METHODS OF SYNTHESIZING AMINE LIPIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/574,028, filed April 3, 2024, the entire contents of which are incorporated by reference herein.

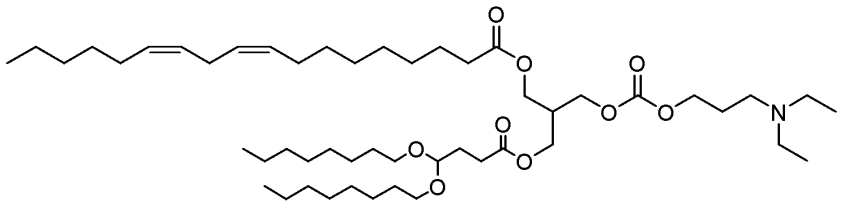
BACKGROUND

Lipid nanoparticles (LNPs) formulated with ionizable amine-containing lipids can serve as vehicles for delivery of cargo, e.g., polynucleotides, into cells. High purity lipids are needed for the preparation of LNPs suitable for *in vivo* delivery. Accordingly, methods of lipid synthesis affording low levels of impurities are of interest.

BRIEF SUMMARY

In certain embodiments, the disclosure relates to a composition, as determined by ultra-performance liquid chromatography (UPLC), comprising:

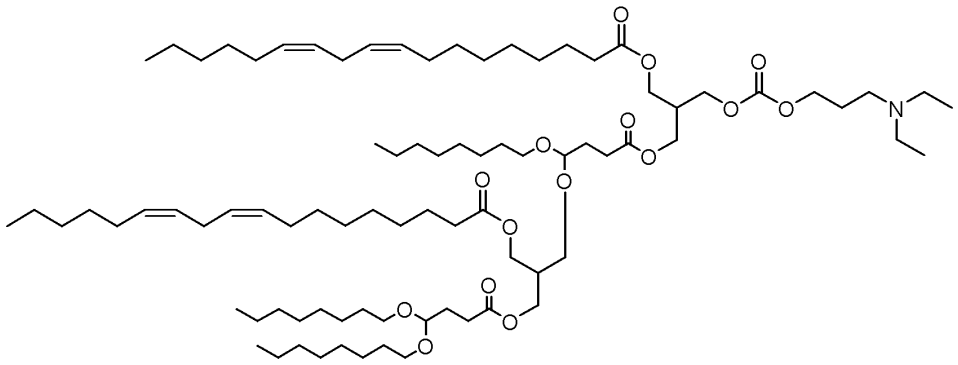
at least 90% of a compound having the structure of formula (IV)



(IV), also referred to herein as Lipid A.

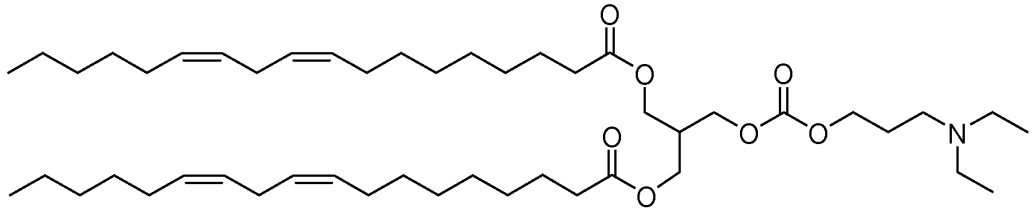
In certain embodiments, the disclosure relates to any one of the compositions described herein, wherein the composition comprises:

(A) less than about 1.1%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (V)



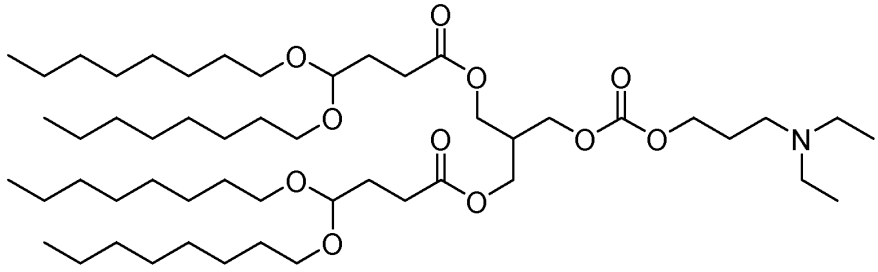
(V), or

(B) less than about 0.75% or less than 0.5% of a compound having the structure of formula (VI)



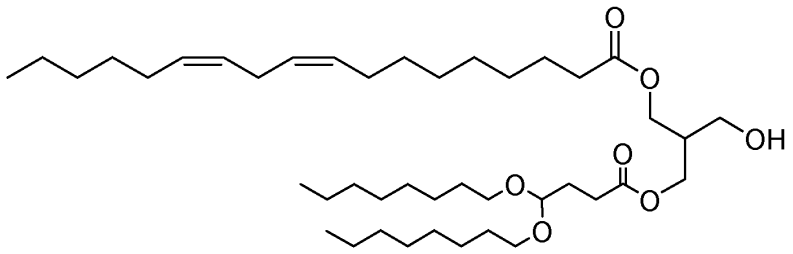
(VI), or

(C) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, or less than about 0.2%, of a compound having the structure of formula (VII)



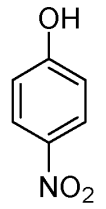
(VII), or

(D) less than about 0.3% or less than 0.2% of a compound having the structure of formula (III)



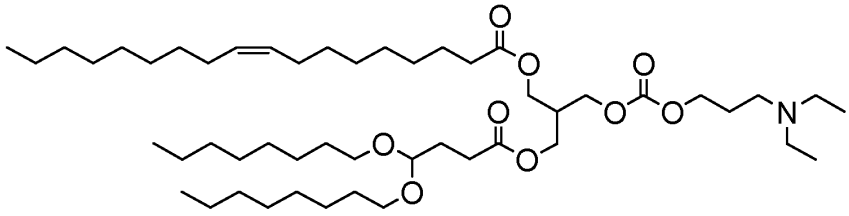
(III), or

(E) less than about 190 ppm, less than about 180 ppm, less than about 170 ppm, less than about 160 ppm, less than about 150 ppm, less than about 140 ppm, less than about 130 ppm, less than about 120 ppm, less than about 110 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



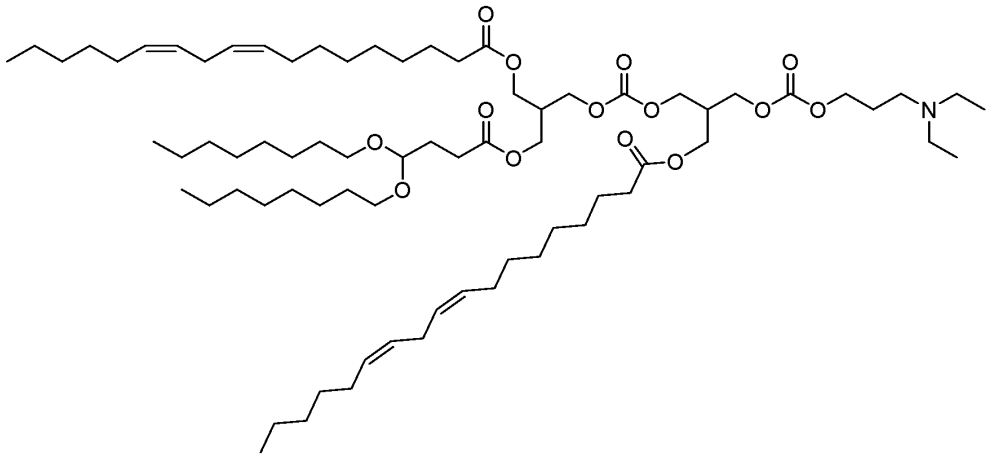
, or

(F) less than about 0.4% or less than 0.3% of a compound having the structure of formula (VIII)



(VIII), and/or

(G) less than about 0.2% or less than 0.1% of a compound having the structure of formula (IX)



(IX).

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) treating Compound-01 with Compound-02 at a temperature not exceeding 100°C to yield Compound-08;

b) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound 09;

c) treating Compound-09 with piperazine to yield Compound-10; and

d) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound-09;

b) treating Compound-09 with piperazine to yield Compound-10; and

c) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) treating Compound-09 with piperazine to yield Compound-10; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) treating Compound-09 with piperazine *p*-xylylenediamine to yield Compound-10; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) treating Compound-03 with Compound-04 and a coupling reagent to yield Compound-11;

b) treating Compound-11 with an ion exchange resin to yield Compound-12; and

c) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any one of the compositions described herein, comprising:

a) treating Compound-11 with an ion exchange resin to yield Compound-12; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing a composition of any one of the compositions described herein, comprising:

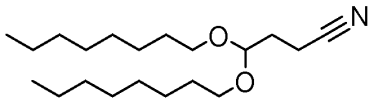
a) treating Compound-13 with *p*NPCF to generate Compound-14 *in situ*;

b) treating Compound-14 *in situ* with 3-dimethylamino-1-propanol;

c) extracting a reaction mixture with heptane; and

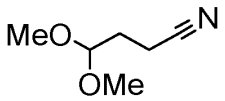
d) performing water and acetonitrile extractions to generate Lipid A.

In certain embodiments, the disclosure relates to a process for preparing 4,4-bis(octyloxy)butanenitrile



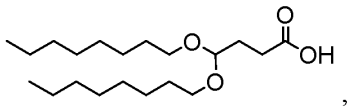
,

comprising combining 4,4-dimethoxybutanenitrile



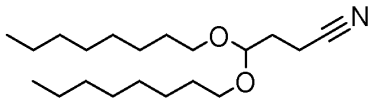
with 1-octanol to form the 4,4-bis(octyloxy)butanenitrile, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 100°C.

In certain embodiments, the disclosure relates to a process for preparing 4,4-bis(octyloxy)butanoic acid



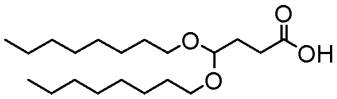
,

comprising hydrolyzing 4,4-bis(octyloxy)butanenitrile



to form the 4,4-bis(octyloxy)butanoic acid, wherein the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 100°C.

In certain embodiments, the disclosure relates to a process for preparing an amine salt of 4,4-bis(octyloxy)butanoic acid

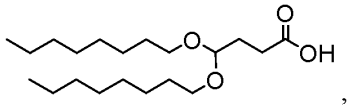


comprising:

combining the 4,4-bis(octyloxy)butanoic acid with piperazine or *p*-xylylenediamine to form a reaction mixture, wherein the amine salt is formed in the reaction mixture; and

filtering the reaction mixture.

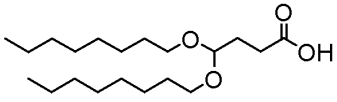
In certain embodiments, the disclosure relates to a compound that is a piperazinium salt or a *p*-xylylenediamine salt of 4,4-bis(octyloxy)butanoic acid



,

wherein the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.3 to about 0.7.

In certain embodiments, the disclosure relates to a process for generating at least 90% free 4,4-bis(octyloxy)butanoic acid

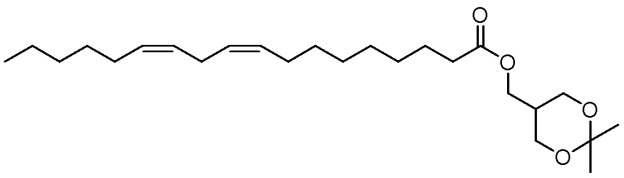


comprising,

preparing an amine salt of the 4,4-bis(octyloxy)butanoic acid by any one of the processes described herein; and

contacting the amine salt with an acid to form free 4,4-bis(octyloxy)butanoic acid.

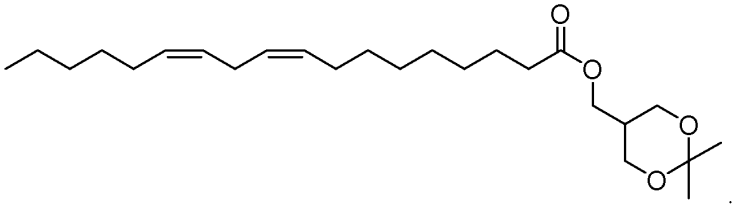
In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (I):



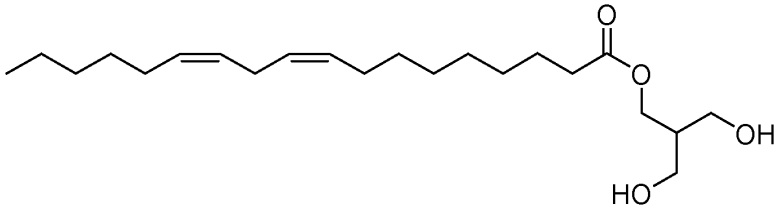
(I),

comprising combining linoleic acid with (2,2-dimethyl-1,3-dioxan-5-yl)methanol and a coupling reagent.

