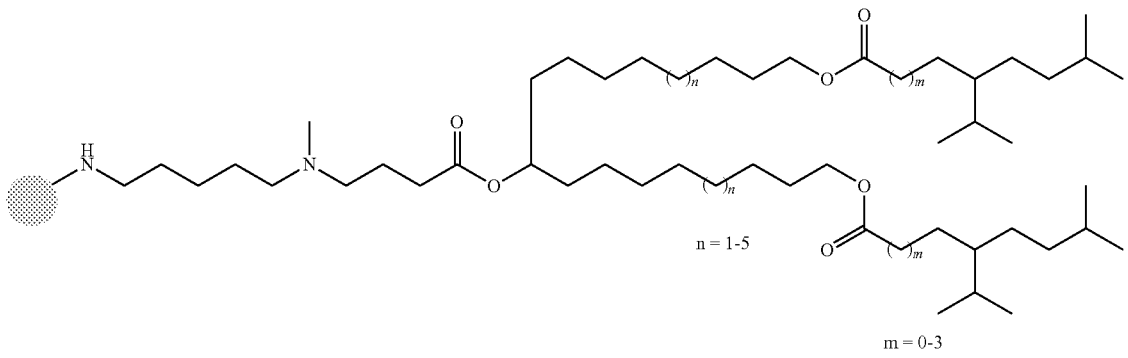
-continued











= Bodipy, Alexa-647 or other label (e.g., other fluorescent label)

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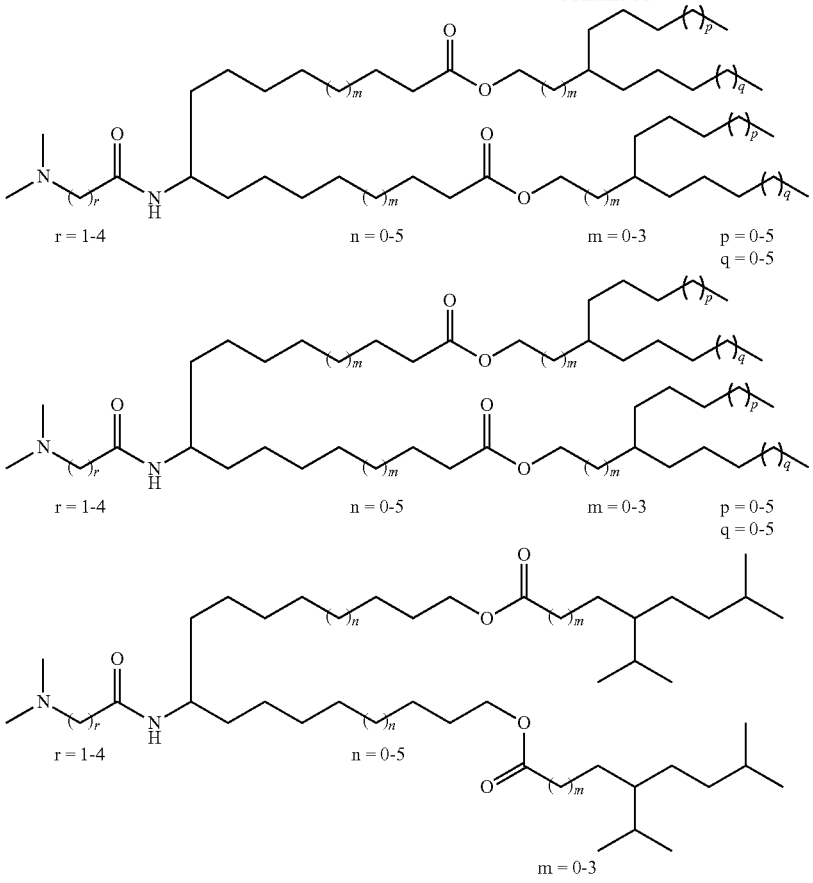
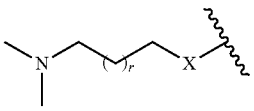
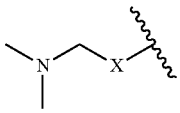


Alternatively, for the compounds above having a head of the formula



(where X can be, for example, —C(O)O—), the head can have one methylene unit between the X group (or other functional group) and nitrogen atom. For example, the head can be:



Cationic lipids include those having alternative fatty acid groups and other dialkylamino groups than those shown, including those in which the alkyl substituents are different (e.g., N-ethyl-N-methylamino-, and N-propyl-N-ethyl-amino-).

In certain embodiments, the cationic lipids have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g. pH 7.4), and neutral at a second pH, preferably at or above physiological pH. Such lipids are also referred to as cationic lipids. It will, of course, be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. The lipids can have more than one protonatable or deprotonatable group, or can be zwitterionic.

In certain embodiments, protonatable lipids (i.e., cationic lipids) have a pK_a of the protonatable group in the range of about 4 to about 11. For example, the lipids can have a pK_a of about 4 to about 7, e.g., from about 5 to about 7, such as from about 5.5 to about 6.8, when incorporated into lipid particles. Such lipids may be cationic at a lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4.

In particular embodiments, the lipids are charged lipids. As used herein, the term "charged lipid" includes, but is not limited to, those lipids having one or two fatty acyl or fatty alkyl chains and a quaternary amino head group. The quaternary amine carries a permanent positive charge. The head group can optionally include an ionizable group, such as a primary, secondary, or tertiary amine that may be protonated at physiological pH. The presence of the quaternary amine can alter the pKa of the ionizable group relative to the pKa of the group in a structurally similar compound that lacks the quaternary amine (e.g., the quaternary amine is replaced by a tertiary amine).

Included in the instant invention is the free form of the cationic lipids described herein, as well as pharmaceutically acceptable salts and stereoisomers thereof. The cationic lipid can be a protonated salt of the amine cationic lipid. The term "free form" refers to the amine cationic lipids in non-salt form. The free form may be regenerated by treating the salt with a suitable dilute aqueous base solution such as dilute aqueous NaOH, potassium carbonate, ammonia and sodium bicarbonate.

The pharmaceutically acceptable salts of the instant cationic lipids can be synthesized from the cationic lipids of this invention which contain a basic or acidic moiety by conventional chemical methods. Generally, the salts of the basic cationic lipids are prepared either by ion exchange chromatography or by reacting the free base with stoichiometric amounts or with an excess of the desired salt-forming inorganic or organic acid in a suitable solvent or various combinations of solvents. Similarly, the salts of the acidic compounds are formed by reactions with the appropriate inorganic or organic base.

Thus, pharmaceutically acceptable salts of the cationic lipids of this invention include non-toxic salts of the cationic lipids of this invention as formed by reacting a basic instant cationic lipids with an inorganic or organic acid. For example, non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxy-benzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and trifluoroacetic (TFA).

When the cationic lipids of the present invention are acidic, suitable "pharmaceutically acceptable salts" refers to salts prepared form pharmaceutically acceptable non-toxic bases including inorganic bases and organic bases. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous, potassium, sodium, and zinc. In one embodiment, the base is selected from ammonium, calcium, magnesium, potassium and sodium. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as arginine, betaine caffeine, choline, N,N^1-dibenzylethylenediamine, diethylamin, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethylmorpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine tripropylamine, and tromethamine.

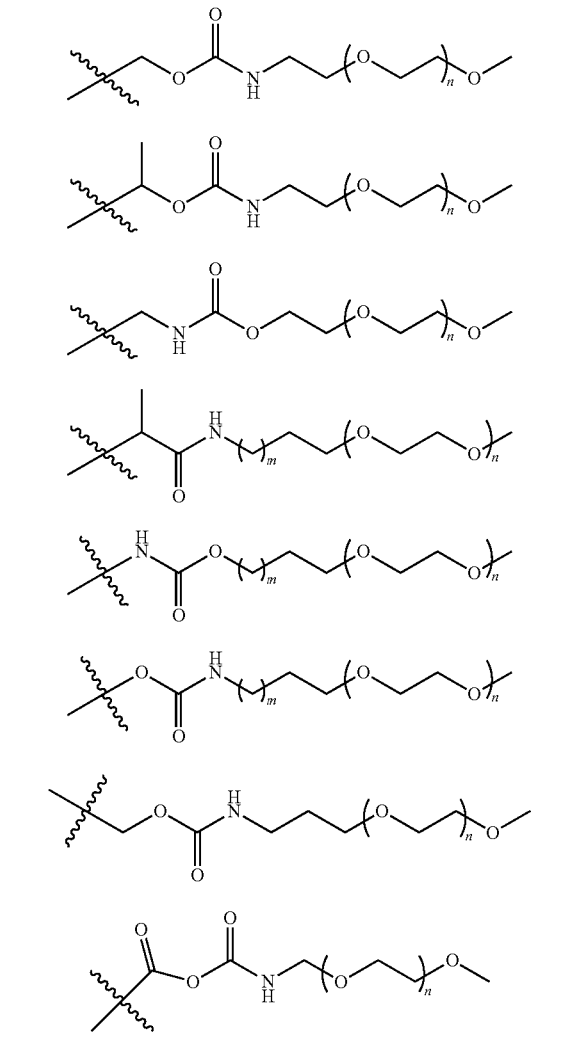
It will also be noted that the cationic lipids of the present invention may potentially be internal salts or zwitterions, since under physiological conditions a deprotonated acidic moiety in the compound, such as a carboxyl group, may be anionic, and this electronic charge might then be balanced off internally against the cationic charge of a protonated or alkylated basic moiety, such as a quaternary nitrogen atom.

One or more additional cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in the lipid particles and compositions described herein. Such cationic lipids include, but are not limited to N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl-N,N—N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N—(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dileoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N,N-dimethyl-2,3-dioleyloxy)propylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids can be used, such as, e.g., LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL).

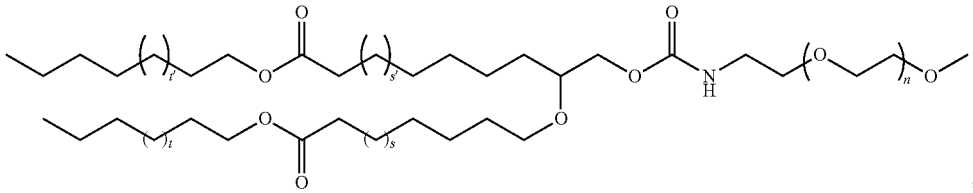
PEG Lipids

Suitable head groups for the PEG lipids include, but are not limited to those shown in Table 3 below.

TABLE 3



Representative PEG lipids include, but are not limited to:



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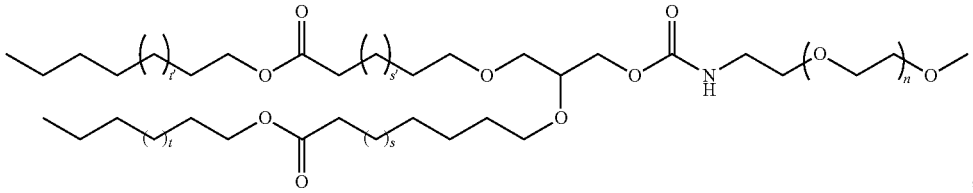

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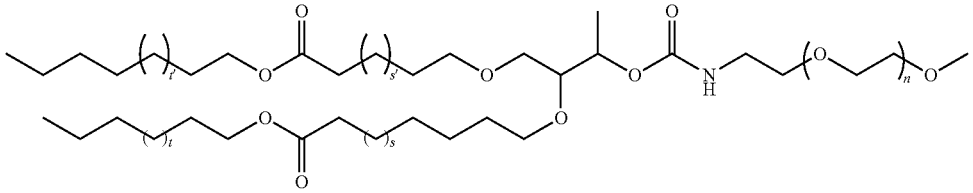

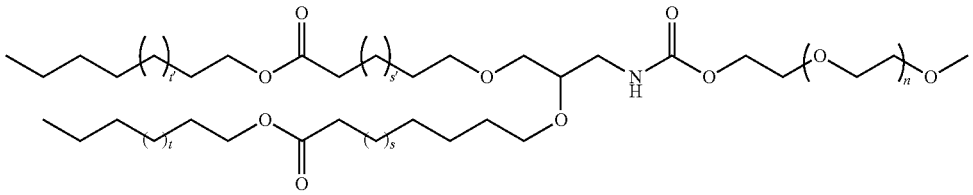

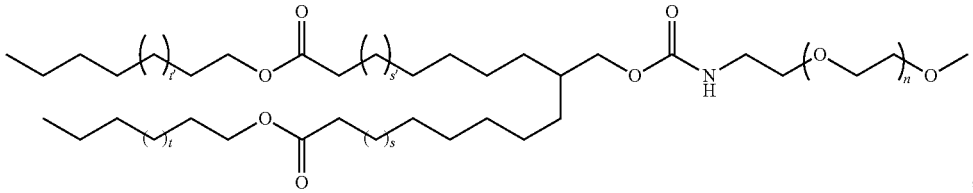

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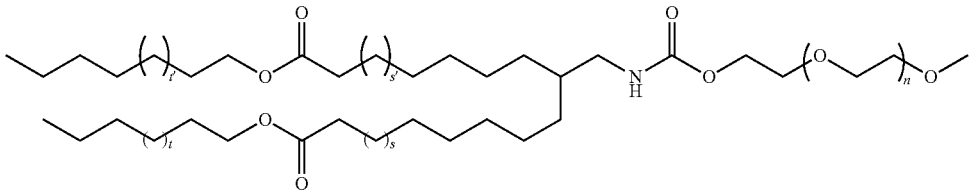

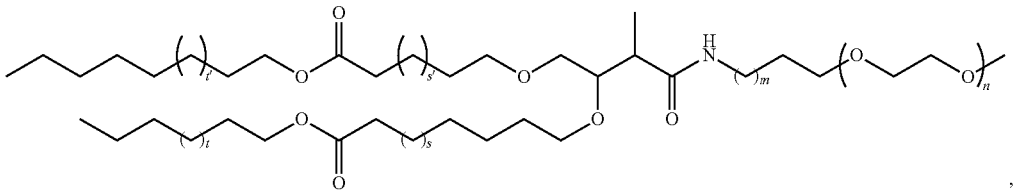

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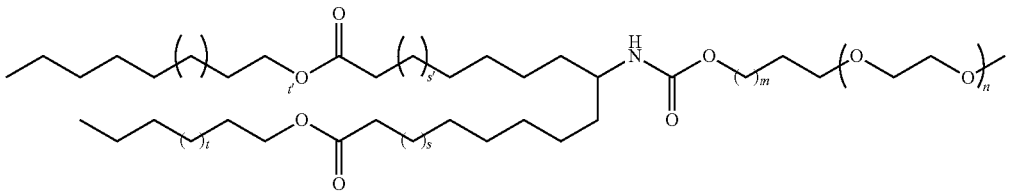

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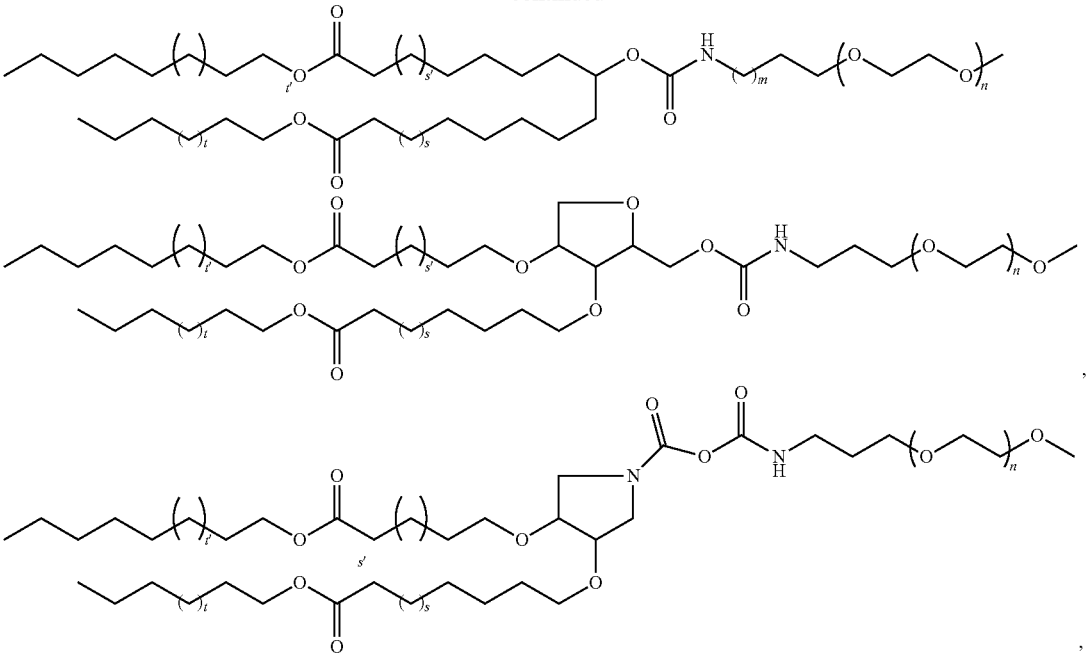
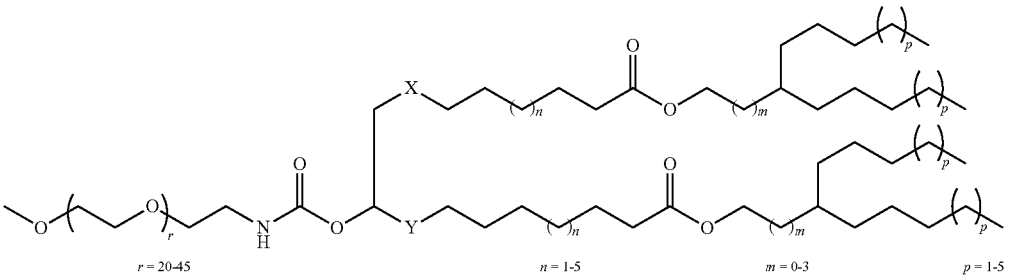


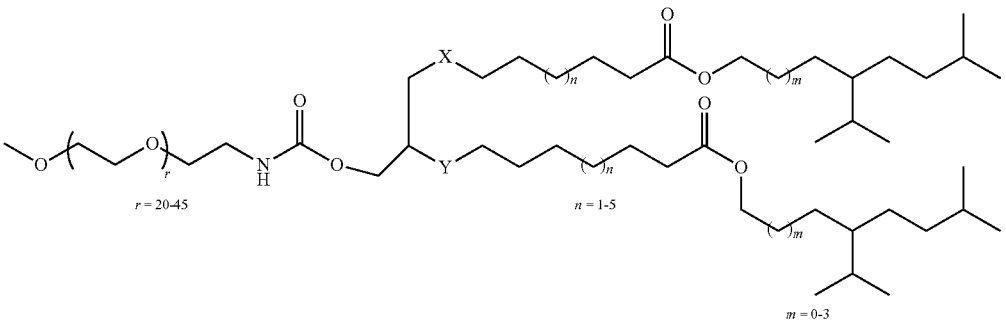
wherein

n is an integer from 10 to 100 (e.g. 20-50 or 40-50);

s, s', t and t' are independently 0, 1, 2, 3, 4, 5, 6 or 7; and m is 1, 2, 3, 4, 5, or 6.

Other representative PEG lipids include, but are not limited to:





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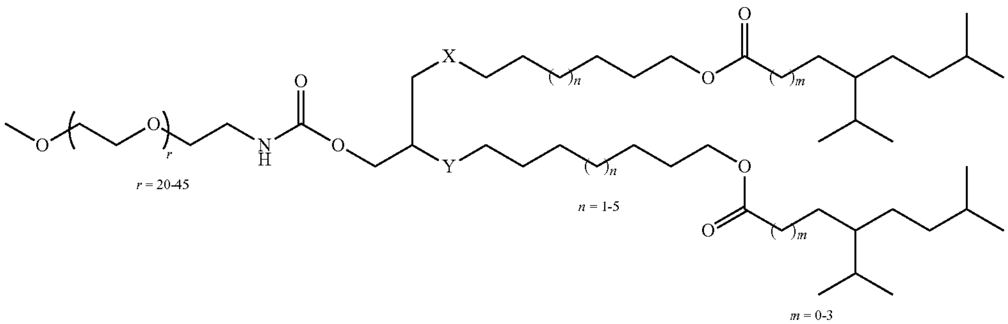
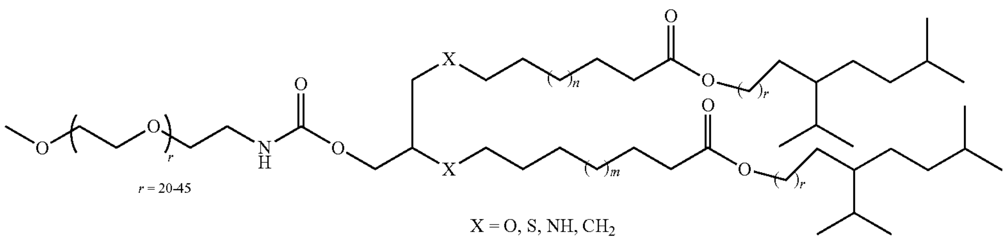
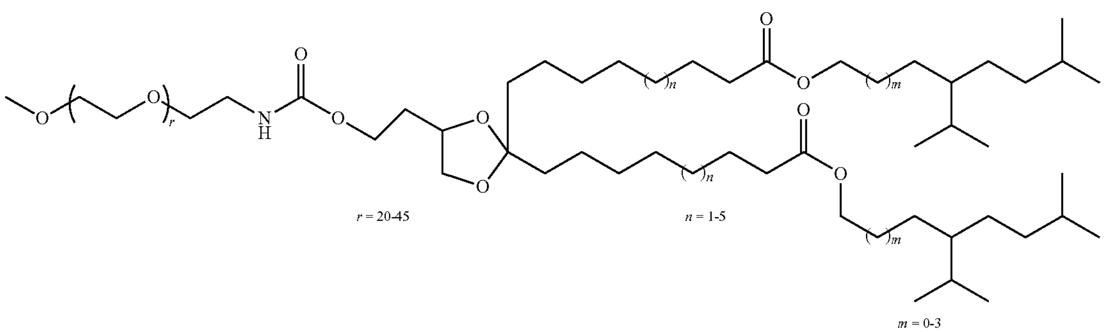


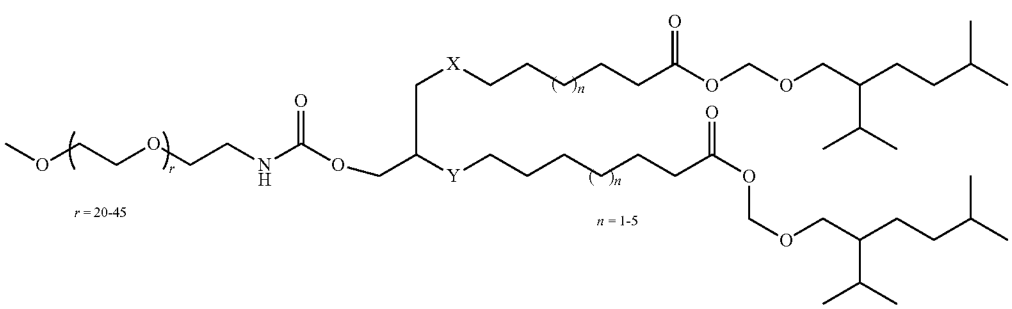
X = Y = CH_2, O, S

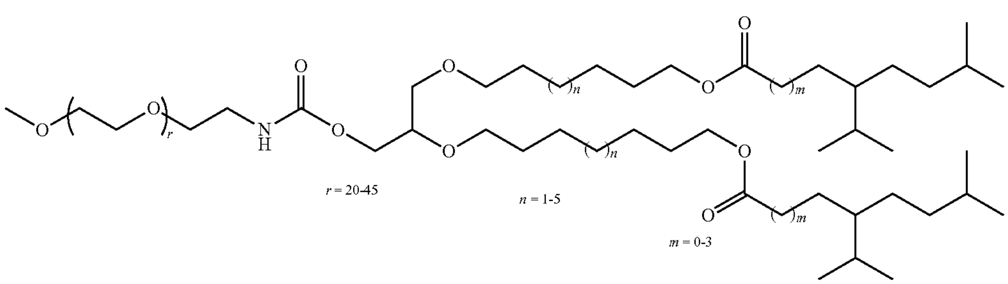


X = O, S, NH, CH_2

m = n = r = 0-10







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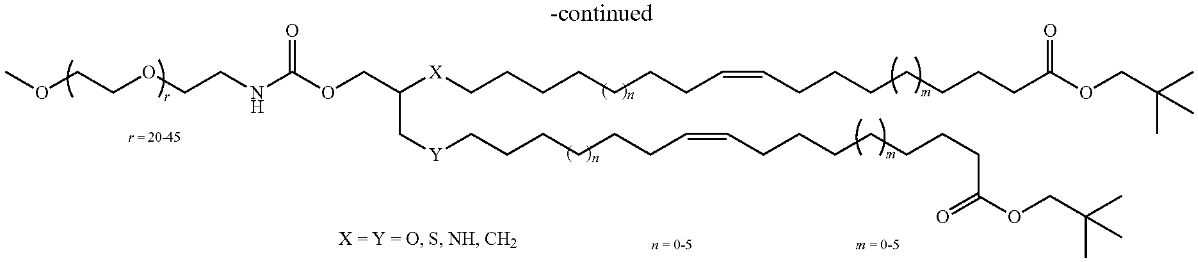
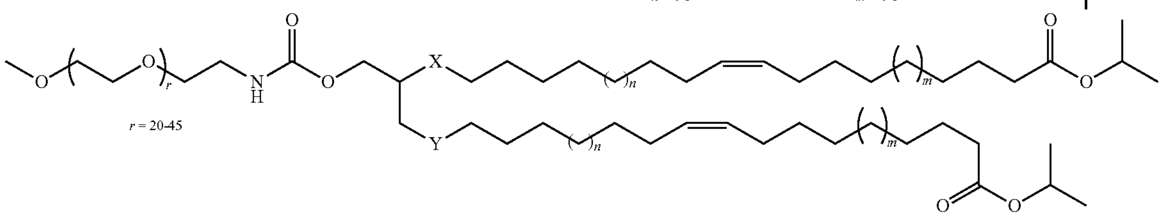


$r = 20\text{-}45$

X = Y = O, S, NH, CH_2 $n = 0\text{-}5$ $m = 0\text{-}5$



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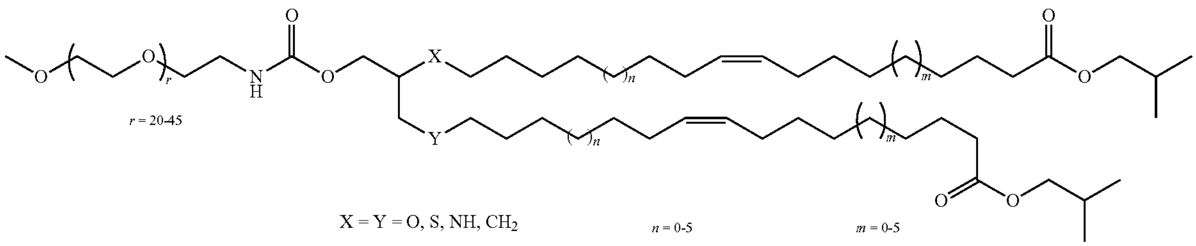
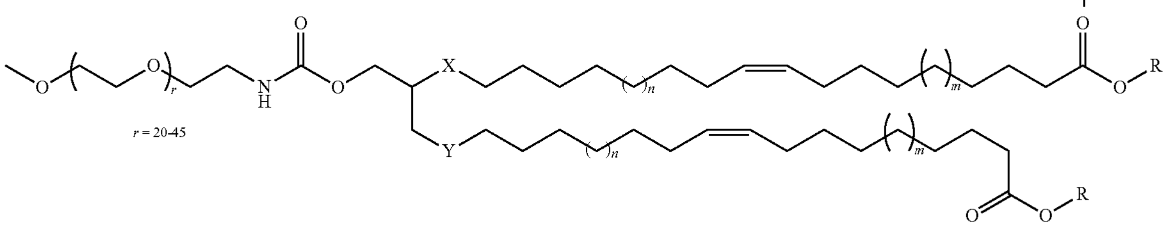


$r = 20\text{-}45$

X = Y = O, S, NH, CH_2 $n = 0\text{-}5$ $m = 0\text{-}5$



$r = 20\text{-}45$

X = Y = O, S, NH, CH_2 $n = 0\text{-}5$ $m = 0\text{-}5$

R = alkyl, substituted alkyl, aryl, benzyl

The Other Lipid Components

The lipid particles and compositions described herein may also include one or more neutral lipids. Neutral lipids, when present, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. In one embodiment, the neutral lipid component is a lipid having two acyl groups (e.g., diacylphosphatidylcholine and diacylphosphatidylethanolamine). In one embodiment, the neutral lipid contains saturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20}. In another embodiment, the neutral lipid includes mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20}. Suitable neutral lipids include, but are not limited to, DSPC, DPPC, POPC, DOPE, DSPC, and SM.

The lipid particles and compositions described herein may also include one or more lipids capable of reducing aggregation. Examples of lipids that reduce aggregation of particles during formation include polyethylene glycol (PEG)-modified lipids (PEG lipids, such as PEG-DMG and PEG-DMA), monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as (described in U.S. Pat. No. 6,320,017, which is incorporated by reference in its entirety). Suitable PEG lipids include, but are not limited to, PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) (such as those described in U.S. Pat. No. 5,820,873, incorporated herein by reference), PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines, PEG-modified diacylglycerols and dialkylglycerols, mPEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE).

The lipid particles and compositions may include a sterol, such as cholesterol.

Lipid Particles

In a further aspect, the present invent relates to lipid particles that include one or more of the cationic lipids described herein. In one embodiment, the lipid particle includes one or more compounds of formula I-VII.

Lipid particles include, but are not limited to, liposomes. As used herein, a liposome is a structure having lipid-containing membranes enclosing an aqueous interior.

Another embodiment is a nucleic acid-lipid particle (e.g., a SNALP) comprising a cationic lipid of the present invention, a non-cationic lipid (such as a neutral lipid), optionally a PEG-lipid conjugate (such as the lipids for reducing aggregation of lipid particles discussed herein), optionally a sterol (e.g., cholesterol), and a nucleic acid. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid

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particle. A SNALP represents a particle made from lipids, wherein the nucleic acid (e.g., an interfering RNA) is encapsulated within the lipids. In certain instances, SNALPs are useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (e.g., sites physically separated from the administration site), and they can mediate silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a SNALP as set forth in International Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety.

For example, the lipid particle may include a cationic lipid, a fusion-promoting lipid (e.g., DPPC), a neutral lipid, cholesterol, and a PEG-modified lipid. In one embodiment, the lipid particle includes the above lipid mixture in molar ratios of about 20-70% cationic lipid: 0.1-50% fusion promoting lipid: 5-45% neutral lipid: 20-55% cholesterol: 0.5-15% PEG-modified lipid (based upon 100% total moles of lipid in the lipid particle).

In another embodiment of the lipid particle, the cationic lipid is present in a mole percentage of about 20% and about 60%; the neutral lipid is present in a mole percentage of about 5% to about 25%; the sterol is present in a mole percentage of about 25% to about 55%; and the PEG lipid is PEG-DMA, PEG-DMG, or a combination thereof, and is present in a mole percentage of about 0.5% to about 15% (based upon 100% total moles of lipid in the lipid particle).

In particular embodiments, the molar lipid ratio, with regard to mol % cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) is approximately 40/10/40/10, 35/15/40/10 or 52/13/30/5. This mixture may be further combined with a fusion-promoting lipid in a molar ratio of 0.1-50%, 0.1-50%, 0.5-50%, 1-50%, 5%-45%, 10%-40%, or 15%-35%. In other words, when a 40/10/40/10 mixture of lipid/DSPC/Chol/PEG-DMG or PEG-DMA is combined with a fusion-promoting peptide in a molar ratio of 50%, the resulting lipid particles can have a total molar ratio of (mol % cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA/fusion-promoting peptide) 20/5/20/5/50. In another embodiment, the neutral lipid, DSPC, in these compositions is replaced with POPC, DPPC, DOPE or SM.

In one embodiment, the lipid particles comprise a cationic lipid of the present invention, a neutral lipid, a sterol and a PEG-modified lipid. In one embodiment, the lipid particles include from about 25% to about 75% on a molar basis of cationic lipid, e.g., from about 35 to about 65%, from about 45 to about 65%, about 60%, about 57.5%, about 57.1%, about 50% or about 40% on a molar basis. In one embodiment, the lipid particles include from about 0% to about 15% on a molar basis of the neutral lipid, e.g., from about 3 to about 12%, from about 5 to about 10%, about 15%, about 10%, about 7.5%, about 7.1% or about 0% on a molar basis. In one embodiment, the neutral lipid is DPPC. In one embodiment, the neutral lipid is DSPC. In one embodiment, the formulation includes from about 5% to about 50% on a molar basis of the sterol, e.g., about 15 to about 45%, about 20 to about 40%, about 48%, about 40%, about 38.5%, about 35%, about 34.4%, about 31.5% or about 31% on a molar basis. In one embodiment, the sterol is cholesterol.

The lipid particles described herein may further include one or more therapeutic agents. In a preferred embodiment, the lipid particles include a nucleic acid (e.g., an oligonucleotide), such as siRNA or miRNA.

In one embodiment, the lipid particles include from about 0.1% to about 20% on a molar basis of the PEG-modified lipid, e.g., about 0.5 to about 10%, about 0.5 to about 5%, about 10%, about 5%, about 3.5%, about 1.5%, about 0.5%, or about 0.3% on a molar basis. In one embodiment, the PEG-modified lipid is PEG-DMG. In one embodiment, the PEG-modified lipid is PEG-c-DMA. In one embodiment, the lipid particles include 25-75% of cationic lipid, 0.5-15% of the neutral lipid, 5-50% of the sterol, and 0.5-20% of the PEG-modified lipid on a molar basis.

In one embodiment, the lipid particles include 35-65% of cationic lipid, 3-12% of the neutral lipid, 15-45% of the sterol, and 0.5-10% of the PEG-modified lipid on a molar basis. In one embodiment, the lipid particles include 45-65% of cationic lipid, 5-10% of the neutral lipid, 25-40% of the sterol, and 0.5-5% of the PEG-modified lipid on a molar basis. In one embodiment, the PEG modified lipid comprises a PEG molecule of an average molecular weight of 2,000 Da. In one embodiment, the PEG modified lipid is PEG-distyryl glycerol (PEG-DSG).

In one embodiment, the ratio of lipid:siRNA is at least about 0.5:1, at least about 1:1, at least about 2:1, at least about 3:1, at least about 4:1, at least about 5:1, at least about 6:1, at least about 7:1, at least about 11:1 or at least about 33:1. In one embodiment, the ratio of lipid:siRNA ratio is between about 1:1 to about 35:1, about 3:1 to about 15:1, about 4:1 to about 15:1, or about 5:1 to about 13:1. In one embodiment, the ratio of lipid:siRNA ratio is between about 0.5:1 to about 12:1.

In one embodiment, the lipid particles are nanoparticles. In additional embodiments, the lipid particles have a mean diameter size of from about 50 nm to about 300 nm, such as from about 50 nm to about 250 nm, for example, from about 50 nm to about 200 nm.

In one embodiment, a lipid particle containing a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 3 hours, such as less than about 2.5 hours, less than about 2 hours, less than about 1.5 hours, less than about 1 hour, less than about 0.5 hour or less than about 0.25 hours.

In another embodiment, a lipid particle containing a cationic lipid of any of the embodiments described herein has an in vivo half life ($t_{1/2}$) (e.g., in the liver, spleen or plasma) of less than about 10% (e.g., less than about 7.5%, less than about 5%, less than about 2.5%) of that for the same cationic lipid without the biodegrable group or groups.

Additional Components

The lipid particles and compositions described herein can further include one or more antioxidants. The antioxidant stabilizes the lipid particle and prevents, decreases, and/or inhibits degradation of the cationic lipid and/or active agent present in the lipid particles. The antioxidant can be a hydrophilic antioxidant, a lipophilic antioxidant, a metal chelator, a primary antioxidant, a secondary antioxidant, salts thereof, and mixtures thereof. In certain embodiments, the antioxidant comprises a metal chelator such as EDTA or salts thereof, alone or in combination with one, two, three, four, five, six, seven, eight, or more additional antioxidants such as primary antioxidants, secondary antioxidants, or other metal chelators. In one preferred embodiment, the antioxidant comprises a metal chelator such as EDTA or salts thereof in a mixture with one or more primary antioxidants and/or secondary antioxidants. For example, the antioxidant may comprise a mixture of EDTA or a salt thereof, a primary antioxidant such as a-tocopherol or a salt thereof, and a secondary antioxidant such as ascorbyl palmitate or a salt thereof. In one embodiment, the antioxidant comprises at least about 100 mM citrate or a salt thereof. Examples of antioxidants include, but are not limited to,

hydrophilic antioxidants, lipophilic antioxidants, and mixtures thereof. Non-limiting examples of hydrophilic antioxidants include chelating agents (e.g., metal chelators) such as ethylenediaminetetraacetic acid (EDTA), citrate, ethylene glycol tetraacetic acid (EGTA), 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA), diethylene triamine pentaacetic acid (DTPA), 2,3-dimercapto-1-propanesulfonic acid (DMPS), dimercap to succinic acid (DMSA), cc-lipoic acid, salicylaldehyde isonicotinoyl hydrazone (SIH), hexyl thioethylamine hydrochloride (HTA), desferrioxamine, salts thereof, and mixtures thereof. Additional hydrophilic antioxidants include ascorbic acid, cysteine, glutathione, dihydrolipoic acid, 2-mercaptoethane sulfonic acid, 2-mercaptobenzimidazole sulfonic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, sodium metabisulfite, salts thereof, and mixtures thereof. Non-limiting examples of lipophilic antioxidants include vitamin E isomers such as α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols; polyphenols such as 2-tert-butyl-4-methyl phenol, 2-fert-butyl-5-methyl phenol, and 2-tert-butyl-6-methyl phenol; butylated hydroxyanisole (BHA) (e.g., 2-teri-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole); butylhydroxytoluene (BHT); tert-butylhydroquinone (TBHQ); ascorbyl palmitate; rc-propyl gallate; salts thereof; and mixtures thereof. Suitable antioxidants and formulations containing such antioxidants are described in International Publication No. WO 2011/066651, which is hereby incorporated by reference.

In another embodiment, the lipid particles or compositions contain the antioxidant EDTA (or a salt thereof), the antioxidant citrate (or a salt thereof), or EDTA (or a salt thereof) in combination with one or more (e.g., a mixture of) primary and/or secondary antioxidants such as α-tocopherol (or a salt thereof) and/or ascorbyl palmitate (or a salt thereof).

In one embodiment, the antioxidant is present in an amount sufficient to prevent, inhibit, or reduce the degradation of the cationic lipid present in the lipid particle. For example, the antioxidant may be present at a concentration of at least about or about 0.1 mM, 0.5 mM, 1 mM, 10 mM, 100 mM, 500 mM, 1 M, 2 M, or 5M, or from about 0.1 mM to about 1 M, from about 0.1 mM to about 500 mM, from about 0.1 mM to about 250 mM, or from about 0.1 mM to about 100 mM.

The lipid particles and compositions described herein can further include an apolipoprotein. As used herein, the term "apolipoprotein" or "lipoprotein" refers to apolipoproteins known to those of skill in the art and variants and fragments thereof and to apolipoprotein agonists, analogues or fragments thereof described below.

In a preferred embodiment, the active agent is a nucleic acid, such as a siRNA. For example, the active agent can be a nucleic acid encoded with a product of interest, including but not limited to, RNA, antisense oligonucleotide, an antagomir, a DNA, a plasmid, a ribosomal RNA (rRNA), a micro RNA (miRNA) (e.g., a miRNA which is single stranded and 17-25 nucleotides in length), transfer RNA (tRNA), a small interfering RNA (siRNA), small nuclear RNA (snRNA), antigens, fragments thereof, proteins, peptides, vaccines and small molecules or mixtures thereof. In one more preferred embodiment, the nucleic acid is an oligonucleotide (e.g., 15-50 nucleotides in length (or 15-30 or 20-30 nucleotides in length)). An siRNA can have, for instance, a duplex region that is 16-30 nucleotides long. In another embodiment, the nucleic acid is an immunostimulatory oligonucleotide, decoy oligonucleotide, supermir, miRNA mimic, or miRNA inhibitor. A supermir refers to a single stranded, double stranded or partially double stranded oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or both or modifications thereof, which has a nucleotide sequence that is substantially identical to an miRNA and that is antisense with respect to its target. miRNA mimics represent a class of molecules that can be used to imitate the gene silencing ability of one or more miRNAs. Thus, the term "microRNA mimic" refers to synthetic non-coding RNAs (i.e. the miRNA is not obtained by purification from a source of the endogenous miRNA) that are capable of entering the RNAi pathway and regulating gene expression.

The nucleic acid that is present in a lipid-nucleic acid particle can be in any form. The nucleic acid can, for example, be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Non-limiting examples of double-stranded RNA include siRNA. Single-stranded nucleic acids include, e.g., antisense oligonucleotides, ribozymes, microRNA, and triplex-forming oligonucleotides. The lipid particles of the present invention can also deliver nucleic acids which are conjugated to one or more ligands.

Pharmaceutical Compositions

The lipid particles, particularly when associated with a therapeutic agent, may be formulated as a pharmaceutical composition, e.g., which further comprises a pharmaceutically acceptable diluent, excipient, or carrier, such as physiological saline or phosphate buffer.

The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, and tonicity adjusting agents, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of lipid particle or lipid-nucleic acid particle in the pharmaceutical formulations can vary, for example, from less than about 0.01%, to at or at least about 0.05-5% to as much as 10 to 30% by weight.

Methods of Manufacture

Methods of making cationic lipids, lipid particles containing them, and pharmaceutical compositions containing the cationic lipids and/or lipid particles are described in, for example, International Publication Nos. WO 2010/054406, WO 2010/054401, WO 2010/054405, WO 2010/054384, WO 2010/042877, WO 2010/129709, WO 2009/086558, and WO 2008/042973, and U.S. Patent Publication Nos. 2004/0142025, 2006/0051405 and 2007/0042031, each of which is incorporated by reference in its entirety.

For example, in one embodiment, a solution of one or more lipids (including a cationic lipid of any of the embodiments described herein) in an organic solution (e.g., ethanol) is prepared. Similarly, a solution of one or more active (therapeutic) agents (such as, for example an siRNA molecule or a 1:1 molar mixture of two siRNA molecules) in an aqueous buffered (e.g., citrate buffer) solution is prepared. The two solutions are mixed and diluted to form a colloidal suspension of siRNA lipid particles. In one embodiment, the

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siRNA lipid particles have an average particle size of about 80-90 nm. In further embodiments, the dispersion may be filtered through 0.45/2 micron filters, concentrated and dia-filtered by tangential flow filtration.

Definitions

As used herein, the term "cationic lipid" inlcudes those lipids having one or two fatty acid or fatty aliphatic chains and an amino acid containing head group that may be protonated to form a cationic lipid at physiological pH. In some embodiments, a cationic lipid is referred to as an "amino acid conjugate cationic lipid."

A subject or patient in whom administration of the complex is an effective therapeutic regimen for a disease or disorder is preferably a human, but can be any animal, including a laboratory animal in the context of a clinical trial or screening or activity experiment. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods, compounds and compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, humans, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, and cats, avian species, such as chickens, turkeys, and songbirds, i.e., for veterinary medical use.