In certain embodiments, the disclosure relates to a compound that is a (2,2-dimethyl-1,3-dioxan-5-yl)methyl (9Z,12Z)-octadeca-9,12-dienoate

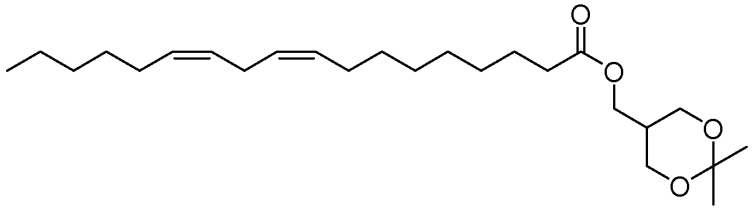


In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (II):



(II),

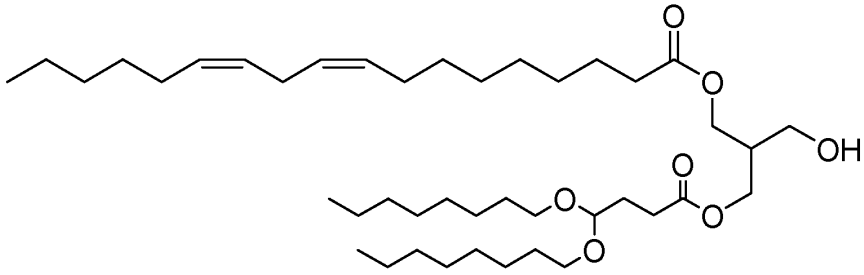
comprising contacting a compound having the structure of formula (I):



(I)

with an ion exchange resin.

In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (III):

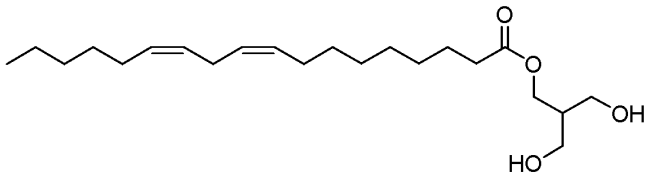


(III),

comprising:

generating free 4,4-bis(octyloxy)butanoic acid by any one of the processes described herein; and

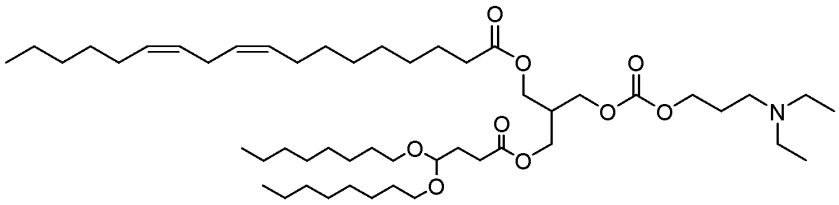
combining the purified 4,4-bis(octyloxy)butanoic acid with a compound having the structure of formula (II):



(II)

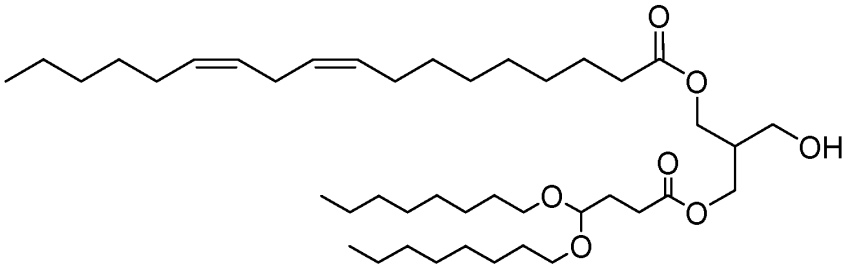
and a coupling reagent.

In certain embodiments, the disclosure relates to a process for preparing Lipid A:



Lipid A,

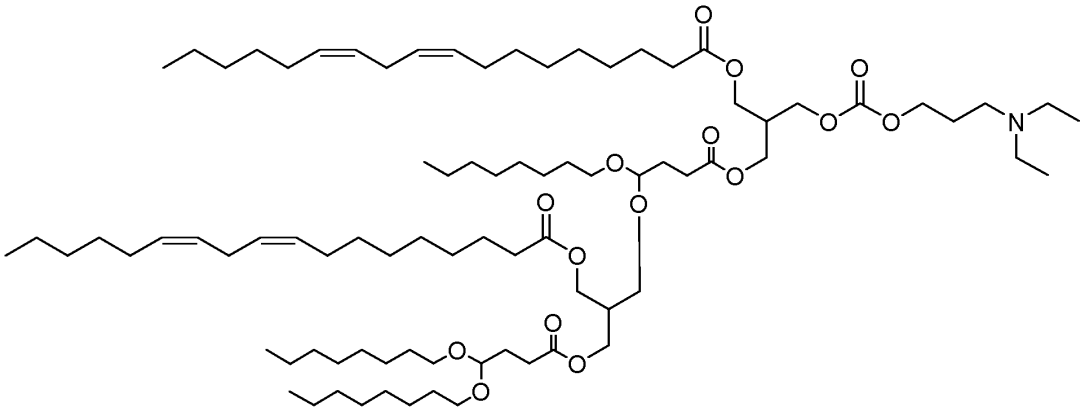
comprising combining a compound having the structure of formula (III):



(III)

with 4-nitrophenyl chloroformate, about 2.2 to about 10 molar equivalents of pyridine, 4-dimethylaminopyridine, and 3-(diethylamino)propan-1-ol, wherein the molar equivalents are relative to the compound of formula (III).

In certain embodiments, the disclosure relates to a composition comprising at least 90% of a compound having the structure of formula (V)



(V).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a UPLC-CAD chromatogram of Lipid A produced using the method of Example 1.

FIG. 2 is a UPLC-CAD overlay of Compound-09 using the method of Example 1. Bottom: Compound-10 material after purification via co-crystallization; Middle: Compound-09 oil after acid/base extractions; Top: Compound-10 mother liquor trace, filtrate from co-crystallization.

FIG. 3 is a UPLC-CAD chromatogram overlay corresponding to samples of linoleic acid (Compound-03), Compound-11, and Compound-12, showing corresponding hydroperoxide impurities.

FIG. 4 is a graph demonstrating the effects of air, temperature, and light exposure on the formation of Impurity-07 relative to the Compound-12 synthesis product.

FIG. 5 is a UPLC-CAD overlay of crude Compound-13 and purified Compound-13.

FIG. 6 is a plot demonstrating the results of a stress study of crude Compound-13, Impurity-06, and Impurity-02 at 40 °C.

FIG. 7 is a plot demonstrating the results of a stress study of crude Compound-13, Impurity-06, and Impurity-02 at 60 °C.

FIG. 8 is a plot demonstrating Impurity-06 and Impurity-02 levels from crude Compound-13 in 15% - 40% wt% of crude Compound-13 in a mixture of DCM and heptane.

FIG. 9 is a plot demonstrating Impurity-06 and Impurity-02 levels from crude Compound-13 in 15% - 40% wt% of crude Compound-13 in a mixture of CPME and heptane.

FIG. 10 is a representative ^{1}H NMR Spectrum of Impurity-11.

FIG. 11 is a scheme of the proposed mechanism for Impurity-11 formation.

FIG. 12 is a response contour plot for the DoE analysis. Effect of pyridine equivalents (x-axis) and R-5 equivalents (y-axis) on Lipid A crude purity (left panel) and levels of total other impurities (right panel).

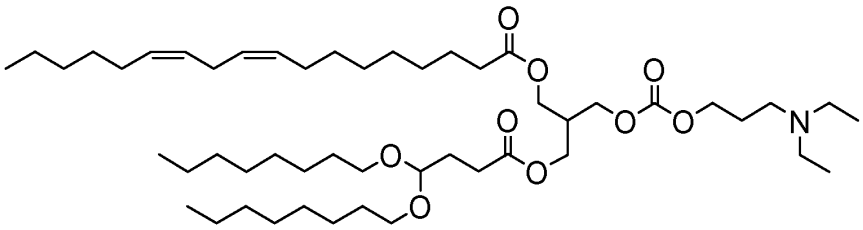
FIG. 13 is a representative ^{1}H NMR Spectrum of Impurity-20.

FIG. 14 is an overlay of the UPLC-CAD chromatograms for the Lipid A product obtained from Process A and Process B.

DETAILED DESCRIPTION

Overview

Described herein are methods of synthesizing ionizable amine lipids, such as Lipid A:



at varying scales, including scales relevant for development and manufacturing of pharmaceutical products with increased overall purity, in particular, with decreased concentration of specific lipid impurities. Lipid A is of particular interest because of its demonstrated use in LNPs for *in vivo* and *in vitro* delivery of cargo, such as components and compositions for gene editing. Methods described in the art for synthesizing Lipid A can result in a product mixture with low purity, which includes specific lipid impurities that cannot be separated from the desired product by conventional purification methods. Indeed, the art does not even recognize the formation of such side products, let alone provide any suggestions as to methods for minimizing or eliminating the formation of such impurities.

Definitions

It should be noted that, as used in this application, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a composition" includes a plurality of compositions and reference to "a cell" includes a plurality of cells and the like. The use of "or" is inclusive and means "and/or" unless stated otherwise.

Unless specifically noted in the above specification, embodiments in the specification that recite "comprising" various components are also contemplated as "consisting of" or "consisting essentially of" the recited components; embodiments in the specification that recite "consisting of" various components are also contemplated as "comprising" or "consisting essentially of" the recited components; embodiments in the specification that recite "about" various components are also contemplated as "at" the recited components; and embodiments in

the specification that recite "consisting essentially of" various components are also contemplated as "consisting of" or "comprising" the recited components (this interchangeability does not apply to the use of these terms in the claims).

Numeric ranges are inclusive of the numbers defining the range. Measured and measurable values are understood to be approximate, taking into account significant digits and the error associated with the measurement. As used in this application, the terms "about" and "approximately" have their art-understood meanings; use of one vs the other does not necessarily imply different scope. Unless otherwise indicated, numerals used in this application, with or without a modifying term such as "about" or "approximately", should be understood to encompass normal divergence and/or fluctuations as would be appreciated by one of ordinary skill in the relevant art. In certain embodiments, the term "approximately" or "about" can refer to a range of values that fall within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1% or less in either direction (greater than or less than) of a stated reference value unless otherwise stated or otherwise evident from the context.

As used herein, the term "contacting" means establishing a physical connection between two or more entities.

"Catalyst" means any compound that is capable of modifying, especially by increasing, the rate of the chemical reaction in which it participates, and which is regenerated at the end of the reaction. Examples of catalysts suitable for the present application include, but are not limited to 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 1,4-diazabicyclo[2.2.2]octane (DABCO), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) , and 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD), and quinuclidine.

"Organic base" as used herein refers to an organic compound capable of accepting a proton, producing a hydroxyl ion in an aqueous solution, or donating an electron pair. Examples of organic bases include, but are not limited to, nitrogen-containing compounds, such as triethylamine (Et_3N), diisopropylethylamine (DIPEA), piperidine, pyridine, 4-dimethylaminopyridine (DMAP), N-methyl-morpholine, dimethylaniline, imidazole, 1-methylpyridine, 2-methylpyridine, 3-methylpyridine, 3,5-dimethylpyridine, 2,4-dimethylpyridine, 2,6-dimethylpyridine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]octane (DABCO) and 2,4,6-trimethylpyridine.

"Inorganic base" as used herein refers to an inorganic compound capable of accepting a proton, producing a hydroxyl group in an aqueous medium, or donating an electron pair. Example of inorganic bases include, but are not limited to, metal hydroxides, such as LiOH, NaOH, KOH, CsOH, $Ca(OH)_2$, $Mg(OH)_2$, and $Ba(OH)_2$. Additional inorganic bases include NaOMe, KOMe, $NaHCO_3$.

Organic amine bases can include β-phenethylamine, dicyclohexylamine, cyclohexylamine, benzylamine, *tert*-butylamine, dibenzylamine, ethylenediamine, *tert*-octylamine, pyrrolidine, piperazine, 1,3-propylenediamine, and *p*-xylylenediamine.

The reaction disclosed herein are typically carried out in one or more organic solvents. Organic solvents can include acetonitrile, dichloromethane, ethanol, toluene, isopropyl acetate, pyridine, isopropyl alcohol, methyl, ethyl ketone, methyl tert-butyl ether, cyclopentyl methyl ether, tetrahydrofuran, 2-methyltetrahydrofuran, diethyl ether, 2,5-dimethyltetrahydrofuran, dimethoxyethane, acetone, and heptanes.

The term "substantially pure" or "substantially free of impurities" means there are not a significant amount of impurities present in the sample of the lipid composition (i.e., the composition has a purity greater than about 90%, preferably greater than about 91%, preferably greater than about 92%, preferably greater than about 93%, preferably greater than about 94%, preferably greater than about 95%, preferably greater than about 96%, preferably greater than about 97%, preferably greater than about 98%, and preferably greater than about 99%). Non-limiting examples of impurities include other lipids that share common features with Lipid A, or residual organic and inorganic molecules such as related intermediates, solvents, water, or salts. Compositions may also include salts of one or more compounds. Salts may be pharmaceutically acceptable salts. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is altered by converting an existing acid or base moiety to its salt form (e.g., by reacting a free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate,

ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, Pharmaceutical Salts: Properties, Selection, and Use, P. H. Stahl and C. G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., Journal of Pharmaceutical Science, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

The terms "polypeptide," "peptide," and "protein," refer a string of at least three amino acids linked together by peptide bonds. Peptide may refer to an individual peptide or a collection of peptides. Peptides can contain natural amino acids, non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain), and/or amino acid analogs. Also, one or more of the amino acids in a peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or

other modification, etc. Modifications may include cyclization of the peptide, the incorporation of D-amino acids, etc.

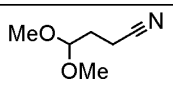
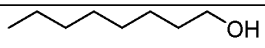
As used herein, the term "an optical isomer" or "a stereoisomer" refers to any of the various stereoisomeric configurations that may exist for a given compound . It is understood that a substituent may be attached at a chiral center of a carbon atom. The term "chiral" refers to molecules that have the property of non-superimposability on their mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner. Therefore, the disclosure includes enantiomers, diastereomers, or racemates of compounds described herein. "Enantiomers" are a pair of stereoisomers that are non-superimposable mirror images of each other. A 1:1 mixture of a pair of enantiomers is a "racemic" mixture. The term “racemate” is used to designate a racemic mixture where appropriate. "Diastereoisomers" are stereoisomers that are not enantiomers. The absolute stereochemistry of a chiral center is specified according to the Cahn-lngold-Prelog R-S system. When a compound is a pure enantiomer the stereochemistry at each chiral carbon may be specified by either *R* or *S*. Resolved compounds whose absolute configuration is unknown can be designated (+) or (-) depending on the direction (dextro- or levorotatory) they rotate plane-polarized light at the wavelength of the sodium D line. Certain compounds described herein contain one or more chiral centers or axes and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)-.

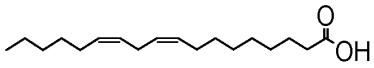
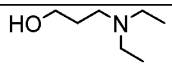
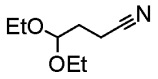
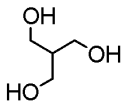
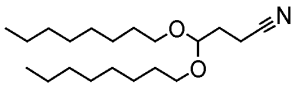
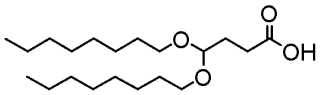
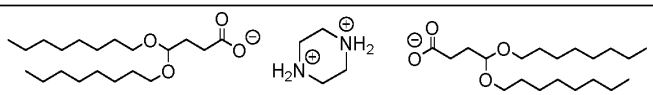
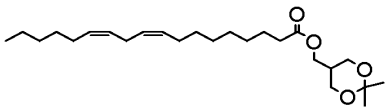
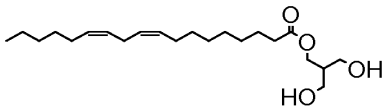
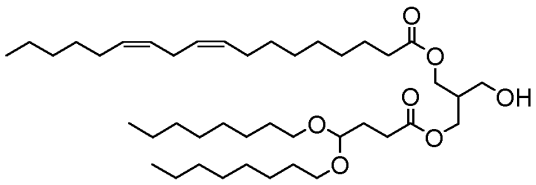
Abbreviations

a%	Area percentage
ACN	Acetonitrile
CAD	Charged Aerosol Detector
CPME	cyclopentyl methyl ether
DABCO	1,4-Diazabicyclo[2.2.2]octane
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DoE	Design of Experiments
DIPEA	*N,N*-Diisopropylethylamine

DMAP	Dimethylaminopyridine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High performance liquid chromatography
HRMS	High-Resolution MS
HS-GC	Headspace Gas Chromatography
KOH	Potassium Hydroxide
IPA	Isopropyl alcohol
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MEK	methyl ethyl ketone
MTBE	methyl tert-butyl ether
MW	Molecular Weight
nm	nanometer
PAC	Powdered Activated Carbon
pNP	*Para*-Nitrophenol, 4-Nitrophenol
pNPCF	*p*-nitrophenyl chloroformate
ppm	parts per million
PPTS	pyridinium-4-toluene sulfonate
Prep HPLC	preparative high performance liquid chromatography
RP	Reversed Phase
RT	Room Temperature
UPLC	Ultra-performance liquid chromatography
UV	ultraviolet

Table A: Compound Shorthand Names and Chemical Structures

Compound-01	
Compound-02	

Compound-03	
Compound-04	
Compound-05	
Compound-06	
Compound-07	
Compound-08	
Compound-09	
Compound-10	
Compound-11 (Formula (I))	
Compound-12 (Formula (II))	
Compound-13 (Formula (III))	

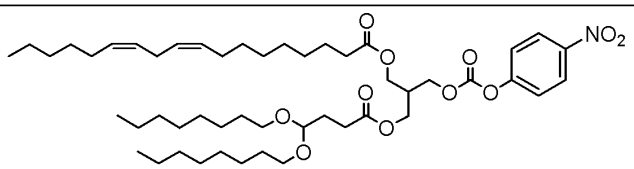
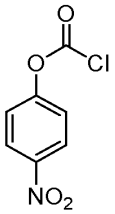
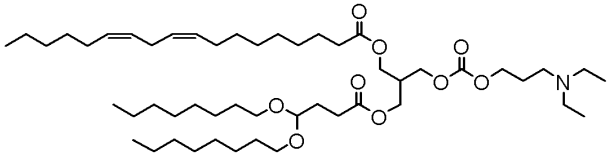
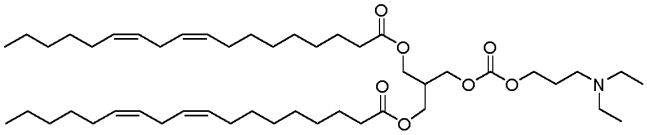
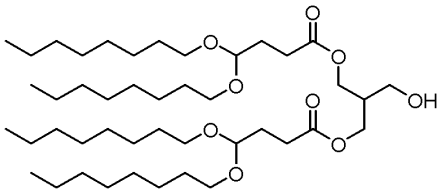
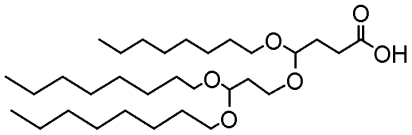
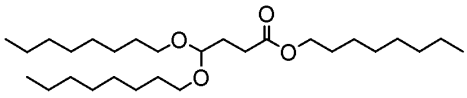
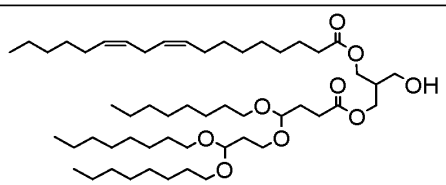
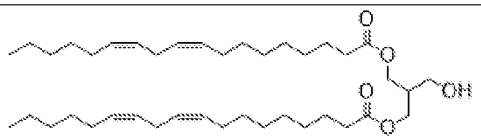
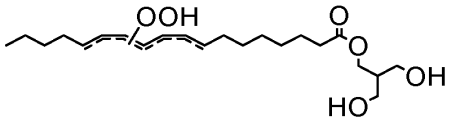
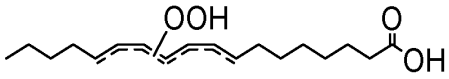
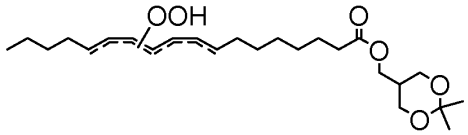
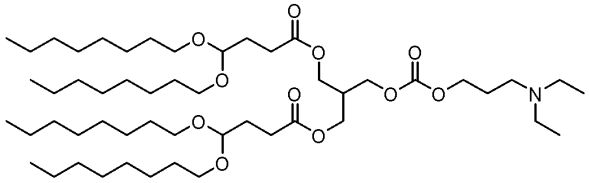
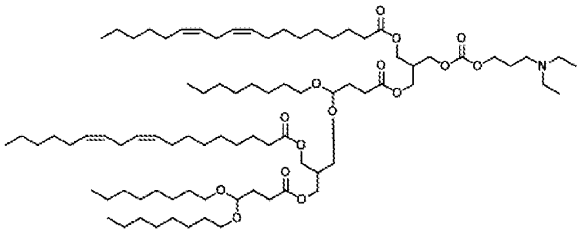
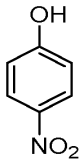
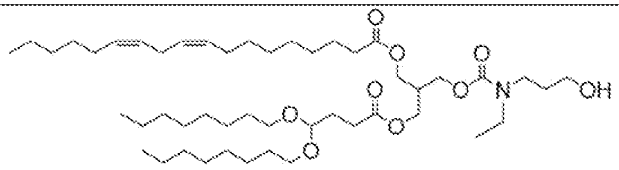
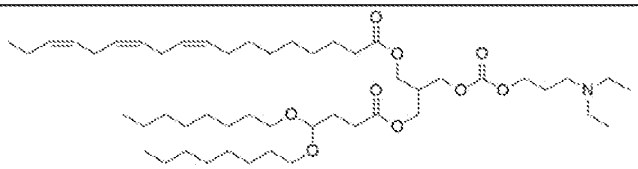
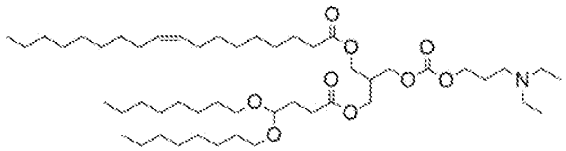
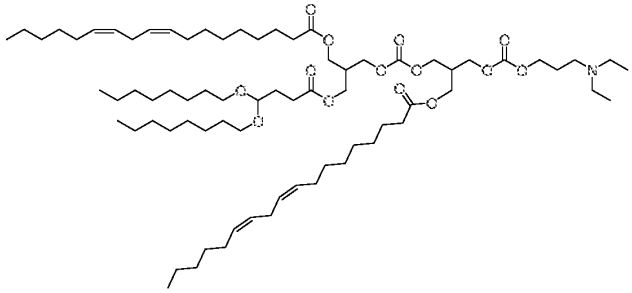
Compound-14	
pNPCF	
Lipid A ((Formula (IV))	

Table B: Impurity Shorthand Names and Chemical Structures

Impurity-01 (Formula (VI))	
Impurity-02	
Impurity-03	
Impurity-04	

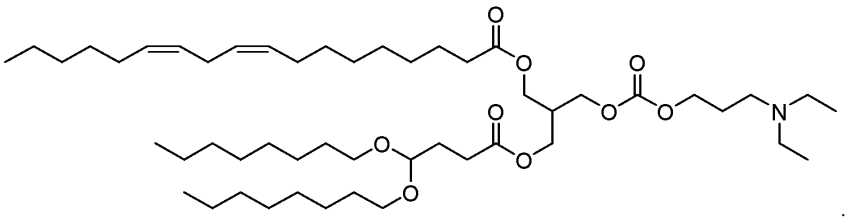
Impurity-05	
Impurity-06	
Compound-12 Hydroperoxide (Proposed Structure for Impurity-07)	
Impurity-08	
Impurity-09	
Impurity-10 (Formula (VII))	
Impurity-11 (Formula (V))	
pNP	

Impurity-20 (Formula (X))	
Impurity-22	
Impurity-23 (Formula (VIII))	
Impurity-24 (Formula (IX))	

Ionizable Lipids

In certain embodiments, lipid compositions, such as LNP compositions, comprise an "amine lipid" or an "ionizable lipid," such as Lipid A or its equivalents, including acetal analogs of Lipid A.