Many of the chemical groups recited in the generic formulas above are written in a particular order (for example, —OC(O)—). It is intended that the chemical group is to be incorporated into the generic formula in the order presented unless indicated otherwise. For example, a generic formula of the form $—(R)_i-(M^1)_k-(R)_m—$ where M^1 is —C(O)O— and k is 1 refers to $—(R)_i—C(O)O—(R)_m—$ unless specified otherwise. It is to be understood that when a chemical group is written in a particular order, the reverse order is also contemplated unless otherwise specified. For example, in a generic formula $—(R)_i-(M^1)_k-(R)_m—$ where M^1 is defined as —C(O)NH— (i.e., $—(R)_i—C(O)—NH—(R)_m—$), the compound where M^1 is —NHC(O)— (i.e., $—(R)_i—NHC(O)—(R)_m—$) is also contemplated unless otherwise specified.

The term "biodegradable cationic lipid" refers to a cationic lipid having one or more biodegradable groups located in the mid- or distal section of a lipidic moiety (e.g., a hydrophobic chain) of the cationic lipid. The incorporation of the biodegradable group(s) into the cationic lipid results in faster metabolism and removal of the cationic lipid from the body following delivery of the active pharmaceutical ingredient to a target area.

As used herein, the term "biodegradable group" refers to a group that include one or more bonds that may undergo bond breaking reactions in a biological environment, e.g., in an organism, organ, tissue, cell, or organelle. For example, the biodegradable group may be metabolizable by the body of a mammal, such as a human (e.g., by hydrolysis). Some groups that contain a biodegradable bond include, for example, but are not limited to esters, dithiols, and oximes. Non-limiting examples of biodegradable groups are —OC(O)—, —C(O)O—, —SC(O)—, —C(O)S—, —OC(S)—, —C(S)O—, —S—S—, $—C(R^5)=N—$, $—N=C(R^5)—$, $—C(R^5)=N—O—$, $—O—N=C(R^5)—$, $—C(O)(NR^5)—$, $—N(R^5)C(O)—$, $—C(S)(NR^5)—$, $—N(R^5)C(O)—$, $—N(R^5)C(O)N(R^5)—$, —OC(O)O—, $—OSi(R^5)_2O—$, $—C(O)(CR^3R^4)C(O)O—$, or $—OC(O)(CR^3R^4)C(O)—$.

As used herein, an "aliphatic" group is a non-aromatic group in which carbon atoms are linked into chains, and is either saturated or unsaturated.

The terms "alkyl" and "alkylene" refer to a straight or branched chain saturated hydrocarbon moiety. In one embodiment, the alkyl group is a straight chain saturated hydrocarbon. Unless otherwise specified, the "alkyl" or "alkylene" group contains from 1 to 24 carbon atoms. Representative saturated straight chain alkyl groups include methyl, ethyl, n-propyl, n-butyl, n-pentyl, and n-hexyl. Representative saturated branched alkyl groups include isopropyl, sec-butyl, isobutyl, tert-butyl, and isopentyl.

The term "alkenyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon double bonds. In one embodiment, the alkenyl group contains 1, 2, or 3 double bonds and is otherwise saturated. Unless otherwise specified, the "alkenyl" group contains from 2 to 24 carbon atoms. Alkenyl groups include both cis and trans isomers.

Representative straight chain and branched alkenyl groups include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, and 2,3-dimethyl-2-butenyl.

The term "alkynyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon triple bonds. Unless otherwise specified, the "alkynyl" group contains from 2 to 24 carbon atoms. Representative straight chain and branched alkynyl groups include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, and 3-methyl-1-butynyl.

Unless otherwise specified, the terms "branched alkyl", "branched alkenyl", and "branched alkynyl" refer to an alkyl, alkenyl, or alkynyl group in which one carbon atom in the group (1) is bound to at least three other carbon atoms and (2) is not a ring atom of a cyclic group. For example, a spirocyclic group in an alkyl, alkenyl, or alkynyl group is not considered a point of branching.

Unless otherwise specified, the term "acyl" refers to a carbonyl group substituted with hydrogen, alkyl, partially saturated or fully saturated cycloalkyl, partially saturated or fully saturated heterocycle, aryl, or heteroaryl. For example, acyl groups include groups such as (C_1-C_{20})alkanoyl (e.g., formyl, acetyl, propionyl, butyryl, valeryl, caproyl, and t-butylacetyl), (C_3-C_{20})cycloalkylcarbonyl (e.g., cyclopropylcarbonyl, cyclobutylcarbonyl, cyclopentylcarbonyl, and cyclohexylcarbonyl), heterocyclic carbonyl (e.g., pyrrolidinylcarbonyl, pyrrolid-2-one-5-carbonyl, piperidinylcarbonyl, piperazinylcarbonyl, and tetrahydrofuranylcarbonyl), aroyl (e.g., benzoyl) and heteroaroyl (e.g., thiophenyl-2-carbonyl, thiophenyl-3-carbonyl, furanyl-2-carbonyl, furanyl-3-carbonyl, 1H-pyrroyl-2-carbonyl, 1H-pyrroyl-3-carbonyl, and benzo[b]thiophenyl-2-carbonyl).

The term "aryl" refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system. Unless otherwise specified, the "aryl" group contains from 6 to 14 carbon atoms. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, anthracenyl, and pyrenyl.

The terms "cycloalkyl" and "cycloalkylene" refer to a saturated monocyclic or bicyclic hydrocarbon moiety such as cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl. Unless otherwise specified, the "cycloalkyl" or "cycloalkylene" group contains from 3 to 10 carbon atoms.

The term "cycloalkylalkyl" refers to a cycloalkyl group bound to an alkyl group, where the alkyl group is bound to the rest of the molecule.

The term "heterocycle" (or "heterocyclyl") refers to a non-aromatic 5- to 8-membered monocyclic, or 7- to

12-membered bicyclic, or 11- to 14-membered tricyclic ring system which is either saturated or unsaturated, and which contains from 1 to 3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized. For instance, the heterocycle may be a cycloalkoxy group. The heterocycle may be attached to the rest of the molecule via any heteroatom or carbon atom in the heterocycle. Heterocycles include, but are not limited to, morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, and tetrahydrothiopyranyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 7-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, where the heteroatoms are selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively). The heteroaryl groups herein described may also contain fused rings that share a common carbon-carbon bond.

The term "substituted", unless otherwise indicated, refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, oxo, thioxy, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy, aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aryloxycarbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxycarbonylalkyl, aminocarbonylalkyl, acyl, aralkoxycarbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and an aliphatic group. It is understood that the substituent may be further substituted. Exemplary substituents include amino, alkylamino, dialkylamino, and cyclic amino compounds.

The term "halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

The following abbreviations may be used in this application:

DSPC: distearoylphosphatidylcholine; DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine;

POPC: 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine; DOPE: 1,2-dileoyl-sn-3-phosphoethanolamine; PEG-DMG generally refers to 1,2-dimyristoyl-sn-glycerol-methoxy polyethylene glycol (e.g., PEG 2000); TBDPSCl: tert-Butylchlorodiphenylsilane; DMAP: dimethylaminopyridine; HMPA: hexamethylphosphoramide; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DIPEA: diisopropylethylamine; DCM: dichloromethane; TEA: triethylamine; TBAF: tetrabutylammonium fluoride

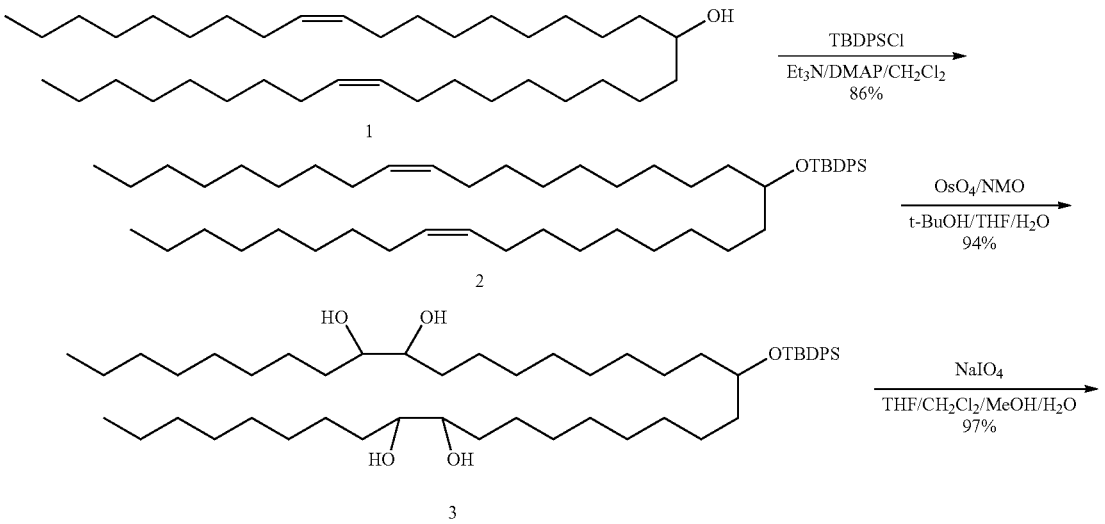
Methods to prepare various organic groups and protective groups are known in the art and their use and modification is generally within the ability of one of skill in the art (see, for example, Green, T. W. et. al., *Protective Groups in Organic Synthesis* (1999); Stanley R. Sandler and Wolf Karo, *Organic Functional Group Preparations* (1989); Greg T. Hermanson, *Bioconjugate Techniques* (1996); and Leroy G. Wade, *Compendium Of Organic Synthetic Methods* (1980)). Briefly, protecting groups are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

The compounds may be prepared by at least one of the techniques described herein or known organic synthesis techniques.

EXAMPLES

Example 1

Scheme 1. ALNY-322/quaternary salts



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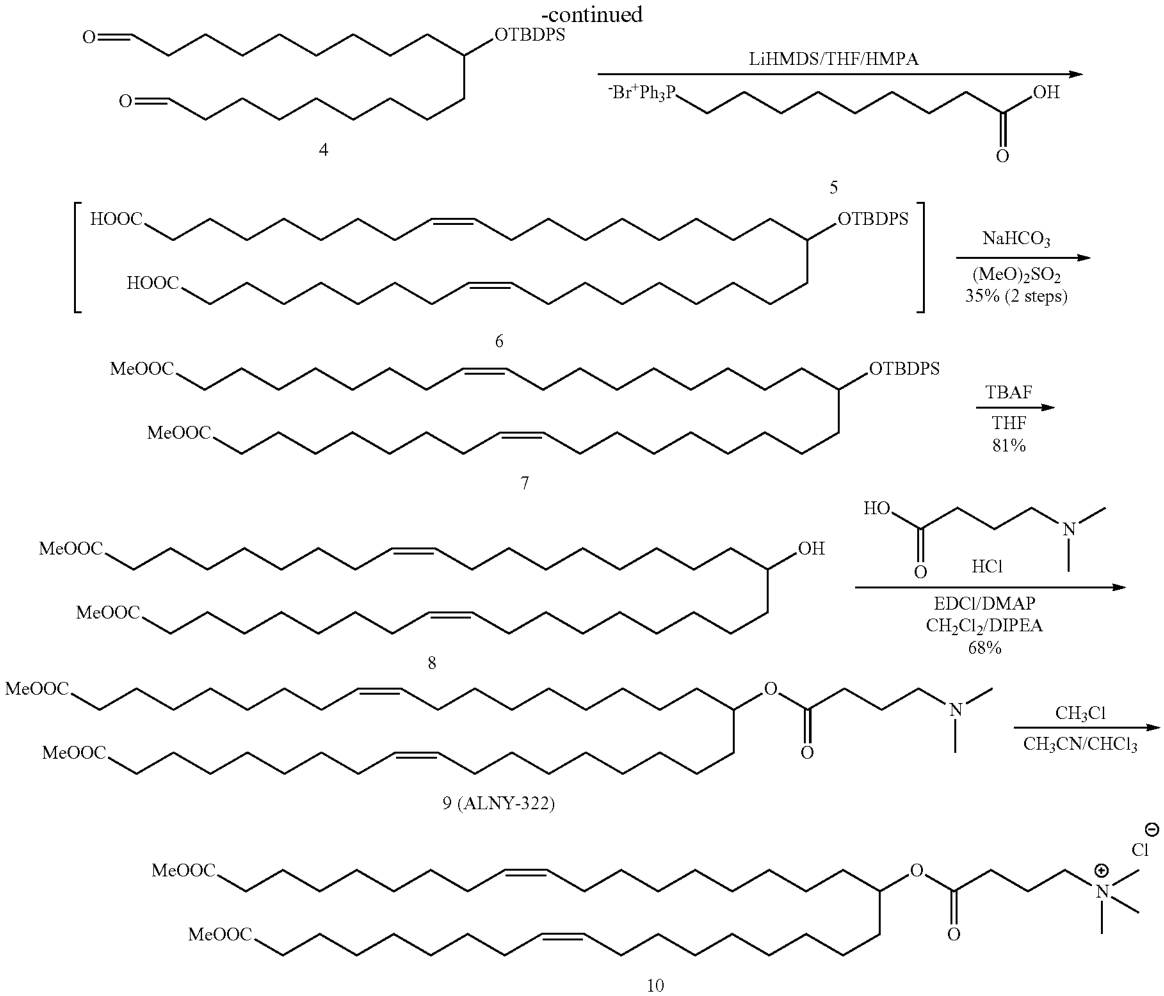


Compound 2: To a solution of compound 1 (10.0 g, 18.8 mmol, see International Publication No. WO 2010/054406) in CH_2Cl_2 (80 mL) were added triethylamine (7.86 mL, 56.4 mmol), DMAP (459 mg, 3.76 mmol) and/e/7-butyl(chloro) diphenylsilane (9.62 mL, 37.6 mmol). The reaction mixture was stirred for 24 hours. The mixture was then diluted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$ solution. The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (0-5% EtOAc in hexane) to afford 2 (12.4 g, 16.1 mmol, 86%, R_f=0.24 with hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66-7.68 (m, 4H), 7.33-7.42 (m, 6H), 5.30-5.39 (m, 4H), 3.67-3.72 (m, 1H), 1.97-2.04 (m, 8H), 1.07-1.42 (m, 52H), 1.05 (s, 9H), 0.88 (t, J=6.8 Hz, 6H).

Compound 3: To a solution of 2 (12.4 g, 16.1 mmol) in tert-butanol (100 mL), THF (30 mL) and H_2O (10 mL) were added 4-methylmorpholine N-oxide (4.15 g, 35.4 mmol) and osmium tetroxide (41 mg, 0.161 mg). The reaction mixture was stirred for 16 hours, then quenched by adding sodium bisulfite. After removing the solvents by evaporation, the residue was extracted with Et_2O (500 mL) and H_2O (300 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude was purified by silica gel column chromatography (hexane: EtOAc=1:1, R_f=0.49) to afford 3 (12.7 g, 15.1 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66-7.68 (m, 4H), 7.33-7.43 (m, 6H), 3.67-3.73 (m, 1H), 3.57-3.62 (m, 4H), 1.82 (t, J=5.0 Hz, 4H), 1.10-1.51 (m, 60H), 1.04 (s, 9H), 0.88 (t, J=6.8 Hz, 6H).

Compound 4: To a solution of 3 (12.6 g, 15.0 mmol) in 1,4-dioxane (220 mL), CH_2Cl_2 (70 mL), MeOH (55 mL), and H_2O (55 mL) was added $NaIO_4$ (7.70 g, 36.0 mmol). The reaction mixture was stirred for 16 hours at room temperature. The mixture was extracted with Et_2O (500 mL) and H_2O (300 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (Hexane:EtOAc=9:1, R_f=0.30) to afford 4 (7.98 g, 14.5 mmol, 97%). Molecular weight for $C_{35}H_{54}NaO_3Si$ $(M+Na)^+$ Calc. 573.3740, Found 573.3.

Compound 7: To a solution of 5 (see, Tetrahedron, 63, 1140-1145, 2006; 1.09 g, 2.18 mmol) in THF (20 mL) and HMPA (4 mL), LiHMDS (1 M THF solution, 4.36 mL, 4.36 mmol) was added at −20° C. The resulting mixture was stirred for 20 minutes at the same temperature, then cooled to −78° C. A solution of 4 (500 mg, 0.908 mmol) in THF (4 mL) was added. The mixture was stirred and allowed to warm to room temperature overnight. MS analysis showed the formation of the di-acid (6; $C_{53}H_{85}O_5Si$ $(M-H)^-$ calc. 829.6166, observed 829.5). To the mixture, $NaHCO_3$ (1.10 g, 13.1 mmol) and dimethyl sulfate (1.24 mL, 13.1 mmol)

were added and stirred for 2 hours at room temperature. The reaction was quenched by adding saturated NH_4Cl aqueous solution (50 mL) then extracted with Et_2O (2×100 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel column chromatography (Hexane: EtOAc=9:1, R_f=0.35) to afford 7 (270 mg, 0.314 mmol, 35%). Molecular weight for $C_{55}H_{90}NaO_5Si$ $(M+Na)^+$ Calc. 881.6455, Found 881.6484.

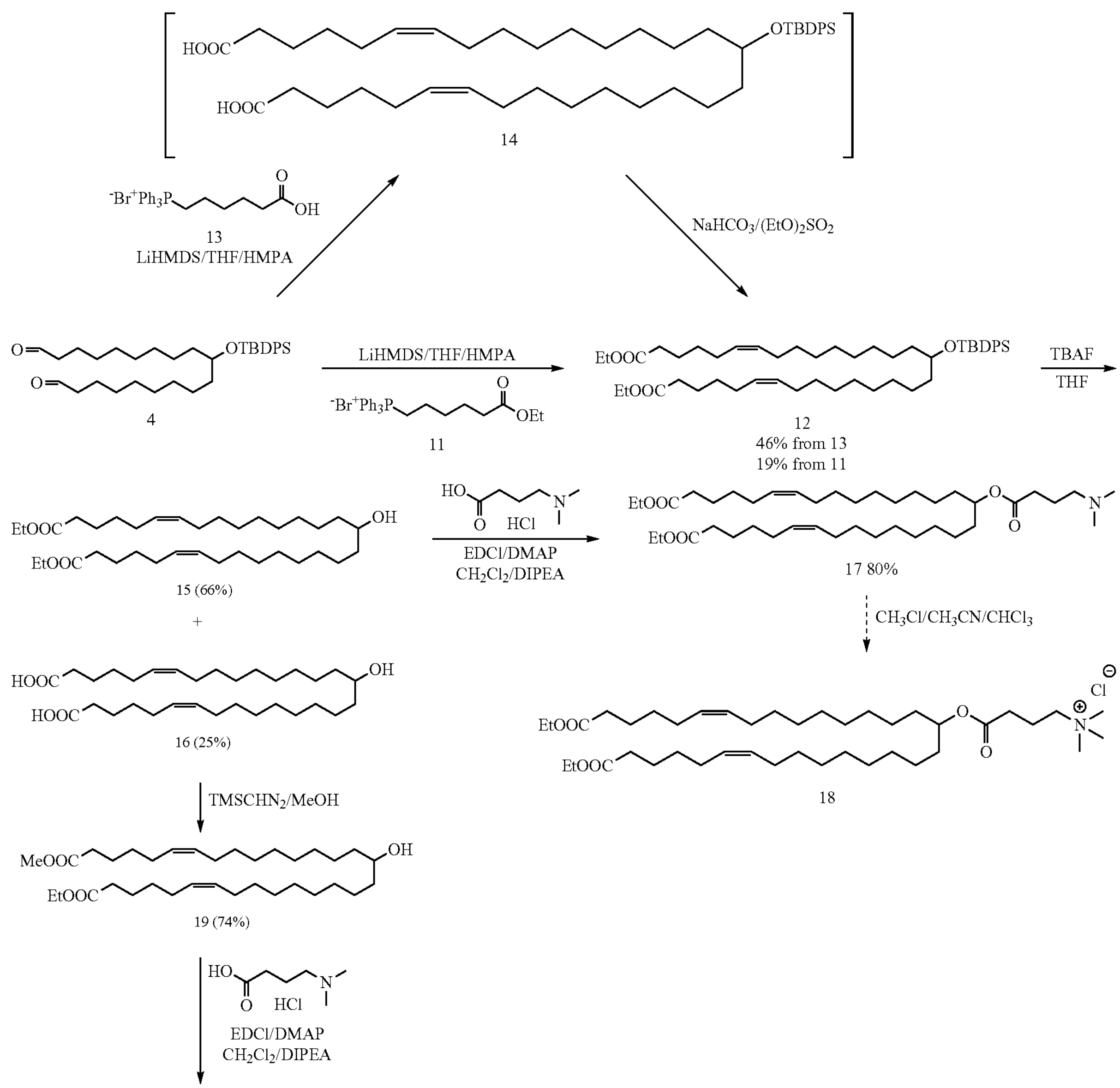
Compound 8: To a solution of 7 (265 mg, 0.308 mmol) in THF (2.5 mL), n-TBAF (1 M THF solution, 0.555 mL, 0.555 mmol) was added. The reaction mixture was stirred for 14 hours at 45° C. After concentration, the mixture was purified by silica gel column chromatography (Hexane: EtOAc=3:1, R_f=0.52) to afford 8 (155 mg, 0.250 mmol, 81%). Molecular weight for $C_{39}H_{72}NaO_5$ $(M+Na)^+$ Calc. 643.5277, Found 643.5273.

Compound 9: To a solution of compound 8 (150 mg, 0.242 mmol) and 4-(dimethylamino)butyric acid hydrochloride (49 mg, 0.290 mmol) in CH_2Cl_2 (5 mL) were added diisopropylethylamine (0.126 mL, 0.726 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (56 mg, 0.290 mmol) and DMAP (6 mg, 0.0484 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was then diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude product was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to afford compound 9 (121 mg, 0.165 mmol, 68%, R_f=0.25 developed with 5% MeOH in CH_2Cl_2). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.5.

Compound 10: Treatment of compound 9 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 10.

Example 2

Scheme 2



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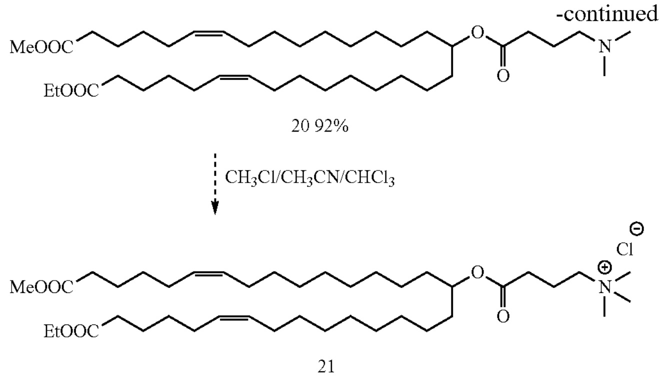


Compound 12: To a solution of 11 (Journal of Medicinal Chemistry (1995), 38, 636-46; 1.25 g, 2.58 mmol) in THF (20 mL) and HMPA (4 mL), LiHMDS (1 M THF solution, 2.58 mL, 2.58 mmol) was added at −20° C. The mixture was stirred for 20 min at the same temperature, then cooled to −78° C. A solution of 4 (500 mg, 0.908 mmol) in THF (9 mL) and HMPA (0.9 mL) was added. The mixture was stirred from −78° C. to room temperature overnight. The reaction was quenched by adding H_2O (40 mL) then extracted with Et_2O (150 mL×3). The organic layer was separated and dried over anhydrous Na_2SO_4. After filtration and concentration, the crude was purified by silica gel column chromatography (Hexane:EtOAc=9:1, R_f=0.35) to give 12 (136 mg, 0.169 mmol, 19%). Molecular weight for $C_{51}H_{82}NaO_5Si$ $(M+Na)^+$ Calc. 825.5829, Found 825.5.

Using 13 in place of 5, a procedure analogous to that described for compound 7 was followed to afford compound 12 (135 mg, 0.168 mmol, 46%).

Compound 15/Compound 16: To a solution of 12 (800 mg, 0.996 mmol) in THF (5 mL), n-TBAF (1 M THF solution, 5 mL, 5.00 mmol) was added. The reaction mixture was stirred for 16 h at 45° C. After concentration, the mixture was purified by silica gel column chromatography to give 15 (Hexane:EtOAc=3:1, R_f=0.46, 372 mg, 0.659 mmol, 66%) and 16 (CH_2Cl_2:MeOH=95:5, R_f=0.36, 135 mg, 0.251 mmol, 25%). Molecular weight for 15; $C_{45}H_{84}NaO_6$ $(M+Na)^+$ Calc. 587.4651, Found 587.4652. Molecular weight for 16; $C_{33}H_{61}O_5$ $(M+H)^+$ Calc. 537.4519, Found 537.5.

Compound 17: To a solution of compound 15 (164 mg, 0.290 mmol) and 4-(dimethylamino)butyric acid hydrochloride (58 mg, 0.348 mmol) in CH_2Cl_2 (5 mL) were added diisopropylethylamine (0.152 mL, 0.870 mmol), A-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (67 mg, 0.348 mmol) and DMAP (7 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to give compound 17 (158 mg, 0.233 mmol, 80%, R_f=0.24 developed with 5% MeOH in CH_2Cl_2). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.5.

Compound 18: Treatment of compound 17 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 18.

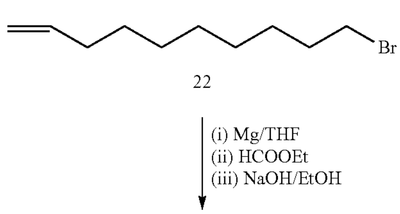
Compound 19: To a solution of 16 (130 mg, 0.242 mmol) in THF (2 mL) and MeOH (2 mL), trimethylsilyldiazomethane (2 M solution in Et_2O, 0.158 mL, 0.315 mmol) was added. The reaction mixture was stirred for 14 h. After evaporation, the residue was purified by silica gel column chromatography (Hexane:EtOAc=3:1, R_f=0.50) to give 19 (99 mg, 0.180 mmol, 74%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.29-5.40 (m, 4H), 4.12 (q, J=7.1 Hz, 2H), 3.66 (s, 3H), 3.55-3.59 (m, 1H), 2.30 (dd, J=14.7, 7.2 Hz, 4H), 1.98-2.07 (m, 8H), 1.60-1.68 (m, 4H), 1.23-1.43 (m, 37H).

Compound 20: To a solution of compound 19 (95 mg, 0.168 mmol) and 4-(dimethylamino)butyric acid hydrochloride (42 mg, 0.252 mmol) in CH_2Cl_2 (3 mL) were added diisopropylethylamine (0.088 mL, 0.504 mmol), A-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (48 mg, 0.504 mmol) and DMAP (4 mg, 0.034 mmol). The reaction mixture was stirred at room temperature for 14 hours. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ aq. (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% MeOH in CH_2Cl_2) to give compound 20 (103 mg, 0.155 mmol, 92%, R_f=0.19 developed with 5% MeOH in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$) δ 5.29-5.40 (m, 4H), 4.83-4.89 (m, 1H), 4.12 (q, J=7.1 Hz, 2H), 3.67 (s, 3H), 2.28-2.34 (m, 8H), 2.23 (s, 6H), 1.98-2.07 (m, 8H), 1.76-1.83 (m, 2H), 1.60-1.68 (m, 4H), 1.23-1.51 (m, 35H).

Compound 21: Treatment of compound 20 with CH_3Cl in CH_3CN and $CHCl_3$ can afford compound 21.

Example 3: Alternate Synthesis for Di-Aldehyde Intermediate 4

Scheme 3



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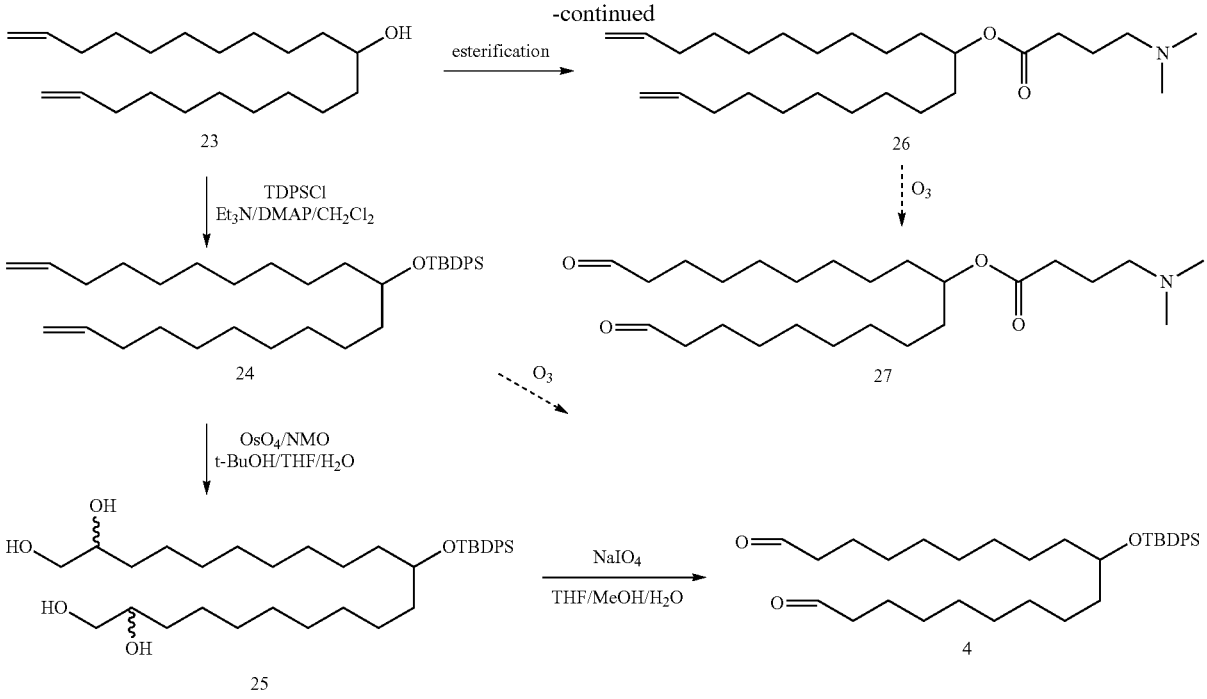
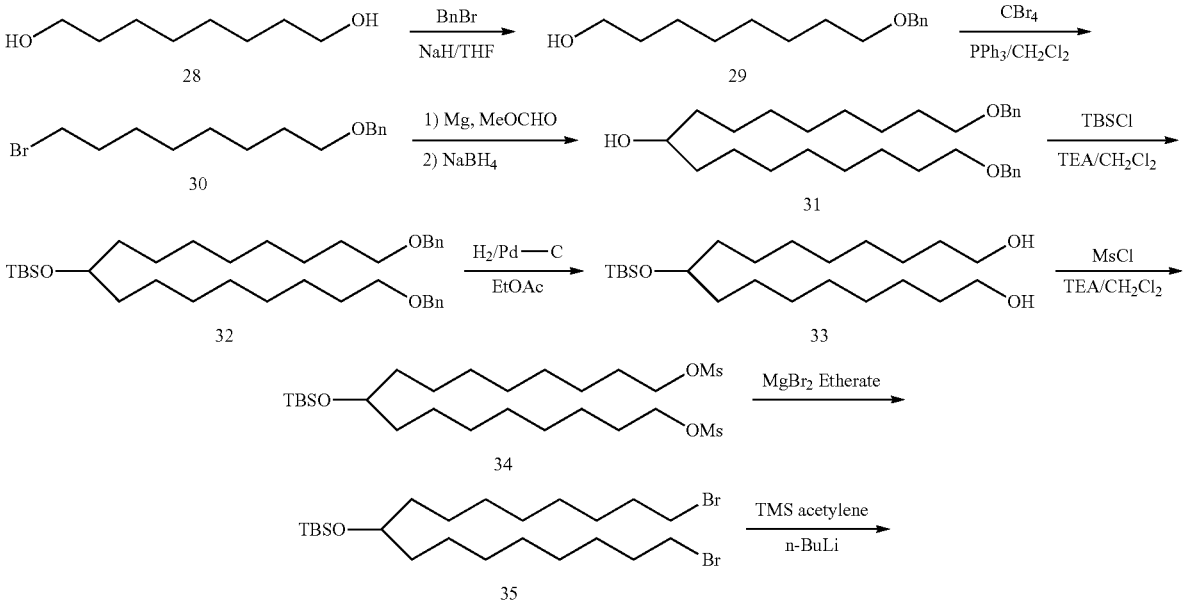


The di-aldehyde 4 can be synthesized as shown in Scheme 3, using 1-bromo-9-decene. Di-aldehyde containing a head group 27 can be useful for the synthesis of terminal ester-substituted lipids using, e.g., a Wittig reaction. Ozonolysis can afford di-aldehyde 4 and 27.

Example 4: Alternate Synthesis for Compound 8

Scheme 4



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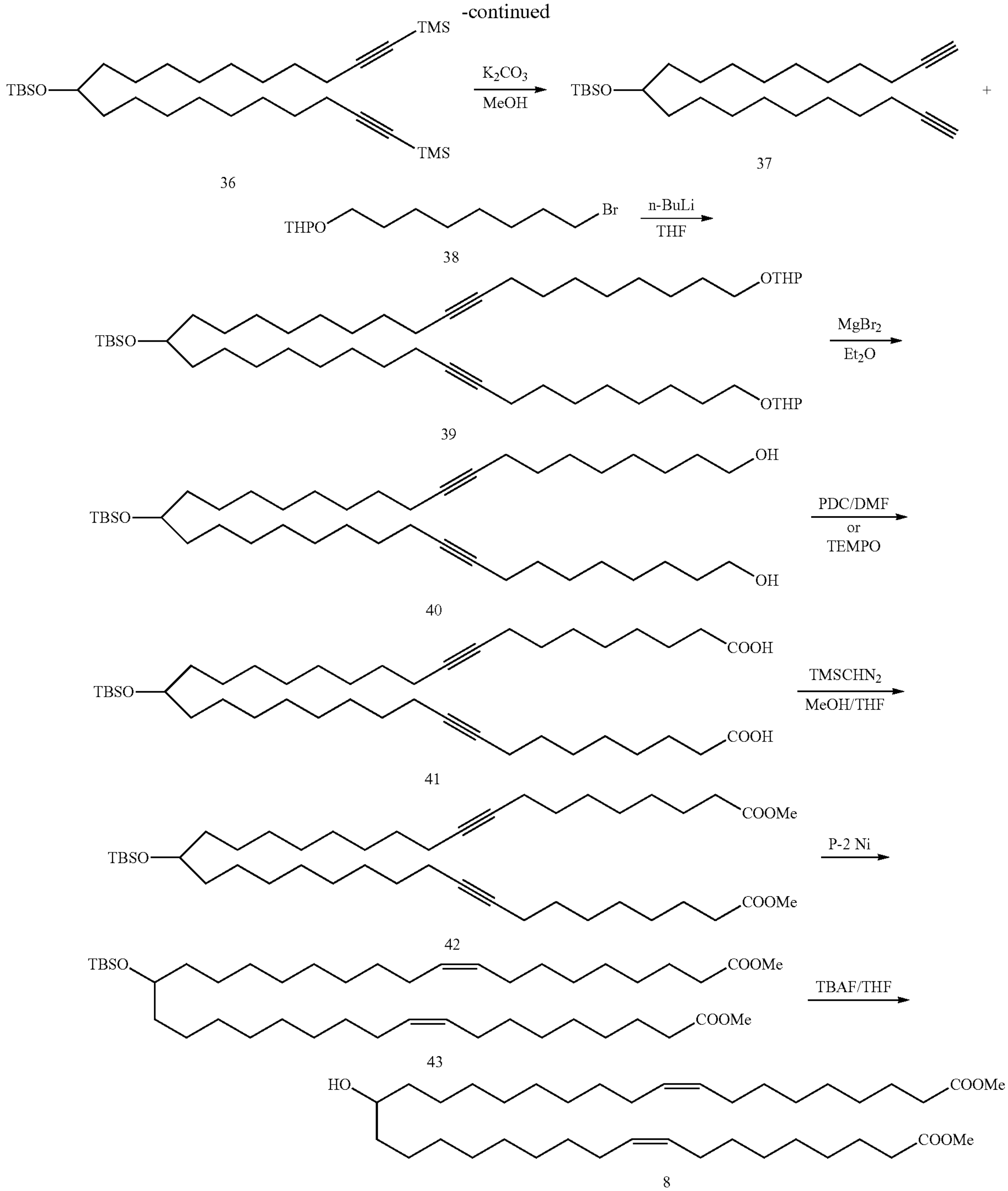


Compound 8 can be synthesized as shown in Scheme 4.

Compound 29: To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 28 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly using a dropping funnel at 0° C. The reaction mixture was stirred for 30 minutes, then benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under an atmosphere of nitrogen. The reaction was then warmed to ambient temperature and stirred for 10 hours. The mixture was then quenched with crushed ice (~2 kg) and extracted with ethyl acetate (2×1 L). The organic layer was washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness in vacuo. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 29 (220 g, 54%) as a pale yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

Compound 30: Compound 29 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM, CBr_4 (280.35 g, 0.8456 mol) was added into this stirring solution and the reaction mixture was cooled to 0° C. under an inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions keeping the temperature below 20° C. After complete addition, the reaction

mixture was stirred for 3 hours at room temperature. After completion of the reaction, the solid (PPh_3O) that precipitated from the reaction mixture was removed by filtration, and the filtrate was diluted with crushed ice (~ 1.5 kg) and extracted with DCM (3×750 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and distilled under vacuum. The resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to afford compound 30 (150 g, 89%) as pale yellow liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

Compound 31: To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF, followed by the addition of pinch of iodine into the mixture under an inert atmosphere. A solution of Compound 30 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly, controlling the exothermic reaction. The reaction was then heated to reflux for 1 hour, then cooled to room temperature. Methyl formate (60.24 g, 1.0033 mol) was then added slowly and the reaction was continued for 2 hours. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with ethyl acetate (3×1 L). The organic layer was taken in 5 litre beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution, an excess of $NaBH_4$ (~ 5 eq) was added in portions to ensure hydrolysis of the formate ester which was not cleaved by addition of HCl. The resulting solution was stirred for an hour and then volatilites were removed under vacuum. The residue was taken in water (1 L) and acidified by 10% HCl solution (pH 4). The product was then extracted with ethyl acetate (3×1 L). the organic phase was then dried and concentrated on rotary evaporator to afford the desired compound 31 (57 g, 24%) as solid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

Compound 32: Compound 31 (56 g, 0.1196 mol) was dissolved in 700 mL dry THF and cooled to 0° C. TBSCl (36.06 g, 0.2396 mol) was added slowly followed by the addition of imidazole (32.55 g, 0.4786 mol) under an inert atmosphere. The reaction was then stirred at room temperature for 18 hours. Upon completion, the reaction was quenched with ice (~1 kg) and extracted with ethyl acetate (3×500 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution to remove acidic impurities, dried over Na_2SO_4 and evaporated under reduce pressure to afford a crude compound that was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to afford (60 g, 82%) of compound 32 as yellowish oil. ^1H NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s, 4H), 3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H). Compound 33: Compound 32 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 minutes. (10 wt %) Pd on carbon (12 g) was added and the reaction was stirred under an atmosphere of hydrogen for 18 hours. After completion, the mixture was filtered through a bed of celite and washed with ethyl acetate. The filtrate was evaporated under vacuum to afford compound 33 (19 g, 46%) that was pure enough to use in the next synthetic sequence. ^1H NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 34: Compound 33 (8.2 g, 0.0199 mol) was dissolved in 100 mL dry DCM and cooled to 0° C. TEA (22.14 mL, 0.1592 mol) was added under an inert atmosphere. After stirring the mixture for 5 minutes, mesyl chloride (4.6 mL, 0.059 mol) was added drop wise and the reaction was stirred further for 3 hours. After completion of the reaction, the mixture was quenched with ice (~200 g) and extracted with DCM (3×75 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated to afford a crude compound which was purified on a 60-120 mesh silica gel column using 0-30% ethyl acetate in hexane as eluting system to afford compound 34 (8.2 g, 73%) as a pale yellow liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=4.22-4.19 (m, 4H), 3.60-3.58 (m, 1H), 2.99 (s, 6H), 1.75-1.69 (m, 4H), 1.38-1.28 (m, 28H), 0.86 (s, 9H), 0.02 (s, 6H).

Compound 35: To a solution of compound 34 (8.2 g, 0.0146 mol) in 400 mL dry ether was added $MgBr_2.Et_2O$ (22.74 g, 0.08817 mol) in portions at 0° C. under a nitrogen atmosphere. After complete addition, the reaction mixture was heated to reflux for 28 hours. After completion of reaction, inorganic material formed in the reaction was removed by filtration. The filtrate was evaporated and the resulting crude compound was purified on 60-120 mesh silica gel column using 0-3% ethyl acetate in hexanes as eluting system to afford compound 35 (6.6 g, 85%) as a colorless liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=3.61-3.58 (m, 1H), 3.41-3.37 (t, 4H, J=6.8 Hz), 1.87-1.80 (m, 4H), 1.42-1.25 (m, 24H), 0.87 (s, 9H), 0.012 (s, 6H). Compound 36: A solution of ethynyl trimethyl silane (5.3 mL, 0.0378 mol) in 60 mL dry THF was cooled to −78° C. and 1.4 M w-BuLi (23 mL, 0.03405 mol) in hexane was added slowly under an inert atmosphere. The reaction was stirred for 10 minutes, then HMPA (2.3 g, 0.01324 mol) was added and the resulting mixture was then stirred for 2 hours at 0° C., then cooled to −78° C. To this a solution of compound 35 (5 g, 0.0094 mol) in 60 mL dry THF was added slowly and after complete addition, the reaction was warmed to room temperature and maintained for 18 hours. The reaction progress was monitored by ^1H NMR. After completion, the reaction mixture was cooled to 0° C. and quenched by careful addition of saturated NH_4Cl solution (50 mL) followed by water (200 mL). The aqueous phase was extracted with hexane (3×250 mL). The organic layer was dried and solvent removed under vacuum to afford compound 36 (5 g, 94%), which was used without further purification. ^1H NMR (400 MHz, $CDCl_3$): δ=3.62-3.56 (m, 1H), 2.21-2.17 (m, 4H), 1.49-1.47 (m, 4H), 1.37-1.26 (m, 24H), 0.87 (s, 9H), 0.13 (s, 18H), 0.021 (s, 6H).