Lipid A is (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. Lipid A can be depicted as:



The terms "a compound of formula (IV)," "a compound having the structure of formula (IV)," and "Lipid A" are used interchangeably herein.

In certain embodiments, an amine lipid is an analog of Lipid A. In certain embodiments, a Lipid A analog is an acetal analog of Lipid A. In particular LNP compositions, the acetal analog is a C4-C12 acetal analog. In some embodiments, the acetal analog is a C5-C12 acetal analog. In additional embodiments, the acetal analog is a C5-C10 acetal analog. In further embodiments, the acetal analog is chosen from a C4, C5, C6, C7, C9, C10, C11, and C12 acetal analog.

Amine lipids such as Lipid A can be biodegradable *in vivo*, and suitable for use in LNP formulations.

Lipid clearance may be measured as described in literature. *See* Maier, M.A., *et al.* Biodegradable Lipids Enabling Rapidly Eliminated Lipid Nanoparticles for Systemic Delivery of RNAi Therapeutics. *Mol. Ther.* 2013, 21(8), 1570-78 ("*Maier*"). For example, in *Maier*, LNP-siRNA systems containing luciferases-targeting siRNA were administered to six- to eight-week old male C57Bl/6 mice at 0.3 mg/kg by intravenous bolus injection *via* the lateral tail vein. Blood, liver, and spleen samples were collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 96, and 168 hours post-dose. Mice were perfused with saline before tissue collection and blood samples were processed to obtain plasma. All samples were processed and analyzed by LC-MS. Further, *Maier* describes a procedure for assessing toxicity after administration of LNP-siRNA formulations. For example, a luciferase-targeting siRNA was administered at 0, 1, 3, 5, and 10 mg/kg (5 animals/group) via single intravenous bolus injection at a dose volume of 5 mL/kg to male Sprague-Dawley rats. After 24 hours, about 1 mL of blood was obtained from the jugular vein of conscious animals and the serum was isolated. At 72 hours post-dose, all animals were euthanized for necropsy. Assessments of clinical signs, body weight, serum chemistry, organ weights and histopathology were performed. Although *Maier* describes methods for assessing

siRNA-LNP formulations, these methods may be applied to assess clearance, pharmacokinetics, and toxicity of administration of LNP compositions of the present disclosure.

Lipids may be ionizable depending upon the pH of the medium they are in. For example, in a slightly acidic medium, the lipid may be protonated and thus bear a positive charge. Conversely, in a slightly basic medium, such as, for example, blood where pH is approximately 7.35, the lipid may not be protonated and thus bear no charge.

The ability of a lipid to bear a charge is related to its intrinsic pKa. In some embodiments, the amine lipids of the present disclosure may each, independently, have a pKa in the range of from about 5.1 to about 7.4. In some embodiments, the amine lipids of the present disclosure may each, independently, have a pKa in the range of from about 5.1 to about 7.4. For example, the amine lipids of the present disclosure may each, independently, have a pKa in the range of from about 5.8 to about 6.5. Lipids with a pKa ranging from about 5.1 to about 7.4 are effective for delivery of cargo in vivo, e.g. to the liver. Further, it has been found that lipids with a pKa ranging from about 5.3 to about 6.4 are effective for delivery in vivo, e.g. to tumors. *See, e.g.*, WO2014/136086.

Depending on the choice of the starting materials or procedures, the amine lipids of the present disclosure can be present in the form of one of the possible isomers or as mixtures thereof, for example as pure optical isomers, or as isomer mixtures, such as racemates and diastereoisomer mixtures, depending on the number of asymmetric carbon atoms. The present disclosure is meant to include all such possible isomers, including racemic mixtures, diasteriomeric mixtures, and optically pure forms. Optically active (*R*)- and (*S*)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques. If the compound contains a double bond, the substituent may be *E* or *Z* configuration.

Any asymmetric atom (e.g., carbon or the like) of the compound(s) of the present disclosure can be racemic or enantiomerically enriched, for example the (*R*)-, or (*S*)- configuration. In certain embodiments, each asymmetric atom has at least 50 % enantiomeric excess, at least 60 % enantiomeric excess, at least 70 % enantiomeric excess, at least 80 % enantiomeric excess, at least 90 % enantiomeric excess, at least 95 % enantiomeric excess, or at least 99 % enantiomeric excess in the (*R*)- or (*S*)- configuration. Substituents at atoms with unsaturated double bonds may, if possible, be present in *cis*- (*Z*)- or *trans*- *(E)*- form.

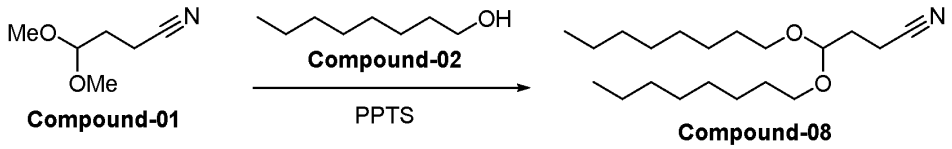
Accordingly, as used herein a compound of the present disclosure can be in the form of one of the possible isomers, rotamers, atropisomers, tautomers or mixtures thereof, for example, as substantially pure geometric (*cis* or *trans*) isomers, diastereomers, optical isomers (antipodes), racemates, or mixtures thereof.

Any resulting mixtures of isomers can be separated on the basis of the physicochemical differences of the constituents, into the pure or substantially pure geometric or optical isomers, diastereomers, racemates, for example, by chromatography and/or fractional crystallization.

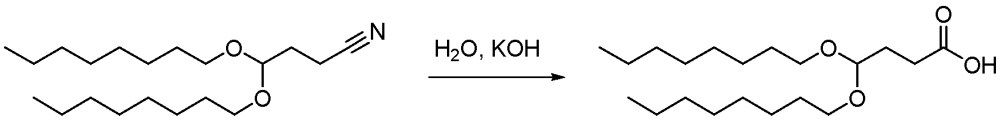
Any resulting racemates of final products or intermediates can be resolved into the optical antipodes by known methods, *e.g.*, by separation of the diastereomeric salts thereof, obtained with an optically active acid or base, and liberating the optically active acidic or basic compound. In particular, a basic moiety may thus be employed to resolve the compounds of the present invention into their optical antipodes, *e.g.*, by fractional crystallization of a salt formed with an optically active acid. Racemic products can also be resolved by chiral chromatography, *e.g.*, high pressure liquid chromatography (HPLC) using a chiral adsorbent.

Methods of Making Lipid A

In certain embodiments, the present disclosure provides methods of making 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate, referred to herein as Lipid A. The steps involved in the synthesis of Lipid A are outlined below.

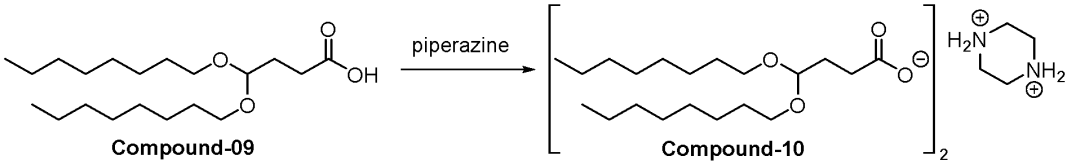


In certain embodiments, Compound-08 is prepared by treating Compound-01 with Compound-02 and an acid catalyst at a temperature between 40 °C and 100 °C for 15 minutes to 6 hours. In preferred embodiments, PPTS is used as the acid catalyst, and the reaction is conducted at 65 °C in toluene for 1 hour. In certain embodiments, Compound-08 is more easily purified when synthesized from Compound-01 as compared to when Compound-08 is synthesized from 4,4-diethoxybutanenitrile; for example, Compound-08 may be purified by partial distillation, such as from toluene.



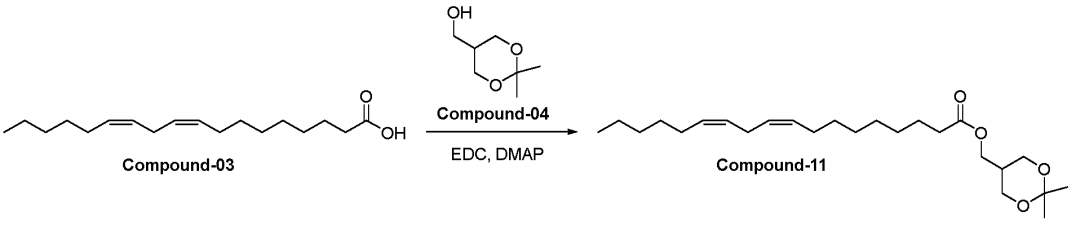
Compound-08 **Compound-09**

In certain embodiments, Compound-09 is prepared by hydrolyzing Compound-08, for example a Compound-08 solution in toluene, under basic conditions at a temperature not exceeding 100 °C for 24 to 72 hours. In preferred embodiments, KOH is used as the base, and the reaction is conducted in a mixture of ethanol and water at 80 °C for 36 to 40 hours. In certain embodiments, synthesizing Compound-09 as described herein results in Compound-09 with little to no octanol byproduct. In preferred embodiments, synthesizing Compound-09 according to the method described herein reduces the relative concentration of Impurity-03 or Impurity-04, or both, as compared to art processes. In certain embodiments, according to the methods described herein, Compound-09 is isolated as an oil at approximately 94% purity.



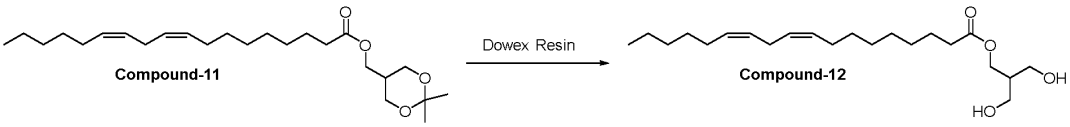
Compound-09 **Compound-10**

In certain embodiments, Compound-09 is purified via co-crystallization as an amine salt. In some embodiments, an amine salt is formed by treating Compound-09 with a diamine at between 0 °C and 70 °C. In preferred embodiments, treatment of Compound-09 with piperazine in isopropyl acetate at 50 °C yields Compound-10 as a crystalline solid. In alternative embodiments, Compound-09 is treated with *p*-xylylenediamine to form a salt. In certain embodiments, Compound-10 may be isolated or purified by filtration. Compound-10 has excellent solubility in water, even at neutral pH. In certain embodiments, Compound-10 has less than 1% 1-octanol, as measured by HS-GC(FID). In certain embodiments, Compound-10 made by the method described herein has a purity >99% by UPLC-CAD.

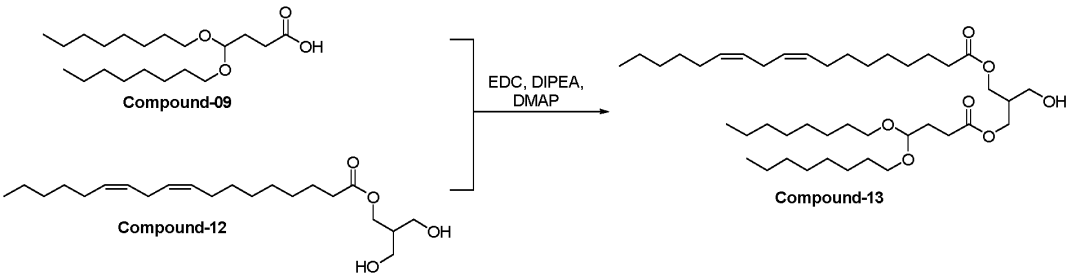


Compound-03 **Compound-11**

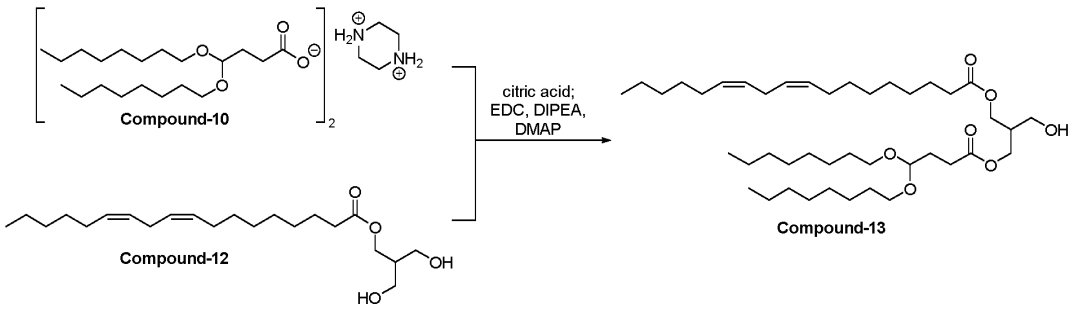
In certain embodiments, Compound-11 is prepared by esterifying Compound-03 and Compound-04 using a coupling reagent for 15 minutes to 24 hours. In preferred embodiments, the reaction to prepare Compound-11 is conducted in acetonitrile or dichloromethane at room temperature for 1 to 4 hours, using EDC and DMAP. In certain embodiments, Compound-11 may be purified by extraction, for example, extraction with heptane.