Compound 37: To a stirred solution of compound 36 (5 g, 0.0088 mol) in 50 mL methanol, was added K_2CO_3 (6.1 g, 0.044 mol) in one portion, and the resulting mixture was stirred for 18 hours at ambient temperature. Volatilities were then removed on a rotary evaporator and the crude mixture was diluted with 100 mL water and extracted with hexane (3×100 mL). The organic layer was dried over Na_2SO_4 and evaporated under vacuum to afford compound 37 (3.5 g, 97%) which was used which was used without further purification. ^1H NMR (400 MHz, $CDCl_3$): δ=3.60-3.58 (m, 1H), 2.19-2.14 (m, 4H), 1.93-1.92 (m, 2H), 1.54-1.49 (m, 4H), 1.37-1.27 (m, 24H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 39: Compound 37 (2.5 g, 0.00598 mol) was dissolved in 25 mL dry THF and cooled to −40° C. n-BuLi (1.4 M in hexane 12.9 mL, 0.01794 mol) was added slowly, followed, after a 10 minute interval, by slow addition of HMPA (25 mL). The resulting mixture was maintained for 30 minutes −40° C. under a nitrogen atmosphere. A solution of compound 38 (3.5 g, 1.01196 mol) in 25 mL dry THF was

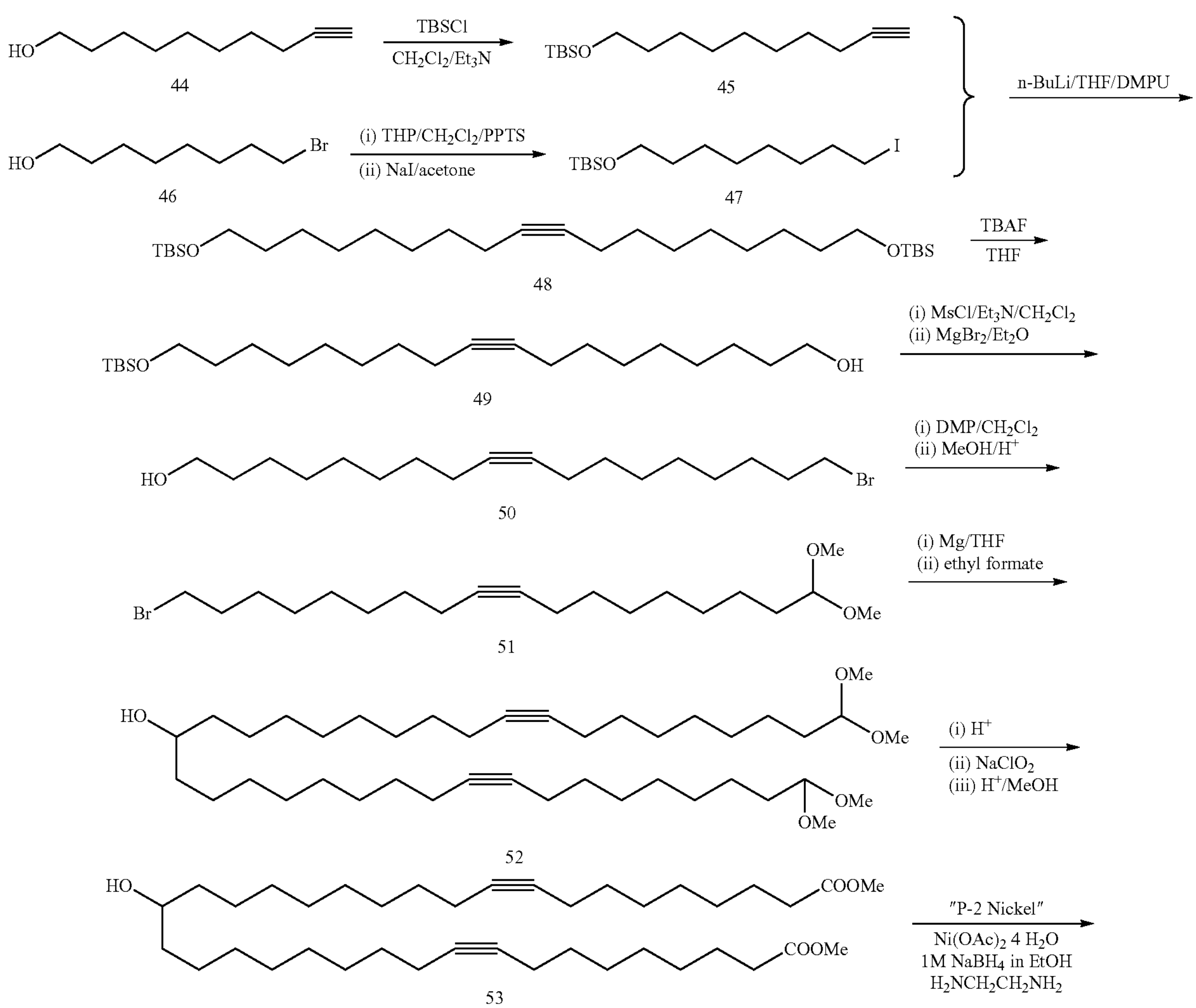
then added drop wise to the cooled reaction mixture. The resulting mixture was warmed to room temperature over 2 hours, then stirred at room temperature for 18 hours. The mixture was then quenched by adding saturated NH_4Cl solution (~50 mL) and the product was extracted with ethyl acetate (3×50 mL). The solvent was removed on a rotary evaporator and the resulting crude product was purified by (100-200 mesh) silica gel column using 0-3% ethyl acetate in dichloromethane as eluting system to afford compound 39 (0.9 g, 18%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ=4.56-4.55 (m, 2H), 3.87-3.83 (m, 2H), 3.74-3.68 (m, 2H), 3.59-3.57 (m, 1H), 3.49-3.46 (m, 2H), 3.39-3.33 (m, 2H), 2.13-2.10 (m, 8H), 1.87-1.75 (m, 2H), 1.74-1.66 (m, 2H), 1.57-1.42 (m, 20H), 1.40-1.19 (m, 40H), 0.87 (s, 9H), 0.02 (s, 6H).

Compound 40: To a solution of compound 39 (504 mg, 0.598 mmol) in 10 mL dry ether was added $MgBr_2Et_2O$ (926 mg, 3.59 mmol). The reaction mixture was stirred for 14 hours, then quenched by adding saturated $NaHCO_3$ aqueous solution. The product was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography to afford compound 40 (307 mg, 0.455 mmol, 76%, R_f=0.36 developed with hexane:EtOAc=2:1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.59-3.66 (m, 5H), 2.14 (t, J=6.6 Hz, 8H), 1.21-1.59 (m, 52H), 0.88 (s, 9H), 0.03 (s, 6H). Compound 41: To a stirred solution of 40 (180 mg, 0.267 mmol) in anhydrous DMF (5 mL) was added pyridinium dichromate (603 mg, 1.60 mmol). The reaction mixture was stirred for 48 hours. After dilution with water (20 mL), the mixture was extracted with Et_2O (3×40 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography to afford compound 41 (53 mg, 0.075 mmol, 28%, R_f=0.25 developed with CH_2Cl_2:MeOH:AcOH=95:4.5:0.5). Molecular weight for $C_{43}H_{77}O_5Si$ $(M-H)^-$ Calc. 701.5540, Found 701.5. This compound can be synthesized by TEMPO oxidation. Compound 42: A procedure analogous to that described for compound 19 afforded compound 42 (23 mg 0.032 mmol, 21% from compound 40). ^1H NMR (400 MHz, $CDCl_3$) δ 3.67 (s, 6H), 3.59-3.62 (m, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.13 (t, J=6.8 Hz, 8H), 1.27-1.64 (m, 48H), 0.88 (s, 9H), 0.03 (s, 6H).

Reduction using P-2 nickel conditions can give compound 43 and subsequent deprotection by TBAF can afford compound 8.

Example 5: Alternate Synthesis for Compound 8

Scheme 5



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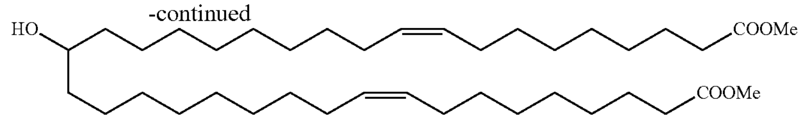


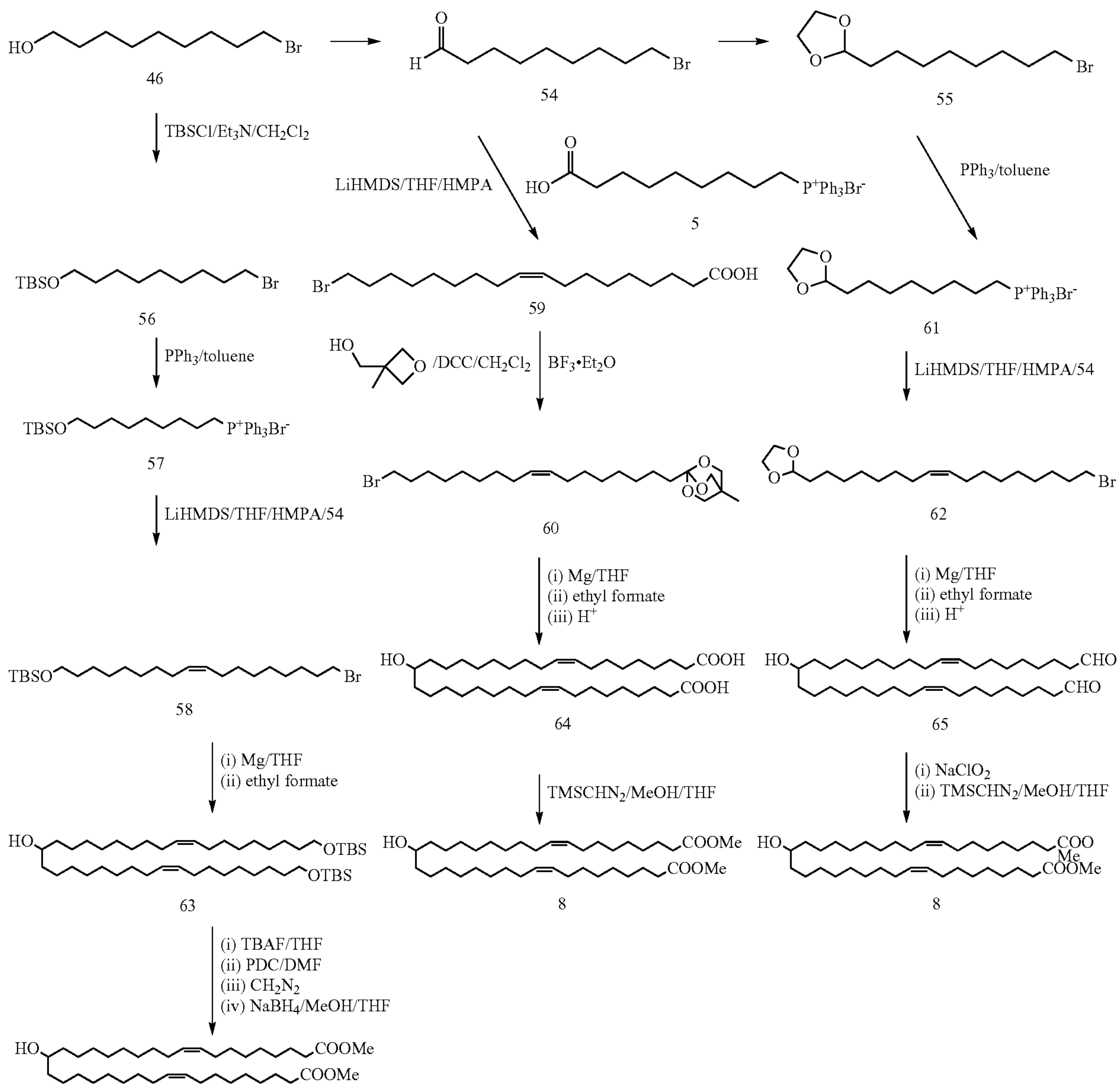
8

Compound 8 can be synthesized as shown in Scheme 5. The bromide 51 can be converted to its Grignard reagent then coupled with ethyl formate to afford compound 52. Subsequent acid treatment, oxidation, and reduction can give compound 8.

Example 6: Alternate Synthesis for Compound 8

Compound 8 can be synthesized as shown in Scheme 6. Either bromides of compound 58, 60, or 62 can be reacted with ethyl formate to generate terminal-functionalized diolefin chain. Compound 8 can then be prepared from the diolefin chain compounds using standard chemical reactions.

Scheme 6



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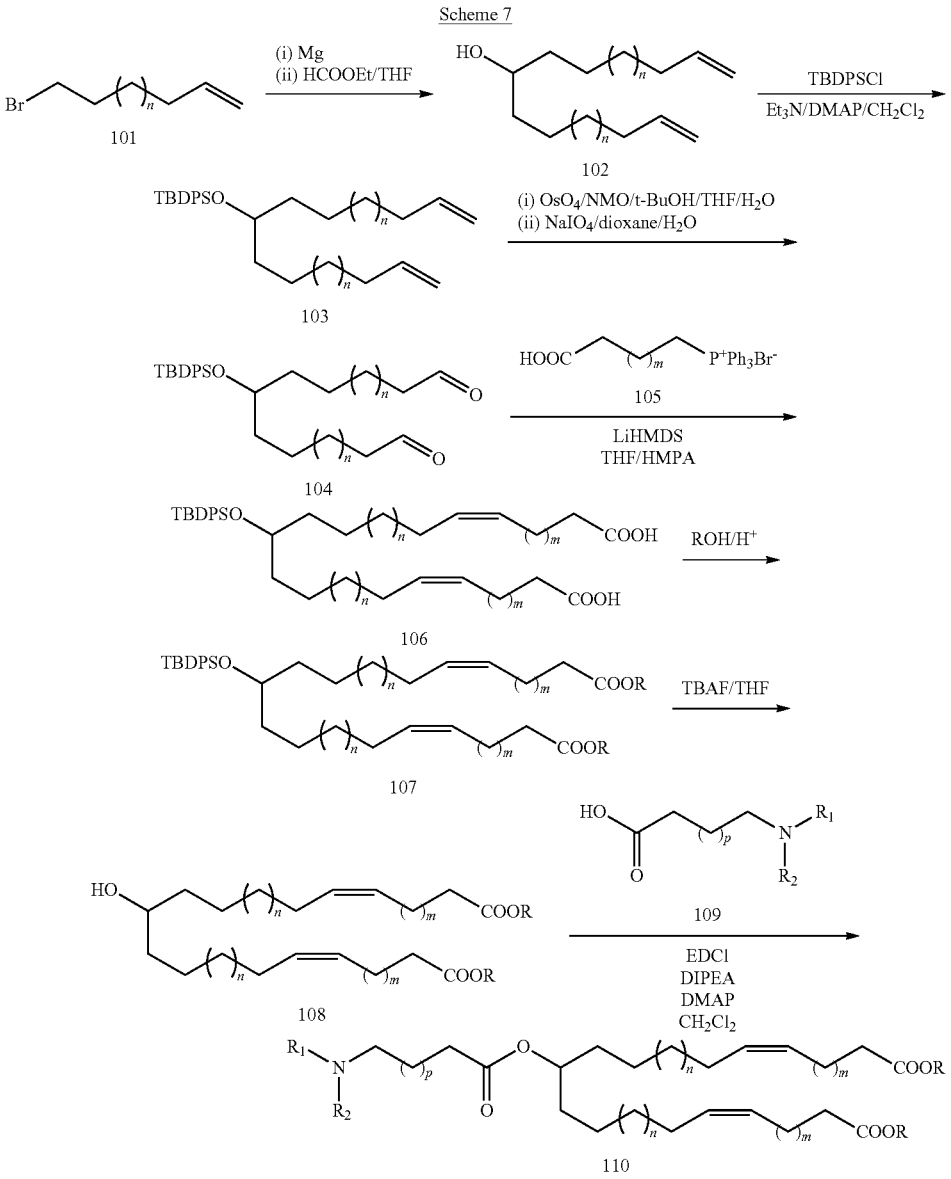
Case 23-2357 Document: 14 Page: 574 Filed: 12/04/2023

Example 7: General Synthetic Scheme for Terminal Ester Lipids

Scheme 7



n = 0-8 m = 0-8 p = 0-3 R = R_1 = R_2 = Me, Et, Pr, Bn, t-Bu, Ph, alkyl, aryl, cycloalkyl, etc.

As shown in Scheme 7, chain length and linker length as well as alkyl groups in ester functionality and substituents on nitrogen atom can be derivatized.

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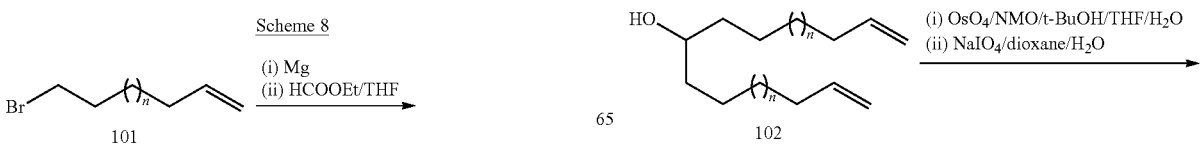
Example 8: General Synthetic Scheme 2 for Terminal Ester Lipids

Scheme 8



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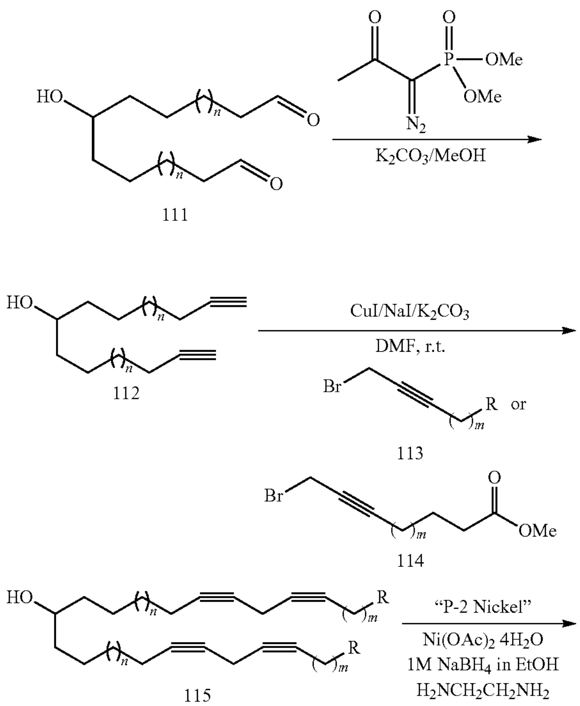

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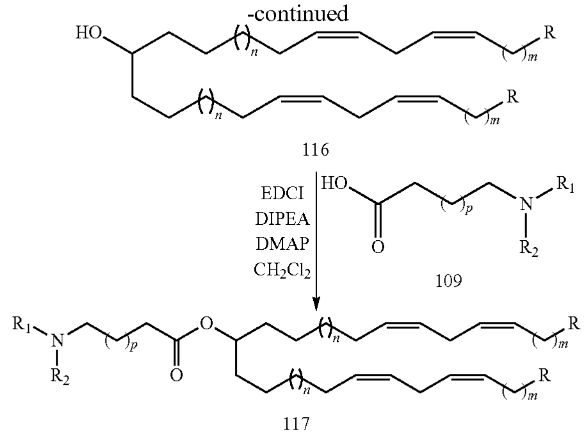
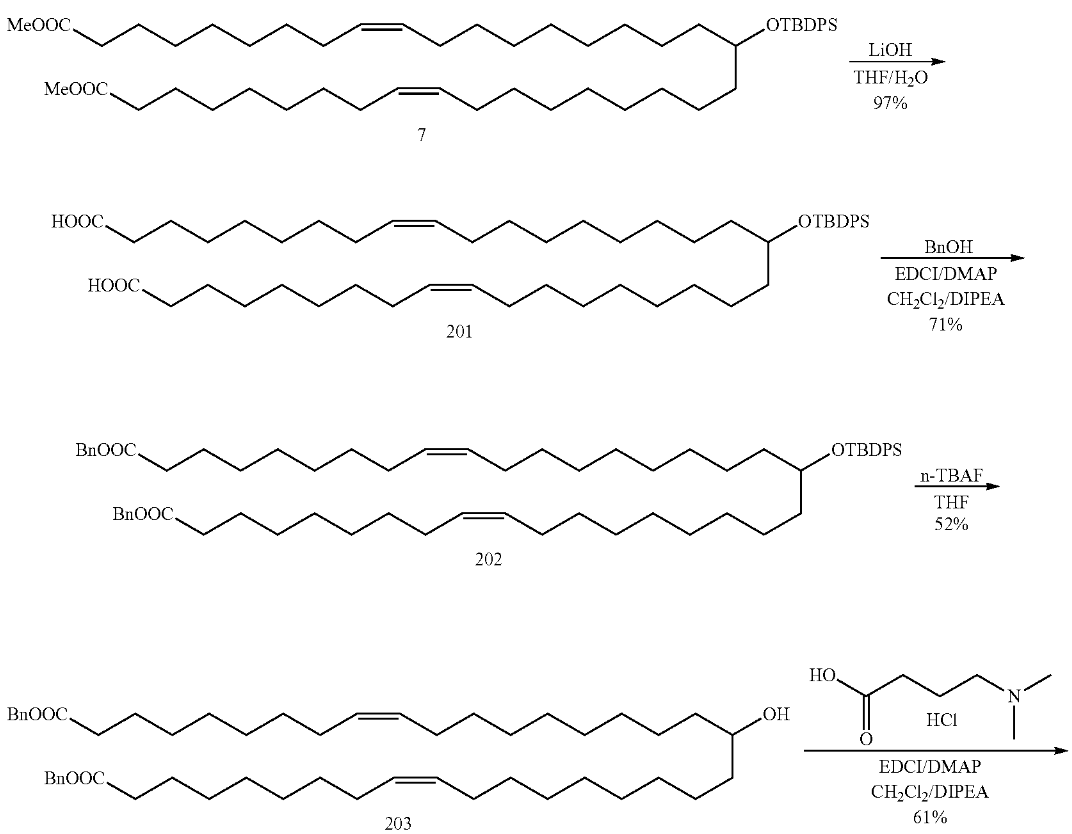


n = 0-8
m = 0-8
p = 0-3
R = Me, Et, Pr, Bn, t-Bu, Ph, alkyl, aryl, cycloalkyl, and alkyl esters, etc.
R_1 = R_2 = Me, Et, Pr, Bn, t-Bu, Ph, alkyl, aryl, cycloalkyl

As shown in Scheme 8, copper-mediated coupling affords di-yne containing lipid chain with terminal functional groups, which can be easily reduced to generate di-ene containing lipid chains. The length of linker and lipid chain as well as functional substituent groups (R, R_1, R_2) can be derivatized.

Example 9: Synthesis of Terminal Benzyl Ester Lipid

Scheme 9



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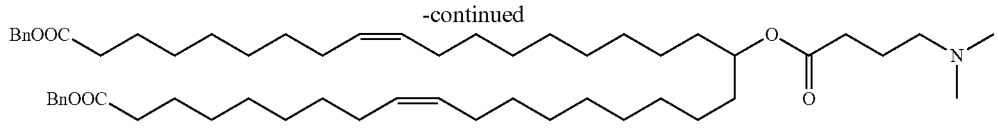


204

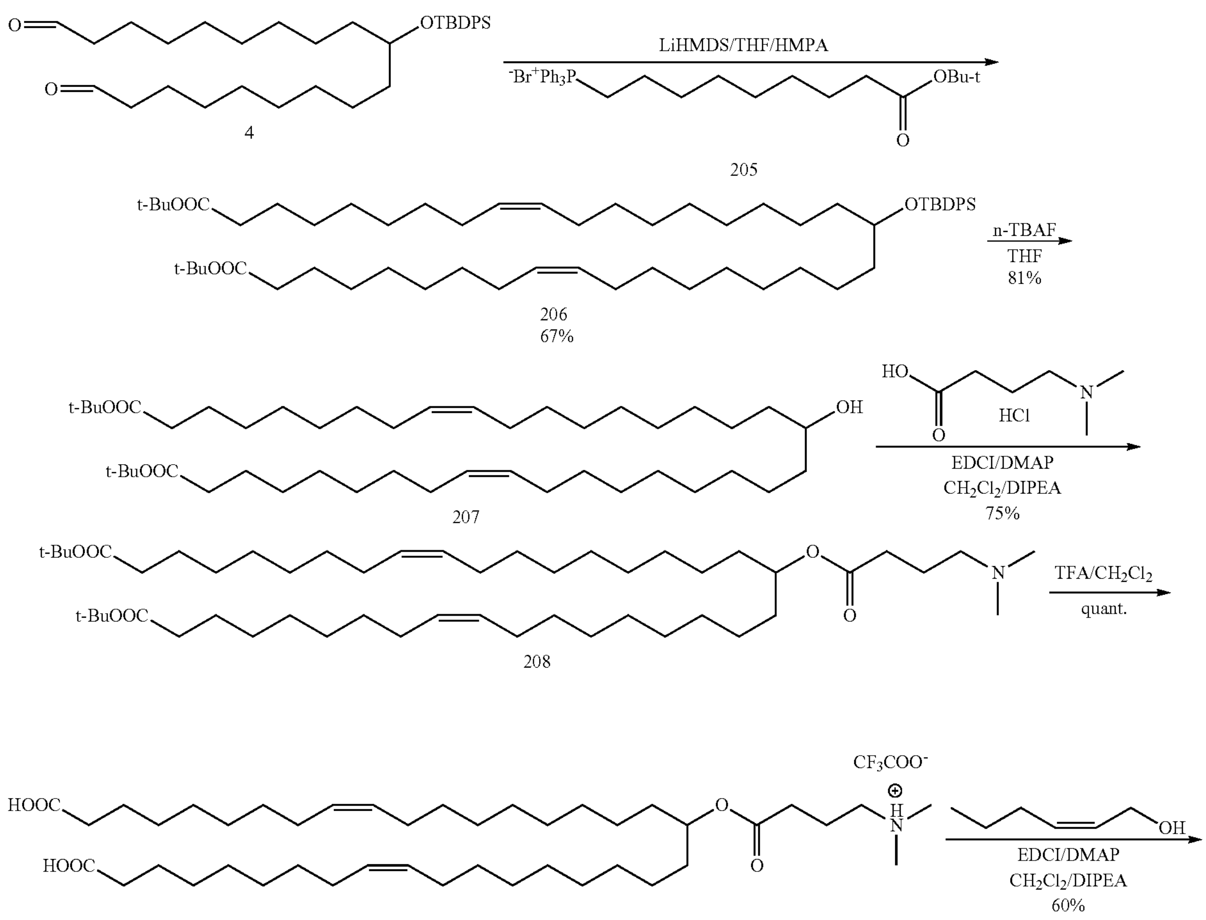
Compound 201: Compound 7 (1.30 g, 1.51 mmol) was treated with lithium hydroxide monohydrate (317 mg, 7.55 mmol) in THF (25 mL) and H_2O (5 mL) for 12 h. Amberlite IR-120 (plus) ion exchange resin was added then stirred for 10 minutes. The resulting clear solution was filtered, washed with THF/H_2O and evaporated. Co-evaporation with toluene gave the compound 201 (1.22 g, 1.47 mmol, 97%). Molecular weight for $C_{53}H_{85}O_5Si$ $(M-H)^-$ Calc. 829.6166, Found 829.5.

Compound 202: A procedure analogous to that described for compound 9 was followed with benzylalcohol and 201 (101 mg, 0.121 mmol) to afford compound 202 (87 mg, 0.0860 mmol, 71%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.30 (m, 16H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 3.71-3.68 (m, 1H), 2.35 (t, J=7.6 Hz, 4H), 2.04-1.97 (m, 8H), 1.66-1.62 (m, 4H), 1.40-1.07 (m, 44H), 1.04 (s, 9H).

Compound 203: A procedure analogous to that described for compound 8 was followed with 202 (342 mg, 0.338 mmol) to afford compound 202 (136 mg, 0.176 mmol, 52%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.38-7.30 (m, 10H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 3.57 (brs, 1H), 2.35 (t, J=7.6 Hz, 4H), 2.01-1.98 (m, 8H), 1.66-1.60 (m, 4H), 1.45-1.25 (m, 44H). Compound 204: A procedure analogous to that described for compound 9 was followed with 203 (133 mg, 0.172 mmol) to afford compound 204 (93 mg, 0.105 mmol, 61%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.38-7.26 (m, 10H), 5.38-5.30 (m, 4H), 5.11 (s, 4H), 4.88-4.83 (m, 1H), 2.37-2.27 (m, 8H), 2.22 (s, 6H), 2.03-1.97 (m, 8H), 1.81-1.26 (m, 50H).

Example 10: Synthesis of Terminal t-Butyl Ester Lipid and the Derivatives

Scheme 10



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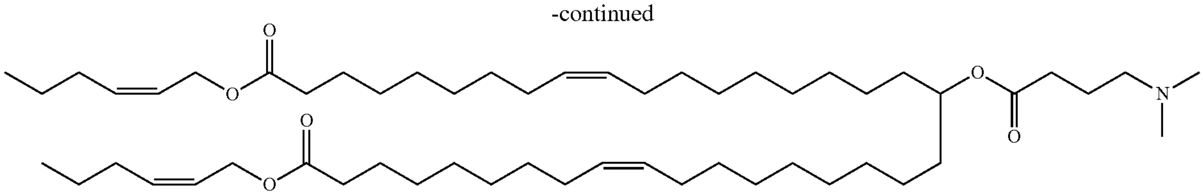


210

Compound 206: A procedure analogous to that described for compound 12 was followed with 205 (3.80 g, 0.7.61 mmol) and 4 (1.75 g, 3.17 mmol) to afford compound 206 (2.00 g, 2.12 mmol, 67%). [1]H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.33 (m, 6H), 5.39-5.31 (m, 4H), 3.71-3.68 (m, 1H), 2.20 (t, J=7.6 Hz, 4H), 2.01-1.98 (m, 8H), 1.59-1.55 (m, 4H), 1.44 (s, 18H), 1.41-1.11 (m, 44H), 1.04 (s, 9H).

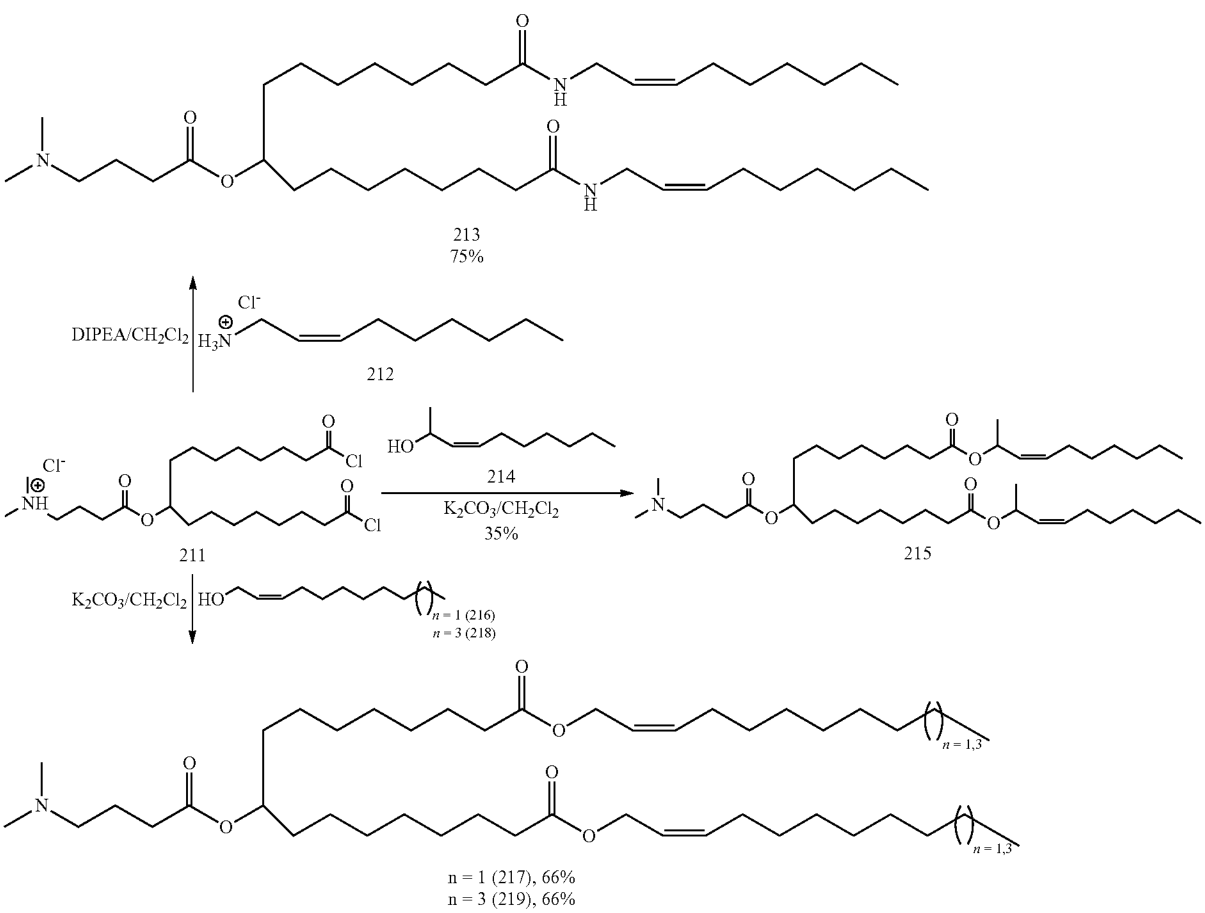
Compound 207: A procedure analogous to that described for compound 8 was followed with 206 (265 mg, 0.281 mmol) to afford compound 207 (161 mg, 0.228 mmol, 81%). [1]H NMR (400 MHz, $CDCl_3$) δ 5.38-5.30 (m, 4H), 3.58 (brs, 1H), 2.20 (t, J=7.4 Hz, 4H), 2.01-1.98 (m, 8H), 1.59-1.55 (m, 4H), 1.44 (s, 18H), 1.35-1.26 (m, 44H).

Compound 208: A procedure analogous to that described for compound 9 was followed with 207 (158 mg, 0.224 mmol) to afford compound 208 (138 mg, 0.169 mmol, 75%). Molecular weight for $C_{51}H_{96}NO_6$ $(M+H)^+$ Calc. 818.7238, Found 818.7.

Compound 209: Compound 208 (148 mg, 0.181 mmol) was treated with TFA (1.5 mL) in CH_2Cl_2 (6 mL) for 2.5 h. After evaporation and co-evaporation with toluene gave the compound 209 (154 mg, quant.). Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5980, Found 706.5. Compound 210: A procedure analogous to that described for compound 9 was followed with 209 (0.061 mmol) and cis-2-Hexen-1-ol (18.3 mg, 0.183 mmol) to afford compound 210 (32 mg, 0.0368 mmol, 60%). Molecular weight for $C_{55}H_{100}NO_6$ $(M+H)^+$ Calc. 870.7551, Found 870.5.

Example 11: Synthesis of Internal Ester/Amide Lipids-1

Scheme 11



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Compound 213: Compound 211 (503 mg, 1.0 mmol) was treated with 212 (533 mg, 3.0 mmol) in CH_2Cl_2 (35 mL) and DIPEA (1.74 mL, 10 mmol) for 14 h. Aqueous work-up then column chromatography gave compound 213 (506 mg, 0.748 mmol, 75%). Molecular weight for $C_{41}H_{78}N_3O_4$ $(M+H)^+$ Calc. 676.5992, Found 676.4.

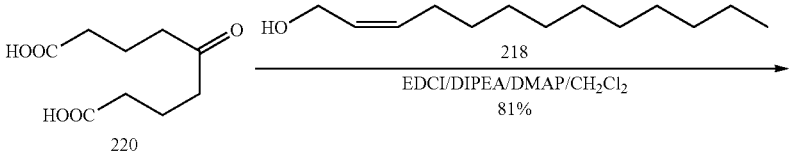
Compound 215: Compound 211 (503 mg, 1.0 mmol) was treated with 214 (469 mg, 3.0 mmol) and K_2CO_3 (1.38 g, 10 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 215 (244 mg, 0.346 mmol, 35%). Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5986, Found 706.4.

Compound 217: Compound 211 (425 mg, 0.845 mmol) was treated with 216 (525 mg, 3.08 mmol) and K_2CO_3 (1.17 g, 8.45 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 217 (407 mg, 0.554 mmol, 66%). Molecular weight for $C_{45}H_{84}NO_6$ $(M+H)^+$ Calc. 734.6299, Found 734.4.

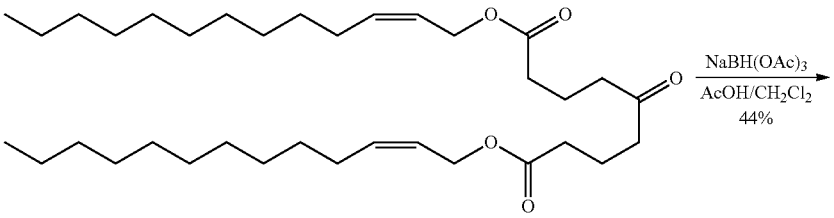
Compound 219: Compound 211 (503 mg, 1.0 mmol) was treated with 218 (595 mg, 3.0 mmol) and K_2CO_3 (1.38 g, 10 mmol) in CH_2Cl_2 (35 mL) for 14 h. Aqueous work-up then column chromatography gave compound 219 (519 mg, 0.657 mmol, 66%). Molecular weight for $C_{49}H_{92}NO_6$ $(M+H)^+$ Calc. 790.6925, Found 790.7.

Example 12: Synthesis of Internal Ester Lipid-223

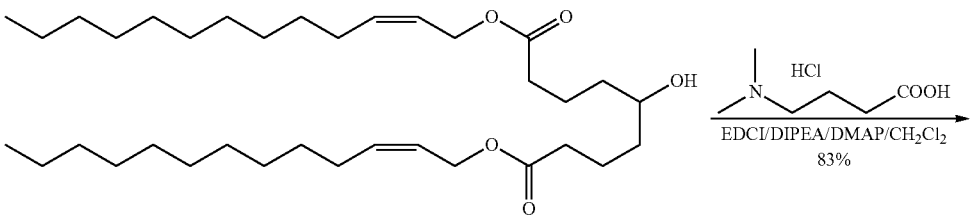
Scheme 12



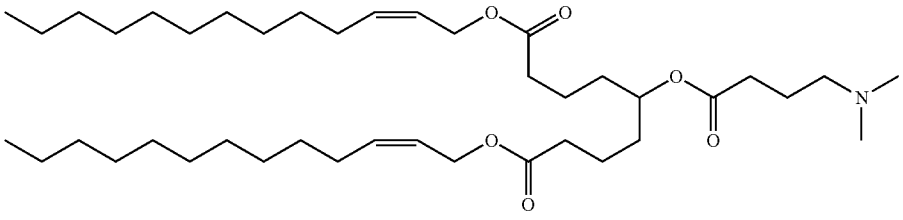
220



221



222



223

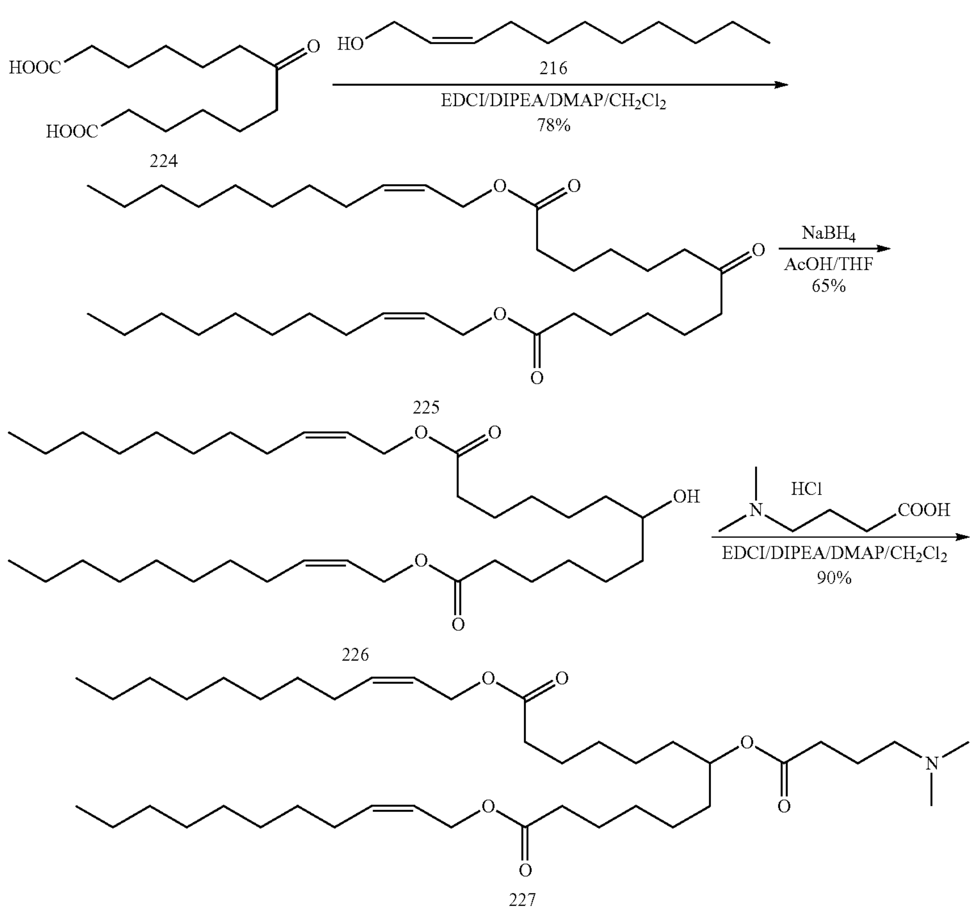
Compound 221: A procedure analogous to that described for compound 9 was followed with 220 (390 mg, 1.93 mmol) and 218 (765 mg, 3.86 mmol) to afford compound 221 (878 mg, 1.56 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.54-5.48 (m, 2H), 4.62 (d, J=6.8 Hz, 4H), 2.47 (t, J=7.2 Hz, 4H), 2.33 (t, J=7.2 Hz, 4H), 2.12-2.06 (m, 4H), 1.93-1.86 (m, 4H), 1.38-1.26 (m, 32H), 0.88 (t, J=6.8 Hz, 6H).

Compound 222: Compound 221 (318 mg, 0.565 mmol) was treated with $NaBH(OAc)_3$ (360 mg, 1.70 mmol) in CH_2Cl_2 (5 mL) and AcOH (0.2 mL) for 16 h. After evaporation, column chromatography gave compound 222 (141 mg, 0.250 mmol, 44%). Molecular weight for $C_{35}H_{65}O_5$ $(M+H)^+$ Calc. 565.4832, Found 565.4.

Compound 223: A procedure analogous to that described for compound 9 was followed with 222 (137 mg, 0.243 mmol) to afford compound 223 (137 mg, 0.202 mmol, 83%). Molecular weight for $C_{41}H_{76}NO_6$ $(M+H)^+$ Calc. 678.5673, Found 678.5.