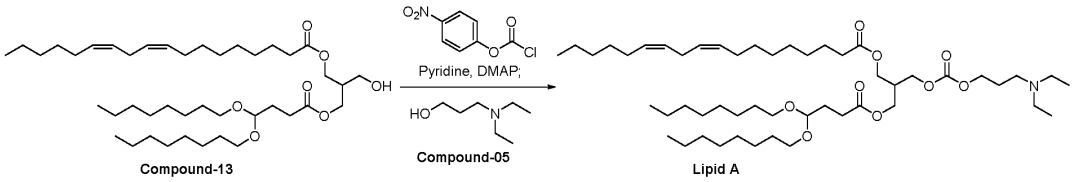
In certain embodiments, Compound-12 is prepared by hydrolyzing Compound-11 using an ion exchange resin. In preferred embodiments, Compound-11 is dissolved in methanol at room temperature and passed through a column bed containing Dowex® 50W ion exchange resin using methanol as the eluent to form Compound-12. In some embodiments, Compound-12 is purified using a powdered activated carbon (PAC) material. In preferred embodiments, Cabot A SUPRA EUR is used as a PAC material. In preferred embodiments, Compound-12 synthesized by the methods described herein does not comprise any Impurity-06. In preferred embodiments, Compound-12 synthesized by the methods described herein comprises less than 6% Impurity-07. In preferred embodiments, the purity of Compound-12 made by the methods described herein is more than 98%.



In certain embodiments, Compound-13 is prepared by esterifying Compound-09 and Compound-12 using a coupling reagent for 1 to 48 hours. In preferred embodiments the reaction to form Compound-13 is conducted in dichloromethane at 20-22 °C for 1 to 20 hours using EDC, DMAP, and DIPEA.



In alternative embodiments, Compound-13 is prepared by treating Compound-10 with an acid to form the corresponding carboxylic acid followed by esterification with Compound-12 using a coupling reagent for 15 minutes to 48 hours. In preferred embodiments, treatment of Compound-10 with citric acid in cyclopentyl methyl ether at room temperature generates Compound-09, which is treated with Compound-12, EDC, DIPEA, and DMAP at 20-22°C for 1 to 20 hours to furnish Compound-13. In preferred embodiments, Compound-13 comprises less than 2% 1-octanol, for example, 1% 1-octanol, as measured by HS-GC(FID). In preferred embodiments, Impurity-04 is not detectable by UPLC-CAD in Compound-13 made by the methods described herein. In preferred embodiments, Impurity-05 is not detectable by UPLC-CAD in Compound-13 made by the methods described herein. In preferred embodiments, Compound-13 comprises less than or equal to 0.15% piperazine (HS-GC(FID)) or less than 0.1% citrate (UPLC-CAD), or both. In preferred embodiments, hydroperoxide impurities are not detectable in Compound-13 made by the methods described herein. In preferred embodiments, the concentration of Impurity-06 is less than 1% or the concentration of Impurity-02 is less than 0.2%, or both, in Compound-13 made by the methods described herein. In preferred embodiments, the purity of Compound-13 made by the methods described herein is >96%.



In certain embodiments, Lipid A is prepared by treating Compound-13 with *p*-nitrophenyl chloroformate at 0 °C to 22 °C to form an activated carbonate, which is treated with Compound-05 for 6 to 24 hours to form Lipid A. In preferred embodiments, Compound-13 is treated with *p*-

nitrophenyl chloroformate, pyridine, and DMAP in acetonitrile at 7 to 11 °C to generate a carbonate intermediate, which is treated with Compound-05 for 15 hours at 20 °C to 24 °C to furnish Lipid A.

Lipid Formulations

In some embodiments, provided herein are compositions comprising a lipid set forth in Table A, e.g., Lipid A, optionally in combination with a carrier or excipient, e.g., a pharmaceutically acceptable carrier or excipient. In some embodiments, a composition can comprise one or more (e.g., two, three, four, five or more) lipids, optionally in combination with a carrier or excipient, e.g., a pharmaceutically acceptable carrier or excipient. In some embodiments, the composition comprises Lipid A. In some embodiments, the composition comprises Lipid A produced by a method set forth herein. For example, in any LNP compositions or formulations comprising Lipid A provided herein, Lipid A may be provided as a composition comprising Lipid A produced by any one or more of the methods set forth herein. In addition, in any LNP compositions or formulations comprising Lipid A provided herein, Lipid A may be provided as a composition comprising Lipid A having a level of purity, and/or a level(s) of one or more impurities as set forth herein. In some embodiments, a lipid set forth in Table A, *e.g.*, Lipid A, can be present in an LNP composition. Said LNP composition can include an ionizable lipid (e.g., Lipid A) optionally in combination with one or more additional lipids, e.g., a neutral lipid, a helper lipid, a stealth lipid, or combinations/subcombinations thereof. Such compositions can also contain an agent, e.g., a biologically active agent. Other lipid components of an LNP composition provided herein can include, but are not limited to, cationic lipids, neutral lipids, anionic lipids, helper lipids, and stealth lipids. Amine lipids, including Lipid A, for use in LNP formulations are described in WO2017/173054, WO2015/095340, and WO2014/136086.

The lipid compositions may be provided as LNP compositions, and LNP compositions described herein may be provided as lipid nanoparticles. Lipid nanoparticles may be, e.g., solid lipid nanoparticles, microspheres (including unilamellar and multilamellar vesicles, e.g. "liposomes"—lamellar phase lipid bilayers that, in some embodiments are substantially spherical, and, in more particular embodiments can comprise an aqueous core, e.g., comprising a

substantial portion of RNA molecules), a dispersed phase in an emulsion, micelles, or an internal phase in a suspension.

The LNP may contain (i) a biodegradable lipid, (ii) an optional neutral lipid, (iii) a helper lipid, and (iv) a stealth lipid, such as a PEG lipid. The LNP may contain a biodegradable lipid and one or more of a neutral lipid, a helper lipid, and a stealth lipid, such as a PEG lipid. The LNP may contain (i) an amine lipid (or ionizable lipid) for encapsulation and for endosomal escape, (ii) a neutral lipid for stabilization, (iii) a helper lipid, also for stabilization, and (iv) a stealth lipid, such as a PEG lipid. In some embodiments, the LNP can contain an amine lipid (e.g., Lipid A) and one or more of a neutral lipid, a helper lipid, and a stealth lipid, such as a PEG lipid.

In certain embodiments, an LNP composition may comprise an amine lipid, a helper lipid, a neutral lipid, and a stealth lipid. In certain embodiments, the amine lipid is Lipid A. In some embodiments, the amine lipid is a Lipid A composition produced by a method provided herein. In certain LNP compositions, the helper lipid is cholesterol. In other compositions, the neutral lipid is DSPC. In additional embodiments, the stealth lipid is PEG2k-DMG or PEG2k-C11. In certain compositions, the amine lipid is Lipid A or an acetal analog thereof, the helper lipid is cholesterol, the neutral lipid is DSPC, and the stealth lipid is PEG2k-DMG. In some embodiments, the amine lipid is a composition comprising Lipid A produced by a method provided herein.

In certain embodiments, lipid compositions are described according to the respective molar ratios of the component lipids. Embodiments of the present disclosure provide lipid compositions described according to the relative molar ratios of the component lipids. In one embodiment, the mol-% of the amine lipid may be from about 30 mol-% to about 60 mol-% among the lipid components. In one embodiment, the mol-% of the amine lipid may be from about 40 mol-% to about 60 mol-%. In one embodiment, the mol-% of the amine lipid may be from about 45 mol-% to about 60 mol-%. In one embodiment, the mol-% of the amine lipid may be from about 50 mol-% to about 60 mol-%. In one embodiment, the mol-% of the amine lipid may be from about 55 mol-% to about 60 mol-%. In one embodiment, the mol-% of the amine lipid may be from about 50 mol-% to about 55 mol-%. In one embodiment, the mol-% of the amine lipid may be about 50 mol-%. In one embodiment, the mol-% of the amine lipid may be

about 55 mol-% of the lipid components. Because the amine lipid constitutes a relatively large portion of the lipid components, controlling impurities present in the amine lipid will necessarily control impurities present in the lipid formulation. In some embodiments, the amine lipid is a composition comprising Lipid A produced by a method provided herein.

In some embodiments, the amine lipid mol-% of the LNP batch will be ±30%, ±25%, ±20%, ±15%, ±10%, ±5%, or ±2.5% of the target mol-%. In some embodiments, the amine lipid mol-% of the LNP batch will be ±4 mol-%, ±3 mol-%, ±2 mol-%, ±1.5 mol-%, ±1 mol-%, ±0.5 mol-%, or ±0.25 mol-% of the target mol-%. All mol-% numbers are given as a fraction of the lipid component of the LNP compositions. In certain embodiments, LNP inter-lot variability of the amine lipid mol-% will be less than 15%, less than 10% or less than 5%.

In one embodiment, the mol-% of the neutral lipid may be from about 5 mol-% to about 15 mol-% of the lipid components. In one embodiment, the mol-% of the neutral lipid may be from about 7 mol-% to about 12 mol-%. In one embodiment, the mol-% of the neutral lipid may be about 9 mol-%. In some embodiments, the neutral lipid mol-% of the LNP batch will be ±30%, ±25%, ±20%, ±15%, ±10%, ±5%, or ±2.5% of the target neutral lipid mol-%. In certain embodiments, LNP inter-lot variability will be less than 15%, less than 10% or less than 5%.

In one embodiment, the mol-% of the helper lipid may be from about 20 mol-% to about 60 mol-% of the lipid components. In one embodiment, the mol-% of the helper lipid may be from about 25 mol-% to about 55 mol-%. In one embodiment, the mol-% of the helper lipid may be from about 25 mol-% to about 50 mol-%. In one embodiment, the mol-% of the helper lipid may be from about 25 mol-% to about 40 mol-%. In one embodiment, the mol-% of the helper lipid may be from about 30 mol-% to about 50 mol-%. In one embodiment, the mol-% of the helper lipid may be from about 30 mol-% to about 40 mol-%. In one embodiment, the mol-% of the helper lipid is adjusted based on amine lipid, neutral lipid, and PEG lipid concentrations to bring the lipid component to 100 mol-%. In some embodiments, the helper mol-% of the LNP batch will be ±30%, ±25%, ±20%, ±15%, ±10%, ±5%, or ±2.5% of the target mol-%. In certain embodiments, LNP inter-lot variability will be less than 15%, less than 10% or less than 5%.

In one embodiment, the mol-% of the PEG lipid may be from about 1 mol-% to about 10 mol-% of the lipid components. In one embodiment, the mol-% of the PEG lipid may be from about 2 mol-% to about 10 mol-%. In one embodiment, the mol-% of the PEG lipid may be from

about 2 mol-% to about 8 mol-%. In one embodiment, the mol-% of the PEG lipid may be from about 2 mol-% to about 4 mol-%. In one embodiment, the mol-% of the PEG lipid may be from about 2.5 mol-% to about 4 mol-%. In one embodiment, the mol-% of the PEG lipid may be about 3 mol-%. In one embodiment, the mol-% of the PEG lipid may be about 2.5 mol-%. In some embodiments, the PEG lipid mol-% of the LNP batch will be ±30%, ±25%, ±20%, ±15%, ±10%, ±5%, or ±2.5% of the target PEG lipid mol-%. In certain embodiments, LNP inter-lot variability will be less than 15%, less than 10% or less than 5%.