Example 13: Synthesis of Internal Ester Lipid-227

Scheme 13



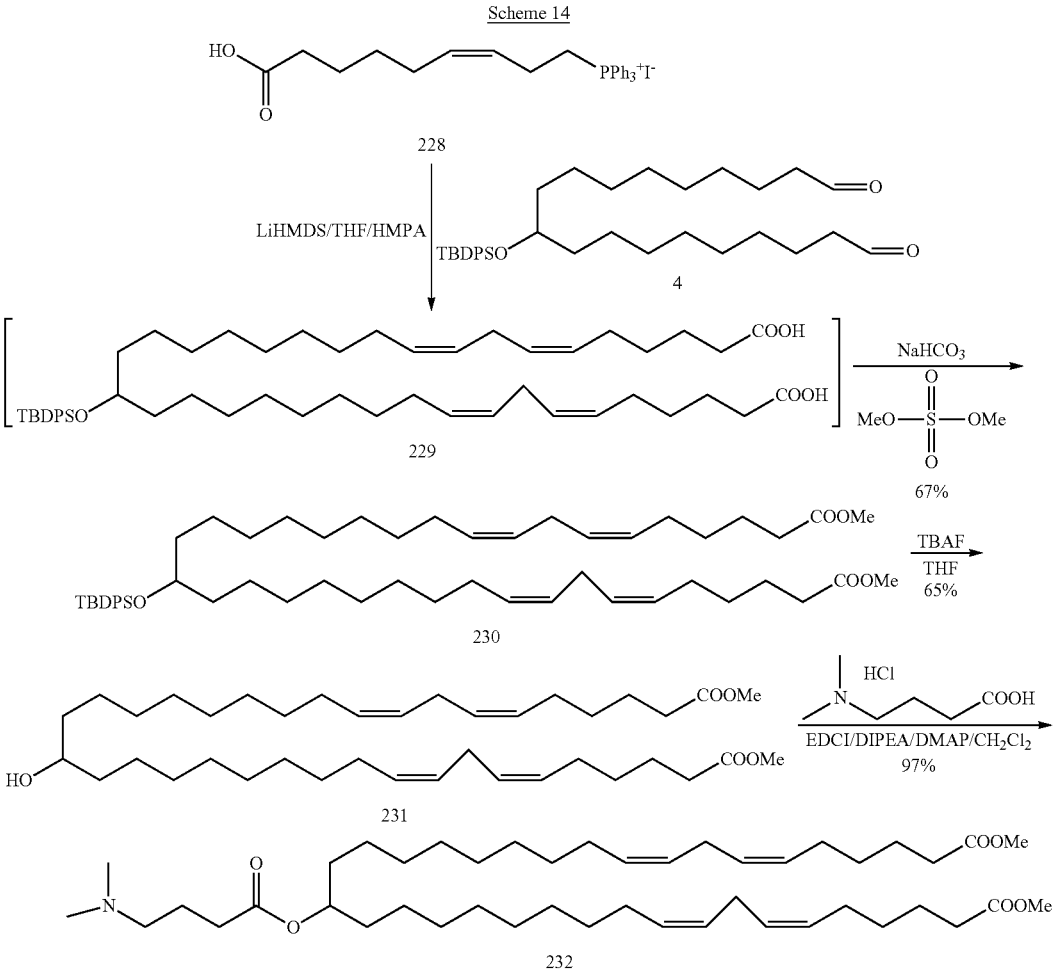
Compound 225: A procedure analogous to that described for compound 9 was followed with 224 (200 mg, 0.774 mmol) and 216 (264 mg, 1.55 mmol) to afford compound 225 (341 mg, 0.606 mmol, 78%). Molecular weight for $C_{35}H_{62}NaO_5$ $(M+Na)^+$ Calc. 585.4495, Found 585.5.

Compound 226: Compound 225 (283 mg, 0.503 mmol) was treated with $NaBH_4$ (57 mg, 1.51 mmol) in THF (5 mL) and AcOH (0.2 mL) for 8 h. After evaporation, column chromatography gave compound 226 (185 mg, 0.328 mmol, 65%). Molecular weight for $C_{35}H_{64}NaO_5$ $(M+Na)^+$ Calc. 587.4651, Found 587.3.

Compound 227: A procedure analogous to that described for compound 9 was followed with 226 (230 mg, 0.407 mmol) to afford compound 227 (248 mg, 0.366 mmol, 90%). Molecular weight for $C_{41}H_{76}NO_6$ $(M+H)^+$ Calc. 678.5673, Found 678.5.

Example 14: Synthesis of Terminal Ester Lipid with Linoleyl Chain-232

Scheme 14



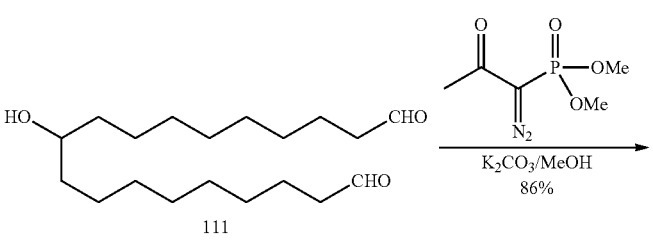
Compound 230: A procedure analogous to that described for compound 7 was followed with 228 (3.27 g, 6.0 mmol) and 4 (1.27 g, 2.30 mmol) to afford compound 230 (1.31 g, 1.53 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.42-7.33 (m, 6H), 5.42-5.29 (m, 8H), 3.71-3.68 (m, 1H), 3.66 (s, 6H), 2.77 (t, J=5.8 Hz, 4H), 2.33-2.28 (m, 4H), 2.11-2.01 (m, 8H), 1.69-1.60 (m, 4H), 1.43-1.10 (m, 32H), 1.04 (s, 9H).

Compound 231: A procedure analogous to that described for compound 8 was followed with 230 (1.30 g, 1.52 mmol) to afford compound 231 (611 mg, 0.990 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.41-5.29 (m, 8H), 3.67 (s, 6H), 3.58 (brs, 1H), 2.77 (t, J=5.8 Hz, 4H), 2.32 (t, J=7.4 Hz, 4H), 2.10-2.00 (m, 8H), 1.69-1.60 (m, 4H), 1.43-1.29 (m, 32H).

Compound 232: A procedure analogous to that described for compound 9 was followed with 231 (520 mg, 0.843 mmol) to afford compound 232 (600 mg, 0.822 mmol, 97%). Molecular weight for $C_{45}H_{80}NO_6$ $(M+H)^+$ Calc. 730.5986, Found 730.5.

Example 15: Synthesis of Terminal Ester Lipid with Linoleyl Chain-232

Scheme 15



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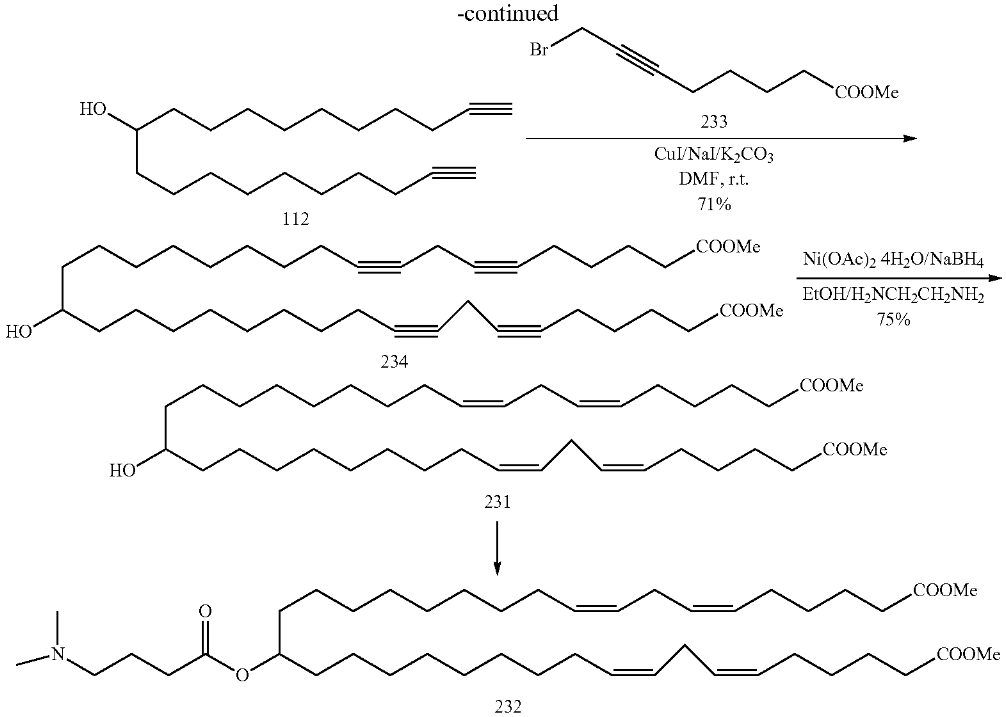


Compound 231 was also synthesized as shown Scheme 15.

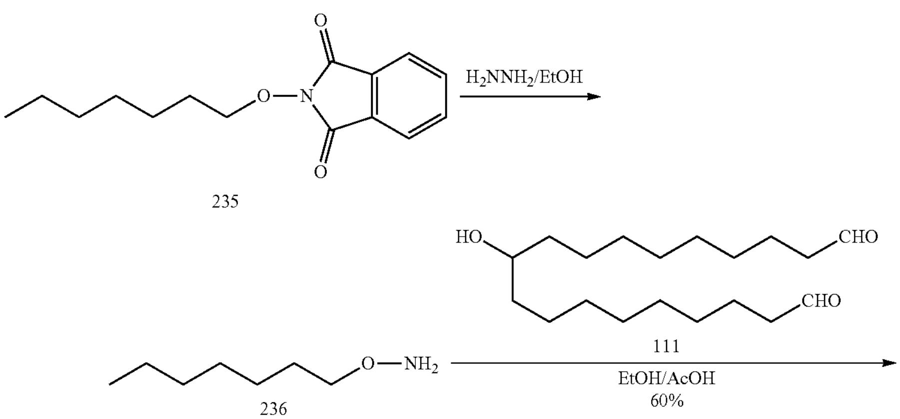
Compound 112: Compound 111 (840 mg, 2.69 mmol) was treated with dimethyl (1-diazo-2-oxopropyl)phosphonate (0.970 mL, 6.46 mmol) and K_2CO_3 (1.49 g, 10.8 mmol) in MeOH (40 mL) for 6 h. Aqueous work-up then column chromatography gave compound 112 (700 mg, 2.30 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.58 (brs, 1H), 2.18 (td, J=7.1, 2.6 Hz, 4H), 1.94 (t, J=2.6 Hz, 2H), 1.56-1.25 (m, 28H).

Compound 234: Compound 112 (207 mg, 0.680 mmol) was treated with 233 (316 mg, 1.36 mmol), K_2CO_3 (282 mg, 2.04 mmol), NaI (408 mg, 2.72 mmol) and CuI (518 mg, 2.72 mmol) in DMF (3.5 mL) for 18 h. Aqueous work-up then column chromatography gave compound 234 (292 mg, 0.480 mmol, 71%). Molecular weight for $C_{39}H_{61}O_5$ $(M+H)^+$ Calc. 609.4519, Found 609.5.

Compound 231: To a stirred solution of nickel(II) acetate tetrahydrate (533 mg, 2.14 mmol) in EtOH (28.5 mL), 1 M solution of $NaBH_4$ in EtOH (2.14 mL) was added at room temperature. After 30 min, ethylenediamine (0.574 mL, 8.57 mmol) and a solution of 234 (290 mg, 0.476 mmol) in EtOH (3 mL) was added then stirred for 1 h. The reaction mixture was filtered through Celite and evaporated. Aqueous work-up then column chromatography gave compound 231 (219 mg, 0.355 mmol, 75%). Molecular weight for $C_{39}H_{69}O_5$ $(M+H)^+$ Calc. 617.5145, Found 617.3.

Example 16: Synthesis of Internal Oxime Lipid-238

Scheme 16



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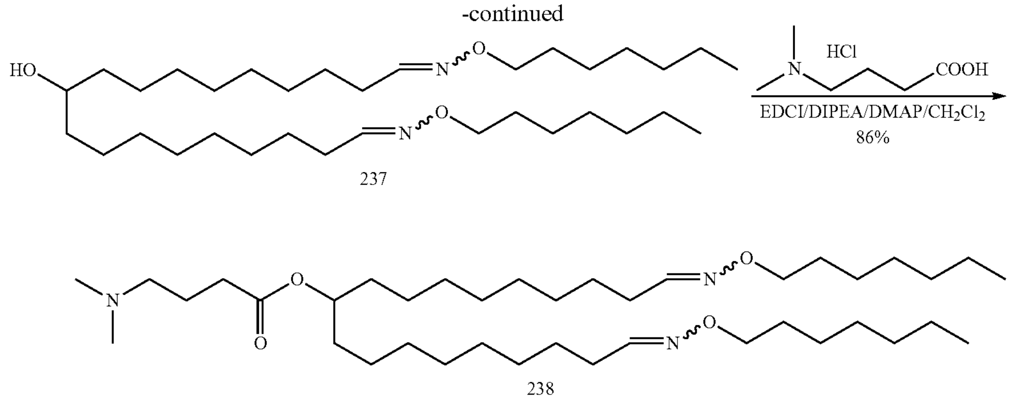
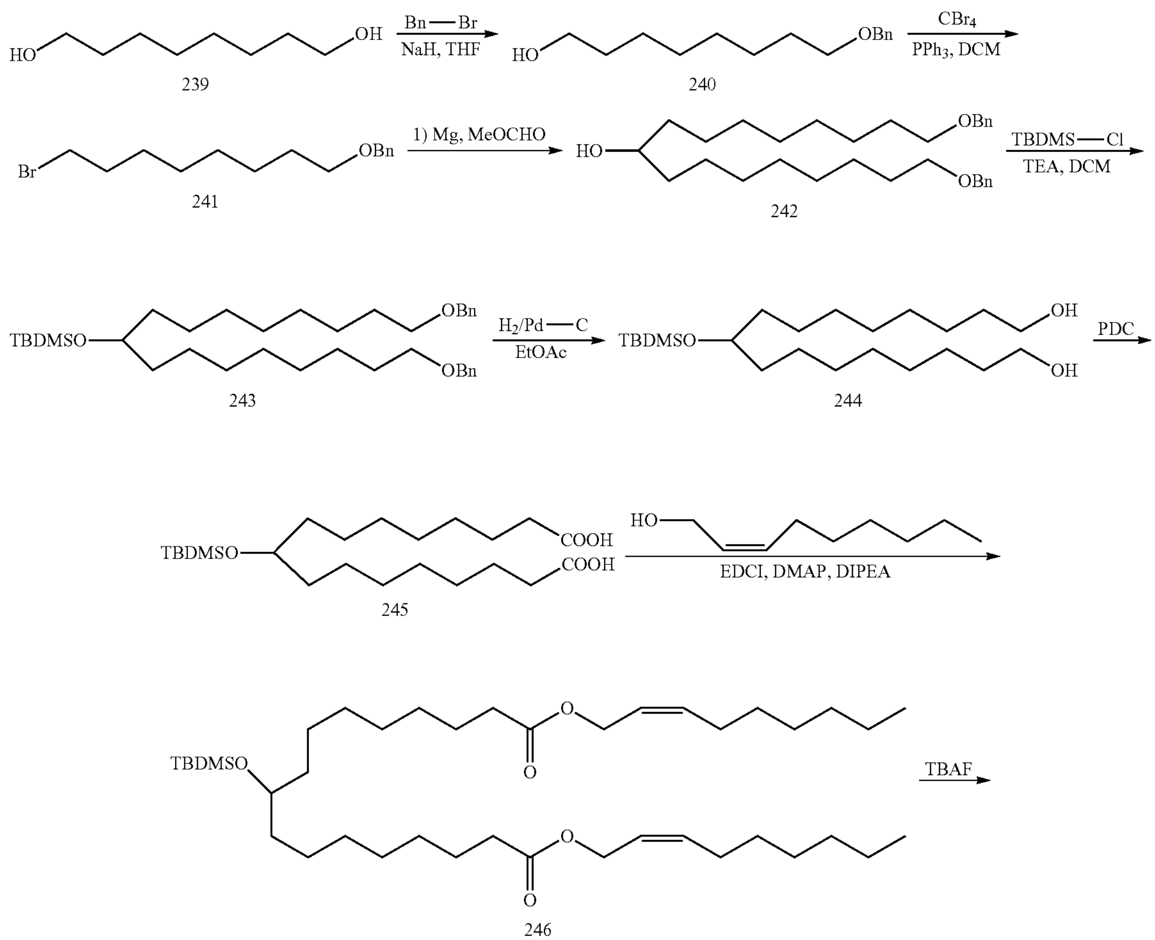


Compound 237: Compound 235 (465 mg, 1.78 mmol) was treated with hydrazine monohydrate (64-65%, 0.135 mL, 1.78 mmol) in EtOH (15 mL) for 4 h. After filtration then evaporation, the crude was re-suspended in EtOH (5 mL). To this solution was added compound 111 (160 mg, 0.512 mmol) and AcOH (a few drops). Aqueous work-up then column chromatography gave compound 237 (165 mg, 0.306 mmol, 60%). Molecular weight for $C_{33}H_{67}N_2O_3$ $(M+H)^+$ Calc. 539.5152, Found 539.3.

Compound 238: A procedure analogous to that described for compound 9 was followed with 237 (162 mg, 0.301 mmol) to afford compound 238 (168 mg, 0.258 mmol, 86%). Molecular weight for $C_{39}H_{78}N_3O_4$ $(M+H)^+$ Calc. 652.5992, Found 652.4.

Example 17

Scheme 17



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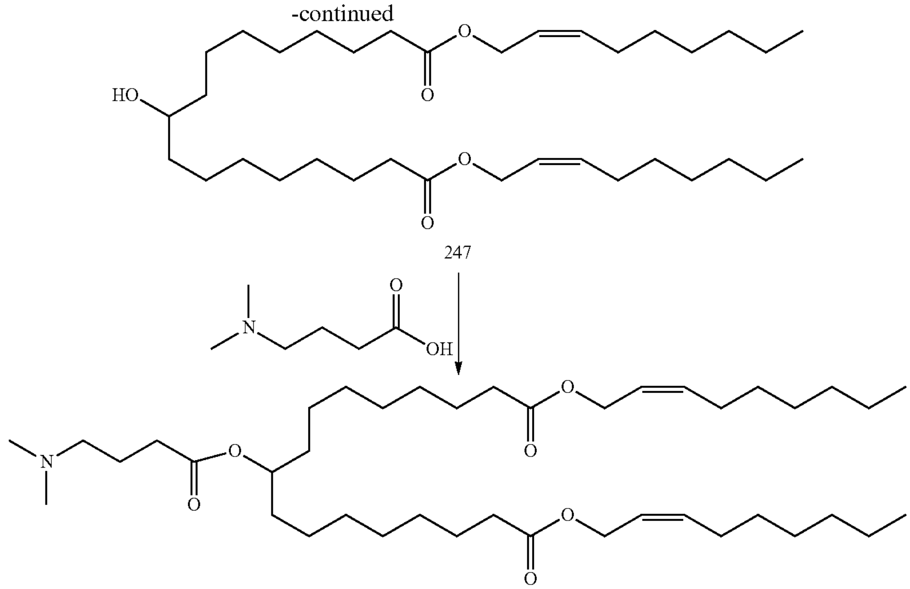


ALNY-319

8-benzyloxy-octan-1-ol (240): To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 239 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly using a dropping funnel at 0° C. The reaction mixture was stirred for 30 minutes, then benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under a nitrogen atmosphere. The reaction was then warmed to ambient temperature and stirred for 10 hours. After completion of reaction, the mixture was quenched with crushed ice (~2 kg) and extracted with ethyl acetate (2×1 L). The organic layer washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness under vacuum. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 240 (220 g, 54%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

(8-bromo-octyloxymethyl)-benzene (241): Compound 240 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM, CBr_4 (280.35 g, 0.8456 mol) was added to this stirring solution and the reaction mixture was cooled to 0° C. under an inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions maintaining the temperature below 20° C. and after complete addition, the reaction mixture was stirred for 3 hours at room temperature. After completion of reaction, solid (PPh_3O) precipitated out from the reaction mixture was isolated by filtration and the filtrate was diluted with crushed ice (~ 1.5 kg) and extracted with DCM (3×750 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and distilled under vacuum. The resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to afford compound 241 (150 g, 89%) as pale yellow liquid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

1,17-bis-benzyloxy-heptadecan-9-ol (242): To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF, followed by the addition of pinch of iodine into the mixture under inert atmosphere. After initiation of the Grignard formation a solution of Compound 241 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly controlling the exothermic reaction. After complete addition, the reaction was heated to reflux for 1 hour, then cooled to room temperature. Methyl formate (60.24 g, 1.0033 mol) was then added slowly and reaction was continued for 2 hours. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with ethyl acetate (3×1 L). The organic layer was taken in 5 litre beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution excess of $NaBH_4$ (~ 5 eq) was added in portions to ensure the hydrolysis of formate ester which was not cleaved by addition of HCl. The resulting solution was stirred for an hour and then volatilites were removed under vacuum. The residue was taken in water (1 L) and acidified by 10% HCl solution (P^H 4). The product was then extracted with ethyl acetate (3×1 L). The organic phase was then dried and concentrated on rotary evaporator to afford compound 242 (57 g, 24%) as solid. ^1H NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). C^{13} NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

[9-benzyloxy-1-(8-benzylozy-octyl)-nonyloxy]-tert-butyl-dimethyl-silane (243): Compound 242 (56 g, 0.1196 mol) was dissolved in 700 mL of anhydrous THF and cooled to 0° C. TBMS-Cl (36.06 g, 0.2396 mol) was added slowly followed by addition of imidazole (32.55 g, 0.4786 mol) under an inert atmosphere. The reaction was then stirred at room temperature for 18 hours, then quenched with ice (~1 kg). The product was extracted with ethyl acetate (3×500 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution to remove the acidic impurity, dried over Na_2SO_4 and evaporated under reduce pressure to obtain crude compound which was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to afford (60 g, 82%) of compound 243 as yellowish oil. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s, 4H),

3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H)

9-(tert-butyl-dimethyl-silanyloxy)-heptadecane-1,17-diol (244): Compound 243 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 min. (10 wt %) Pd on carbon (12 g) was added and reaction was stirred under an atmosphere of hydrogen for 18 hours. After completion, the mixture was filtered through a bed of celite and washed with ethyl acetate. The filtrate was evaporated under vacuum. Compound 244 (19 g, 46%) thus obtained was pure enough to carry out the next reaction. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

9-(tert-butyl-dimethyl-silanyloxy)-heptadecanedioic acid (245): To a stirred solution of 244 (2 g, 0.0049 mol) in anhydrous DMF (40 mL) was added pyridinium dirchromate (2.7 g, 0.0074 mol) at 0° C. under an inert atmosphere. The reaction mixture was then allowed to warm to room temperature over a period of 10-15 minutes and continued for 24 hours. Then, the reaction was diluted with water (100 mL). The aqueous phase was extracted using DCM (3×40 mL). The organic phase was washed with brine (1×25 mL) and concentrated under vacuum to afford crude acid which was then purified by (100-200 mesh) silica gel column using 0-30% ethyl acetate in hexanes system. Pure product (245) was obtained (0.7 g, 33%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.61-3.56 (m, 1H), 2.35-2.32 (m, 4H), 1.64-1.59 (m, 4H), 1.40-1.19 (m, 24H), 0.86 (s, 9H), 0.017 (s, 6H); LC-MS [M+H]—431.00; HPLC (ELSD) purity—96.94%

Di((Z)-non-2-en-1-yl) 9-((tert-butyldimethylsilyl)oxy) heptadecanedioate (246): The diacid 245 (0.42 g, 0.97 mmol) was dissolved in 20 mL of dichloromethane and to it cis-2-nonen-1-ol (0.35 g, 2.44 mmol) was added followed by Hunig's base (0.68 g, 4.9 mmol) and DMAP (12 mg). To this mixture EDCI (0.47 g, 2.44 mmol) was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash R_f purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to afford the pure product 246 (0.35 g, 53%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.58-5.43 (m, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.71-3.48 (m, 1H), 2.30 (t, J=7.6 Hz, 4H), 2.20-1.98 (m, 4H), 1.71-1.53 (m, 4H), 1.31 (ddd, J=8.3, 7.0, 3.7 Hz, 34H), 1.07-0.68 (m, 14H), 0.02 (s, 5H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.18, 139.81, 127.78, 81.73, 81.42, 81.10, 76.72, 64.59, 41.52, 41.32, 38.76, 36.09, 34.10, 33.93, 33.80, 33.70, 33.59, 33.55, 33.26, 31.95, 30.34, 29.69, 29.58, 29.39, 27.01, 22.56, 18.48, 0.01.

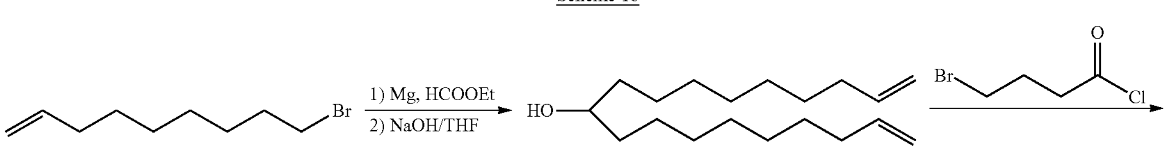
Di((Z)-non-2-en-1-yl) 9-hydroxyheptadecanedioate (247): The silyl protected diester 246 (0.3 g, 0.44 mmol) was dissolved in 1 M solution of TBAF in THE (6 mL) and the solution was kept at 40° C. for two days. The reaction mixture was diluted with water (60 mL) and extracted with ether (2×50 mL). The combined organic layers were concentrated and the thus obtained crude product was purified by column to isolate the pure product (0.097 g, 39%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.52 (dt, J=11.0, 6.8 Hz, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.57 (s, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.09 (q, J=7.1 Hz, 4H), 1.75-1.53 (m, 4H), 1.53-1.06 (m, 36H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.98, 135.64, 123.57, 77.54, 77.22, 76.91, 72.14, 60.41, 37.69, 34.54, 31.89, 29.70, 29.60, 29.44, 29.29, 29.07, 27.76, 25.80, 25.15, 22.82, 14.29.

Di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl) oxy)heptadecanedioate: The alcohol 247 (0.083 g, 0.147 mmol) was dissolved in 20 mL of dichloromethane and to it dimethylaminobutyric acid hydrochloride (0.030 g, 0.176 mmol) was added followed by Hunig's base (0.045 g, 0.44 mmol) and DMAP (2 mg). To this mixture EDCI (0.034 g, 0.176 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 10% MeOH in CH_2Cl_2) showed complete disappearance of the starting alcohol. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash R_f purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product (0.062 g, 62%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74-5.58 (m, 2H), 5.51 (dtt, J=9.7, 6.8, 1.3 Hz, 2H), 4.95-4.75 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 2.35-2.24 (m, 8H), 2.22 (d, J=7.9 Hz, 6H), 2.09 (q, J=6.9 Hz, 4H), 1.83-1.72 (m, 2H), 1.60 (dd, J=14.4, 7.2 Hz, 4H), 1.49 (d, J=5.7 Hz, 4H), 1.41-1.13 (m, 30H), 0.88 (t, J=6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.72, 173.36, 135.40, 123.35, 74.12, 60.18, 58.95, 45.46, 34.30, 34.11, 32.45, 31.67, 29.38, 29.35, 29.17, 29.07, 28.84, 27.53, 25.28, 24.93, 23.16, 22.59, 14.06. MW calc, for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

Example 18

The following shorter route was used for the synthesis of analogs of Compound 1 of the present invention The commercial 9-bromonon-1-ene 248 was treated with magnesium to form the corresponding Grignard reagent which was reacted with ethylformate to give the corresponding adduct 249 which on treatment with bromobutyryl chloride to provide the bromoester 250. The bromoester 250 on treatment with RuO_4 provided the diacid 251. The bromodiacid 251 on treatment with dimethylamine provided the amino diacid 252. The diacid 252 on treatment with oxalyl chloride in the presence of DML provided the diacid chlorides 253. The lipids 254a-n were synthesized by treating the acid Chloride 253 with respective alcohols.

Scheme 18



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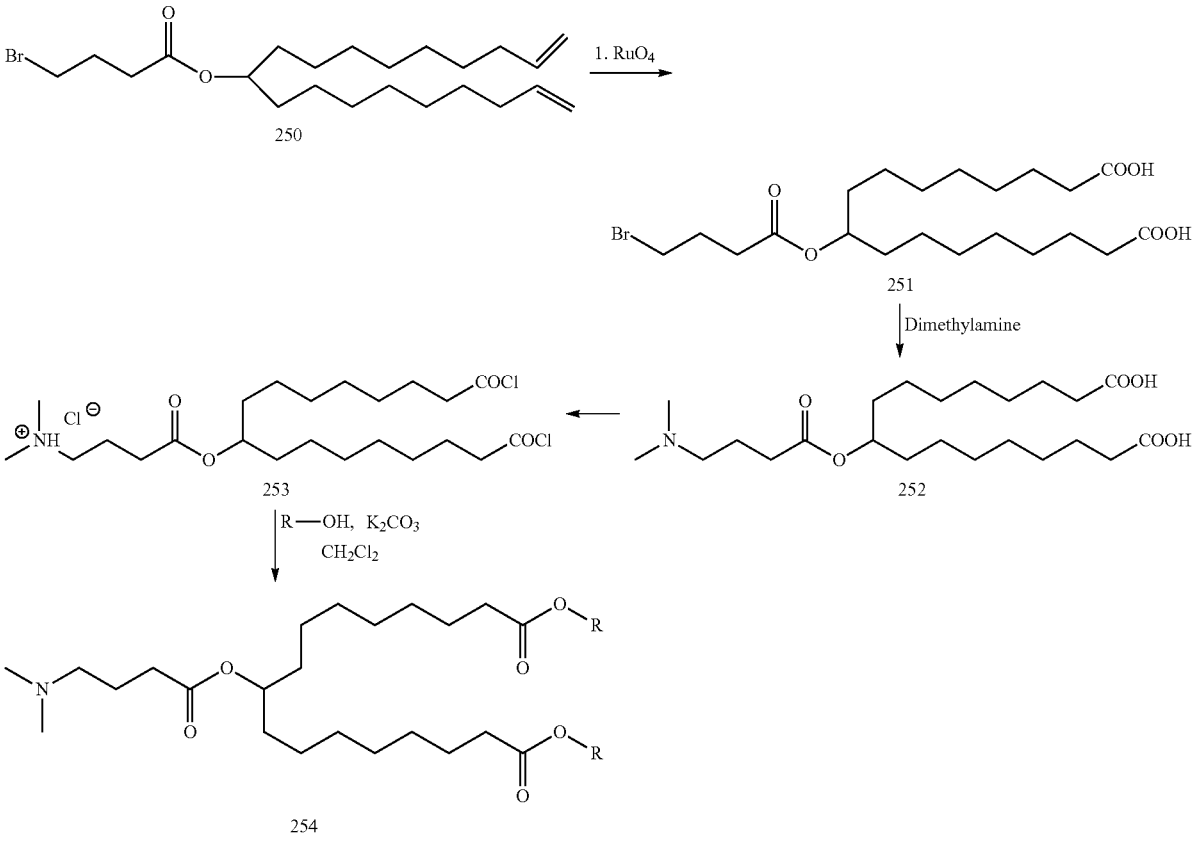

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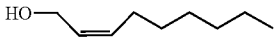
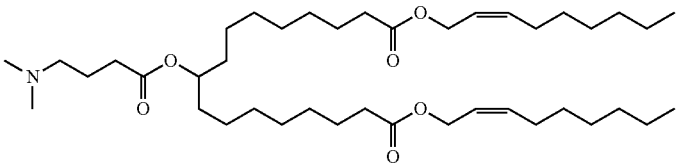
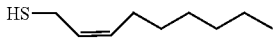
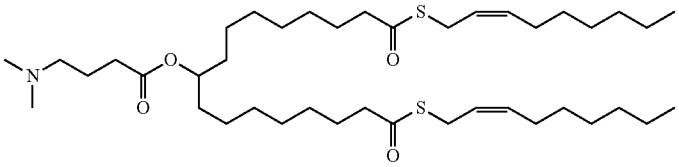
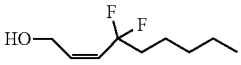
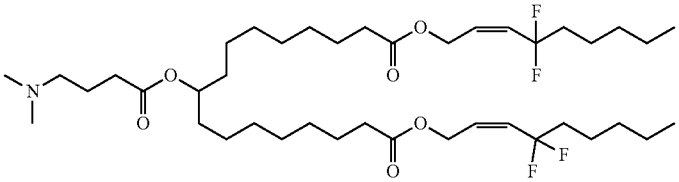
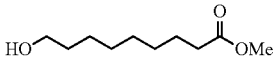
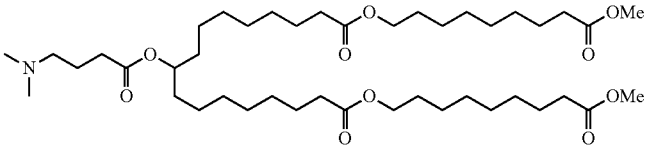
254a		
254aS		
254aF		
254b		

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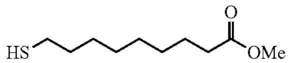
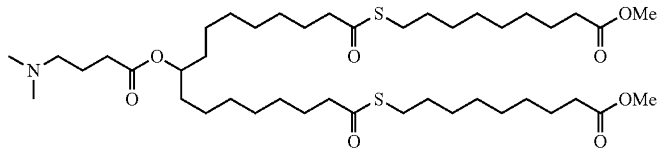
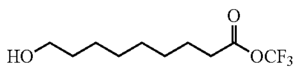
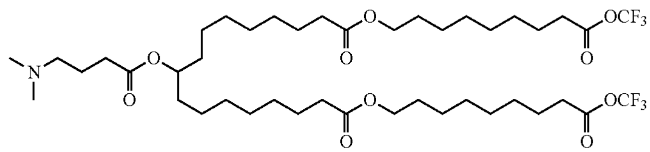
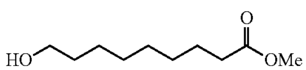
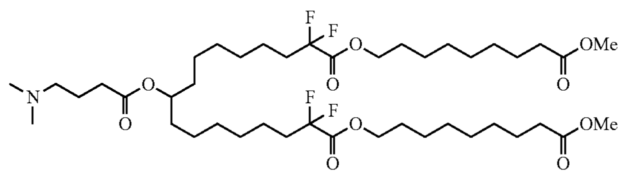
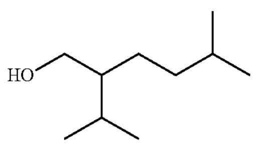
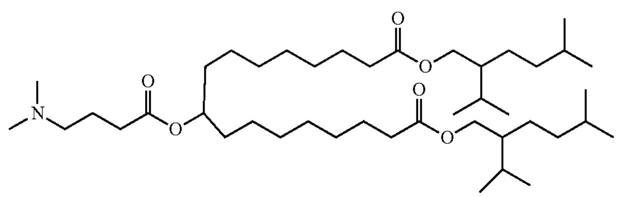
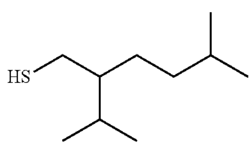
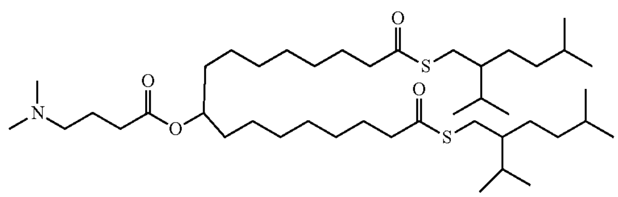
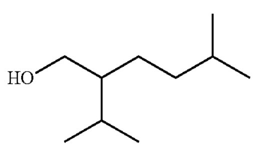
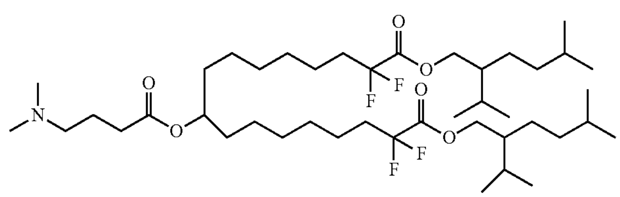
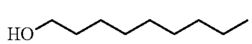
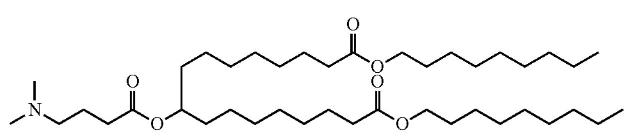
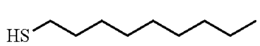
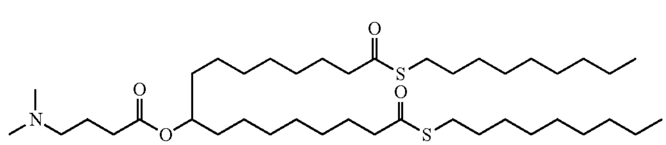
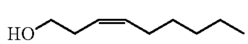
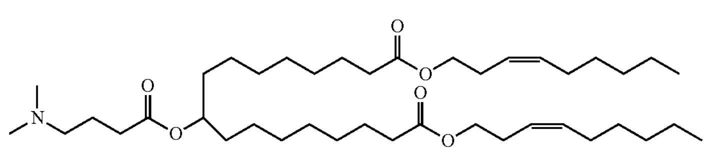
No	Starting Alcohol (ROH)	Product
254bS		
254bF		
254bF2		
254c		
254cS		
254cF		
254d		
254ds		
254e		

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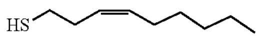
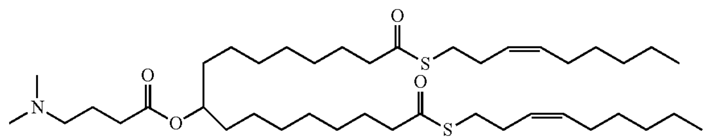
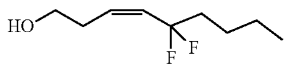
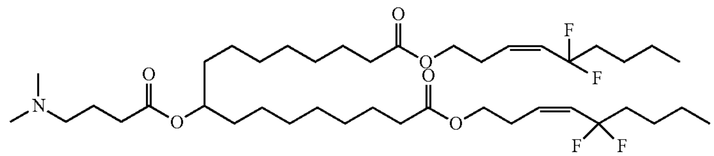
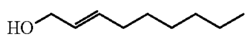
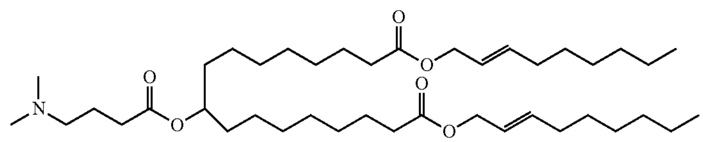
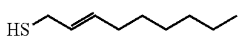
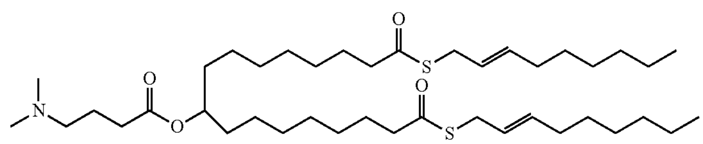
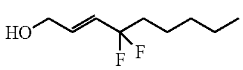
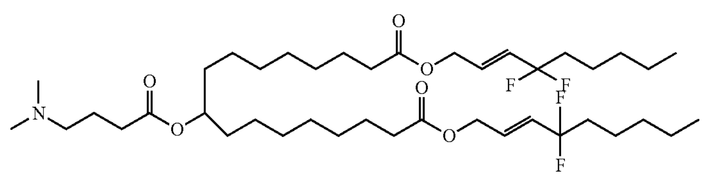
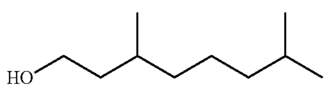
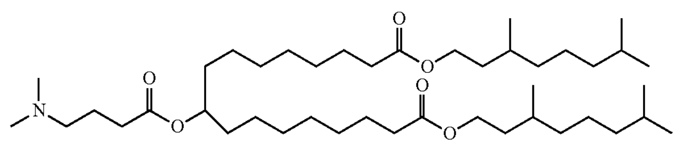
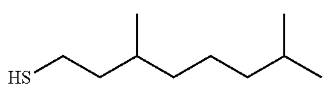
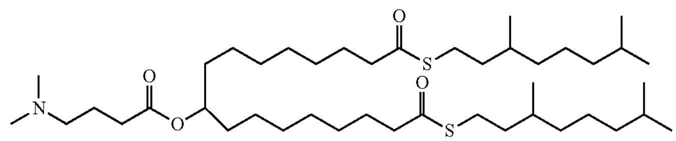
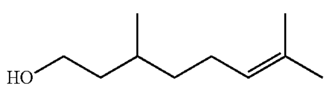
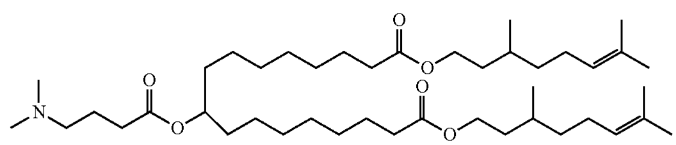
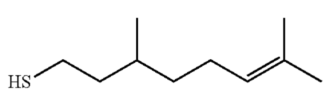
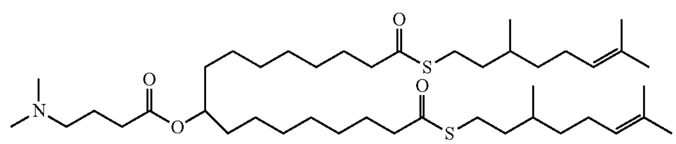
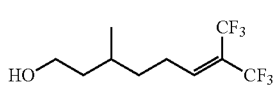
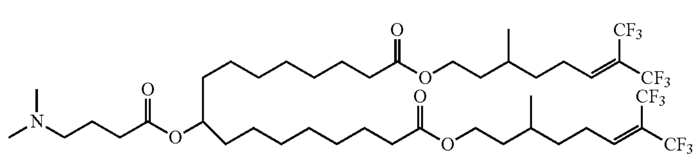
254es		
254eF		
254f		
254fs		
254fF		
254g		
254gs		
254h		
254hs		
254hF		

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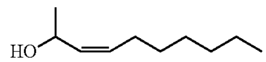
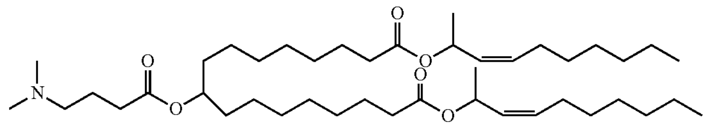
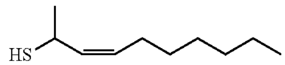
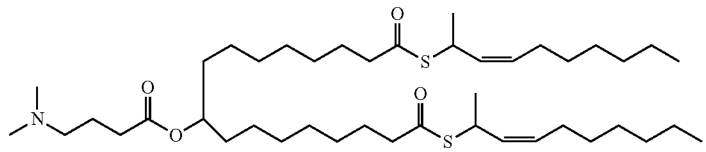
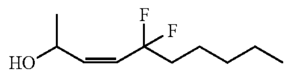
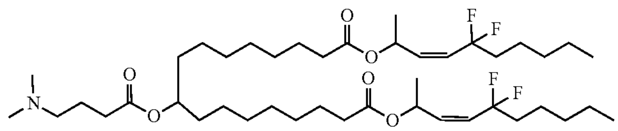
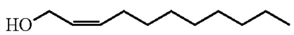
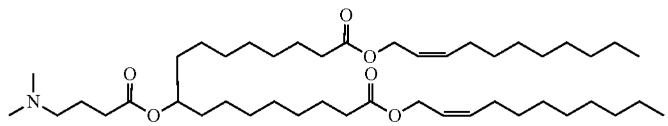
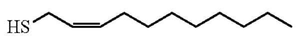
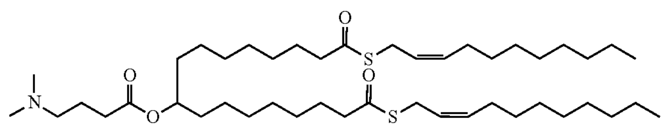
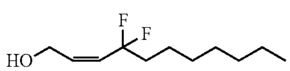
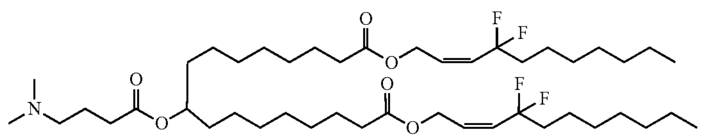
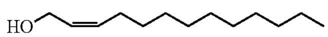
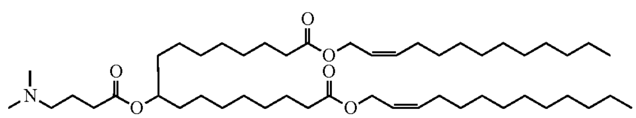
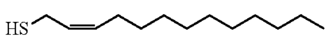
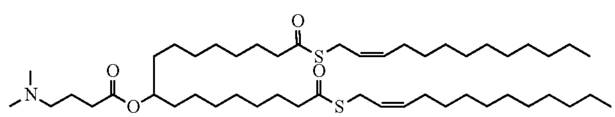
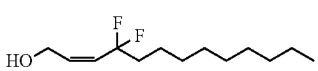
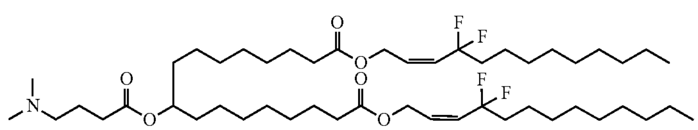
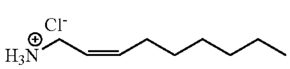
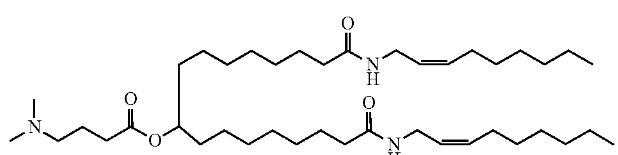
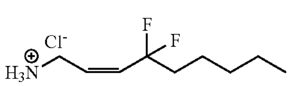
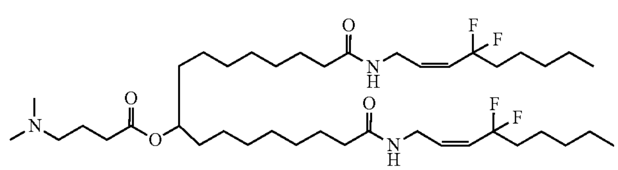
254i		
254is		
254iF		
254j		
254js		
254jF		
254k		
254ks		
254kS		
254l		
254lF		

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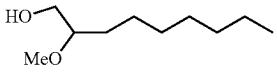
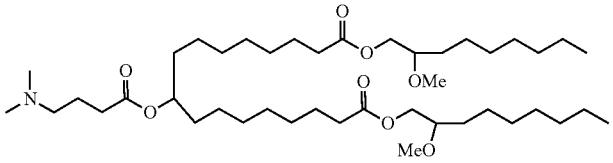
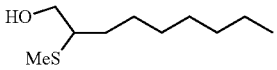
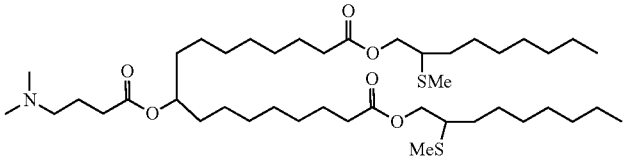
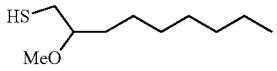
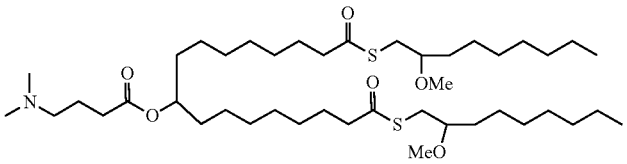
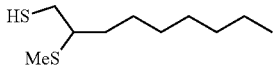
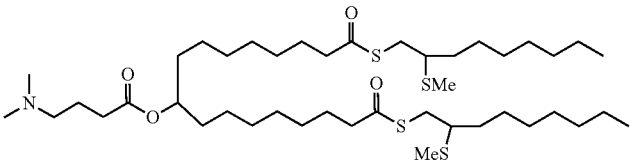
254m		
254ms		
254ns		
254os		

Synthesis of nonadeca-1,18-dien-10-ol (249)

To a flame dried 500 mL RB flask, freshly activated Mg turnings (9 g) were added and the flask was equipped with a magnetic stir bar, an addition funnel and a reflux condenser. This set-up was degassed and flushed with argon and 100 mL of anhydrous ether was added to the flask via syringe. The bromide 3 (51.3 g, 250 mmol) was dissolved in anhydrous ether (100 mL) and added to the addition funnel. About 5 mL of this ether solution was added to the Mg turnings while stirring vigorously. An exothermic reaction was noticed (to confirm/accelerate the Grignard reagent formation, 5 mg of iodine was added and immediate decolorization was observed confirming the formation of the Grignard reagent) and the ether started refluxing. The rest of the solution of the bromide was added dropwise while keeping the reaction under gentle reflux by cooling the flask in water. After the completion of the addition the reaction mixture was kept at 35° C. for 1 hour and then cooled in ice bath. Ethyl formate (9 g, 121 mmol) was dissolved in anhydrous ether (100 mL) and transferred to the addition funnel and added dropwise to the reaction mixture with stirring. An exothermic reaction was observed and the reaction mixture started refluxing. After the initiation of the reaction the rest of the ethereal solution of formate was quickly added as a stream and the reaction mixture was stirred for a further period of 1 h at ambient temperature. The reaction was quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture was treated with aq. H_2SO_4 (10% by volume, 300 mL) until the solution became homogeneous and the layers were separated. The aq. phase was extracted with ether (2×200 mL). The combined ether layers were dried (Na_2SO_4) and concentrated to afford the crude product which was purified by column (silica gel, 0-10% ether in hexanes) chromatography. The product fractions were evaporated to provide the pure product 249 as a white solid (30.6 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 1H), 5.81 (ddt, J=16.9, 10.2, 6.7 Hz, 8H), 5.04-4.88 (m, 16H), 3.57 (dd, J=7.6, 3.3 Hz, 4H), 2.04 (q, J=6.9 Hz, 16H), 1.59 (s, 1H), 1.45 (d, J=7.5 Hz, 8H), 1.43-1.12 (m, 94H), 0.88 (t, J=6.8 Hz, 2H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 139.40, 114.33, 77.54, 77.22, 76.90, 72.21, 37.70, 34.00, 29.86, 29.67, 29.29, 29.12, 25.85.

Synthesis of nonadeca-1,18-dien-10-yl 4-bromobutanoate (250)

To a solution of the alcohol 249 (5.6 g, 20 mol) in anhydrous DCM (300 mL) was added slowly and carefully Bromobutryl chloride (20 mmol) at 0° C. under inert atmosphere. The reaction mixture was warmed to room temperature, stirred for 20 h and monitored by TLC (silica gel, 10% ethyl acetate in hexanes). Upon completion of the reaction, mixture was diluted with water (400 mL) and organic layer was separated out. Organic phase was then washed with sat. solution of $NaHCO_3$ (1×400 mL) followed by brine (1×100 mL) and concentrated under vacuum. Crude product was then purified by silica gel (100-200 mesh) column, eluted with 2-3% ethyl acetate in hexane solution to give 6 g (90%) of desired product 250 as colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (ddt, J=16.9, 10.2, 6.7 Hz, 2H), 5.05-4.81 (m, 5H), 3.46 (t, J=6.5 Hz, 2H), 2.48 (t, J=7.2 Hz, 2H), 2.17 (p, J=6.8 Hz, 2H), 2.11-1.93 (m, 4H), 1.65-1.44 (m, 4H), 1.43-1.17 (m, 19H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 172.51, 139.37, 114.35, 77.54, 77.23, 76.91, 74.86, 34.31, 33.99, 33.01, 32.96, 29.65, 29.56, 29.24, 29.09, 28.11, 25.52.

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Synthesis of 9-((4-bromobutanoyl)oxy)heptadecanedioic acid (251)

To a solution of the bromoester 250 (12.1 g, 28.2 mmol) in dichloromethane (300 mL) and acetonitrile (300 mL), $RuCl_3$ (1.16 g, 5 mol %) was added and the mixture was cooled to 10° C. and sodium metaperiodate (60 g) in water (400 mL) was added dropwise. It was stirred at 10° C. for 20 hr. The reaction mixture was diluted with water, The layers were separated and to the organic layer, was added saturated brine solution with stirring followed by 3% sodium sulfide solution drop wise for the decolourisation (dark green to pale yellow). The layers were separated, the organic layer was dried over sodium sulfate and evaporated at reduced pressure to afford pure product. MW calcd for $C_{20}H_{35}BrO_7$ 467.39; Found 465.4 (M−2H). ^{1}H NMR (400 MHz, DMSO) δ 11.94 (s, 2H), 4.88-4.69 (m, 1H), 3.53 (t, J=6.6 Hz, 2H), 2.43 (t, J=7.2 Hz, 2H), 2.17 (t, J=7.4 Hz, 4H), 2.09-1.95 (m, 2H), 1.90 (s, 3H), 1.46 (s, 7H), 1.23 (s, 15H).

Synthesis of 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioic acid (252)

The Bromoacid 251 (2 mmol) is dissolved in 2M solution of dimethylamine in THF (20 mL) and to it 1 g of anhdrous K_2CO_3 was added and the mixture was heated in a pressure bottle at 50° C. overnight. The TLC showed the completion of the reaction. The reaction mixture was acidified with acetic acid and diluted with water (100 mL) and extracted with dichloromethane (2×60 mL). The combined organic layers were concentrated dried and used as such in the next reaction. MW calcd for $C_{23}H_{43}NO_6$ 429.59; Found 430.6 $(MH)^+$. 1H NMR (400 MHz, DMSO) δ 11.87-11.82 (m, 7H), 5.75 (d, J=0.7 Hz, 15H), 4.85-4.69 (m, 38H), 3.64-3.55 (m, 12H), 3.35-2.83 (m, 106H), 3.01-2.90 (m, 59H), 2.94 (ddd, J=30.6, 7.7, 4.0 Hz, 63H), 2.90-2.73 (m, 9H), 2.70 (s, 221H), 2.57-2.46 (m, 91H), 2.44-2.30 (m, 76H), 2.17 (t, J=7.3 Hz, 147H), 1.89 (tq, J=15.5, 7.6 Hz, 88H), 1.79-1.69 (m, 13H), 1.65-1.32 (m, 311H), 1.28 (d, J=46.0 Hz, 598H).

Synthesis of 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioyl chloride (253)

The diacid 252 is converted to the corresponding diacid chloride 253 by treating it with oxalyl chloride in dichloromethane in the presence of catalytic DMF and the crude acid chloride obtained after the concentration of the reaction mixture was used as such for the coupling with different alcohols.

General Procedure for the Synthesis of Cationic Lipids 254a-n

To a solution of the acid chloride 253 (500 mg, 1 mmol) in dichloromethane (30 mL) the corresponding alcohol (5 equivalent) was added at room temperature followed by solid K_2CO_3 (1 g) and the solution was stirred for 16 h at room temperature. The reaction mixture was diluted with dichloromethane (100 mL) and washed with satd. $NaHCO_3$ (100 mL) and the organic layer was dried (Anhyd. Na_2SO_4) and concentrated to obtain the crude product which was purified by Combiflash R_f purification system.

Compound 254b: By using the above procedure the lipid 254b was isolated in 72% yield (554 mg). 1H NMR (400 MHz, CDCl3) δ 4.91-4.78 (m, 1H), 4.05 (t, J=6.7 Hz, 4H), 3.81 (s, 6H), 3.63 (t, J=6.4 Hz, 1H), 2.29 (dt, J=15.2, 7.5 Hz, 8H), 2.21 (s, 6H), 1.84-1.69 (m, 2H), 1.57 (dt, J=13.4, 5.2 Hz, 9H), 1.53-1.40 (m, 4H), 1.27 (s, 43H). 13C NMR (101 MHz, cdcl3) δ 174.45, 174.13, 173.59, 77.54, 77.22, 76.91, 74.34, 64.54, 59.17, 51.65, 45.67, 34.56, 34.35, 34.27, 32.67, 29.59, 29.40, 29.33, 29.31, 29.25, 28.83, 26.06, 25.51, 25.18, 25.11, 23.38. MW calcd for $C_{43}H_{79}NO_{10}$ 770.09; Found 770.68.

Compound 254c: By using the above procedure the lipid 254c was isolated in 69% (490 mg). 1H NMR (400 MHz, CDCl3) δ 5.71-5.36 (m, 4H), 4.89-4.72 (m, 1H), 4.59 (d, J=6.8 Hz, 4H), 2.26 (ddd, J=22.3, 13.0, 8.6 Hz, 9H), 2.19 (s, 6H), 2.12-1.95 (m, 4H), 1.82-1.68 (m, 2H), 1.63-1.37 (m, 8H), 1.37-1.00 (m, 32H), 0.85 (t, J=6.8 Hz, 6H). 13C NMR (101 MHz, cdcl3) δ 173.94, 173.57, 135.61, 123.57, 77.54, 77.22, 76.91, 74.34, 60.40, 59.16, 45.65, 34.52, 34.33, 32.66, 31.88, 29.59, 29.57, 29.38, 29.28, 29.06, 27.75, 25.49, 25.14, 23.35, 22.81, 14.28. MW calcd for $C_{43}H_{83}NO_6$: 710.12; Found 710.81.

Compound 254d: By using the above procedure the lipid 254d was isolated in 67% yield (456 mg). 1H NMR (400 MHz, CDCl3) δ 4.92-4.78 (m, 1H), 4.05 (t, J=6.7 Hz, 4H), 3.63 (t, J=6.4 Hz, 1H), 2.39-2.24 (m, 8H), 2.21 (s, 6H), 1.89-1.70 (m, 2H), 1.69-1.54 (m, 8H), 1.51 (dd, J=17.2, 6.3 Hz, 4H), 1.27 (s, 42H), 0.88 (t, J=6.8 Hz, 6H). MW calcd for: $C_{41}H_{79}NO_6$: 682.07; Found 682.96.

Compound 254e: By using the above procedure the lipid 254e was isolated in 70% (474 mg). 1H NMR (400 MHz, CDCl3) δ 5.49 (ddd, J=12.9, 9.8, 7.3 Hz, 2H), 5.40-5.23 (m, 2H), 4.92-4.77 (m, 1H), 4.05 (t, J=6.9 Hz, 4H), 2.32 (ddd, J=23.4, 14.5, 7.1 Hz, 12H), 2.21 (s, 6H), 2.07-1.91 (m, 4H), 1.84-1.70 (m, 2H), 1.66-1.39 (m, 8H), 1.40-1.15 (m, 26H), 0.88 (t, J=6.8 Hz, 5H). MW calc, for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

Compound 254f: By using the above procedure the lipid 254f was isolated in 73% (559 mg). 1H NMR (400 MHz, CDCl3) δ 5.87-5.62 (m, 2H), 5.55 (dtt, J=9.1, 6.4, 1.3 Hz, 2H), 4.93-4.75 (m, 1H), 4.50 (dd, J=6.5, 0.6 Hz, 4H), 2.40-2.17 (m, 13H), 2.12-1.95 (m, 4H), 1.89-1.67 (m, 2H), 1.69-1.44 (m, 7H), 1.41-1.12 (m, 25H), 0.88 (t, J=6.9 Hz, 5H). MW calc, for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

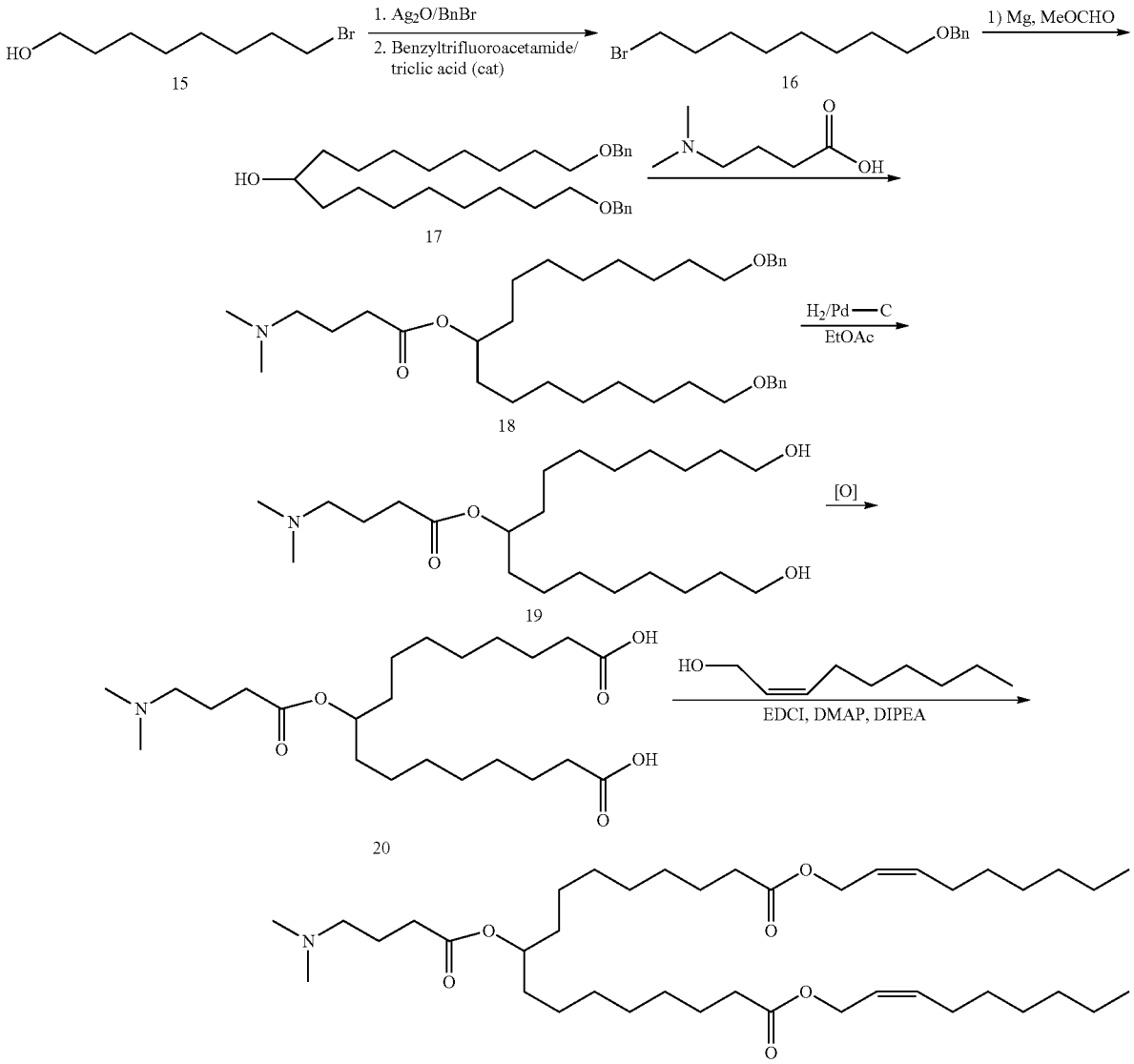
Compound 254 g: By using the above procedure the lipid 254 g was isolated in 63% (432 mg). 1H NMR (400 MHz, CDCl3) δ 4.93-4.77 (m, 1H), 4.20-3.95 (m, 4H), 2.44-2.23 (m, 8H), 2.21 (s, 6H), 1.84-1.66 (m, 3H), 1.68-1.34 (m, 15H), 1.35-1.17 (m, 20H), 1.17-1.04 (m, 5H), 0.88 (dd, J=12.4, 6.6 Hz, 16H). MW calcd for $C_{43}H_{83}NO_6$: 710.12; Found 710.81.

Compound 254h: By using the above procedure the lipid 254 h was isolated in 66% (466 mg). 1H NMR (400 MHz, CDCl3) δ 5.08 (ddd, J=7.1, 5.9, 1.3 Hz, 2H), 4.91-4.75 (m, 1H), 4.22-3.97 (m, 4H), 2.39-2.22 (m, 8H), 2.23 (d, J=16.7 Hz, 7H), 2.09-1.84 (m, 4H), 1.86-1.71 (m, 3H), 1.71-1.02 (m, 44H), 0.91 (t, J=4.9 Hz, 6H). MW calcd for $C_{43}H_{79}NO_6$: 706.12; Found 706.81.

Example 19

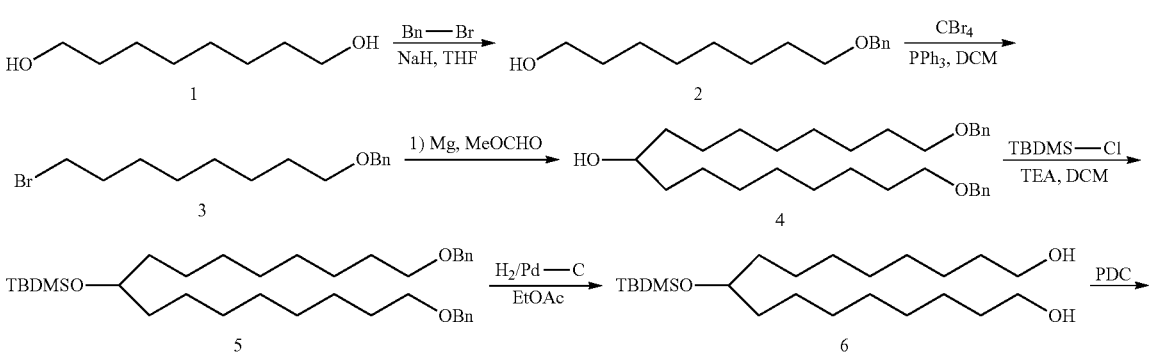
In another approach the following synthetic approach is used for the synthesis of Compound 1 of the present invention.

Scheme 19



Example 20

Scheme 20



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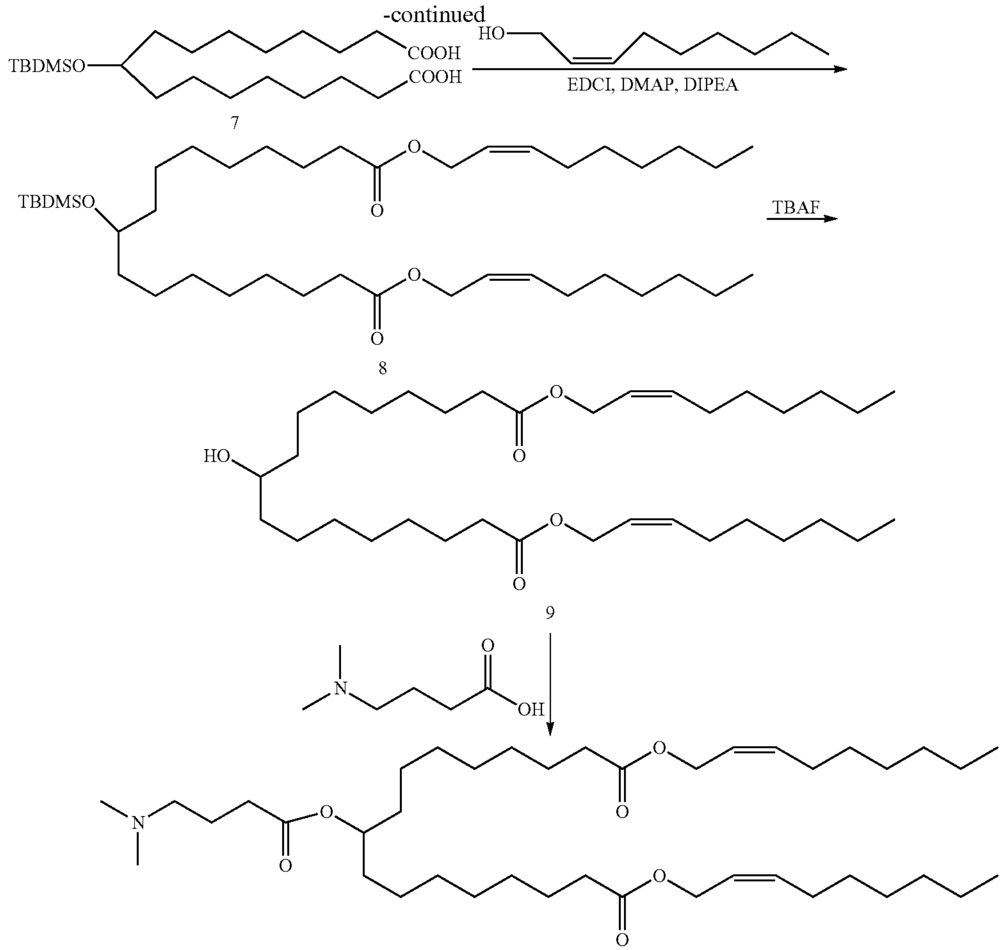

8-benzyloxy-octan-1-ol (2): To a stirred suspension of NaH (60% in oil, 82 g, 1.7096 mol) in 500 mL anhydrous DMF, a solution of compound 1 (250 g, 1.7096 mol) in 1.5 L DMF was added slowly with dropping funnel at 0° C. Reaction mixture was stirred for 30 min and to it Benzyl bromide (208.86 mL, 1.7096 mol) was added slowly under nitrogen atmosphere. Reaction was then warmed to ambient temperature and stirred for 10 h. After completion of reaction, mixture was quenched with crushed ice (~2 kg) and extracted with Ethyl acetate (2×1 L). Organic layer washed with water (1 L) to remove unwanted DMF, dried over Na_2SO_4 and evaporated to dryness under vacuum. The crude compound was purified on 60-120 silica gel, eluted with 0-5% MeOH in DCM to afford compound 2 (220 g, 54%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 5H), 4.49 (s, 2H), 3.63-3.60 (m, 2H), 3.47-3.43 (m, 2H), 1.63-1.51 (m, 4H), 1.39-1.23 (m, 8H).

(8-bromo-octyloxymethyl)-benzene (3): Compound 2 (133 g, 0.5635 mol) was dissolved in 1.5 L of DCM, CBr_4 (280.35 g, 0.8456 mol) was added into this stirring solution and reaction mixture was cooled to 0° C. under inert atmosphere. PPh_3 (251.03 g, 0.9571 mol) was then added in portions keeping the temperature below 20° C. and after complete addition reaction was stirred for 3 h at room temperature and monitored by TLC. After completion of reaction, solid (PPh_3O) precipitated out from the reaction mixture was filtered off and filtrate was diluted with crushed ice (~ 1.5 kg) and extracted with DCM (3×750 mL). Organic layer was separated, dried over an. Na_2SO_4 and distilled under vacuum. Resulting crude compound was chromatographed on 60-120 mesh silica gel column using 0-5% ethyl acetate in hexanes as eluting system to give compound 3 (150 g, 89%) as pale yellow liquid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.25 (m, 5H), 4.49 (s, 2H), 3.47-3.41 (m, 2H), 3.41-3.37 (m, 2H), 1.86-1.80 (m, 4H), 1.62-1.56 (m, 2H), 1.42-1.29 (m, 8H).

1,17-bis-benzyloxy-heptadecan-9-ol (4): To freshly activated Mg turnings (24.08 g, 1.003 mol) was added 200 mL anhydrous THF was added followed by the addition of pinch of iodine into the mixture under inert atmosphere. After initiation of the Grignard formation a solution of Compound 3 (150 g, 0.5016 mol) in 1 L of dry THF was added slowly controlling the exothermic reaction. After complete addition reaction was refluxed for 1 h and then cooled to room temperature. (60.24 g, 1.0033 mol) methyl formate was then added slowly and reaction was continued for 2 h. After completion, the reaction was quenched by slow addition of 10% HCl followed by water (1 L) and extracted with Ethyl Acetate (3×1 L). Organic layer was taken in 5 lit beaker, diluted with 500 mL of methanol and cooled to 0° C. To this solution excess of $NaBH_4$ (~ 5 eq) was added in portions to ensure the hydrolysis of formate ester which was not cleaved by addition of HCl. Resulting solution was stirred for an hour and then volatilites were stripped off under vacuum.

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Residue was taken in water (1 L) and acidified by 10% HCl solution (P^H 4). Product was then extracted out with ethyl acetate (3×1 L). Organic phase was then dried and concentrated on rotary evaporator to get the desired compound 4 (57 g, 24%) as solid. H^1 NMR (400 MHz, $CDCl_3$): δ=7.35-7.32 (m, 8H), 7.29-7.24 (m, 2H), 4.49 (s, 4H), 3.56 (m, 1H), 3.46-3.43 (m, 4H), 1.63-1.56 (m, 4H), 1.44-1.34 (m, 28H). C^{13} NMR (100 MHz, $CDCl_3$): δ=138.56, 128.21, 127.49, 127.34, 72.72, 71.76, 70.37, 37.37, 29.64, 29.56, 29.47, 29.33, 26.07, 25.54.

[9-benzyloxy-1-(8-benzylozy-octyl)-nonyloxy]-tert-butyl-dimethyl-silane (5): Compound 4 (56 g, 0.1196 mol) was dissolved in 700 mL of anhydrous THF and cooled to 0° C. TBMS-Cl (36.06 g, 0.2396 mol) was added slowly followed by addition of Imidazole (32.55 g, 0.4786 mol) under inert atmosphere. Reaction was then stirred at room temperature for 18 h. Reaction was judged complete by TLC and then quenched with ice (~1 kg) and extracted with Ethyl acetate (3×500 mL). Organic layer was separated, washed with Sat $NaHCO_3$ solution to remove the acidic impurity, dried over Na_2SO_4 and evaporated under reduce pressure to obtain crude compound which was purified by silica gel (60-120 mesh) and eluted with 0-10% ethyl acetate hexane to yield (60 g, 82%) of compound 5 as yellowish oil. H^1 NMR (400 MHz, $CDCl_3$): δ=7.33-7.24 (m, 10H), 4.49 (s, 4H), 3.60-3.57 (m, 1H), 3.46-3.43 (m, 4H), 1.61-1.54 (m, 4H), 1.41-1.26 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H)

9-(tert-butyl-dimethyl-silanyloxy)-heptadecane-1,17-diol (6): Compound 5 (60 g, 0.1030 mol) was dissolved in 500 mL ethyl acetate and degassed with N_2 for 20 min. (10 wt %) Pd on carbon (12 g) was added and reaction was stirred under H_2 atmosphere for 18 h. After completion of reaction (by TLC) mixture was filtered through celite bed and washed with ethyl acetate. Filtrate was evaporated under vacuum. The compound 6 (19 g, 46%) thus obtained was pure enough to carry out the next reaction. H^1 NMR (400 MHz, $CDCl_3$): δ=3.64-3.58 (m, 5H), 1.59 (br, 2H), 1.57-1.51 (m, 4H), 1.38-1.22 (m, 28H), 0.87 (s, 9H), 0.02 (s, 6H).

9-(tert-butyl-dimethyl-silanyloxy)-heptadecanedioic acid (7): To a stirred solution of 6 (2 g, 0.0049 mol) in anhydrous DMF (40 mL) was added pyridinium dirchromate (2.7 g, 0.0074 mol) at 0° C. under inert atmosphere. Reaction mixture was then allowed to warm to room temperature over a period of 10-15 minutes and continued for 24 h. Progress of the reaction was monitored by TLC. After complete oxidation reaction was diluted with water (100 mL). Aqueous phase was extracted with DCM (3×40 mL). Organic phase was washed with brine (1×25 mL) and concentrated under vacuum to afford crude acid which was then purified by (100-200 mesh) silica gel column using 0-30% ethyl acetate in hexanes system. Pure product 26-003 was obtained (0.7 g, 33%) as pale yellow oil. H^1 NMR (400 MHz, $CDCl_3$): δ=3.61-3.56 (m, 1H), 2.35-2.32 (m, 4H), 1.64-1.59 (m, 4H), 1.40-1.19 (m, 24H), 0.86 (s, 9H), 0.017 (s, 6H); LC-MS [M+H]—431.00; HPLC (ELSD) purity—96.94%

Di((Z)-non-2-en-1-yl) 9-((tert-butyldimethylsilyl)oxy) heptadecanedioate (8): The diacid 7 (0.42 g, 0.97 mmol) was dissolved in 20 mL of dichloromethane and to it cis-2-nonen-1-ol (0.35 g, 2.44 mmol) was added followed by Hunig's base (0.68 g, 4.9 mmol) and DMAP (12 mg). To this mixture EDCI (0.47 g, 2.44 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 5% MeOH in CH_2Cl_2) showed complete disappearance of the starting acid. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash R_f purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product 8 (0.35 g, 53%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$): δ ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.58-5.43 (m, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.71-3.48 (m, 1H), 2.30 (t, J=7.6 Hz, 4H), 2.20-1.98 (m, 4H), 1.71-1.53 (m, 4H), 1.31 (ddd, J=8.3, 7.0, 3.7 Hz, 34H), 1.07-0.68 (m, 14H), 0.02 (s, 5H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.18, 139.81, 127.78, 81.73, 81.42, 81.10, 76.72, 64.59, 41.52, 41.32, 38.76, 36.09, 34.10, 33.93, 33.80, 33.70, 33.59, 33.55, 33.26, 31.95, 30.34, 29.69, 29.58, 29.39, 27.01, 22.56, 18.48, 0.01.

Di((Z)-non-2-en-1-yl) 9-hydroxyheptadecanedioate (9): The silyl protected diester 8 (0.3 g, 0.44 mmol) was dissolved in 1 M solution of TBAL in THE (6 mL) and the solution was kept at 40° C. for two days after which the TLC showed the completion of the reaction. The reaction mixture was diluted with water (60 mL) and extracted with ether (2×50 mL). The combined organic layers were concentrated and the thus obtained crude product was purified by column to isolate the pure product (0.097 g, 39%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (dt, J=10.9, 7.4 Hz, 2H), 5.52 (dt, J=11.0, 6.8 Hz, 2H), 4.61 (d, J=6.8 Hz, 4H), 3.57 (s, 1H), 2.30 (t, J=7.5 Hz, 4H), 2.09 (q, J=7.1 Hz, 4H), 1.75-1.53 (m, 4H), 1.53-1.06 (m, 36H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.98, 135.64, 123.57, 77.54, 77.22, 76.91, 72.14, 60.41, 37.69, 34.54, 31.89, 29.70, 29.60, 29.44, 29.29, 29.07, 27.76, 25.80, 25.15, 22.82, 14.29.

Di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl) oxy)heptadecanedioate: The alcohol 9 (0.083 g, 0.147 mmol) was dissolved in 20 mL of dichloromethane and to it dimethylaminobutyric acid hydrochloride (0.030 g, 0.176 mmol) was added followed by Hunig's base (0.045 g, 0.44 mmol) and DMAP (2 mg). To this mixture EDCI (0.034 g, 0.176 mmol) was added and the reaction mixture was stirred at room temperature overnight and the TLC (silica gel, 10% MeOH in CH_2Cl_2) showed complete disappearance of the starting alcohol. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with saturated $NaHCO_3$ (50 mL), water (60 mL) and brine (60 mL). The combined organic layers were dried over anhyd. Na_2SO_4 and solvents were removed in vacuo. The crude product thus obtained was purified by Combiflash R_f purification system (40 g silicagel, 0-10% MeOH in CH_2Cl_2) to isolate the pure product (0.062 g, 62%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 5.74-5.58 (m, 2H), 5.51 (dtt, J=9.7, 6.8, 1.3 Hz, 2H), 4.95-4.75 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 2.35-2.24 (m, 8H), 2.22 (d, J=7.9 Hz, 6H), 2.09 (q, J=6.9 Hz, 4H), 1.83-1.72 (m, 2H), 1.60 (dd, J=14.4, 7.2 Hz, 4H), 1.49 (d, J=5.7 Hz, 4H), 1.41-1.13 (m, 30H), 0.88 (t, J=6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.72, 173.36, 135.40, 123.35, 74.12, 60.18, 58.95, 45.46, 34.30, 34.11, 32.45, 31.67, 29.38, 29.35, 29.17, 29.07, 28.84, 27.53, 25.28, 24.93, 23.16, 22.59, 14.06. MW calc, for $C_{41}H_{75}NO_6$ (MH^+): 678.04, found: 678.5.

In another embodiment the following shorter route was used for the synthesis of the di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate. The commercial 9-bromonon-1-ene 10 was treated with magnesium to form the corresponding Grignard reagent which was reacted with ethylformate to give the corresponding adduct 11 which on treatment with bromobutyryl chloride to provide the bromoester 12. The bromoester 12 on treatment with RuO_4 provided the diacid 13. The bromodiacid 13 on treatment with dimethylamine provided the amino diacid 14. The aminodiacid 14 on coupling with the alcohol 15 provided the product in good yields.

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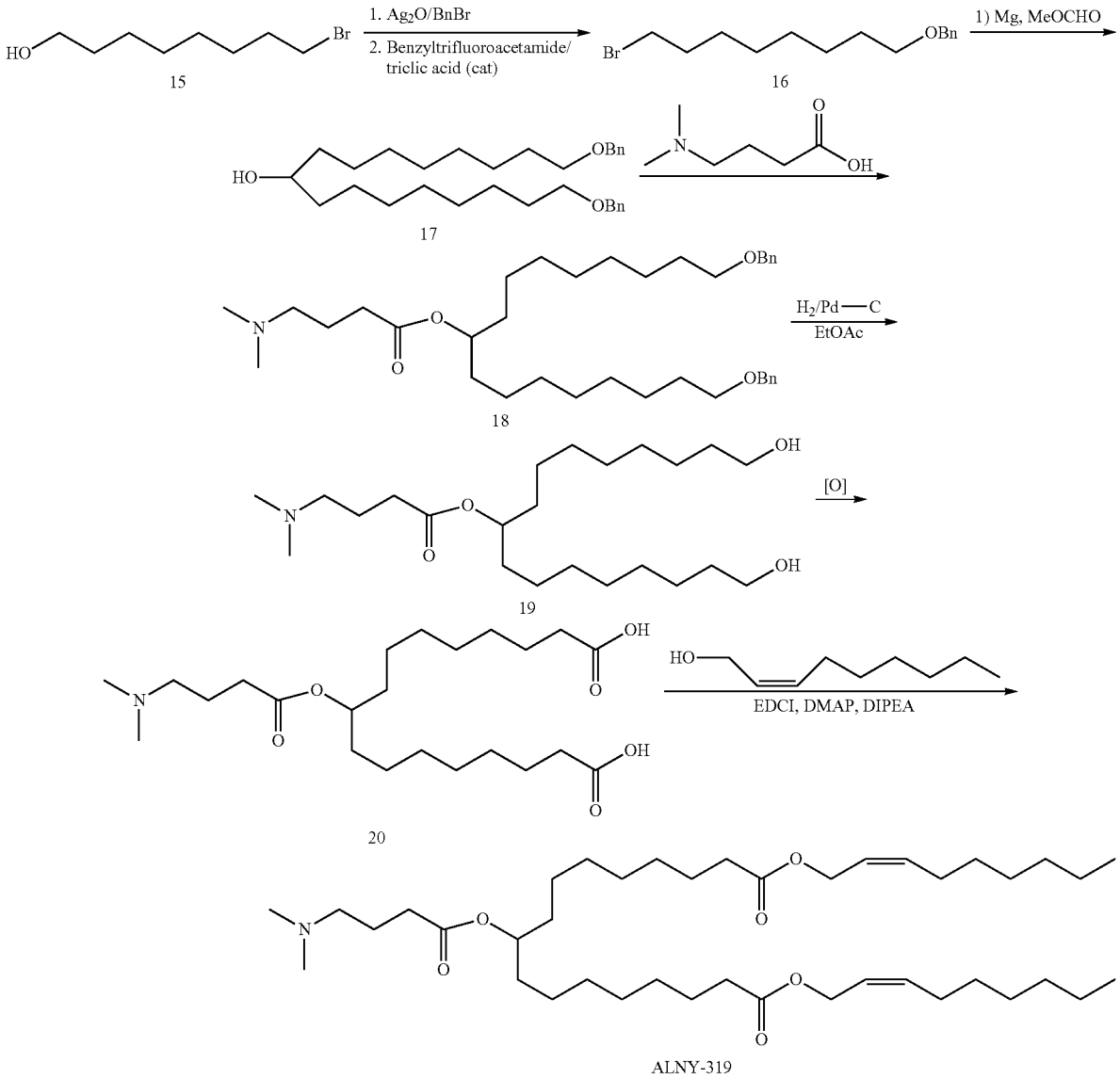
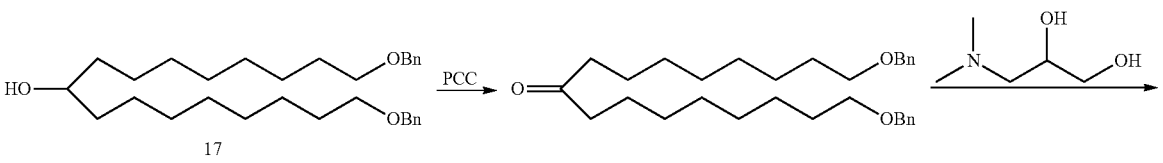
Example 21

Scheme 21



Example 22

Scheme 22



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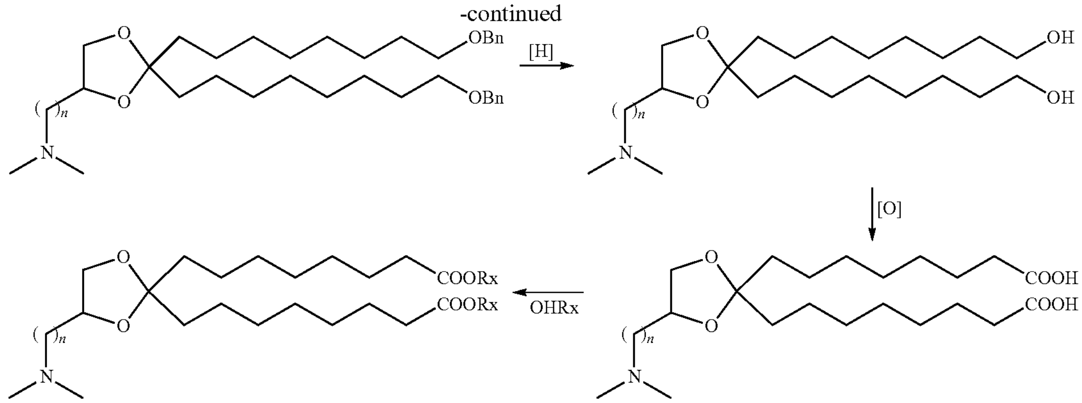
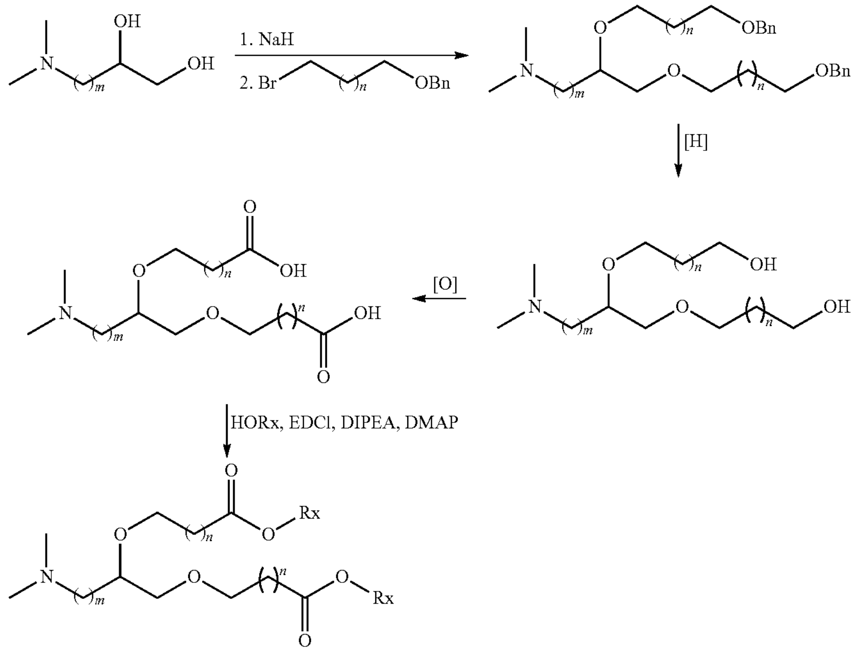


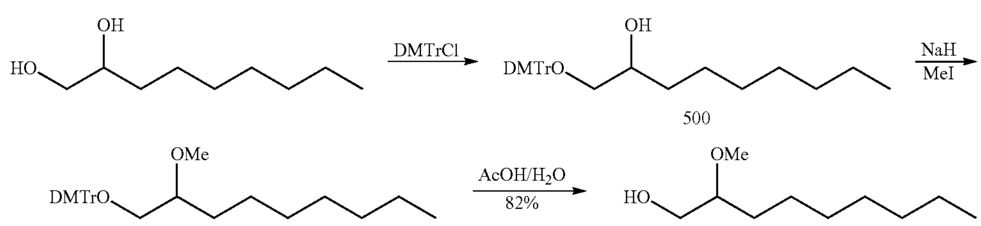
Example 23

Scheme 23



Example 24

Scheme 24



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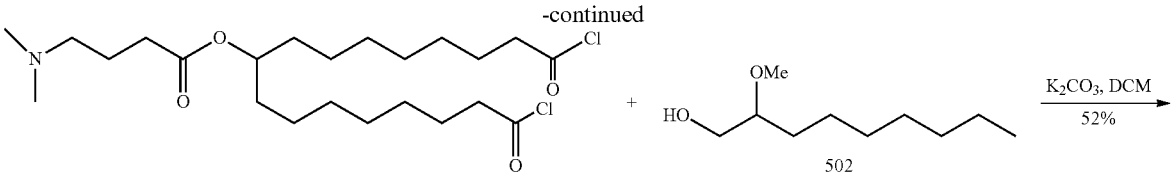
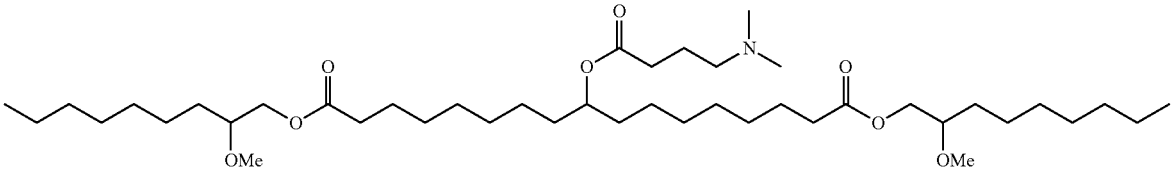




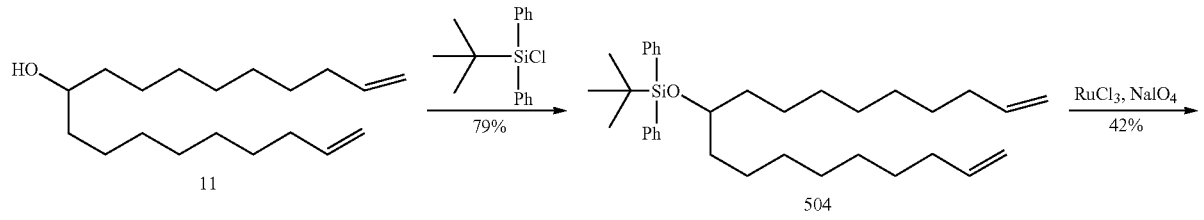
Compound 501: To a stirred solution of 2-hydroxy 1-octanol 5 g (31.25 mmol), DMAP 0.38 g (3.1 mmol) in dry pyridine (100 mL) was added DMTr-Cl and stirred at room temperature for 14 h. 10 mL of water was added and extracted with ethyl acetate, washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentration of the solvent gave 20 g of crude product 500 which was co-evaporated with toluene twice and used for the next step without further purification. To the above crude DMTr ether in dry THF (250 mL) were added NaH and iodo methane at 0° C. and then brought to room temperature over 30 min. and then stirred for two days. 5 mL of water was added and concentrated followed by column chromatography (0-30% ethyl acetate in hexane) gave the corresponding product 501 (10.25 g, R_f: 0.45, 20% ethyl acetate in hexane) and 8.4 g of recovered starting material 500. ^1H NMR (400 MHz, $CDCl_3$) δ 7.47-6.8 (m, 13H), 3.79 (s, 6H), 3.42 (s, 3H), 3.29-3.26 (m, 1H), 3.13-3.04 (m, 2H), 1.55-1.47 (m, 2H), 1.3-1.2 (m, 10H), 0.89 (t, J=6.4 Hz, 3H).