In various embodiments, an LNP composition comprises an amine lipid, a neutral lipid, a helper lipid, and a PEG lipid. In certain embodiments, the helper lipid is cholesterol. In certain embodiments, the neutral lipid is DSPC. In specific embodiments, PEG lipid is PEG2k-DMG. In some embodiments, an LNP composition may comprise a Lipid A, a helper lipid, a neutral lipid, and a PEG lipid. In some embodiments, an LNP composition comprises an amine lipid, DSPC, cholesterol, and a PEG lipid. In some embodiments, the LNP composition comprises a PEG lipid comprising DMG. In certain embodiments, the amine lipid is selected from Lipid A, and an equivalent of Lipid A, including an acetal analog of Lipid A. In additional embodiments, an LNP composition comprises Lipid A, cholesterol, DSPC, and PEG2k-DMG.

Embodiments of the present disclosure also provide lipid compositions described according to the molar ratio between the positively charged amine groups of the amine lipid (N) and the negatively charged phosphate groups (P) of a nucleic acid to be encapsulated. This may be mathematically represented by the equation N/P. In some embodiments, an LNP composition may comprise a lipid component that comprises an amine lipid, a helper lipid, a neutral lipid, and a helper lipid; and a nucleic acid component, wherein the N/P ratio is about 3 to 10. In some embodiments, an LNP composition may comprise a lipid component that comprises an amine lipid, a helper lipid, a neutral lipid, and a helper lipid; and an RNA component, wherein the N/P ratio is about 3 to 10. In one embodiment, the N/P ratio may about 5-7. In one embodiment, the N/P ratio may about 4.5-8. In one embodiment, the N/P ratio may about 6. In one embodiment, the N/P ratio may be 6 ±1. In one embodiment, the N/P ratio may about 6 ± 0.5. In some embodiments, the N/P ratio will be ±30%, ±25%, ±20%, ±15%, ±10%, ±5%, or ±2.5% of the target N/P ratio. In certain embodiments, LNP inter-lot variability will be less than 15%, less than 10% or less than 5%.

In some embodiments, LNPs are formed by mixing an aqueous solution with an organic solvent-based lipid solution, e.g., 100% ethanol. Suitable solutions or solvents include or may contain: water, PBS, Tris buffer, NaCl, citrate buffer, ethanol, chloroform, diethylether, cyclohexane, tetrahydrofuran, methanol, isopropanol. A pharmaceutically acceptable buffer, e.g., for *in vivo* administration of LNPs, may be used. In certain embodiments, a buffer is used to maintain the pH of the composition comprising LNPs at or above pH 6.5. In certain embodiments, a buffer is used to maintain the pH of the composition comprising LNPs at or above pH 7.0. In certain embodiments, the composition has a pH ranging from about 7.2 to about 7.7. In additional embodiments, the composition has a pH ranging from about 7.3 to about 7.7 or ranging from about 7.4 to about 7.6. In further embodiments, the composition has a pH of about 7.2, 7.3, 7.4, 7.5, 7.6, or 7.7. The pH of a composition may be measured with a micro pH probe. In certain embodiments, a cryoprotectant is included in the composition. Non-limiting examples of cryoprotectants include sucrose, trehalose, glycerol, DMSO, and ethylene glycol. Exemplary compositions may include up to 10% cryoprotectant, such as, for example, sucrose. In certain embodiments, the LNP composition may include about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10% cryoprotectant. In certain embodiments, the LNP composition may include about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10% sucrose. In some embodiments, the LNP composition may include a buffer. In some embodiments, the buffer may comprise a phosphate buffer (PBS), a Tris buffer, a citrate buffer, and mixtures thereof. In certain exemplary embodiments, the buffer comprises NaCl. In certain embodiments, NaCl is omitted. Exemplary amounts of NaCl may range from about 20 mM to about 45 mM. Exemplary amounts of NaCl may range from about 40 mM to about 50 mM. In some embodiments, the amount of NaCl is about 45 mM. In some embodiments, the buffer is a Tris buffer. Exemplary amounts of Tris may range from about 20 mM to about 60 mM. Exemplary amounts of Tris may range from about 40 mM to about 60 mM. In some embodiments, the amount of Tris is about 50 mM. In some embodiments, the buffer comprises NaCl and Tris. Certain exemplary embodiments of the LNP compositions contain 5% sucrose and 45 mM NaCl in Tris buffer. In other exemplary embodiments, compositions contain sucrose in an amount of about 5% w/v, about 45 mM NaCl, and about 50 mM Tris at pH 7.5. The salt, buffer, and cryoprotectant amounts may be varied such that the osmolality of the overall formulation is maintained. For example, the final osmolality may be maintained at less than 450

mOsm/L. In further embodiments, the osmolality is between 350 and 250 mOsm/L. Certain embodiments have a final osmolality of 300 +/- 20 mOsm/L.

In some embodiments, microfluidic mixing, T-mixing, or cross-mixing is used. In certain aspects, flow rates, junction size, junction geometry, junction shape, tube diameter, solutions, and/or RNA and lipid concentrations may be varied. LNPs or LNP compositions may be concentrated or purified, *e.g.*, via dialysis, tangential flow filtration, or chromatography. The LNPs may be stored as a suspension, an emulsion, or a lyophilized powder, for example. In some embodiments, an LNP composition is stored at 2-8° C, in certain aspects, the LNP compositions are stored at room temperature. In additional embodiments, an LNP composition is stored frozen, for example at -20° C or -80° C. In other embodiments, an LNP composition is stored at a temperature ranging from about 0° C to about -80° C. Frozen LNP compositions may be thawed before use, for example on ice, at 4° C, at room temperature, or at 25° C. Frozen LNP compositions may be maintained at various temperatures, for example on ice, at 4° C, at room temperature, at 25° C, or at 37° C.

"Neutral lipids" suitable for use in a lipid composition of the disclosure include, for example, a variety of neutral, uncharged or zwitterionic lipids. Examples of neutral phospholipids suitable for use in the present disclosure include, but are not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), phosphocholine (DOPC), dimyristoylphosphatidylcholine (DMPC), phosphatidylcholine (PLPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DAPC), phosphatidylethanolamine (PE), egg phosphatidylcholine (EPC), dilauryloylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), 1-myristoyl-2-palmitoyl phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl phosphatidylcholine (PSPC), 1,2-diarachidoyl-sn-glycero-3-phosphocholine (DBPC), 1-stearoyl-2-palmitoyl phosphatidylcholine (SPPC), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (DEPC), palmitoyloleoyl phosphatidylcholine (POPC), lysophosphatidyl choline, dioleoyl phosphatidylethanolamine (DOPE), dilinoleoylphosphatidylcholine, distearoylphosphatidylethanolamine (DSPE), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE), lysophosphatidylethanolamine and combinations thereof. In certain embodiments, the neutral phospholipid may be selected from

distearoylphosphatidylcholine (DSPC) and dimyristoyl phosphatidyl ethanolamine (DMPE), preferably distearoylphosphatidylcholine (DSPC).

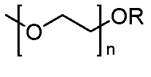
"Helper lipids" include steroids, sterols, and alkyl resorcinols. Helper lipids suitable for use in the present disclosure include, but are not limited to, cholesterol, 5-heptadecylresorcinol, and cholesterol hemisuccinate. In certain embodiments, the helper lipid may be cholesterol or a derivative thereof, such as cholesterol hemisuccinate.

In some embodiments, the LNP compositions include polymeric lipids, such as PEG lipids which can affect the length of time the nanoparticles can exist *in vivo* or *ex vivo* (*e.g.*, in the blood or medium). PEG lipids may assist in the formulation process by, for example, reducing particle aggregation and controlling particle size. PEG lipids used herein may modulate pharmacokinetic properties of the LNPs. Typically, the PEG lipid comprises a lipid moiety and a polymer moiety based on PEG (sometimes referred to as poly(ethylene oxide)) (a PEG moiety). PEG lipids suitable for use in a lipid composition with a compound of Formula (I) or (II) of the present disclosure and information about the biochemistry of such lipids can be found in Romberg et al., Pharmaceutical Research 25(1), 2008, pp. 55-71 and Hoekstra et al., Biochimica et Biophysica Acta 1660 (2004) 41-52. Additional suitable PEG lipids are disclosed, e.g., in WO 2015/095340 (p. 31, line 14 to p. 37, line 6), WO 2006/007712, and WO 2011/076807 ("stealth lipids"), each of which is incorporated by reference in its entirety.

In some embodiments, the lipid moiety may be derived from diacylglycerol or diacylglycamide, including those comprising a dialkylglycerol or dialkylglycamide group having alkyl chain length independently comprising from about C4 to about C40 saturated or unsaturated carbon atoms, wherein the chain may comprise one or more functional groups such as, for example, an amide or ester. In some embodiments, the alkyl chain length comprises about C10 to C20. The dialkylglycerol or dialkylglycamide group can further comprise one or more substituted alkyl groups. The chain lengths may be symmetrical or asymmetric.

Unless otherwise indicated, the term "PEG" as used herein means any polyethylene glycol or other polyalkylene ether polymer, such as an optionally substituted linear or branched polymer of ethylene glycol or ethylene oxide. In certain embodiments, the PEG moiety is unsubstituted. Alternatively, the PEG moiety may be substituted, *e.g.*, by one or more alkyl, alkoxy, acyl, hydroxy, or aryl groups. For example, the PEG moiety may comprise a PEG

copolymer such as PEG-polyurethane or PEG-polypropylene (see, *e.g.*, J. Milton Harris, Poly(ethylene glycol) chemistry: biotechnical and biomedical applications (1992)); alternatively, the PEG moiety may be a PEG homopolymer. In certain embodiments, the PEG moiety has a molecular weight of from about 130 to about 50,000, such as from about 150 to about 30,000, or even from about 150 to about 20,000. Similarly, the PEG moiety may have a molecular weight of from about 150 to about 15,000, from about 150 to about 10,000, from about 150 to about 6,000, or even from about 150 to about 5,000. In certain preferred embodiments, the PEG moiety has a molecular weight of from about 150 to about 4,000, from about 150 to about 3,000, from about 300 to about 3,000, from about 1,000 to about 3,000, or from about 1,500 to about 2,500.

In certain preferred embodiments, the PEG moiety is a "PEG-2K," also termed "PEG 2000," which has an average molecular weight of about 2,000 daltons. PEG-2K is represented herein by the following formula (III),  (III), wherein n is about 45, meaning that the number averaged degree of polymerization comprises about 45 subunits. However, other PEG embodiments known in the art may be used, including, *e.g.*, those where the number-averaged degree of polymerization comprises about 23 subunits (n=23), and/or 68 subunits (n=68). In some embodiments, n may range from about 30 to about 60. In some embodiments, n may range from about 35 to about 55. In some embodiments, n may range from about 40 to about 50. In some embodiments, n may range from about 42 to about 48. In some embodiments, n may be 45. In some embodiments, R may be selected from H, substituted alkyl, and unsubstituted alkyl. In some embodiments, R may be unsubstituted alkyl, such as methyl.

In any of the embodiments described herein, the PEG lipid may be selected from PEG-dilauroylglycerol, PEG-dimyristoylglycerol (PEG-DMG) (catalog # GM-020 from NOF, Tokyo, Japan), PEG-dipalmitoylglycerol, PEG-distearoylglycerol (PEG-DSPE) (catalog # DSPE-020CN, NOF, Tokyo, Japan), PEG-dilaurylglycamide, PEG-dimyristylglycamide, PEG-dipalmitoylglycamide, and PEG-distearoylglycamide, PEG-cholesterol (1-[8'-(Cholest-5-en-3[beta]-oxy)carboxamido-3',6'-dioxaoctanyl]carbamoyl-[omega]-methyl-poly(ethylene glycol), PEG-DMB (3,4-ditetradecoxylbenzyl-[omega]-methyl-poly(ethylene glycol)ether), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DMPE), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG2k-

DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSPE) (cat. #880120C from Avanti Polar Lipids, Alabaster, Alabama, USA), 1,2-distearoyl-sn-glycerol, methoxypolyethylene glycol (PEG2k-DSG; GS-020, NOF Tokyo, Japan), poly(ethylene glycol)-2000-dimethacrylate (PEG2k-DMA), and 1,2-distearyloxypropyl-3-amine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSA). In certain such embodiments, the PEG lipid may be PEG2k-DMG. In some embodiments, the PEG lipid may be PEG2k-DSG. In other embodiments, the PEG lipid may be PEG2k-DSPE. In some embodiments, the PEG lipid may be PEG2k-DMA. In yet other embodiments, the PEG lipid may be PEG2k-C-DMA. In certain embodiments, the PEG lipid may be compound S027, disclosed in WO2016/010840 (paragraphs [00240] to [00244]). In some embodiments, the PEG lipid may be PEG2k-DSA. In other embodiments, the PEG lipid may be PEG2k-C11. In some embodiments, the PEG lipid may be PEG2k-C14. In some embodiments, the PEG lipid may be PEG2k-C16. In some embodiments, the PEG lipid may be PEG2k-C18.