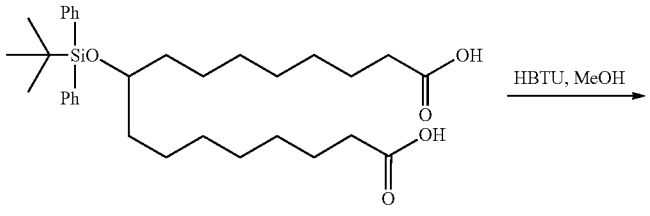
Alcohol 502: The compound 501 (10.25 g, 21.5 mmol) was dissolved in 75 mL of 80% acetic acid and stirred at room temperature for 14 h. 10 mL of methanol was added and concentrated, followed by column chromatography (0-50% ethyl acetate in hexane) yielded the expected product 502 as colorless oil (1.8 g, 82%, R_f: 0.3, 30% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 3.71-3.65 (m, 1H), 3.5-3.45 (m, 1H), 3.41 (s, 3H), 3.28-3.25 (m, 1H), 1.93-1.9 (m, 1H), 1.45-1.41 (m, 2H), 1.39-1.27 (m, 10H), 0.88 (s, J=6.8 Hz, 3H).

Compound 503: Compound 503 was synthesized following general experimental procedure for compound 213. 0.3 g as pale yellow oil (52%, R_f=0.2, 5% methanol in dichloromethane). ^1H NMR (400 MHz, $CDCl_3$) δ 4.87-4.84 (m, 1H), 4.18-4.00 (m, 4H), 3.4 (s, 6H), 3.37-3.19 (m, 2H), 2.34-2.26 (m, 6H), 2.2 (s, 6H), 1.8-1.6 (m, 2H), 1.63-1.2 (m, 50H), 0.88 (s, J=6.8 Hz, 6H).

Example 25

Scheme 25





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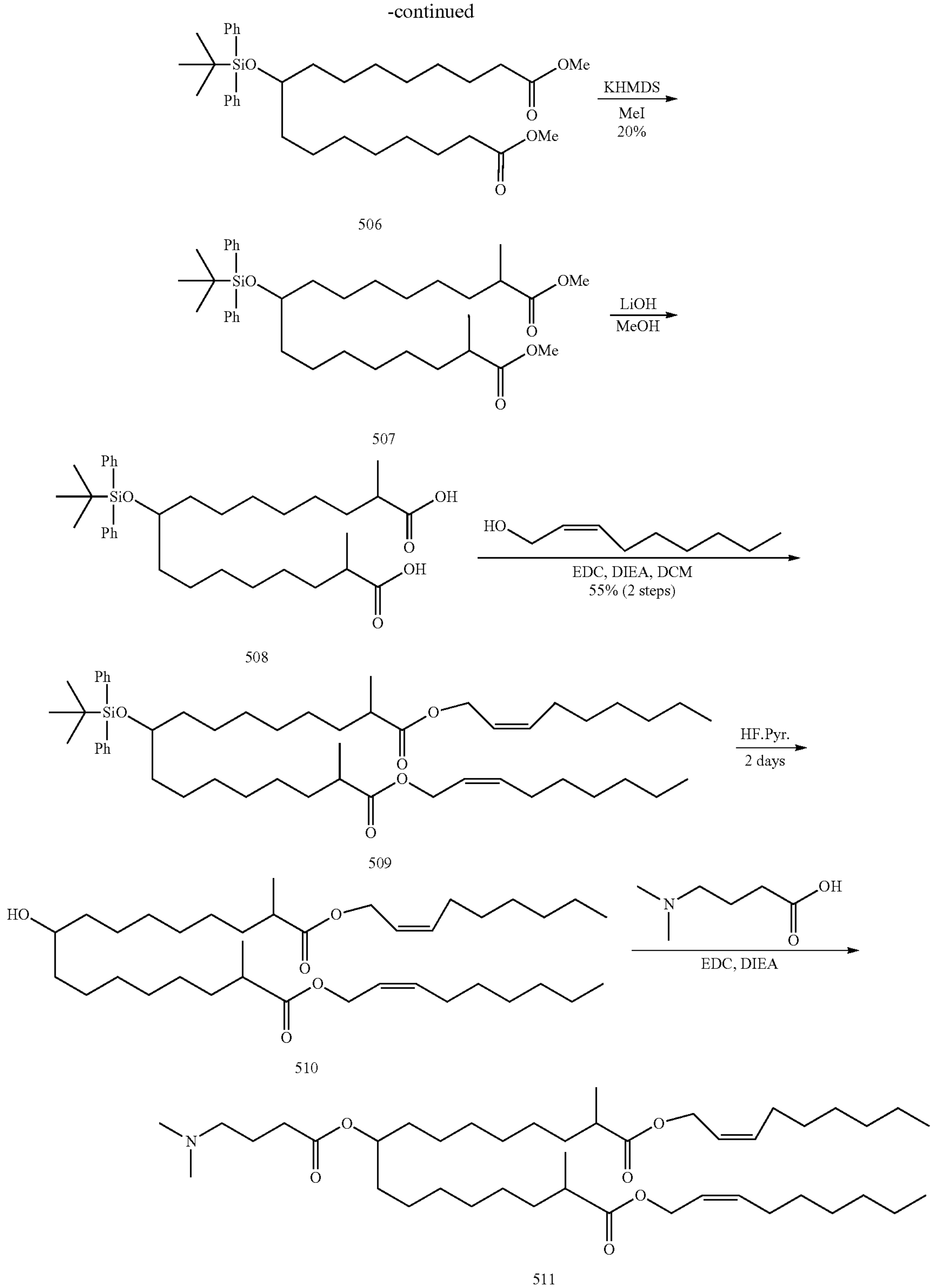


167 mg, 66% (2 steps)

Compound 504: To a stirred solution of alcohol 11 (4.01 g, 22.25 mmol), TBDPS-Cl (12.24 g, 44.5 mmol) and DMAP (0.54 g, 4.42 mmol) was added triethyl amine (8.99 g, 90 mmol) and stirred at room temper for 14 h. To the above solution was added imidazole (1.51 g, 22.25 mmol) and continued to stir for 14 h at room temperature. 20 mL of water was added and extracted with DCM followed by washing with 2N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10%

ethyl acetate in hexane) to yield compound 504 (7.38 g, 79%, R_f: 0.8, 5% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.66 (m, 4H), 7.43-7.33 (m, 6H), 5.86-5.76 (m, 2H), 5.02-4.91 (m, 4H), 3.73-3.67 (m, 1H), 2.04-1.99 (m, 4H), 1.42-1.08 (m, 24H), 1.05 (s, 9H).

Compound 505: To a stirred solution of diene 504 (7.38 g, 17.6 mmol) and $RUCl_3$ (0.18 g, 0.88 mmol) in 400 mL of DCM/CH_3CN (1:1) was added $NaIO_4$ (37.6 g, 176 mmol) dissolved in 400 mL of water drop wise around 5° C. over 30 min. and stirred at room temperature for 3 h. The organic layer was separated followed by washing with 3% Na_2S solution (100 mL), water (250 mL) brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product 505 (4 g, 42%, R_f: 0.3, 40% ethyl acetate in hexane), which was used for the next step without further purification.

Compound 506: To a stirred solution of the acid 505 (4 g, 7.22 mmol), HBTU (6.02 g, 15.88 mmol), HOBt (2.14 g, 15.88 mmol) and DMAP (88 mg, 0.72 mmol) in 75 mL of dry DCM was added 5 mL of methanol and stirred at room temperature for 14 h. 10 mL of water was added followed by extraction with DCM (3×50 mL), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in hexane) to yield compound 506 (2 g, 47.6%, R_f: 0.3, 10% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (m, 4H), 7.41-7.33 (m, 6H), 3.70-3.64 (m, 1H), 3.66 (s, 6H), 2.28 (t, J=7.2 Hz, 4H), 1.63-1.07 (m, 24H), 1.04 (s, 9H).

Compound 507: To a stirred solution of dimethyl ester 506 (1.0 g, 1.79 mmol) in dry THF (20 mL) were added KHMDS (0.752 g, 3.76 mmol) and methyl iodide (0.762 g, 5.37 mmol) at 0° C. and then brought to room temperature over 30 min. and stirred for 24 h. 10 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×50 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was purified by column chromatography (0-5% ethyl acetate in hexane) to obtain the product 507 (0.218 g, 20%, R_f: 0.8, 5% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68-7.65 (m, 4H), 7.41-7.33 (m, 6H), 3.70-3.67 (m, 1H), 3.67 (s, 6H), 2.43-2.38 (m, 2H), 1.59-1.07 (m, 24H), 1.13 (d, J=7.2 Hz, 6H), 1.04 (s, 9H).

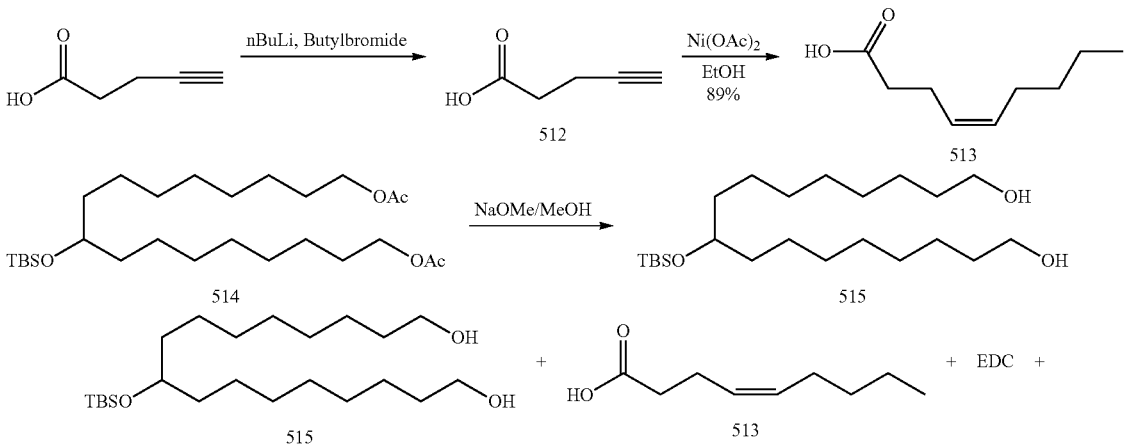
Compound 509: To a stirred solution of methyl ester 507 (0.4 g, 0.66 mmol) in 10 mL of MeOH/THF (1:1) was added LiOH (0.079 g, 3.27 mmol) in 1 mL of water and stirred at room temperature for 24 h. To the above solution was added KOH (0.183 g, 3.27 mmol) in 1 mL of water and stirred for another 2 days. 2 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×25 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product 508 (0.45 g, R_f: 0.2, 10% ethyl acetate in hexane), which was used for the next step without further purification. To a stirred solution of the above di-acid 508 (0.45 g), cis-2-Nonen-1-ol (0.66 g, 4.6 mmol) and EDC.HCl (0.82 g, 4.6 mmol) in dry DCM (15 mL) was added DIEA (1.2 g, 9.24 mmol) and stirred at room temperature for 3 days. 10 mL of water was added followed by extraction with DCM followed by washing with 2N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 509 (0.3 g, 55%, R_f: 0.5, 3% ethyl acetate in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67-7.65 (m, 4H), 7.42-7.33 (m, 6H), 5.67-5.6 (m, 2H), 5.55-5.49 (m, 2H), 4.615 (d, J=4 Hz, 4H), 3.71-3.65 (m, 1H), 2.44-2.35 (m, 2H), 2.10 (q, J=8.0 Hz, 4H), 1.64-1.07 (m, 40H), 1.13 (d, J=8.0 Hz, 6H), 1.04 (s, 9H), 0.86 (t, J=10 Hz, 6H).

Compound 511: To a stirred solution of silyl ether 509 (0.3 g, 0.36 mmol) in dry THF were added pyridine (1 mL) and HF.Pyr., (1 mF) drop wise and stirred at 4° C. for 48 h. The solvent was evaporated and used for the next step without purification.

To a stirred solution of the above crude alcohol 510, N,N-Dimethyl amino butyric acid (0.34 g, 2.04 mmol), EDC.HCl (0.39 g, 2.04 mmol) and DMAP (0.06 g, 0.51 mmol) in dry DCM (10 mF) was added DIEA (0.5 g, 3.88 mmol) and stirred at room temperature for 2 days. 10 mF of water was added followed by extraction with DCM (3×25 mF), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in 1% TEA containing hexane) to yield compound 511 (0.167 g, 66%, R_f: 0.4, 10% MeOH in DCM). Molecular weight for $C_{43}H_{79}NO_6$ $(M+H)^+$ Calc. 706.59, Found 706.5.

Example 26

Scheme 26



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Case: 23-2357 Document: 14 Page: 299 Filed: 12/04/2023

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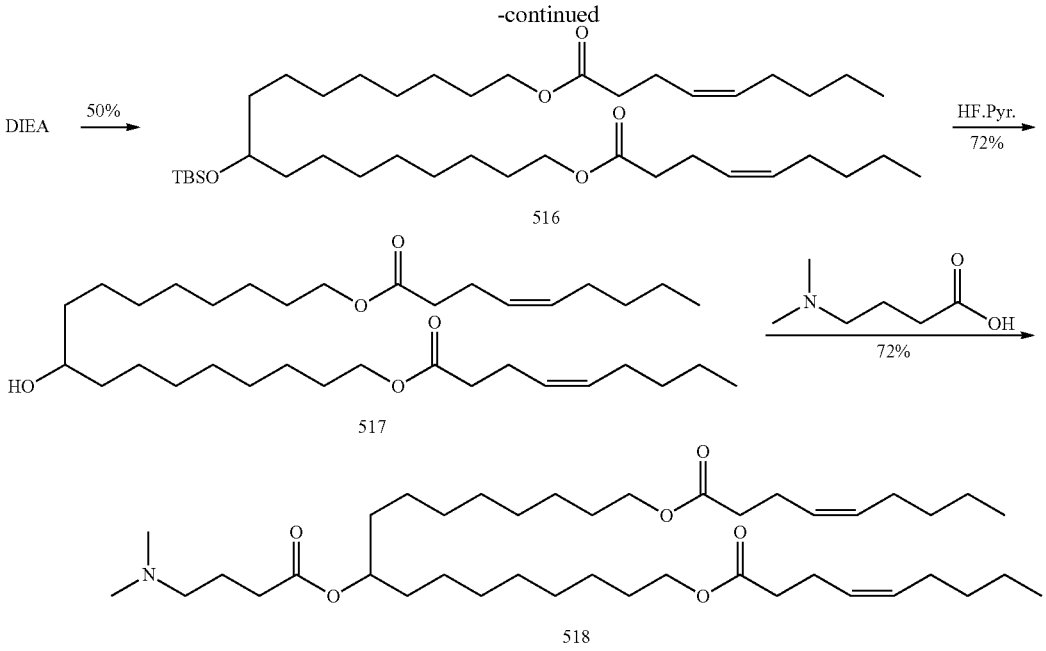


Compound 512: To a stirred solution of 4-Pentynoic acid in 100 mL of THF/HMPA (4:1) at −78° C. was added nBuLi (3.1 g, 49 mmol) drop wise and stirred for 30 min. Then the reaction mixture was brought to 0° C. and stirred for 2 h. Again, the reaction mixture was cooled to −78° C. and n-butyl bromide (3.07 g, 22.44 mmol) was added drop wise and stirred at room temperature for 14 h. 10 mL of sat. NH_4Cl solution was added followed by extraction with ethyl acetate (3×25 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was purified by column chromatography (0-30% ethyl acetate in hexane) to yield compound 512 (0.4 g, R_f: 0.8, 30% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 2.59-2.55 (m, 2H), 2.49-2.44 (m, 2H), 2.16-2.11 (m, 2H), 1.49-1.34 (m, 4H), 0.9 (t, J=6.0 Hz, 3H).

Compound 513: To a suspension of $Ni(OAc)_2$ (0.45 g, 2.53 mmol) in EtOH (20 mL) was added $NaBH_4$ (0.096 g, 12.65 mmol) portion wise at room temperature and stirred for 15 min. under H_2 atm. Filtered off the solid followed by concentration of the solvent gave compound 513 (0.35 g, 88.6%, R_f: 0.6, 20% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 10.88 (br s, 1H), 5.47-5.41 (m, 1H), 5.35-5.31 (m, 1H), 2.43-2.33 (m, 4H), 2.07-2.03 (m, 2H), 1.36-2.27 (m, 4H), 0.9 (t, J=8.0 Hz, 3H).

Compound 515: To a stirred solution of di-acetate 514 (1.5 g, 3.09 mmol) in MeOH (100 mL) was added a piece of sodium metal (0.05 g, 2.17 mmol) and stirred at room temperature for 14 h. Neutralized with dry ice and concentrated followed by extraction with ethyl acetate (3×50 mL), washing with water, dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product 515 (1.1 g, 88.7%), which was used for the next step without purification.

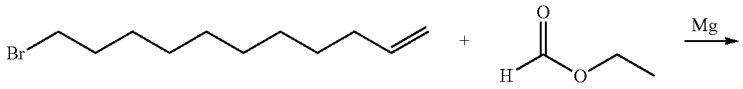
Compound 516: To a stirred solution of the above diol 515 (0.4 g, 1 mmol), 513 (0.341 g, 2.19 mmol), DMAP (0.1 g, 0.82 mmol) and EDC.HCl (0.57 g, 2.98 mmol) in dry DCM (15 mL) was added DIEA (5.97 g, 6 mmol) and stirred at room temperature for 2 days. 10 mL of water was added followed by extraction with ethyl acetate followed by washing with ^1N HCl, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 516 (0.335 g, 50%, R_f: 0.6, 5% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.45-5.38 (m, 2H), 5.36-5.29 (m, 2H), 4.06 (t, J=8 Hz, 4H), 3.63-3.589 m, 1H), 2.39-2.31 (m, 8H), 2.07-2.02 (m, 4H), 1.65-1.57 (m, 4H), 1.4-1.28 (m, 32H), 0.9 (t, J=6.0 Hz, 6H), 0.88 (s, 9H), 0.03 (s, 6H).

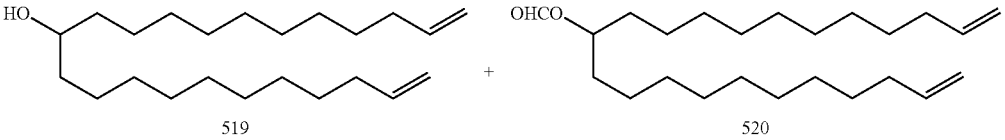
Compound 517: To a stirred solution of silyl ether 516 (0.3 g, 0.36 mmol) in dry THF (5 mL) were added pyridine (1 mL) and HF.Pyr. (1 mL) drop wise and stirred at 45° C. for 24 h. The solvent was evaporated followed by purification by column chromatography gave product 517 (0.2 g, 72%, R_f: 0.4, 10% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.43-5.36 (m, 2H), 5.34-5.27 (m, 2H), 4.04 (t, J=8 Hz, 4H), 3.59-3.53 (m, 1H), 2.37-2.3 (m, 8H), 2.05-2.0 (m, 4H), 1.61-1.29 (m, 37H), 0.88 (t, J=8.0 Hz, 6H).

Compound 518: To a stirred solution of the alcohol 517 (0.2 g, 0.355 mmol), N,N-Dimethyl amino butyric acid (0.36 g, 2.14 mmol), EDC.HCl (0.406 g, 2.14 mmol) and DMAP (0.043 g, 0.36 mmol) in dry DCM (10 mL) was added DIEA (0.55 g, 4.26 mmol) and stirred at room temperature for 2 days. 10 mL of water was added followed by extraction with DCM (3×25 mL), washing with saturated $NaHCO_3$, water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-30% ethyl acetate in 1% TEA containing hexane) to yield compound 518 (0.172 g, 72%, R_f: 0.2, 5% MeOH in DCM). ^1H NMR (400 MHz, $CDCl_3$) δ 5.43-5.36 (m, 2H), 5.32-5.27 (m, 2H), 4.87-4.83 (m, 1H), 4.03 (t, J=6 Hz, 4H), 2.36-2.2 (m, 6H), 2.32 (s, 6H), 2.03-1.25 (m, 40H), 0.88 (t, J=6.0 Hz, 6H).

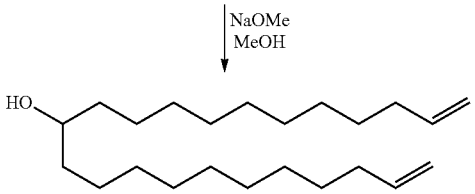
Example 27

Scheme 27

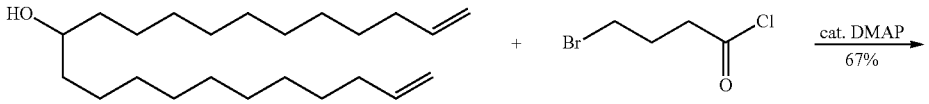


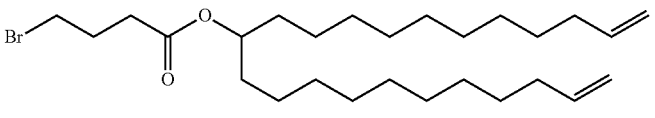


519 520

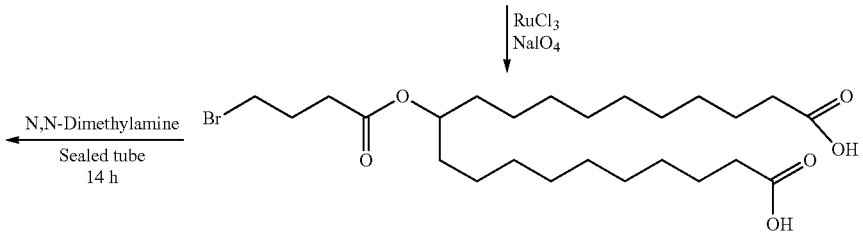


521
94%





522



523
83%

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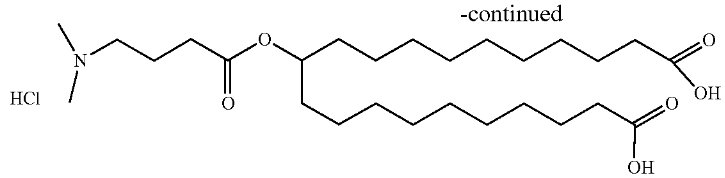
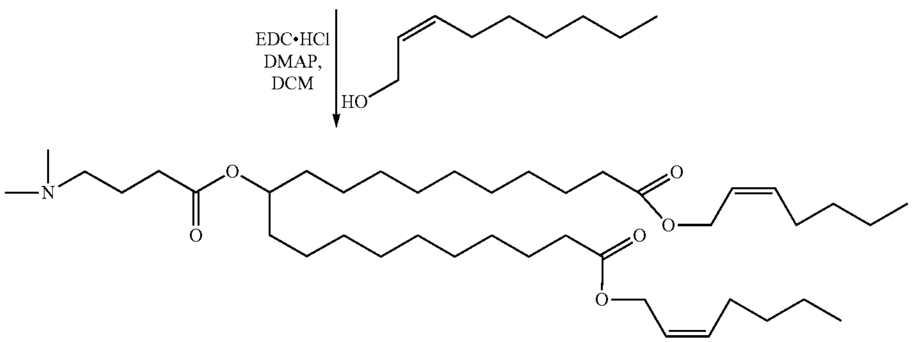


524
82%



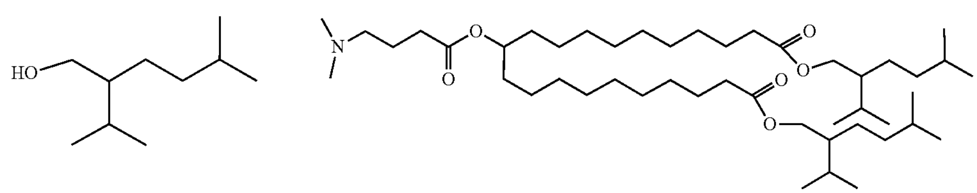
525
60%

Compound 521: To a suspension of Mg in Et_2O was added alkyl bromide (25 g, 107.7 mmol) drop wise at 40° C. over one hour. Ethyl formate was added to the above reaction mixture at 0-5° C. and then the reaction mixture was stirred at room temperature for 14 h. The reaction mixture was poured onto the ice cold sat. NH_4Cl solution followed by extraction with Et_2O (3×250 mL), washing with water, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was re-dissolved in MeOH (250 mL) and a small piece of sodium (0.1 g) was added and stirred at room temperature for 14 h. The solvent was evaporated and 100 mL of water was added followed by filtration of the solid, washing with water (2×100 mL) gave pale yellow powder 521 (17 g, 94%, %, R_f: 0.8, 10% ethyl acetate in hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 5.84-5.74 (m, 2H), 5.0-4.89 (m, 4H), 3.64-3.49 (m, 1H), 2.04-1.99 (m, 4H), 1.79 (br s, 1H), 1.44-1.23 (m, 32H).

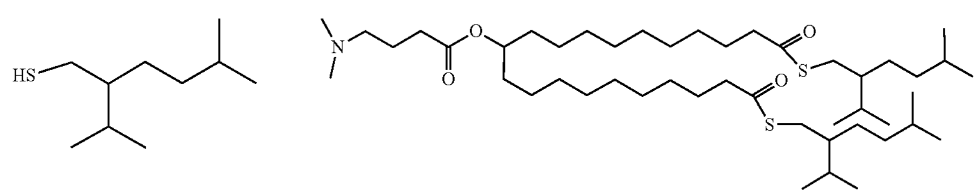
Compound 522: To a stirred solution of 521 (10 g, 29.73 mmol) and DMAP (0.1 g, 0.82 mmol) in dry DCM (50 mL) was added 4-bromo butyryl chloride (6.56 g, 35.68 mmol) and stirred at room temperature for 14 h. 5 mL of saturated $NaHCO_3$ was added and the organic layer was separated and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product which was purified by column chromatography (0-10% ethyl acetate in hexane) to yield compound 522 (9.6 g, 66.7%, R_f: 0.9, 5% ethyl acetate in hexane).

Compound 524: Oxidation was carried out to get compound 523 (8.6 g, 83.5%, R_f: 0.1, 5% MeOH in DCM) following same experimental procedure as for compound 505. This crude material was dissolved in 2N N,N-dimethyl amine in THF (20 mL) and heated to 60° C. in a sealed tube for 14 h. Concentrated the reaction mixture and then pH of the reaction mixture was brought to 3. This mixture was freeze-dried to obtain compound 524 as HCl salt (4 g, 82%). Molecular weight for C27H51NO6 $(M+H)^+$ Calc. 486.37, Found 486.2. ^1H NMR (400 MHz, $CDCl_3$) δ 4.94-4.89 (m, 1H), 3.32-3.3 (m, 2H), 3.2-3.16 (m, 2H), 2.91 (s, 6H), 2.47 (t, J=8 Hz, 2H), 2.28 (t, J=8 Hz, 4H), 2.05-1.97 (m, 2H), 1.61-1.56 (8H), 1.4-1.25 (m, 22H).

526



526s



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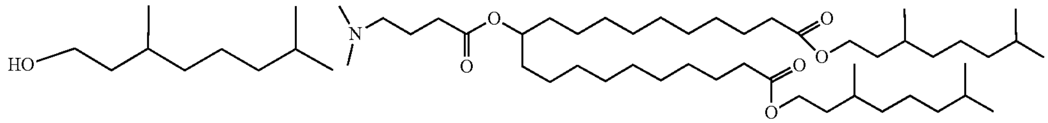
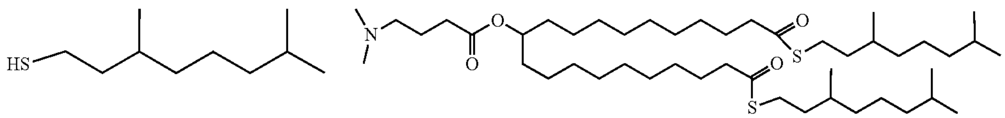
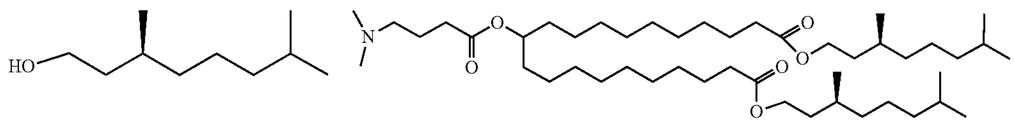
527



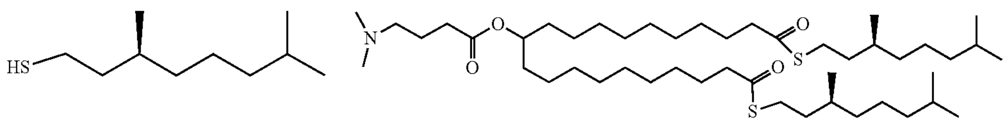
527s



528



528s



Synthesis of Ester 525, 526, 527 and 528

The title compounds were synthesized following the experimental procedure as for compound 516.

Compound 525: (0.75 g, 60%, R_f: 0.3, 5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.65-5.59 (m, 2H), 5.53-5.47 (m, 2H), 4.87-4.81 (m, 1H), 4.595 (d, J=4.0 Hz, 4H), 2.43-2.25 (m, 8H), 2.2 (s, 6H), 2.1-2.03 (m, 4H), 1.81-1.73 (m, 2H), 1.61-1.56 (m, 4H), 1.48-1.47 (m, 4H), 1.36-1.23 (m, 32H), 0.86 (t, J=8.0 Hz, 6H).

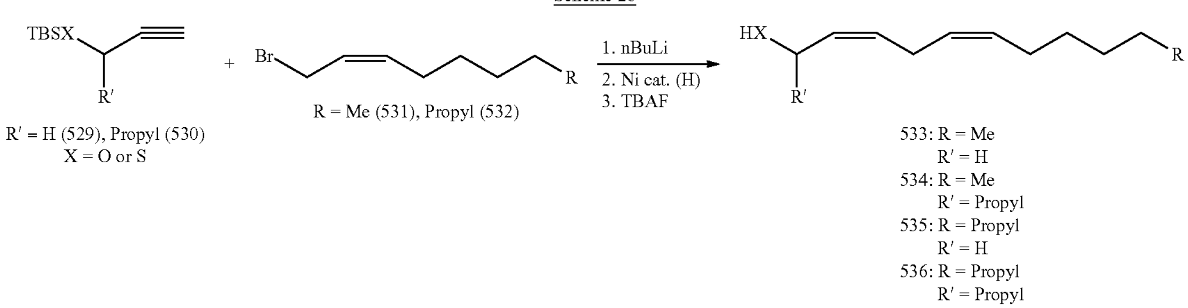
Compound 526: (0.358 g, 60.9%, R_f: 0.5, 5% MeOH in DCM). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.87-4.81 (m, 1H), 4.07-3.95 (m, 4H), 2.32-2.24 (m, 6H), 2.2 (s, 6H), 1.80-1.69 (m, 4H), 1.6-1.14 (m, 46H), 0.88-0.84 (m, 24H).

Compound 527: (0.258 g, 56.8%, R_f: 0.5, 5% MeOH in DCM). Molecular weight for C47H91NO6 $(M+H)^+$ Calc. 766.23; Found: 766.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.86-4.80 (m, 1H), 4.12-4.02 (m, 4H), 2.31-2.23 (m, 8H), 2.19 (s, 6H), 1.80-1.72 (m, 2H), 1.66-1.06 (m, 52H), 0.87 (d, J=8.0 Hz, 6H), 0.84 (d, J=8.0 Hz, 12H).

Compound 528: (0.3 g, 68.1%, R_f: 0.5, 5% MeOH in DCM). Molecular weight for C47H91NO6 $(M+H)^+$ Calc. 766.23; Found: 766.7. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.86-4.80 (m, 1H), 4.12-4.02 (m, 4H), 2.31-2.21 (m, 8H), 2.19 (s, 6H), 1.79-1.72 (m, 2H), 1.66-0.98 (m, 52H), 0.87 (d, J=8.0 Hz, 6H), 0.835 (d, J=4.0 Hz, 12H).

Example 28

Scheme 28



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Case 23-2357 Document: 14 Page: 203 Filed: 12/04/2023

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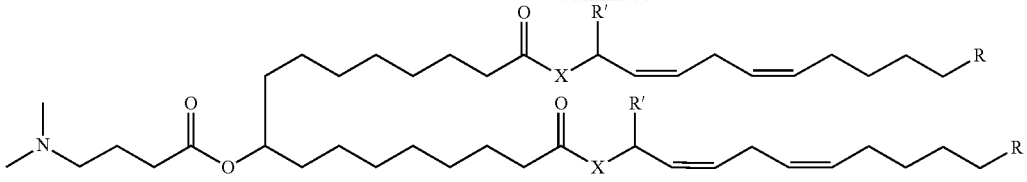


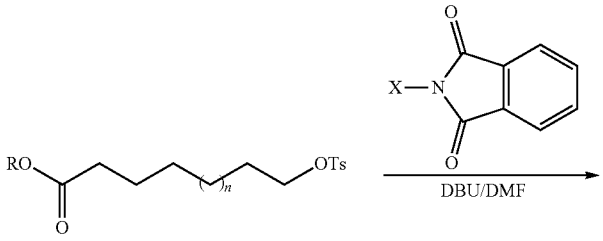
537: R = Me
R′ = H
538: R = Me
R′ = Propyl
539: R = Propyl
R′ = H
540: R = Propyl
R′ = Propyl

Synthesis of compounds 533, 534, 535 and 536: The title compounds (1 mmol) are synthesized following the experimental procedure of compound 513 except de-silylation step and it is done using TBAF in THF at room temperature.

Synthesis of compounds 537, 538, 539 and 540: The title compounds (1 mmol) are synthesized following the experimental procedure of compound 525.

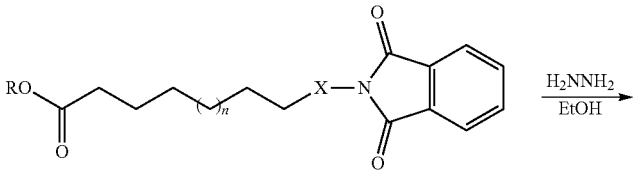
Example 29

Scheme 29



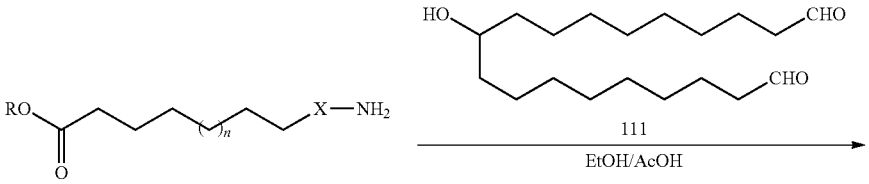
239

R = alkyl/aryl
n = 0-10



240

X = O/NH
R = alkyl/aryl
n = 0-10

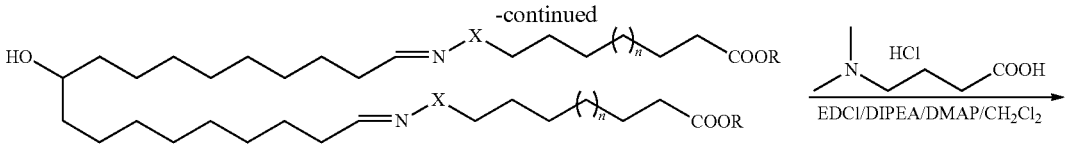


241

X = O/NH
R = alkyl/aryl
n = 0-10

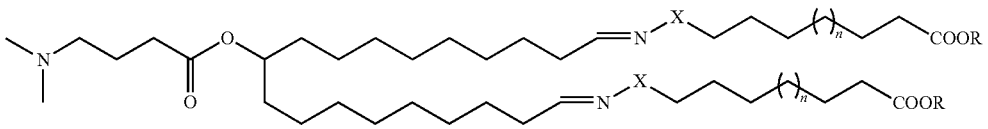
Case 1:22-cv-00835-CFC Document 55-1 Filed 02/15/24 Page 5 of 204 PageID #: 2876
Case: 23-2357 Document: 14 Page: 204 Filed: 12/04/2023

-continued



242

X = O/NH
R = alkyl/aryl
n = 0-10

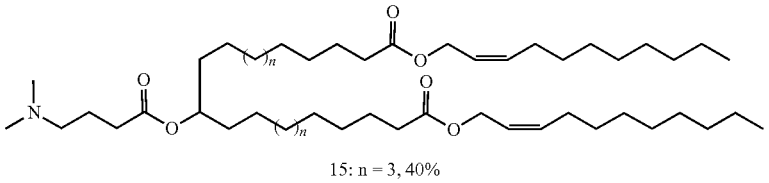


243

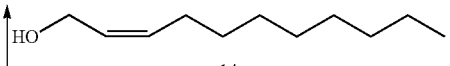
X = O/NH
R = alkyl/aryl
n = 0-10

Compound 243 (X=O/NH, R=alkyl/aryl) can be synthesized as shown in Scheme 16-2. Tosyl group of 239 can be replaced with phthalimide group by nucleophilic substitution. After deprotection followed by coupling with 111 under acidic conditions, 242 can be synthesized. Standard esterification gives cationic lipid 243 and its analogs.

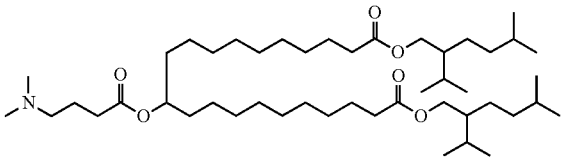
Example 30: Synthesis of Ester-Containing Lipids



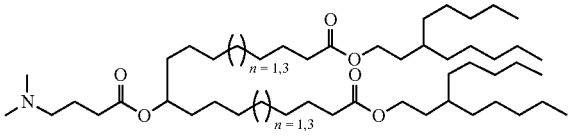
15: n = 3, 40%



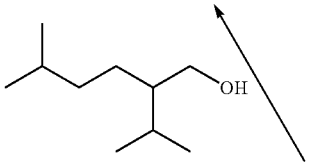
14

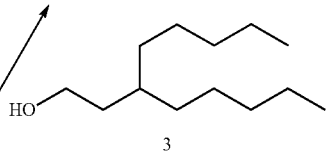


16

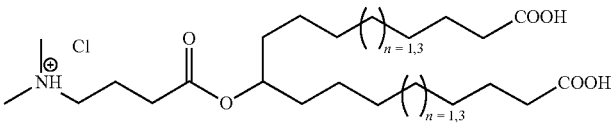


17: n = 1
18: n = 3



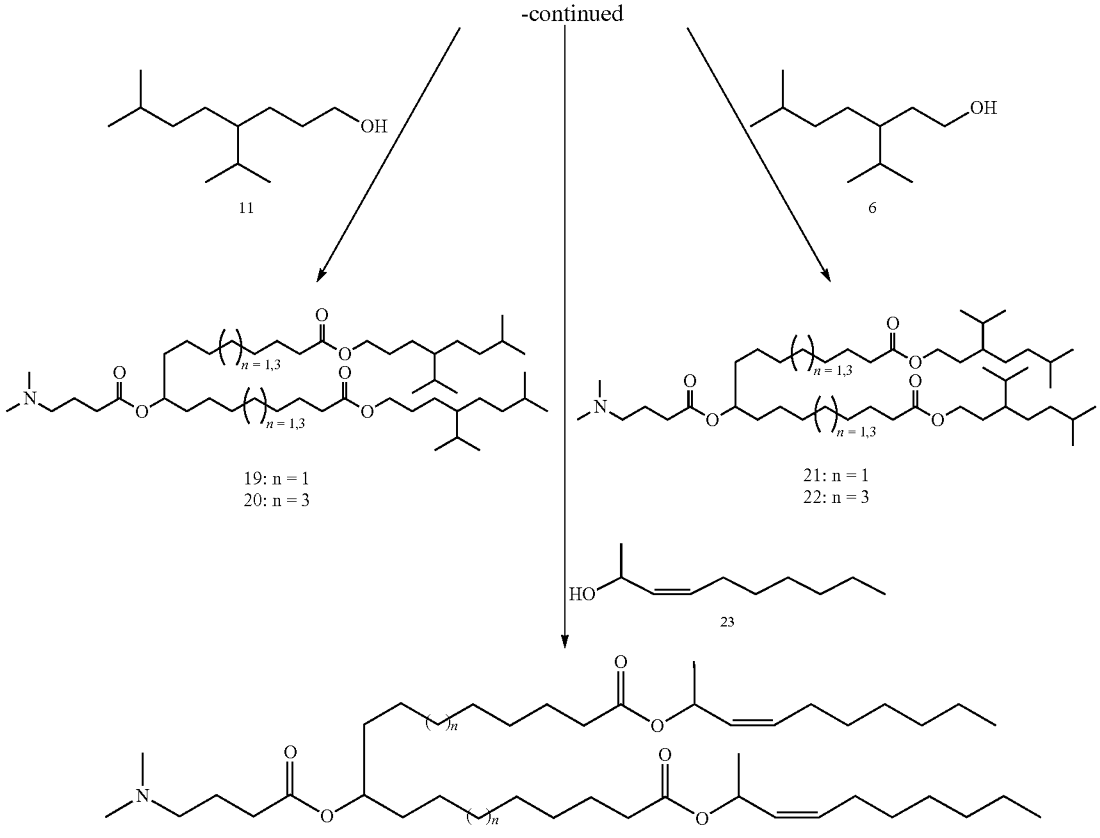


3



12: n = 1
13: n = 3

-continued



Compound 15: Compound 13 (503 mg, 1.0 mmol) was treated with 14 (469 mg, 3.0 mmol) in the presence of EDCI (2.30 g, 12.0 mmol), DMAP (235 mg, 1.92 mmol) and DIEA (8.34 mL, 47.9 mmol) in CH_2Cl_2 (50 mL) for 14 h. Aqueous work-up then column chromatography gave compound 15 (1.22 g, 1.54 mmol, 40%).

Molecular weight for $C_{49}H_{92}NO_6$ $(M+H)^+$ Calc. 790.6925, Found 790.7.

Compound 16: This compound was synthesized from 13 and tetrahydrolavandulol using a procedure analogous to that described for compound 15. Yield: 0.358 g, 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.87-4.81 (m, 1H), 4.07-3.95 (m, 4H), 2.32-2.24 (m, 6H), 2.2 (s, 6H), 1.80-1.69 (m, 4H), 1.6-1.14 (m, 46H), 0.88-0.84 (m, 24H).

Compound 17: This compound was synthesized from 12 (1.0 g, 2.15 mmol) and 3 (1.03 g, 5.16 mmol) using a procedure analogous to that described for compound 15.

Yield: 856 mg (50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.91-4.79 (m, 1H), 4.08 (t, J=7.1 Hz, 4H), 2.35-2.25 (m, 14H), 1.89-1.76 (m, 2H), 1.67-1.13 (m, 62H), 0.88 (t, J=7.0 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.08, 74.45, 63.08, 45.27, 34.76, 34.56, 34.28, 33.70, 32.61, 32.39, 29.54, 29.36, 29.28, 26.36, 25.47, 25.13, 22.83, 14.26. Molecular weight for $C_{49}H_{96}NO_6$ $(M+H)^+$ Calc. 794.7238, Found 794.6.

Compound 18: This compound was synthesized from 13 (1.0 g, 2.15 mmol) and 3 (1 g) using a procedure analogous to that described for compound 15.

Yield: 1 g (59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.94-4.74 (m, 1H), 4.17-3.85 (m, 4H), 2.46-2.19 (m, 12H), 1.93-1.79 (m, 2H), 1.74-1.45 (m, 10H), 1.37 (d, J=20.2 Hz, 2H), 1.35-1.13 (m, 44H), 0.88 (t, J=6.9 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.19, 77.53, 77.21, 76.90, 63.12, 34.81, 34.66, 34.35, 33.76, 32.66, 32.45, 29.76, 29.73, 29.63, 29.48, 29.39, 26.42, 25.57, 25.23, 22.89, 14.32. Molecular weight for $C_{53}H_{103}NO_6$ $(M+H)^+$ Calc. 850.38, Found 850.7.

Compound 19: This compound was synthesized from 12 and 11 using a procedure analogous to that described for compound 15.

Yield: 860 mg (51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.90-4.81 (m, 1H), 4.04 (t, J=6.8 Hz, 4H), 2.37-2.17 (m, 14H), 1.84-1.06 (m, 48H), 0.93-0.78 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.06, 74.35, 65.51, 64.91, 59.05, 45.51, 43.77, 37.10, 34.55, 34.29, 32.55, 29.54, 29.37, 29.34, 29.28, 28.58, 28.19, 26.99, 26.74, 25.47, 25.15, 22.90, 22.82, 19.60, 19.41, 19.28. Molecular weight for $C_{47}H_{92}NO_6$ $(M+H)^+$ Calc. 766.6925, Found 766.5.

Compound 20: This compound was synthesized from 13 and 11 using a procedure analogous to that described for compound 15.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.86 (p, J=6.2 Hz, 1H), 4.04 (t, J=6.7 Hz, 4H), 2.38-2.17 (m, 14H), 1.84-1.07 (m, 56H), 0.93-0.76 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.11, 173.46, 74.44, 64.90, 59.06, 45.51, 43.77, 37.11, 34.59, 34.32, 32.57, 29.71, 29.67, 29.57, 29.43, 29.34, 28.58, 28.20, 27.00, 26.75, 25.51, 25.20, 22.90, 22.82, 19.41, 19.28. Molecular weight for $C_{51}H_{100}NO_6$ $(M+H)^+$ Calc. 822.7551, Found 822.6.

Compound 21: This compound was synthesized from 12 and 6 using a procedure analogous to that described for compound 15.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.91-4.78 (m, 1H), 4.15-3.98 (m, 4H), 2.39-2.18 (m, 14H), 1.84-1.11 (m, 44H), 0.92-0.77 (m, 24H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.06, 173.44, 74.36, 63.73, 59.03, 45.48, 41.00, 36.98, 34.56, 34.29, 32.54, 29.60, 29.54, 29.49, 29.36, 29.28, 28.52, 25.47, 25.13, 23.15, 22.85, 22.81, 19.49, 18.89. Molecular weight for $C_{45}H_{88}NO_6$ $(M+H)^+$ Calc. 738.6612, Found 738.6.

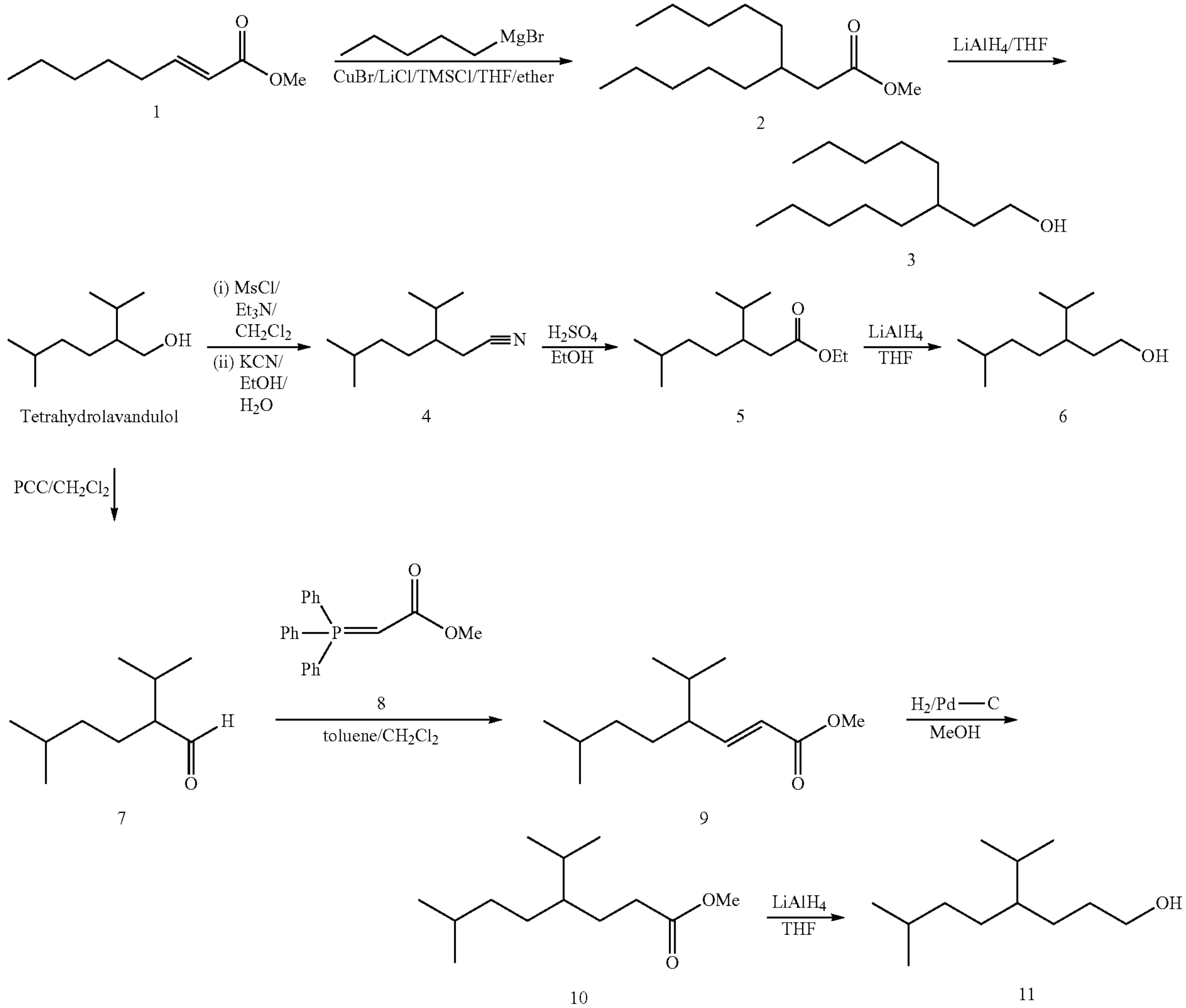
Compound 22: This compound was synthesized from 13 and 6 using a procedure analogous to that described for compound 15.

Yield: 900 mg (57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.92-4.78 (m, 1H), 4.15-3.91 (m, 4H), 3.33-3.08 (m, 1H), 2.36-2.15 (m, 14H), 1.79 (dq, J=14.3, 7.2 Hz, 2H), 1.74-1.55 (m, 8H), 1.55-1.37 (m, 9H), 1.35-0.95 (m, 36H), 0.96-0.61 (m, 27H). ^{13}C NMR (101 MHZ $CDCl_3$) δ 174.16, 173.52, 77.54, 77.22, 76.91, 74.48, 63.76, 59.10, 45.55, 42.02, 41.04, 38.75, 37.09, 37.02, 34.65, 34.36, 32.62, 30.71, 29.75, 29.72, 29.64, 29.62, 29.53, 29.48, 29.44, 29.38, 28.56, 28.45, 25.56, 25.23, 23.59, 23.23, 22.90, 22.86, 19.54, 19.03, 18.94. Molecular weight for $C_{49}H_{95}NO_6$ $(M+H)^+$ Calc. 794.2817, Found 794.7.

Compound 24: This compound was synthesized from 13 and 23 using a procedure analogous to that described for compound 15.

Yield: 0.567 g (30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.85 (p, J=6.1 Hz, 1H), 4.20-3.93 (m, 4H), 2.41-2.18 (m, 13H), 1.92-1.72 (m, 2H), 1.56 (ddd, J=27.4, 16.4, 5.8 Hz, 12H), 1.39 (s, 2H), 1.25 (s, 54H), 0.91 (dt, J=13.7, 6.4 Hz, 11H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.18, 173.51, 77.54, 77.23, 76.91, 74.50, 63.12, 59.10, 45.55, 34.81, 34.66, 34.38, 33.76, 32.67, 32.62, 32.45, 29.77, 29.73, 29.64, 29.49, 29.39, 26.42, 25.57, 25.24, 23.23, 22.89, 14.32. Molecular weight for $C_{47}H_{88}NO_6$ $(M+H)^+$ Calc. 762.6612, Found 762.5.

Example 31: Synthesis of Alcohol Components



Compound 2: Compound 2 was synthesized from 1 using a procedure analogous to that described in *Journal of the Organic Chemistry,* 2009, 1473.

^{1}n NMR (400 MHz, $CDCl_3$) δ 3.66 (s, 3H), 2.23 (d, J=6.9 Hz, 2H), 1.84 (brs, 1H), 1.27 (d, J=11.5 Hz, 16H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.29, 51.49, 39.25, 35.22, 34.00, 32.24, 26.34, 22.77, 14.22.

Compound 3: To a suspension of $LiAlH_4$ (2.84 g, 74.9 mmol) in THF (85 mL) was added a solution of compound

2 (8.55 g, 37.4 mmol) in THF (25 mL). The reaction mixture was refluxed overnight. Aqueous workup then column chromatography gave pure compound 3 (7.35 g, 36.7 mmol, 98%) as a colorless oil.

^{1}n NMR (400 MHz, $CDCl_3$) δ 3.66 (t, J=7.0 Hz, 2H), 1.59-1.12 (m, 19H), 0.88 (t, J=6.9 Hz, 6H).

Compound 4: Tetrahydrolavandulol (10.1 g, 63.8 mmol) was treated with methansulfonyl chloride (6.38 mL) in CH_2Cl_2 (200 mL) and Et_3N (17.6 mL). Aqueous workup gave the crude mesylate, which was treated with KCN (4.98 g, 76.5 mmol) in EtOH (90 mL) and H_2O (10 mL). Aqueous workup then column chromatography gave pure compound 4 (8.36 g, 50.0 mmol, 72%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.38-2.23 (m, 2H), 1.86-1.78 (m, 1H), 1.59-1.42 (m, 3H), 1.40-1.07 (m, 3H), 0.93-0.89 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.73, 41.69, 36.46, 30.10, 28.44, 28.33, 22.82, 22.59, 19.62, 19.11, 19.05.

Compound 6: The cyano derivative 4 was converted to the ethyl ester under acidic conditions to give compound 5 and the ester was reduced by $LiAlH_4$ in THF to give compound 6.

Compound 7: Tetrahydrolavandulol (98.1 g, 51.2 mmol) was oxidized with PCC (16.6 g, 76.8 mmol) in CH_2Cl_2 (200 mL). Aqueous workup then column chromatography gave pure compound 7 (6.19 g, 39.6 mmol, 77%) as a colorless oil.

1H), 1.72-1.64 (m, 1H), 1.54-1.40 (m, 2H), 1.37-1.22 (m, 1H), 1.18-0.97 (m, 2H), 0.94-0.78 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.19, 152.54, 121.70, 51.53, 49.66, 36.95, 31.76, 29.49, 28.29, 22.92, 22.54, 20.84, 19.24.

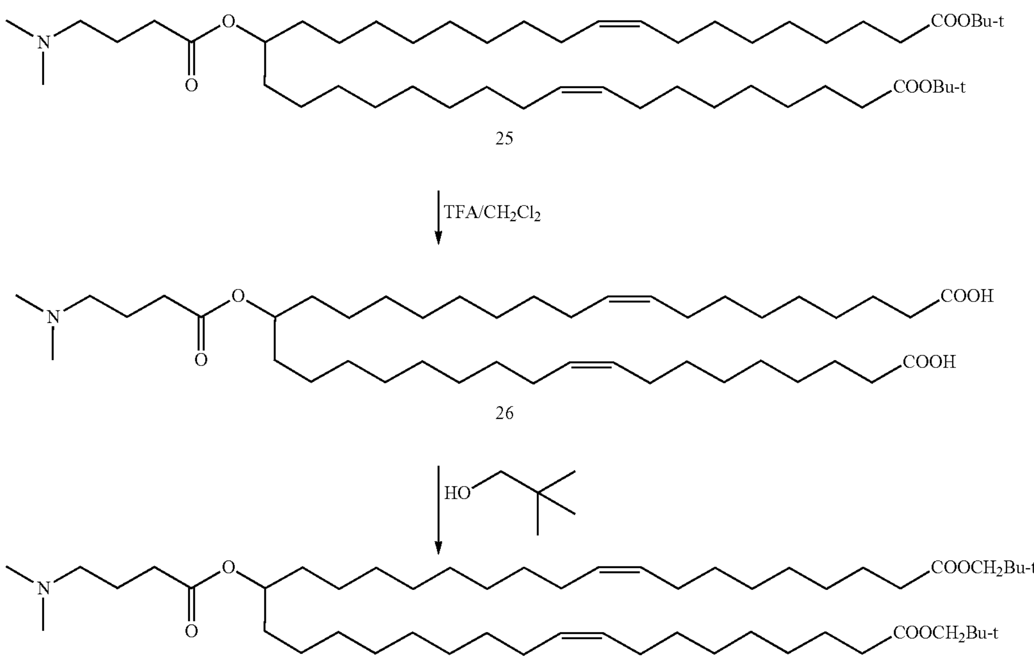
Compound 10: To a solution of compound 9 (1.0 g, 4.71 mmol) in MeOH (15 mL) was added Pd—C (125 mg). The mixture was stirred under H_2 atmosphere overnight. The mixture was filtered over Celite then evaporated to give pure compound 10 (924 mg, 4.31 mmol, 92%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67 (s, 3H), 2.41-2.16 (m, 2H), 1.74-1.57 (m, 2H), 1.57-1.42 (m, 2H), 1.33-1.02 (m, 5H), 0.88-0.83 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.78, 51.62, 43.71, 36.97, 32.69, 29.23, 28.56, 27.94, 25.92, 22.85, 22.79, 19.32, 19.19.

Compound 11: To a suspension of $LiAlH_4$ (444 mg, 11.7 mmol) in THF (12 mL) was added a solution of compound 10 (1.25 g, 5.83 mmol) in THF (8 mL). The reaction mixture was refluxed overnight. Aqueous workup gave the crude compound 11 (1.1 g) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.63 (t, J=6.7 Hz, 2H), 1.74-1.66 (m, 1H), 1.60-1.45 (m, 3H), 1.37-1.05 (m, 7H), 0.88-0.82 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 63.75, 44.00, 37.16, 31.22, 29.40, 28.61, 28.28, 26.62, 22.90, 22.82, 19.43, 19.28.

Example 32: Synthesis of Ester-Containing Lipids



^{1}H NMR (400 MHz, $CDCl_3$) δ 9.60 (d, J=3.1 Hz, 1H), 2.05-1.79 (m, 1H), 1.71-1.36 (m, 4H), 1.23-1.04 (m, 2H), 1.02-0.82 (m, 12H).

Compound 9: To a solution of compound 7 (2.0 g, 12.8 mmol) in toluene (40 mL) and CH_2Cl_2 (18 mL) and was added 8 (3.96 g, 11.8 mmol). The mixture was heated at 70° C. overnight. Column chromatography gave pure compound 9 (1.40 g, 6.59 mmol, 51%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.77 (dd, J=15.6, 9.9 Hz, 1H), 5.76 (d, J=15.6 Hz, 1H), 3.73 (s, 3H), 1.97-1.83 (m,

Compound 26: Compound 25 (840 mg, 1.03 mmol) was stirred in TFA (9 mL) and CH_2Cl_2 (36 mL) for 3 h at room temperature. Evaporation of the solvents and co-evaporation with toluene 3 times gave compound 26.

Molecular weight for $C_{43}H_{80}NO_6$ $(M+H)^+$ Calc. 706.5986, Found 706.4.

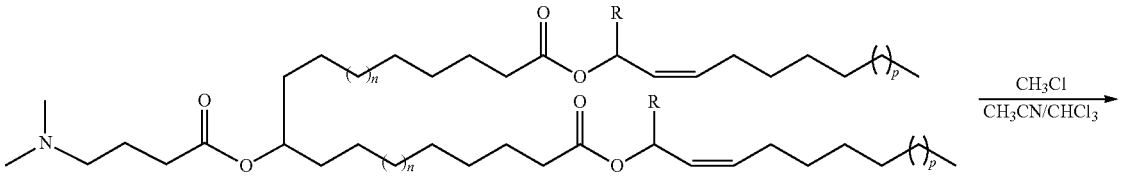
Compound 27: Compound 26 from the previous step was treated with 2,2-dimethylpropanol (363 mg, 4.12 mmol) in the presence of EDCI (592 mg, 3.09 mmol), DMAP (50 mg, 0.412 mmol) and DIEA (1.44 mL, 8.24 mmol) in CH_2Cl_2

(10 mL) for 14 h. Aqueous work-up then column chromatography gave compound 27 (575 mg, 0.679 mmol, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40-5.28 (m, 4H), 4.91-4.81 (m, 1H), 3.76 (s, 4H), 2.34-2.27 (m, 8H), 2.22 (s, 6H), 2.03-1.97 (m, 8H), 1.83-1.26 (m, 50H), 0.94 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.14, 173.53, 130.09, 129.92, 74.41, 73.72, 59.12, 45.61, 34.60, 34.32, 32.64, 31.45, 29.93, 29.85, 29.71, 29.68, 29.48, 29.32, 29.28, 27.39, 27.33, 26.62, 25.52, 25.22, 23.32.

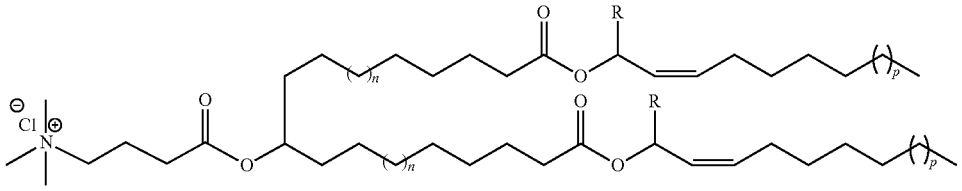
Molecular weight for $C_{53}H_{100}NO_6$ $(M+H)^+$ Calc. 846.7551, Found 846.5.

Example 33: Synthesis of Quaternary Lipids

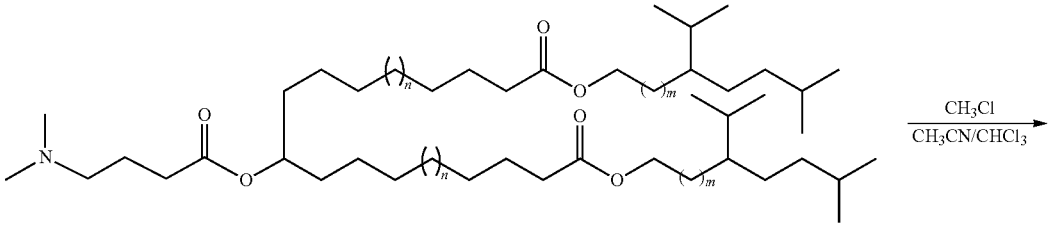
A. The amino lipids synthesized in Examples 31 and 32 can be converted to the corresponding quaternary lipids as shown below by treatment with CH_3Cl in CH_3CN and $CHCl_3$.



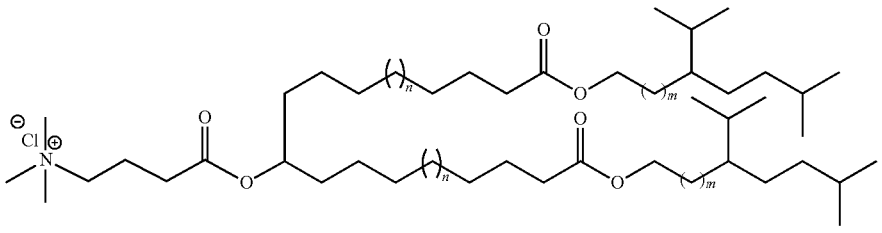
15: n = 3, p = 3, R = H
24: n = 3, p = 1, R = Me



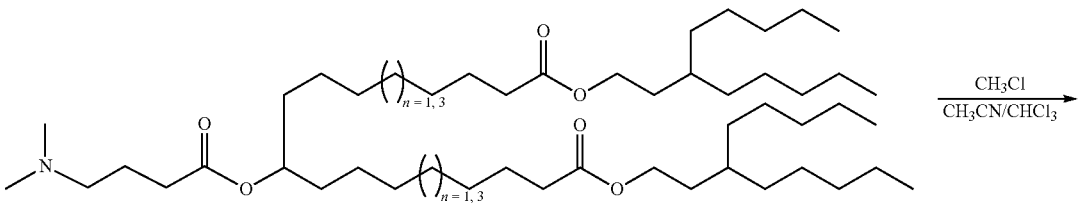
15Q: n = 3, p = 3, R = H
24Q: n = 3, p = 1, R = Me



16: m = 0, n = 3
19: m = 2, n = 1
20: m = 2, n = 3
21: m = 1, n = 1
22: m = 1, n = 3



16Q: m = 0, n = 3
19Q: m = 2, n = 1
20Q: m = 2, n = 3
21Q: m = 1, n = 1
22Q: m = 1, n = 3



17: n = 1
18: n = 3

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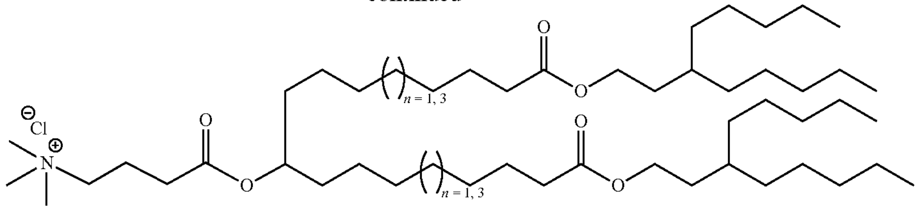
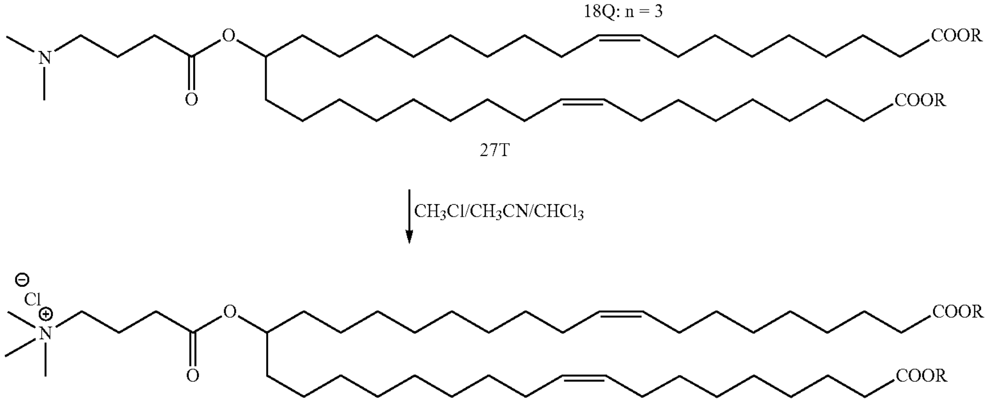


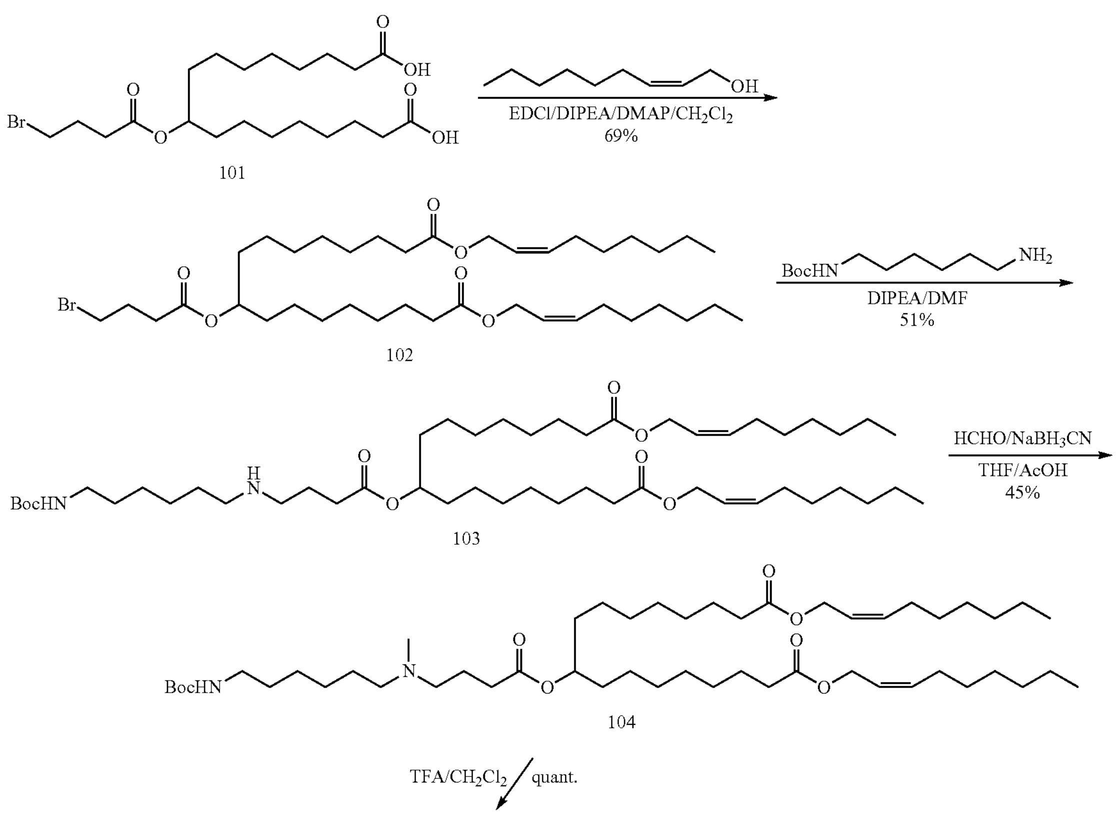
17Q: n = 1
18Q: n = 3



R = Me, Et, iPr, t-Bu, other alkyl/allyl groups

B. Synthesis of BODIPY-Lipid Conjugates

Synthesis of BODIPY-Labeled Lipid



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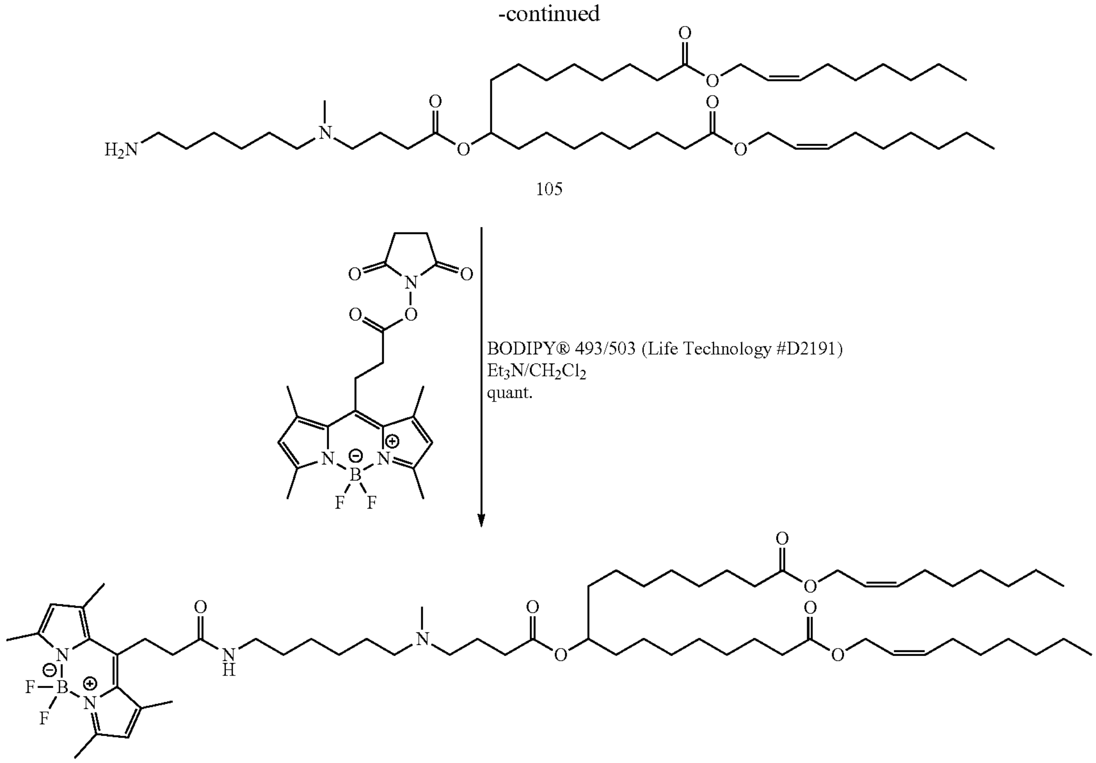

105

106

Compound 102: To a solution of compound 101 (2.00 g, 4.30 mmol) and cis-2-nonen-1-ol (1.81 mL, 10.7 mmol) in CH_2Cl_2 (20 mL) were added diisopropylethylamine (3.00 mL, 17.2 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (2.06 g, 10.7 mmol) and DMAP (106 mg, 0.868 mmol). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with saturated $NaHCO_3$ aq. (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The crude was purified by silica gel column chromatography (0-5% EtOAc in Hexane) to give compound 102 (2.11 g, 2.96 mmol, 69%, R_f=0.45 developed with 10% EtOAc in Hexane).

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.54-5.49 (m, 2H), 4.89-4.84 (m, 1H), 4.62 (d, J=6.5 Hz, 4H), 3.46 (t, J=6.5 Hz, 2H), 2.48 (t, J=7.3 Hz, 2H), 2.30 (t, J=7.5 Hz, 4H), 2.20-2.14 (m, 2H), 2.12-2.04 (m, 4H), 1.63-1.60 (m, 4H), 1.51-1.50 (m, 4H), 1.37-1.27 (m, 32H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.90, 172.45, 135.58, 123.51, 74.74, 60.36, 34.47, 34.24, 32.93, 32.91, 31.83, 29.54, 29.48, 29.31, 29.21, 29.01, 28.03, 27.70, 25.43, 25.08, 22.76, 14.23.

Molecular weight for $C_{39}H_{69}BrNaO_6$ $(M+Na)^+$ Calc. 735.42, Found 735.2.

Compound 103: To a solution of 102 (2.11 g, 2.96 mmol) in DMF (20 mL) was added a solution of N-Boc-1,6-diaminohexane (670 mg, 3.10 mmol) in DMF (20 mL) at 0° C. The mixture was stirred for 18 hours at room temperature. Then additional N-Boc-1,6-diaminohexane (160 mg, 0.740 mmol) in DMF (1 mL) was added and the mixture was stirred for 12 hour. The reaction was quenched by adding saturated $NaHCO_3$ aq. (100 mL) then extracted with Et_2O (150 mL×3). The organic layer was separated and dried over anhydrous $MgSO_4$. After filtration and concentration, the crude was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2, R_f=0.24) to give 103 (1.28 g, 1.51 mmol, 51%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67-5.61 (m, 2H), 5.55-5.50 (m, 2H), 4.88-4.81 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 4.54 (brs, 1H), 3.11-3.08 (m, 2H), 2.67-2.59 (m, 4H), 2.35 (t, J=7.4 Hz, 2H), 2.29 (t, J=7.6 Hz, 4H), 2.10-2.07 (m, 4H), 1.84-1.81 (m, 4H), 1.63-1.57 (m, 4H) 1.50-1.47 (m, 8H), 1.44 (s, 9H), 1.38-1.27 (m, 34H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.90, 173.53, 135.57, 123.50, 74.49, 60.36, 49.82, 49.29, 40.64, 34.47, 34.24, 32.68, 31.83, 30.16, 29.89, 29.54, 29.50, 29.33, 29.23, 29.01, 28.58, 27.69, 27.11, 26.80, 25.44, 25.37, 25.09, 22.76, 14.23.

Molecular weight for $C_{50}H_{93}N_2O_8$ $(M+H)^+$ Calc. 849.69, Found 849.5.

Compound 104: To a solution of 103 (1.16 g, 1.37 mmol) in THF (20 mL) were added formaldehyde (37 wt. % in H_2O, 0.306 mL, 4.11 mmol), sodium cyanoborohydride (1 M solution in THF, 2.06 mL, 2.06 mmol) and acetic acid (0.008 mL, 0.137 mmol) at 0° C. The mixture was stirred at room temperature for 17 hours. The reaction was quenched by adding saturated $NaHCO_3$ aq. (50 mL) then extracted with Et_2O (100 mL×3). The organic layer was separated and dried over anhydrous $MgSO_4$. After filtration and concentration, the crude was purified by silica gel column chromatography (8% MeOH in CH_2Cl_2, R_f=0.46) to give 104 (531 mg, 0.615 mmol, 45%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.66-5.60 (m, 2H), 5.53-5.47 (m, 2H), 4.86-4.80 (m, 1H), 4.61-4.59 (m, 5H), 3.12-3.07 (m, 2H), 2.89-2.78 (m, 4H), 2.62 (s, 3H), 2.40 (t, J=6.8 Hz, 2H), 2.28 (t, J=7.4 Hz, 4H), 2.11-2.06 (m, 4H), 1.99-1.92 (m, 2H), 1.69-1.27 (m, 57H), 0.87 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.86, 172.45, 156.18,

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135.55, 123.45, 75.24, 60.32, 56.68, 55.83, 40.72, 40.36, 34.40, 34.09, 31.79, 31.29, 29.92, 29.49, 29.41, 29.26, 29.17, 28.96, 28.55, 27.65, 26.49, 26.30, 25.41, 25.02, 24.79, 22.71, 20.12, 14.19.

Molecular weight for $C_{51}H_{95}N_2O_8$ $(M+H)^+$ Calc. 863.71, Found 863.6.

Compound 105: To a solution of compound 104 (525 mg, 0.608 mmol) in CH_2Cl_2 (8 mL) was added trifluoroacetic acid (2 mL) at 0° C. The reaction mixture was stirred at 0° C. for 1 hour and at room temperature for 3 hours. The reaction mixture was evaporated and co-evaporated with toluene 3 times then dried in vacuo overnight to give compound 105 (603 mg, 0.603 mmol calculated as 2 TFA salt, quantitatively, R_f=0.24 developed with 8% MeOH in CH_2Cl_2).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (brs, 1H), 5.68-5.61 (m, 2H), 5.55-5.49 (m, 2H), 4.87-4.81 (m, 1H), 4.62 (d, J=6.8 Hz, 4H), 4.28 (brs, 3H), 3.20-3.02 (m, 6H), 2.82 (d, J=4.0 Hz, 3H), 2.45-2.40 (m, 2H), 2.30 (t, J=7.4 Hz, 4H), 2.12-2.00 (m, 6H), 1.78-1.22 (m, 52H), 0.88 (t, J=6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.04, 172.08, 161.84, 161.47, 135.63, 123.44, 117.60, 114.71, 75.56, 60.41, 55.69, 55.27, 39.94, 39.64, 34.44, 34.06, 31.82, 30.72, 29.53, 29.43, 29.28, 29.19, 29.00, 27.69, 26.58, 25.42, 25.27, 25.05, 24.60, 23.06, 22.75, 19.00, 14.22.

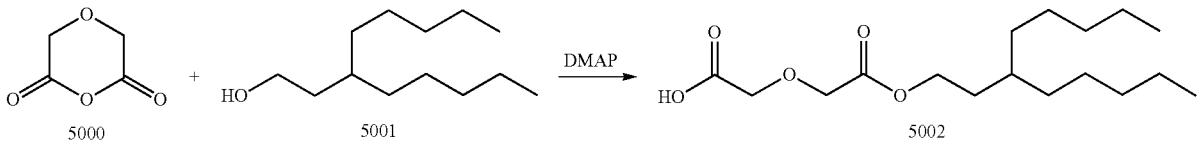
Molecular weight for $C_{46}H_{87}N_2O_6$ $(M+H)^+$ Calc. 763.66, Found 763.4.

Compound 106: To a solution of 105 (23.8 mg, 0.0240 mmol, calculated as 2TFA salt) in CH_2Cl_2 (1 mL) and Et_3N (0.050 mL, 0.360 mmol) was added a solution of BODIPY®493/503 (10 mg, 0.0240 mmol, Life Technology #D2191) in CH_2Cl_2 (2 mL). The reaction mixture was stirred for 1 h. The reaction mixture was loaded onto silica gel column chromatography and eluted with 0-5% MeOH in CH_2Cl_2. The product color fractions were collected (5% MeOH in CH_2Cl_2, R_f=0.36) to give 106 (26 mg, 0.024 mmol, quantitatively).

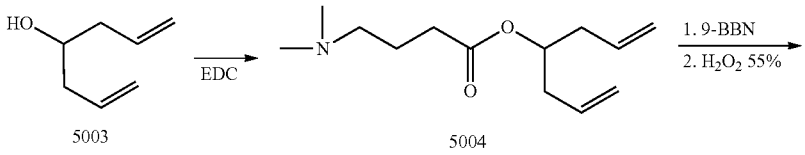
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.05 (s, 2H), 5.67-5.61 (m, 2H), 5.54-5.48 (m, 2H), 4.85-4.82 (m, 1H), 4.61 (d, J=6.8 Hz, 4H), 3.37-3.32 (m, 2H), 3.27-3.22 (m, 2H), 2.51-2.44 (m, 17H), 2.34-2.27 (m, 8H), 2.12-2.06 (m, 4H), 1.60-1.21 (m, 52H), 0.88 (t, J=6.8 Hz, 6H).

Molecular weight for $C_{62}H_{104}BF_2N_4O_7$ $(M+H)^+$ Calc. 1065.80, Found 1065.5.