In preferred embodiments, the PEG lipid includes a glycerol group. In preferred embodiments, the PEG lipid includes a dimyristoylglycerol (DMG) group. In preferred embodiments, the PEG lipid comprises PEG-2k. In preferred embodiments, the PEG lipid is a PEG-DMG. In preferred embodiments, the PEG lipid is a PEG-2k-DMG. In preferred embodiments, the PEG lipid is 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000. In preferred embodiments, the PEG-2k-DMG is 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000.

Cargo

The LNP compositions described herein can be used in a broad range of applications to deliver cargo across a lipid bilayer, e.g., a cell membrane. In some embodiments, the LNP compositions can be used to deliver cargo to a cell. The cell can be provided *in vitro* or *in vivo*. The cargo delivered via a lipid composition described herein can include a biologically active agent. The biologically active agent may be a nucleic acid, such as an mRNA, a DNA, a guide RNA (gRNA), etc. In other embodiments, the agent may be a protein, a peptide, and/or a small molecule. In certain embodiments, the cargo is or comprises one or more biologically active agent, such as mRNA, gRNA, expression vector, RNA-guided DNA-binding agent, antibody

(e.g. , monoclonal, chimeric, humanized, nanobody, and fragments thereof etc.), cholesterol, hormone, peptide, protein, chemotherapeutic and other types of antineoplastic agent, low molecular weight drug, vitamin, co-factor, nucleoside, nucleotide, oligonucleotide, enzymatic nucleic acid, antisense nucleic acid, triplex forming oligonucleotide, antisense DNA or RNA composition, chimeric DNA:RNA composition, allozyme, aptamer, ribozyme, decoys and analogs thereof, plasmid and other types of vectors, and small nucleic acid molecule, RNAi agent, short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA) and "self-replicating RNA" (encoding a replicase enzyme activity and capable of directing its own replication or amplification in vivo) molecules, peptide nucleic acid (PNA), a locked nucleic acid ribonucleotide (LNA), morpholino nucleotide, threose nucleic acid (TNA), glycol nucleic acid (GNA), sisiRNA (small internally segmented interfering RNA), and iRNA (asymmetrical interfering RNA). The above list of biologically active agents is exemplary only, and is not intended to be limiting. Such compounds may be purified or partially purified, and may be naturally occurring or synthetic, and may be chemically modified.

The cargo delivered via LNP composition may be an RNA, such as an mRNA molecule encoding a protein of interest. In some embodiments, an mRNA for expressing a protein such as green fluorescent protein (GFP), an RNA-guided DNA-binding agent, or a Cas nuclease is provided. LNP compositions that include a Cas nuclease mRNA, for example a Class 2 Cas nuclease mRNA that allows for expression in a cell of a Class 2 Cas nuclease such as a Cas9 or Cpf1 (also referred to as Cas12a) protein are provided. Further, the cargo may contain one or more gRNAs or nucleic acids encoding gRNAs. A template nucleic acid, *e.g.*, for repair or recombination, may also be included with the compositions or a template nucleic acid may be used in the methods described herein. In a sub-embodiment, the cargo comprises an mRNA that encodes a *Streptococcus pyogenes* Cas9, and/or an *S. pyogenes* gRNA. In a further sub-embodiment, the cargo comprises an mRNA that encodes a *Neisseria meningitidis* Cas9, and/or an Nme (*Neisseria meningitidis)* gRNA.

"mRNA" refers to a polynucleotide and comprises an open reading frame that can be translated into a polypeptide (*i.e.*, can serve as a substrate for translation by a ribosome and amino-acylated tRNAs). mRNA can comprise a phosphate-sugar backbone including ribose

residues or analogs thereof, *e.g.*, 2'-methoxy ribose residues. In some embodiments, the sugars of an mRNA phosphate-sugar backbone consist essentially of ribose residues, 2'-methoxy ribose residues, or a combination thereof. In general, mRNAs do not contain a substantial quantity of thymidine residues (*e.g.*, 0 residues or fewer than 30, 20, 10, 5, 4, 3, or 2 thymidine residues; or less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 4%, 3%, 2%, 1%, 0.5%, 0.2%, or 0.1% thymidine content). An mRNA can contain modified uridines (e.g., pseudouridine (Ψ) or N1-methyl-pseudouridine (m1Ψ)) at some or all of its uridine positions.

In some embodiments, the LNP composition is a lipid nucleic acid assembly, also referred to as a lipid nucleic acid composition. In some embodiments, the lipid nucleic acid composition or LNP composition comprises a genome editing tool or a nucleic acid encoding the same. As used herein, the term "genome editing tool" (or "gene editing tool") is any component of "genome editing system" (or "gene editing system") necessary or helpful for producing an edit in the genome of a cell. In some embodiments, the present disclosure provides for methods of delivering genome editing tools of a genome editing system (for example a zinc finger nuclease system, a TALEN system, a meganuclease system or a CRISPR/Cas system) to a cell (or population of cells). Genome editing tools include, for example, nucleases capable of making single or double strand break in the DNA or RNA of a cell, *e.g.*, in the genome of a cell. The genome editing tools, *e.g.* nucleases, may optionally modify the genome of a cell without cleaving the nucleic acid, or nickases. A genome editing nuclease or nickase may be encoded by an mRNA. Such nucleases include, for example, RNA-guided DNA binding agents, and CRISPR/Cas components. Genome editing tools include fusion proteins, including e.g., a nickase fused to an effector domain such as an editor domain. Genome editing tools include any item necessary or helpful for accomplishing the goal of a genome edit, such as, for example, guide RNA, sgRNA, dgRNA, donor nucleic acid, and the like.

Various suitable gene editing systems comprising genome editing tools for delivery with the lipid nucleic acid assembly compositions are described herein, including but not limited to the CRISPR/Cas system; zinc finger nuclease (ZFN) system; and the transcription activator-like effector nuclease (TALEN) system. Generally, the gene editing systems involve the use of engineered cleavage systems to induce a double strand break (DSB) or a nick (e.g., a single strand break, or SSB) in a target DNA sequence. Cleavage or nicking can occur through the use

of specific nucleases such as engineered ZFN, TALENs, or using the CRISPR/Cas system with an engineered guide RNA to guide specific cleavage or nicking of a target DNA sequence. Further, targeted nucleases are being developed based on the Argonaute system (e.g., from T. thermophilus, known as 'TtAgo', see Swarts et al (2014) Nature 507(7491): 258-261), which also may have the potential for uses in genome editing and gene therapy.

In preferred embodiments, the disclosed compositions comprise an mRNA encoding an RNA-guided DNA-binding agent, such as a Cas nuclease. In particular embodiments, the disclosed compositions comprise an mRNA encoding a Class 2 Cas nuclease, such as *S. pyogenes* Cas9.

As used herein, an "RNA-guided DNA-binding agent" means a polypeptide or complex of polypeptides having RNA and DNA-binding activity, or a DNA-binding subunit of such a complex, wherein the DNA-binding activity is sequence-specific and depends on the sequence of the RNA. Exemplary RNA-guided DNA-binding agents include Cas cleavases/nickases and inactivated forms thereof ("dCas DNA-binding agents"). "Cas nuclease", as used herein, encompasses Cas cleavases, Cas nickases, and dCas DNA-binding agents. Cas cleavases/nickases and dCas DNA-binding agents include a Csm or Cmr complex of a type III CRISPR system, the Cas10, Csm1, or Cmr2 subunit thereof, a Cascade complex of a type I CRISPR system, the Cas3 subunit thereof, and Class 2 Cas nucleases. As used herein, a "Class 2 Cas nuclease" is a single-chain polypeptide with RNA-guided DNA-binding activity. Class 2 Cas nucleases include Class 2 Cas cleavases/nickases (e.g., H840A, D10A, or N863A variants), which further have RNA-guided DNA cleavases or nickase activity, and Class 2 dCas DNA-binding agents, in which cleavase/nickase activity is inactivated. Class 2 Cas nucleases that may be used with the LNP compositions described herein include, for example, Cas9, Cpf1, C2c1, C2c2, C2c3, HF Cas9 (e.g., N497A, R661A, Q695A, Q926A variants), HypaCas9 (e.g., N692A, M694A, Q695A, H698A variants), eSPCas9(1.0) (e.g, K810A, K1003A, R1060A variants), and eSPCas9(1.1) (e.g., K848A, K1003A, R1060A variants) proteins and modifications thereof. Cpf1 protein, Zetsche et al., *Cell*, 163: 1-13 (2015), is homologous to Cas9, and contains a RuvC-like nuclease domain. Cpf1 sequences of Zetsche are incorporated by reference in their entirety. *See*, *e.g.*, Zetsche, Tables 2 and 4. See, *e.g.*, Makarova et al., *Nat Rev Microbiol*, 13(11): 722-36 (2015); Shmakov et al., *Molecular Cell,* 60:385-397 (2015).

In some embodiments, the RNA-guided DNA binding agent comprises an editor. An exemplary editor is BC22n which comprises a *H. sapiens* APOBEC3A fused to *S. pyogenes*-D10A Cas9 nickase by an XTEN linker. In some embodiments, the editor is provided with a uracil glycosylase inhibitor ("UGI"). In some embodiments, the editor is fused to the UGI. In some embodiments, the mRNA encoding the editor and an mRNA encoding the UGI are formulated together in an LNP. In other embodiments, the editor and UGI are provided in separate LNPs.

In some embodiments, the RNA-guided DNA-binding agent comprises one or more heterologous functional domains (*e.g.*, is or comprises a fusion polypeptide).

In some embodiments, the heterologous functional domain may facilitate transport of the RNA-guided DNA-binding agent into the nucleus of a cell. For example, the heterologous functional domain may be a nuclear localization signal (NLS).

In some embodiments, the heterologous functional domain may be capable of modifying the intracellular half-life of the RNA-guided DNA binding agent. In some embodiments, the half-life of the RNA-guided DNA binding agent may be increased. In some embodiments, the half-life of the RNA-guided DNA-binding agent may be reduced. In some embodiments, the heterologous functional domain may be capable of increasing the stability of the RNA-guided DNA-binding agent. In some embodiments, the heterologous functional domain may be capable of reducing the stability of the RNA-guided DNA-binding agent. In some embodiments, the heterologous functional domain may act as a signal peptide for protein degradation. In some embodiments, the protein degradation may be mediated by proteolytic enzymes, such as, for example, proteasomes, lysosomal proteases, or calpain proteases. In some embodiments, the heterologous functional domain may comprise a PEST sequence. In some embodiments, the RNA-guided DNA-binding agent may be modified by addition of ubiquitin or a polyubiquitin chain. In some embodiments, the ubiquitin may be a ubiquitinlike protein (UBL). Non-limiting examples of ubiquitin-like proteins include small ubiquitinlike modifier (SUMO), ubiquitin cross-reactive protein (UCRP, also known as interferonstimulated gene-15 (ISG15)), ubiquitin-related modifier-1 (URM1), neuronal-precursor-cellexpressed developmentally downregulated protein-8 (NEDD8, also called Rub1 in *S. cerevisiae*), human leukocyte antigen F-associated

(FAT10), autophagy-8 (ATG8) and -12 (ATG12), Fau ubiquitin-like protein (FUB1), membrane-anchored UBL (MUB), ubiquitin fold-modifier-1 (UFM1), and ubiquitin-like protein-5 (UBL5).

The nuclease may comprise at least one domain that interacts with a guide RNA ("gRNA"). Additionally, the nuclease may be directed to a target sequence by a gRNA. In Class 2 Cas nuclease systems, the gRNA interacts with the nuclease as well as the target sequence, such that it directs binding to the target sequence. In some embodiments, the gRNA provides the specificity for the targeted cleavage, and the nuclease may be universal and paired with different gRNAs to cleave different target sequences. Class 2 Cas nuclease may pair with a gRNA scaffold structure of the types, orthologs, and exemplary species listed above.