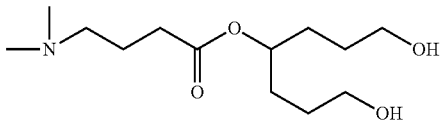
Example 34: Multi-Ester Containing Lipids and Acetal Linked Lipids



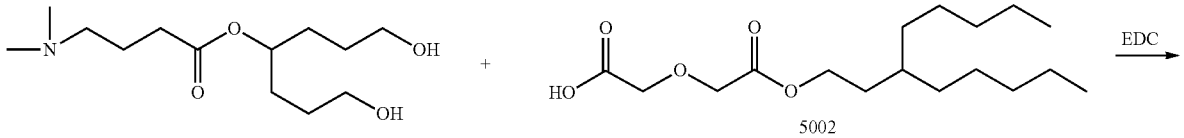
5000 5001 5002



5003 5004



5005



5005 5002

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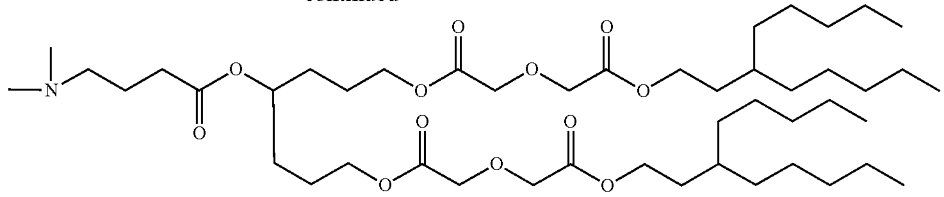
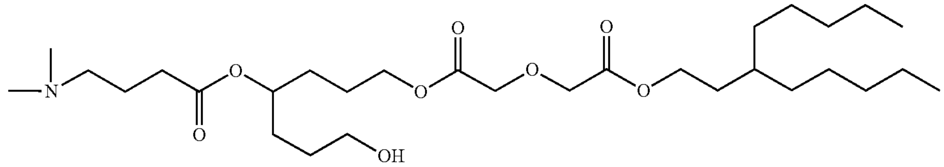


5006



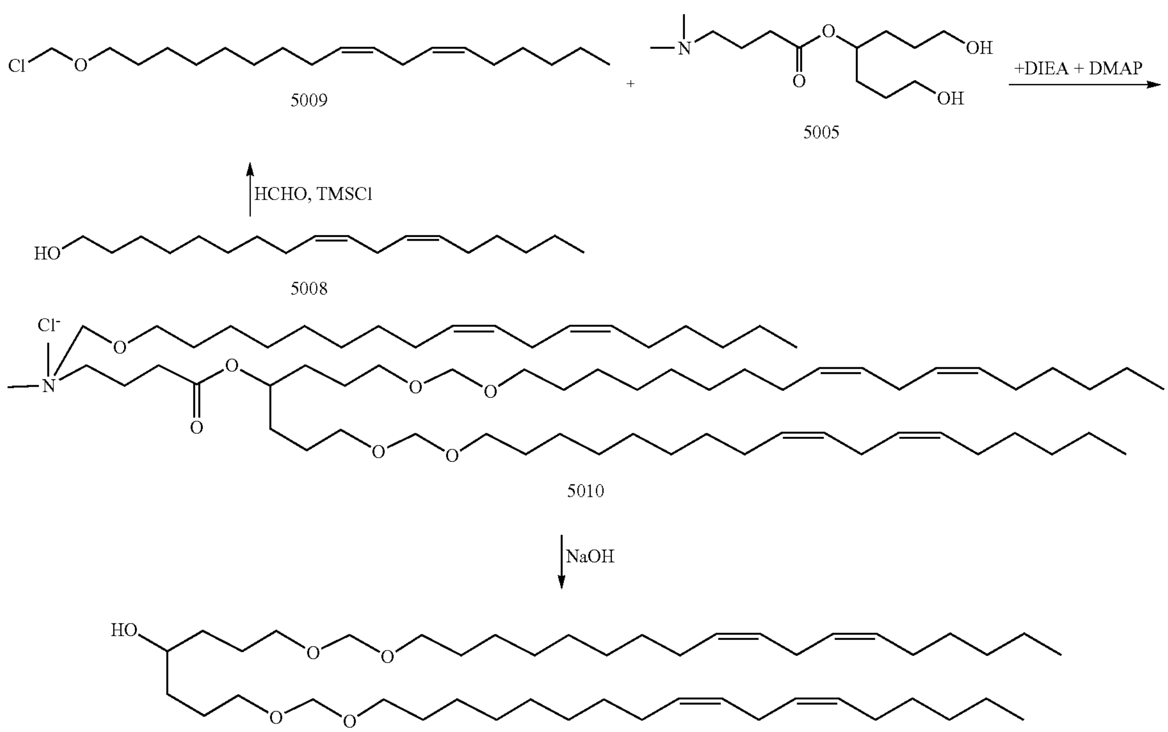
5007

Synthesis of compound 5002: To a stirred solution of alcohol 5001 (1.0 g, 5.15 mmol), Glycolic anhydride 5000 (5.66 mmol) in DCM (20 mL) was added DMAP (1.26 g, 10.41 mmol) and stirred at room temperature for 48 h. The reaction mixture was concentrated followed by column purification gave the corresponding product 5002 (1.4 g, 86%) as DMAP salt. LCMS: Calculated: 316.22 (M^+), Found: 315.1 (M^+−1).

Synthesis of compound 5004: To a stirred solution of alcohol 5003 (5.0 g, 44.6 mmol), 4-(Dimethylamino)butyric acid hydrochloride (8.1 g, 48.3 mmol) and EDC (10.3 g, 53.6 mmol) in DCM (100 mL) was added DIEPA (23 g, 178.3 mmol) and stirred at room temperature overnight. After usual work up, the crude product was purified by column chromatography (9.0 g, 90%).

Synthesis of compound 5005: To a stirred solution of diene 5004 (4.0 g, 18 mmol) in 10 mL of THF was added 9-BBN and stirred overnight. To the above solution was added 6.6 mL of 3M NaOAc and 7.4 mL of 30% H_2O_2 at 0-5° C. The reaction mixture was stirred at room temperature overnight. After usual work up, the crude material was purified by column chromatography to get 5005 (2.6 g, 55%) as viscous oil. LCMS: Calculated: 261.19 (M^+), Found: 262.1 (M^++1).

Synthesis of compound 5006 and 5007: To a stirred solution of diene 5005 (260 mg, 1 mmol), acid 5002 (1.0 g, 2.28 mmol), EDC (387 mg, 2 mmol) in 10 mL of DCM was added DIEA (516 mg, 4 mmol) and stirred overnight. After usual work up, the crude material was purified by column chromatography to get 5006 (0.1 g, 12%) and 5007 (0.2 g, 36%). LCMS for compound 5006: Calculated: 857.62 (M^+), Found: 858.5 (M^++1), 880.5 (M^++Na). LCMS for compound 5007: Calculated: 559.4 (M^+), Found: 560.4 (M^++1).



5011

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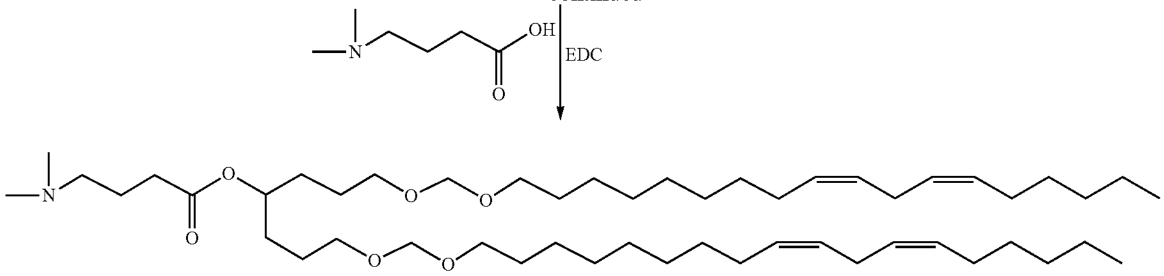
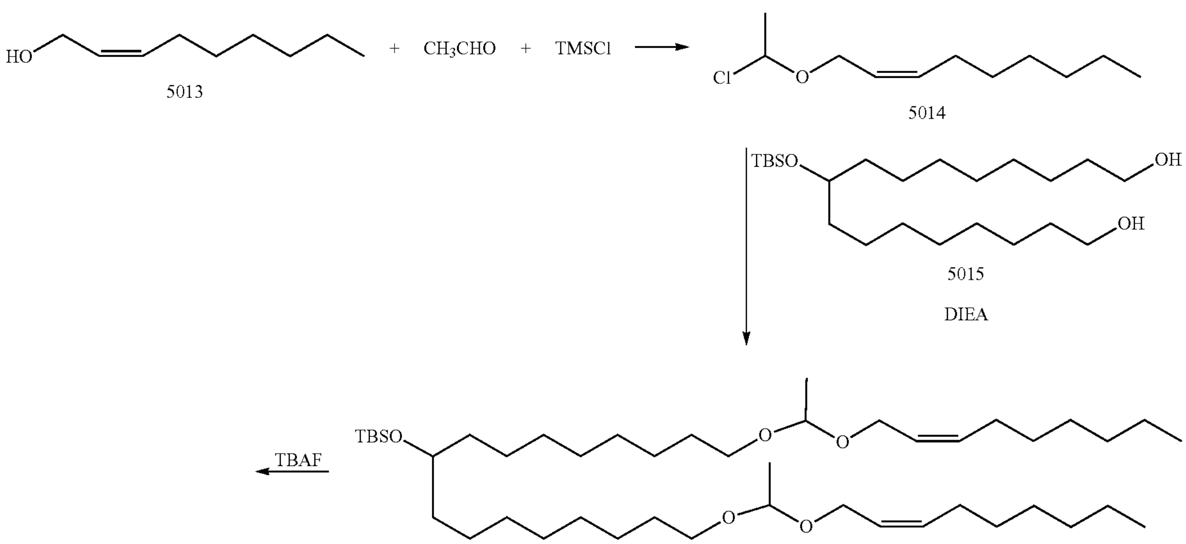


5012

Synthesis of compound 5011: To a stirred solution of alcohol 5008 (2.66 g 10 mmol) in 5 mL of Chlorotrimethylsilane was added paraformaldehyde (0.3 g, 10 mmol) and stirred at room temperature overnight. The excess Chlorotrimethylsilane was evaporated followed by drying under reduced pressure gave the corresponding product 5009 and used for next step without purification. The compound 5009 was added dropwise to the solution of diol (261 mg, 1 mmol), DIEA (2.5 g, 19.4 mmol) and DMAP (20 mg, 0.16 mmol) in DCM (10 mL) and stirred overnight. Concentration of the solvent gave the crude product 5010, which was dissolved in 5 mL of THF and 2 mL of 1N NaOH was added and stirred for 2 days at room temperature. After usual work up, the crude material was purified by column chromatography to get the corresponding product 5011 (200 mg, 28%). LCMS for compound 5010: Calculated: 1131.95 (M^+), Found: 1096.98 (M^+-Cl^-)). LCMS for compound 5011: Calculated: 704.63 (M^+), Found: 727.5 (M^++Na).

Synthesis of compound 5012: To a stirred solution of alcohol 5011 (200 mg, 0.284 mmol), 4-(Dimethylamino) butyric acid hydrochloride (103 mg, 0.57 mmol), EDC (109 mg, 0.57 mmol) in 10 mL of DCM was added DIEA (294 mg, 4 mmol) and stirred overnight. After usual work up, the crude material was purified by column chromatography to get 5012 (190 mg, 85%). LCMS for compound 5012: Calculated: 817.72 (M^+), Found: 818.5 (M^++Na).



5016

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Case: 23-2357 Document: 14 Page: 234 Filed: 12/04/2023

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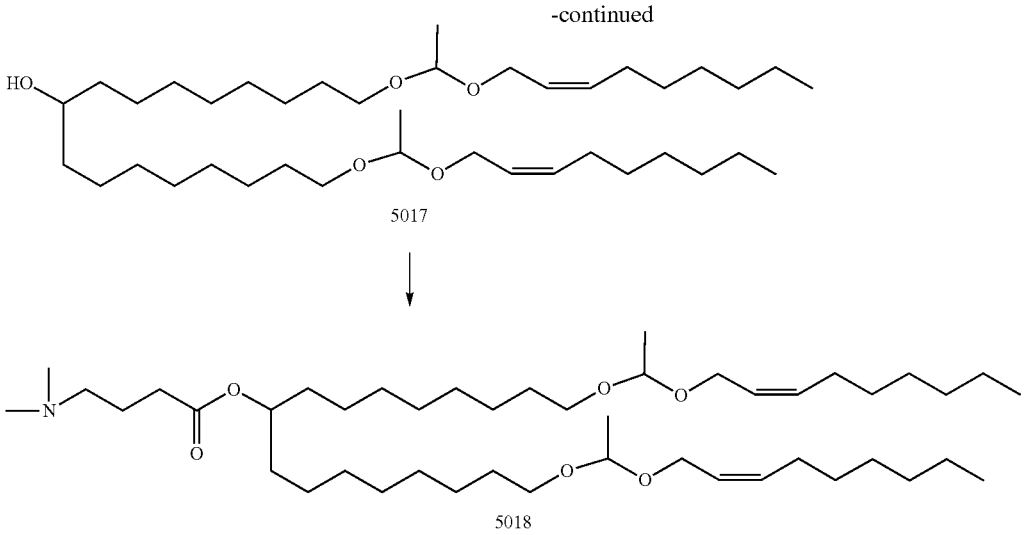


Synthesis of compound 5016: To a stirred solution of alcohol 5013 (1.0 g 7.03 mmol) in 5 mL of Chlorotrimethylsilane was added acetaldehyde (0.3 g, 7.03 mmol) and stirred at room temperature for 2 h. The excess Chlorotrimethylsilane was evaporated followed by drying under reduced pressure gave the corresponding product 5014 and used for next step without purification. The compound 5014 was added dropwise to the solution of diol 5015 (223 mg, 0.55 mmol), DIEA (2 mL g, 11.5 mmol) and DMAP (20 mg, 0.16 mmol) in DCM (10 mL) and stirred overnight. 10 mL of water was added followed by extraction with DCM (3×30 mL), washed with water, saturated $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. Concentration of the solvent gave the crude product, which was used for the next step without purification. LCMS for compound 5016: Calculated: 738.66 (M^+), Found: 761.5 (M^++Na).

Synthesis of compound 5017: To a stirred solution of alcohol 5016 in 5 mL of THE was added 0.54 mL of 1M TBAL in THE (0.54 mmol) and stirred for 2 days at room temperature. After usual work up, the crude material was purified by column chromatography to get 5017. However, it contains some inseparable impurity and hence used for next step without further purification. LCMS for compound 5017: Calculated: 624.57 (M^+), Found: 647.5 (M^++Na).

Synthesis of compound 5018: To a stirred solution of alcohol 5017 (0.55 mmol), 4-(Dimethylamino)butyric acid hydrochloride (116 mg, 0.64 mmol), EDC (123 mg, 0.64 mmol) in 10 mL of DCM was added DIEA (165 mg, 1.28 mmol) and stirred for 2 days. After usual work up, the crude material is purified by column chromatography (0-10% MeOH in 1% Et_3N containing DCM) to get 5018 (300 mg, 75% from 5015). LCMS for compound 5018: Calculated: 737.65 (M^+), Found: 738.6 (M^++1), 760.5 (M^++Na^+).

Example 35: Preparation of Lipid Nanoparticles

The cationic lipids described herein are used to formulate liposomes containing the AD-1661 duplex (shown in the table below) using an in-line mixing method as described in International Publication No. WO 2010/088537, which is incorporated by reference in its entirety. The lipid nanoparticles had the formulation shown in the table below.

Component	Mole Percentage (Based on 100% of the lipid components in the LNP)
Cationic lipid	50%
Distearoylphosphatidylcholine (DSPC)	10%
Cholesterol	38.5%
1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) (with an average PEG molecular weight of 2000)	1.5%
siRNA (AD-1661)	—

The siRNA AD-1661 duplex has the sequence shown below.

Duplex	Sequence 5'-3'	SEQ ID NO:	Target
AD-1661	GGAfUfCAfUfCfUfCAAGfUfCfUfUAfCdTsdT	1	FVII
	GfUAAGAfCfUfUGAGAfUGAfUfCfCdTsdT	2	

Lower case is 2'OMe modification and Nf is a 2'F modified nucleobase, dT is deoxythymidine, s is phosphothioate

The lipid nanoparticles was prepared as follows. Cationic lipid, DSPC, cholesterol, and PEG-DMG in the ratio recited in the table above were solubilized in ethanol at a total lipid concentration of 25 mg/mL.

A siRNA stock solution was prepared by solubilizing the siRNA AD-1661 in a low pH acetate or citrate buffer (pH=4) at 0.8 mg/mL.

The stock solutions should be completely clear and the lipids should be completely solubilized before combining with the siRNA. Therefore, if it was determined appropriate, the stock solutions were heated to completely solubilize the lipids.

The individual stock solutions were combined by pumping each solution to a T-junction (i.e., by in-line mixing). Specifically, the ethanol solution (at 5 ml/min, via 0.01 in. PEEK tube) and aqueous buffer solution (at 15 mL/min, via 0.02 in. PEEK tube) were mixed through a T-junction (PEEK Tee body, IDEX).

After the T-junction a single tubing is placed where the combined stream will emit. Ethanol is removed and exchanged for PBS by dialysis. The lipid formulations are then concentrated using centrifugation or diafiltration to an appropriate working concentration.

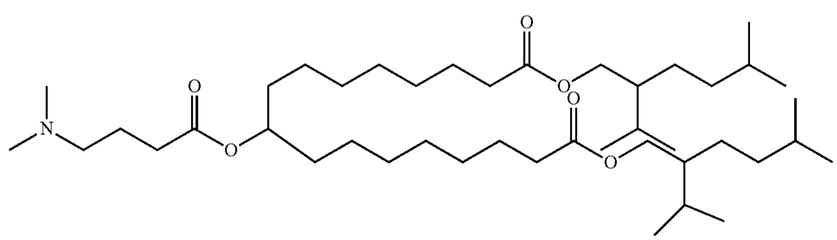
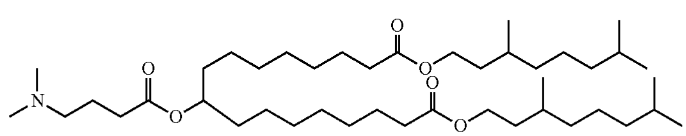
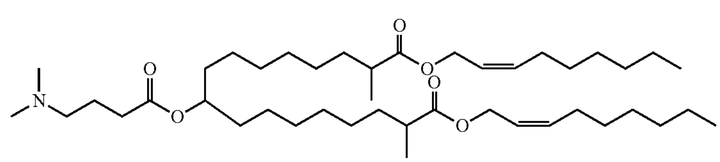
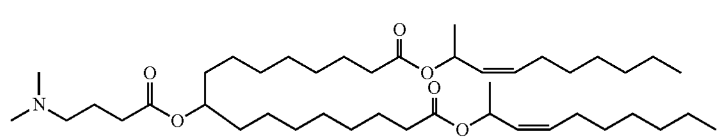
Lipid nanoparticles containing the cationic lipids listed in the table in Example 36 were prepared as described above.

Example 36: Efficacy of Lipid Nanoparticles

Factor VII (FVII), a prominent protein in the coagulation cascade, is synthesized in the liver (hepatocytes) and secreted into the plasma. FVII levels in plasma can be determined by a simple, plate-based colorimetric assay. As such, FVII represents a convenient model for determining siRNA-mediated downregulation of hepatocyte-derived proteins.

Test formulations of the lipid nanoparticles prepared in Example 35 were initially assessed for their FVII knockdown in female 7-9 week old, 15-25 g, female C57Bl/6 mice at 0.1, 0.3, 1.0 and 5.0 mg/kg with 3 mice per treatment group. All studies included animals receiving either phosphate-buffered saline (PBS, control group) or a benchmark formulation. Formulations were diluted to the appropriate concentration in PBS immediately prior to testing. Mice were weighed and the appropriate dosing volumes calculated (10 μl/g body weight). Test and benchmark formulations as well as PBS (for control animals) were administered intravenously via the lateral tail vein. Animals were anesthetised 24 hours later with an intraperitoneal injection of ketamine/xylazine and 500-700 μl of blood was collected by cardiac puncture into serum separator tubes (BD Microtainer). Blood was centrifuged at 2,000×g for 10 minutes at 15° C. and serum was collected and stored at −70° C. until analysis. Serum samples were thawed at 37° C. for 30 minutes, diluted in PBS and aliquoted into 96-well assay plates. Factor VII levels were assessed using a chromogenic assay (Biophen FVII kit, Hyphen BioMed) according to the manufacturer's instructions and absorbance was measured in a microplate reader equipped with a 405 nm wavelength filter. Plasma FVII levels were quantified and ED_{50} values (dose resulting in a 50% reduction in plasma FVII levels compared to control animals) were calculated using a standard curve generated from a pooled sample of serum from control animals. Those formulations of interest showing high levels of FVII knockdown (ED_{50}<<0.1 mg/kg) were re-tested in independent studies at a lower dose range to confirm potency and establish ED_{50} levels.

The following table shows ED_{50} values for some of the cationic lipids described herein. Two asterisks (**) indicates an ED_{50} value between 0.001 and 0.10. One asterisk (*) indicates an ED_{50} value greater than 0.10.

ED_{50}	Cationic Lipid
**	
**	
**	
**	

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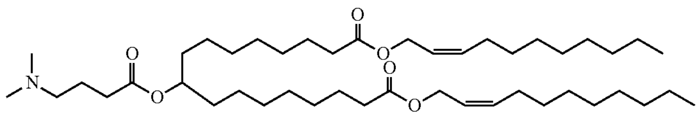
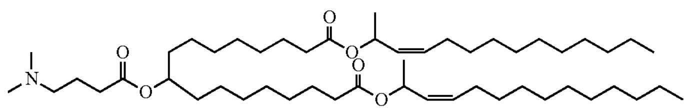
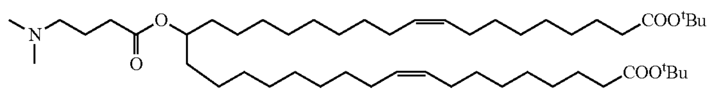
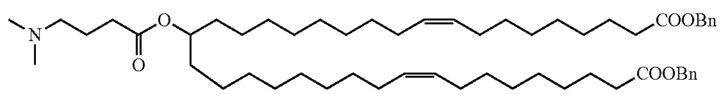
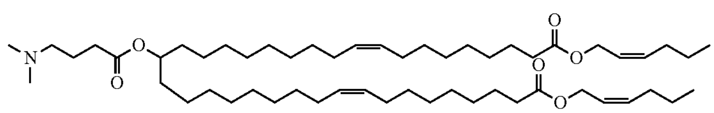
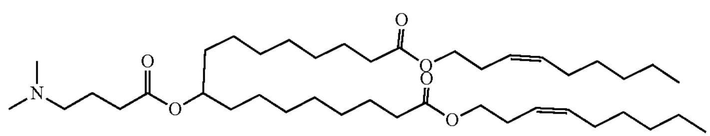
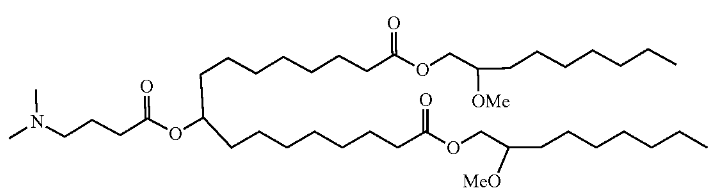
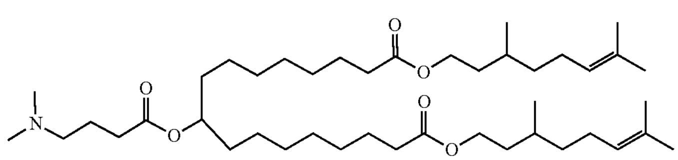
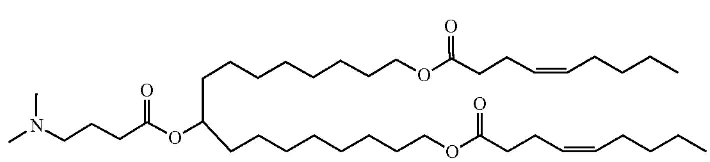
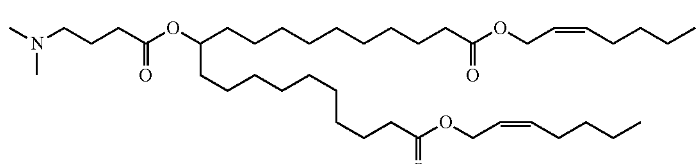
ED_{50}	Cationic Lipid
**	
**	
**	
**	
*	
**	
*	
**	
**	
**	

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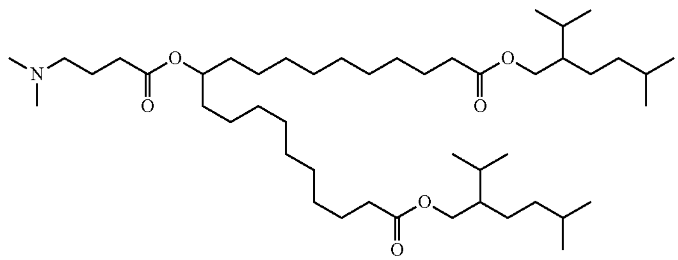
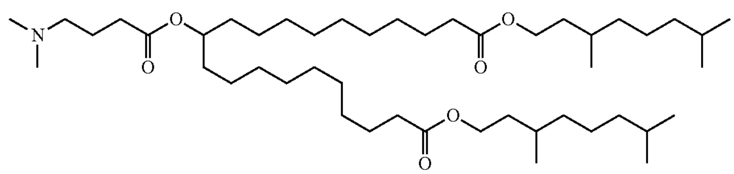
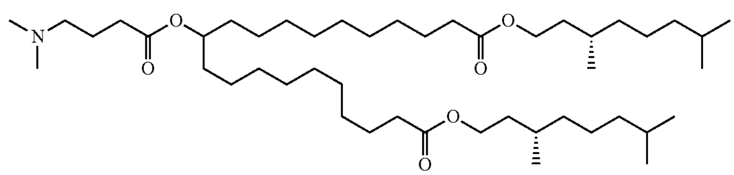
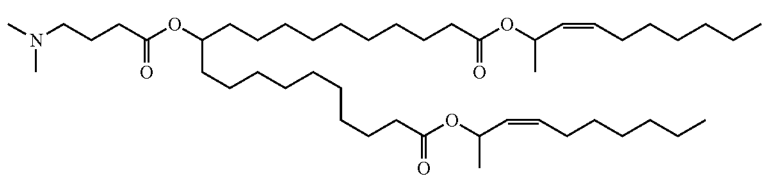
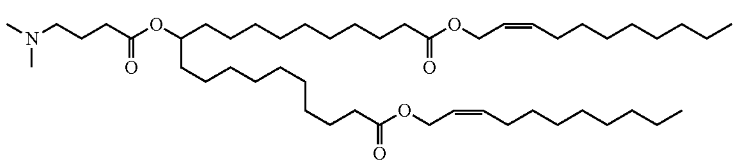
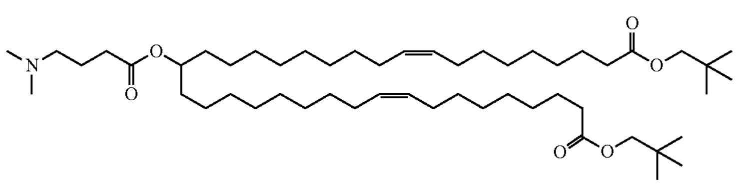
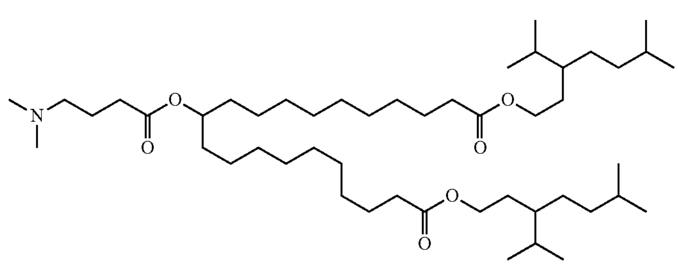
ED_{50}	Cationic Lipid
**	
**	
**	
**	
**	
**	
**	

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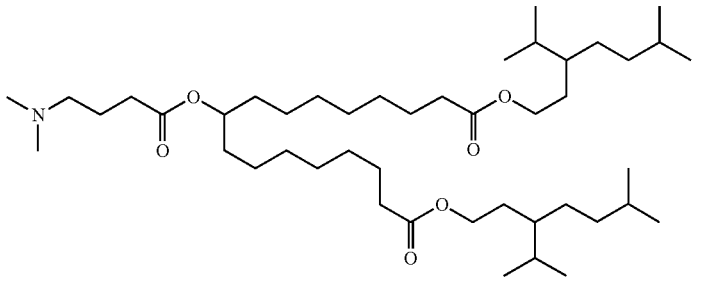
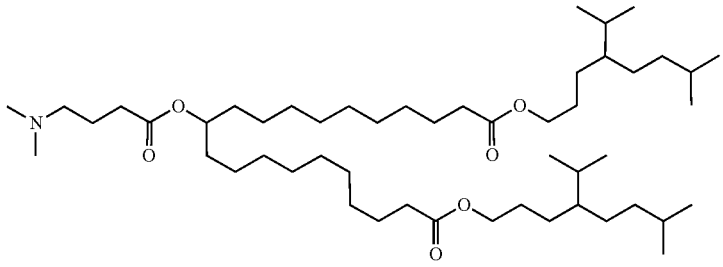
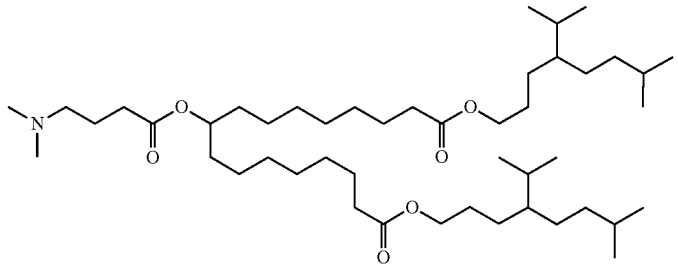
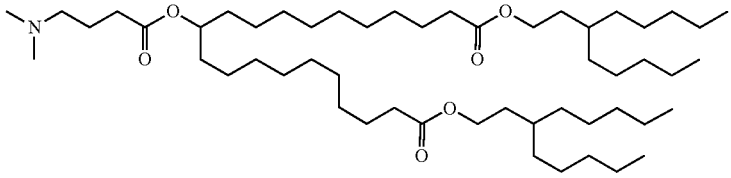
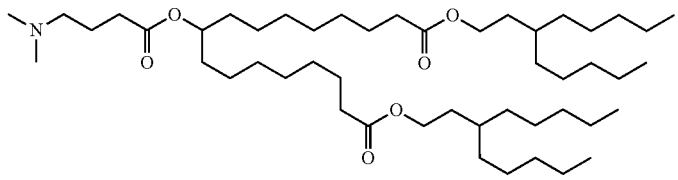
**	
**	
**	
**	
**	

Example 37: Hydrophobicity and Stability

The log P values for the biodegradable cationic lipids listed in the table below were calculated using the software available at http://www.molinspiration.com/services/logp.html from Molinspiration Cheminformatics of Slovensky Grob, Slovak Republic.

Furthermore, the HPLC retention time for each biodegradable cationic lipid was measured in lipid nanoparticles prepared from them. The lipid nanoparticles were prepared as described in Example 35 using AD-1661 as the payload. The retention times are reported in the table below relative to the retention time for cholesterol.

The HPLC buffer used was a mixture of two solutions (Solution #1 and Solution #2).

Solution #1: 80% methanol/20% 10 mM NH_4HCO_3

Solution #2: 80% methanol/20% isopropanol

The ratios of the two solutions in the mixture changed over time as indicated in the table below.

Time (min)	Solution #1 (vol %)	Solution #2 (vol %)
0	70	30
4	10	90
6	10	90

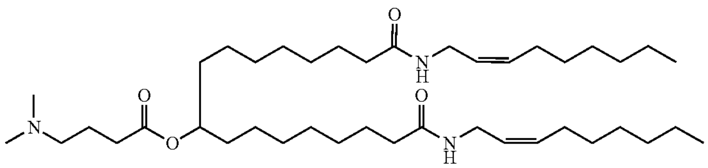
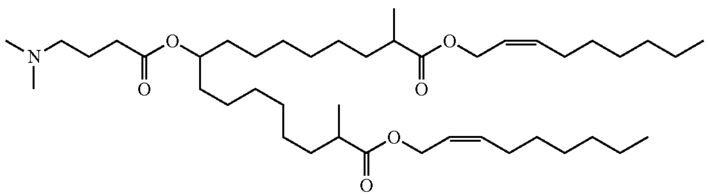
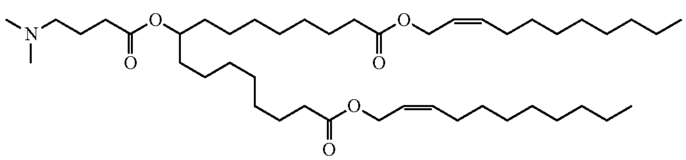
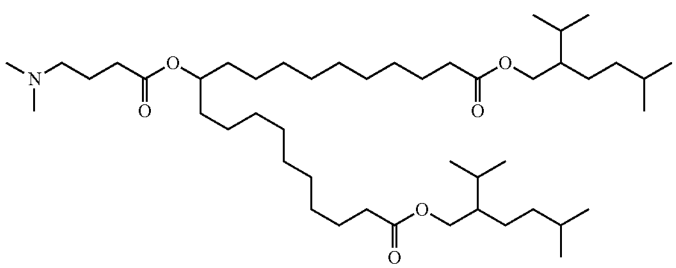
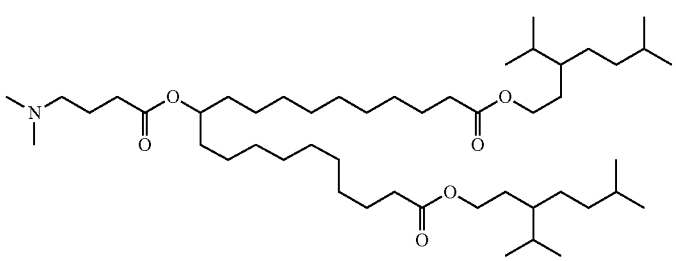
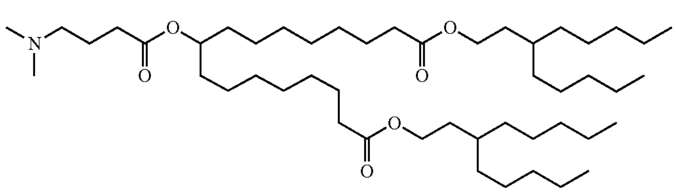
-continued

Time (min)	Solution #1 (vol %)	Solution #2 (vol %)
6.1	70	30
8	70	30

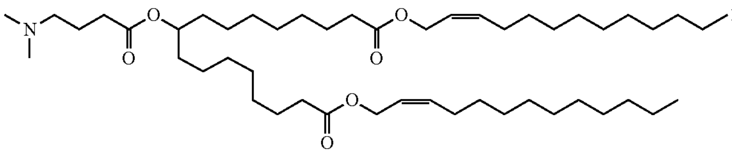
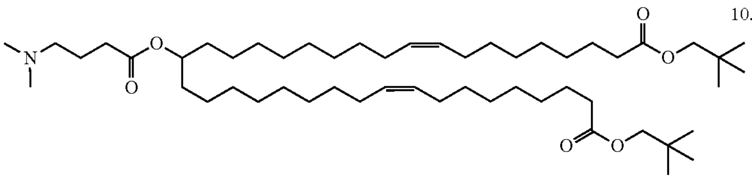
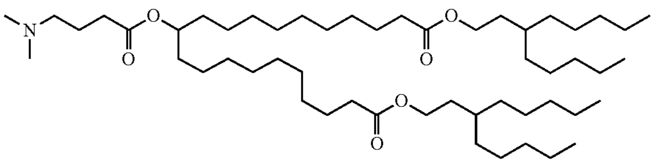
The size of the lipid nanoparticles was measured before and after undergoing dialysis overnight. In general, greater changes in lipid nanoparticle size are indicative of lesser stability.

Dynamic laser light scattering was used to determine the lipid nanoparticle size (expressed as the intensity weighted diameter) with a Zetasizer (Malvern Instruments, Inc. of Westborough, Mass.). All measurements were made at 532 nm wavelength at the scattering angle of 173° using normal resolution mode as the analysis model.

The results of these experiments are provided in the table below.

Cationic Lipid	logP	t(lipid)-t(chol)	LNPs Size (nm) change
	9.647	−1.4	170 −> 260
	9.972	0.848	73 −> 77
	10.093	1.44	60 −> 67
	10.201	1.751	59 −> 60
	10.259	2.106	
	10.313	2.365	56 −> 56

-continued

Cationic Lipid	logP	t(lipid)-t(chol)	LNPs Size (nm) change
	10.315	2.219	68 –> 67
	10.416	2.707	
	10.495	3.178	

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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-continued

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guaagacuug agaugaucct t 21

What is claimed is:

1. A lipid particle comprising:

(i) a nucleic acid,

(ii) 35-65 mol % of a cationic lipid,

(iii) 3-12 mol % distearoylphosphatidylcholine (DSPC),

(iv) 15-45 mol % cholesterol, and

(v) 0.5-10 mol % of a PEG-modified lipid,

wherein the mol % is based on 100% total moles of lipids in the lipid particle; and

the cationic lipid comprises a head group, two hydrophobic tails, and a central moiety to which the head group and the two hydrophobic tails are directly bonded, wherein

(a) the central moiety is a central carbon or nitrogen atom;

(b) each hydrophobic tail independently has the formula -(hydrophobic chain)-(ester group)-(hydrophobic chain), wherein the ester group is —OC(O)— or —C(O)O—; and

(c) for at least one hydrophobic tail,

(I) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the ester group;

(II) the hydrophobic tail has the formula —R^{12}-M^1-R^{13}, wherein R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the ester group, and R^{13} is a branched C_{10}-C_{20} alkyl;

(III) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26; and

(IV) the ester group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms.

2. The lipid particle of claim **1**, wherein the central moiety is a nitrogen atom.

3. The lipid particle of claim **1**, wherein the cationic lipid has a pKa in the range of about 4 to about 11 and a log P of at least 10.1.

4. The lipid particle of claim **1**, wherein the ester group is —OC(O)—.

5. The lipid particle of claim **1**, wherein the ester group is —C(O)O—.

6. The lipid particle of claim **1**, wherein the PEG-modified lipid is 1-(monomethoxy-polyethylene glycol)-2,3-dimyristoylglycerol (PEG-DMG).

7. The lipid particle of claim **1**, wherein the PEG-modified lipid comprises a PEG molecule having an average molecular weight of 2,000 Da.

8. The lipid particle of claim **7**, wherein the PEG-modified lipid is 1-(monomethoxy-polyethylene glycol)-2,3-dimyristoylglycerol (PEG-DMG) comprising the PEG molecule having an average molecular weight of 2,000 Da.

9. The lipid particle of claim **1**, comprising 45-65 mol % of the cationic lipid, 5-10 mol % DSPC, 25-40 mol % cholesterol, and 0.5-5 mol % of the PEG-modified lipid.

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10. The lipid particle of claim 1, comprising about 50 mol % of the cationic lipid, about 10 mol % of DSPC, about 38.5 mol % cholesterol, and about 1.5 mol % of the PEG-modified lipid.

11. The lipid particle of claim 1, wherein the nucleic acid comprises an RNA.

12. The lipid particle of claim 11, wherein both hydrophobic tails have the formula —R^{12}-M^1-R^{13}.

13. The lipid particle of claim 12, wherein the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms from the first atom after the central moiety to a terminus of the tail.

14. The lipid particle of claim 11, wherein, in at least one hydrophobic tail,

(i) the number of carbon atoms between the central moiety and the ester group in the hydrophobic tail ranges from 5 to 10;

(ii) the total number of carbon atoms between the central moiety and a terminus of the hydrophobic tail ranges from 15 to 20; and

(iii) the ester group is separated from a terminus of the hydrophobic tail by from 8 to 12 carbon atoms.

15. The lipid particle of claim 11, wherein in both hydrophobic tails,

(I) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl group, where the branching occurs at the α-position relative to the ester group;

(II) the ester group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms;

(III) the hydrophobic tail has the formula —R^{12}-M^1-R^{13}, where R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the ester group, and R^{13} is a branched C_{10}-C_{20} alkyl; and

(IV) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26.

16. The lipid particle of claim 15, wherein the PEG-modified lipid comprises a PEG molecule having an average molecular weight of 2,000 Da.

17. The lipid particle of claim 15, wherein the chain length of —R^{12}-M^1-R^{13} is at most 21 atoms from the first atom after the central moiety to a terminus of the tail.

18. A method for preparing a lipid particle mixture comprising mixing a first solution comprising an organic solvent, a cationic lipid, distearoylphosphatidylcholine (DSPC), cholesterol, and a PEG-modified lipid, with a second solution comprising a nucleic acid and water to form a mixture containing lipid particles, wherein each lipid particle comprises

(i) the nucleic acid,

(ii) 35-65 mol % of the cationic lipid,

(iii) 3-12 mol % distearoylphosphatidylcholine (DSPC),

(iv) 15-45 mol % cholesterol, and

(v) 0.5-10 mol % of the PEG-modified lipid, and

wherein the mol % is based on 100% total moles of lipids in the lipid particle, and

the cationic lipid comprises a head group, two hydrophobic tails and a central moiety to which the head group and the two hydrophobic tails are directly bonded, wherein

(a) the central moiety is a central carbon or nitrogen atom;

(b) each hydrophobic tail independently has the formula -(hydrophobic chain)-(ester group)-(hydrophobic chain), wherein the ester group is —OC(O)— or —C(O)O—; and

(c) for at least one hydrophobic tail,

(I) the terminal hydrophobic chain in the hydrophobic tail is a branched alkyl, where the branching occurs at the α-position relative to the ester group;

(II) the hydrophobic tail has the formula —R^{12}-M^1-R^{13}, wherein R^{12} is a C_4-C_{14} alkylene or C_4-C_{14} alkenylene, M^1 is the ester group, R^{13} is a branched C_{10}-C_{20} alkyl;

(III) the total carbon atom content of the tail —R^{12}-M^1-R^{13} is 21 to 26; and

(IV) the ester group is separated from a terminus of the hydrophobic tail by from 6 to 12 carbon atoms.

19. The method of claim 18, wherein the organic solvent comprises ethanol and the second solution comprises an aqueous buffer.

20. The method of claim 19, wherein the aqueous buffer is a citrate buffer.

21. The method of claim 18, wherein the mixing is by in-line mixing.

22. The method of claim 18, wherein the PEG-modified lipid is 1-(monomethoxy-polyethylene glycol)-2,3-dimyristoylglycerol (PEG-DMG).

23. The method of claim 18, wherein the PEG-modified lipid comprises a PEG molecule having an average molecular weight of 2,000 Da.

24. The method of claim 23, wherein the PEG-modified lipid is 1-(monomethoxy-polyethylene glycol)-2,3-dimyristoylglycerol (PEG-DMG) comprising the PEG molecule having an average molecular weight of 2,000 Da.

25. The method of claim 18, wherein the lipid particle comprises 45-65 mol % of the cationic lipid, 5-10 mol % DSPC, 25-40 mol % cholesterol, and 0.5-5 mol % of the PEG-modified lipid.

26. The method of claim 18, wherein the lipid particle comprises about 50 mol % of the cationic lipid, about 10 mol % of distearoylphosphatidylcholine (DSPC), about 38.5 mol % cholesterol, and about 1.5 mol % of the PEG-modified lipid.

27. The method of claim 18, wherein the nucleic acid comprises an RNA.

28. The method of claim 18, wherein the central moiety is a nitrogen atom.

29. The method of claim 18, further comprising diafiltering or dialyzing the lipid particle mixture.

30. The method of claim 29, wherein the lipid particle mixture is dialyzed with phosphate-buffered saline.

* * * * *

CERTIFICATE OF COMPLIANCE

Pursuant to Federal Rule of Appellate Procedure 32(a)(7)(C), the undersigned counsel for Appellant certifies that this brief:

(i) complies with the type-volume limitation of Rule 32(a)(7)(B) because it contains 12,382 words, including footnotes and excluding the parts of the brief exempted by Rule 32(a)(7)(B)(iii); and

(ii) complies with the typeface requirements of Rule 32(a)(5) and the type style requirements of Rule 32(a)(6) because it has been prepared using Microsoft Office Word 365 and is set in Century Schoolbook font in a size equivalent to 14 points or larger.

Dated: December 4, 2023

/s/ *William G. Gaede, III*
William G. Gaede, III

CERTIFICATE OF SERVICE

I hereby certify that digital versions of Appellant's Principal Brief were served electronically via the Court's CM/ECF system.

Dated: December 4, 2023

/s/ *William G. Gaede, III*
William G. Gaede, III