As used herein, "ribonucleoprotein" (RNP) or "RNP complex" refers to a gRNA together with an RNA-guided DNA-binding agent, such as a Cas nuclease, e.g., a Cas cleavase, Cas nickase, or dCas DNA-binding agent (e.g., Cas9). In some embodiments, the gRNA guides the RNA-guided DNA-binding agent such as Cas9 to a target sequence, and the gRNA hybridizes with and the agent binds to the target sequence; in cases where the agent is a cleavase or nickase, binding can be followed by cleaving or nicking.

In some embodiments of the present disclosure, the cargo for the LNP composition includes at least one gRNA comprising guide sequences that direct an RNA-guided DNA-binding agent, which can be a nuclease (e.g., a Cas nuclease such as Cas9), to a target DNA. The gRNA may guide the Cas nuclease or Class 2 Cas nuclease to a target sequence on a target nucleic acid molecule. In some embodiments, a gRNA binds with and provides specificity of cleavage by a Class 2 Cas nuclease. In some embodiments, the gRNA and the Cas nuclease may form a ribonucleoprotein (RNP), *e.g.*, a CRISPR/Cas complex such as a CRISPR/Cas9 complex. In some embodiments, the CRISPR/Cas complex may be a Type-II CRISPR/Cas9 complex. In some embodiments, the CRISPR/Cas complex may be a Type-V CRISPR/Cas complex, such as a Cpf1/gRNA complex. Cas nucleases and cognate gRNAs may be paired. The gRNA scaffold structures that pair with each Class 2 Cas nuclease vary with the specific CRISPR/Cas system.

"Guide RNA", "gRNA", and simply "guide" are used herein interchangeably to refer to a cognate guide nucleic acid for an RNA-guided DNA-binding agent. Guide RNAs can include modified RNAs as described herein. A gRNA may be, for example, either a single guide RNA (sgRNA), or the combination of a crRNA and a trRNA (also known as tracrRNA). The crRNA

and trRNA may be associated as a single RNA molecule (as a single guide RNA, sgRNA) or, for example, in two separate RNA strands (dual guide RNA, dgRNA). In some systems a gRNA may be a crRNA (also known as a CRISPR RNA). "Guide RNA" or "gRNA" refers to each type. The trRNA may be a naturally occurring sequence, or a trRNA sequence with modifications or variations compared to naturally occurring sequences.

In some embodiments, an mRNA encoding a RNA-guided DNA binding agent is formulated in a first LNP composition and a gRNA nucleic acid is formulated in a second LNP composition. In some embodiments, the first and second LNP compositions are administered simultaneously. In other embodiments, the first and second LNP compositions are administered sequentially. In some embodiments, the first and second LNP compositions are combined prior to the preincubation step. In other embodiments, the first and second LNP compositions are preincubated separately.

In some embodiments, the cargo may comprise a DNA molecule. In some embodiments, the nucleic acid may comprise a nucleotide sequence encoding a crRNA. In some embodiments, the nucleotide sequence encoding the crRNA comprises a targeting sequence flanked by all or a portion of a repeat sequence from a naturally occurring CRISPR/Cas system. In some embodiments, the nucleic acid may comprise a nucleotide sequence encoding a tracr RNA. In certain embodiments, the crRNA and the tracr RNA may be encoded by two separate nucleic acids. In other embodiments, the crRNA and the tracr RNA may be encoded by a single nucleic acid. In some embodiments, the crRNA and the tracr RNA may be encoded by opposite strands of a single nucleic acid. In other embodiments, the crRNA and the tracr RNA may be encoded by the same strand of a single nucleic acid. In some embodiments, the gRNA nucleic acid encodes an sgRNA. In some embodiments, the gRNA nucleic acid encodes a Cas9 nuclease sgRNA. In come embodiments, the gRNA nucleic acid encodes a Cpf1 nuclease sgRNA.

The nucleotide sequence encoding the guide RNA may be operably linked to at least one transcriptional or regulatory control sequence, such as a promoter, a 3' UTR, or a 5' UTR. In one example, the promoter may be a tRNA promoter, *e.g.*, tRNALys3, or a tRNA chimera. *See* Mefferd et al., *RNA*. 2015 21:1683-9; Scherer et al., *Nucleic Acids Res.* 2007 35: 2620–2628. In some embodiments, the promoter may be recognized by RNA polymerase III (Pol III). Non-limiting examples of Pol III promoters also include U6 and H1 promoters. In some embodiments,

the nucleotide sequence encoding the guide RNA may be operably linked to a mouse or human U6 promoter. In some embodiments, the gRNA nucleic acid is a modified nucleic acid. In some embodiments, the gRNA nucleic acid includes a modified nucleoside or nucleotide. In some embodiments, the gRNA nucleic acid includes a 5' end modification, for example a modified nucleoside or nucleotide to stabilize and prevent integration of the nucleic acid. In other embodiments, the gRNA nucleic acid comprises a double-stranded DNA having a 5' end modification on each strand. In some embodiments, the gRNA nucleic acid includes an inverted dideoxy-T or an inverted abasic nucleoside or nucleotide as the 5' end modification. In some embodiments, the gRNA nucleic acid includes a label such as biotin, desthiobiotin- TEG, digoxigenin, and fluorescent markers, including, for example, FAM, ROX, TAMRA, and AlexaFluor.

As used herein, a "guide sequence" refers to a sequence within a gRNA that is complementary to a target sequence and functions to direct a gRNA to a target sequence for binding and/or modification (*e.g.*, cleavage) by an RNA-guided DNA-binding agent. A "guide sequence" may also be referred to as a "targeting sequence," or a "spacer sequence." A guide sequence can be 20 base pairs in length, *e.g.*, in the case of *Streptococcus pyogenes* (*i.e.*, Spy Cas9) and related Cas9 homologs/orthologs. Shorter or longer sequences can also be used as guides, *e.g.*, 15-, 16-, 17-, 18-, 19-, 21-, 22-, 23-, 24-, or 25-nucleotides in length. In some embodiments, the target sequence is in a gene or on a chromosome, for example, and is complementary to the guide sequence. In some embodiments, the degree of complementarity or identity between a guide sequence and its corresponding target sequence may be about or at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the guide sequence and the target region may be 100% complementary or identical over a region of at least 15, 16, 17, 18, 19, or 20 contiguous nucleotides. In other embodiments, the guide sequence and the target region may contain at least one mismatch. For example, the guide sequence and the target sequence may contain 1, 2, 3, or 4 mismatches, where the total length of the target sequence is at least 17, 18, 19, 20 or more base pairs. In some embodiments, the guide sequence and the target region may contain 1-4 mismatches where the guide sequence comprises at least 17, 18, 19, 20 or more nucleotides. In some embodiments, the guide sequence and the target region may contain 1, 2, 3, or 4 mismatches where the guide sequence comprises 20 nucleotides.

In certain embodiments, multiple LNP compositions may be used collaboratively and/or for separate purposes. In some embodiments, a cell may be contacted with first and second LNP compositions described herein. In some embodiments, the first and second LNP compositions each independently comprise one or more of an mRNA, a gRNA, and a guide RNA nucleic acid. In some embodiments, the first and second LNP compositions are administered simultaneously. In some embodiments, the first and second LNP compositions are administered sequentially.

Target sequences for RNA-guided DNA-binding proteins such as Cas proteins include both the positive and negative strands of genomic DNA (*i.e.*, the sequence given and the sequence's reverse complement), as a nucleic acid substrate for a Cas protein is a double stranded nucleic acid. Accordingly, where a guide sequence is said to be "complementary to a target sequence", it is to be understood that the guide sequence may direct a gRNA to bind to the reverse complement of a target sequence. Thus, in some embodiments, where the guide sequence binds the reverse complement of a target sequence, the guide sequence is identical to certain nucleotides of the target sequence (*e.g.*, the target sequence not including the PAM) except for the substitution of U for T in the guide sequence.

In certain embodiments, at least one of the foregoing lipid compositions comprises a nucleic acid genome editing tool as described herein. In some embodiments, a further lipid composition comprises an RNA-guided DNA binding agent. In some embodiments, the RNA-guided DNA binding agent is Cas9.

In some embodiments, the methods of the present disclosure further comprise contacting the cell with a donor nucleic acid. In some embodiments, a further lipid composition comprises a donor nucleic acid. The donor nucleic acid may be inserted in a target sequence. In some embodiments, a donor nucleic acid sequence is provided as a vector. In some embodiments, the donor nucleic acid encodes a targeting receptor.

The length of the targeting sequence of a guide RNA (e.g., sgRNA) may depend on the CRISPR/Cas system and components used. For example, different Class 2 Cas nucleases from different bacterial species have varying optimal targeting sequence lengths. Accordingly, the targeting sequence may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or more than 50 nucleotides in length. In some embodiments, the targeting sequence length is 0, 1, 2, 3, 4, or 5 nucleotides longer or shorter

than the guide sequence of a naturally occurring CRISPR/Cas system. In certain embodiments, the Cas nuclease and gRNA scaffold will be derived from the same CRISPR/Cas system. In some embodiments, the targeting sequence may comprise or consist of 18-24 nucleotides. In some embodiments, the targeting sequence may comprise or consist of 19-21 nucleotides. In some embodiments, the targeting sequence may comprise or consist of 20 nucleotides.

In some embodiments, the sgRNA is a "Cas9 sgRNA" capable of mediating RNA-guided DNA cleavage by a Cas9 protein. In some embodiments, the sgRNA is a "Cpf1 sgRNA" capable of mediating RNA-guided DNA cleavage by a Cpf1 protein. In certain embodiments, the gRNA comprises a crRNA and tracr RNA sufficient for forming an active complex with a Cas9 protein and mediating RNA-guided DNA cleavage. In certain embodiments, the gRNA comprises a crRNA sufficient for forming an active complex with a Cpf1 protein and mediating RNA-guided DNA cleavage. See Zetsche 2015.

Certain embodiments also provide nucleic acids, *e.g.*, expression cassettes, encoding the gRNA described herein. A "guide RNA nucleic acid" is used herein, in some embodiments, to refer to a gRNA (*e.g.* an sgRNA or a dgRNA) and a gRNA expression cassette, which is a nucleic acid that encodes one or more gRNAs.

In certain embodiments, the lipid compositions, such as LNP compositions comprise modified nucleic acids, including modified RNAs.

Modified nucleosides or nucleotides can be present in an RNA, for example a gRNA or mRNA. A gRNA or mRNA comprising one or more modified nucleosides or nucleotides, for example, is called a "modified" RNA to describe the presence of one or more non-naturally and/or naturally occurring components or configurations that are used instead of or in addition to the canonical A, G, C, and U residues. In some embodiments, a modified RNA is synthesized with a non-canonical nucleoside or nucleotide, here called "modified."

Modified nucleosides and nucleotides can include one or more of: (i) alteration, *e.g.*, replacement, of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens in the phosphodiester backbone linkage (an exemplary backbone modification); (ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar (an exemplary sugar modification); (iii) wholesale replacement of the phosphate moiety with "dephospho" linkers (an exemplary backbone modification); (iv)

modification or replacement of a naturally occurring nucleobase, including with a non-canonical nucleobase (an exemplary base modification); (v) replacement or modification of the ribose-phosphate backbone (an exemplary backbone modification); (vi) modification of the 3' end or 5' end of the polynucleotide, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, cap or linker (such 3' or 5' cap modifications may comprise a sugar and/or backbone modification); and (vii) modification or replacement of the sugar (an exemplary sugar modification). Certain embodiments comprise a 5' end modification to an mRNA, gRNA, or nucleic acid. Certain embodiments comprise a modification to an mRNA, gRNA, or nucleic acid. Certain embodiments comprise a 3' end modification to an mRNA, gRNA, or nucleic acid. A modified RNA can contain 5' end and 3' end modifications. A modified RNA can contain one or more modified residues at non-terminal locations. In certain embodiments, a gRNA includes at least one modified residue. In certain embodiments, an mRNA includes at least one modified residue. In certain embodiments, the modified gRNA comprises a modification at one or more of the first five nucleotides at a 5' end. In certain embodiments, the modified gRNA comprises a modification at one or more of the last five nucleotides at a 3' end.

Unmodified nucleic acids can be prone to degradation by, *e.g.*, intracellular nucleases or those found in serum. For example, nucleases can hydrolyze nucleic acid phosphodiester bonds. Accordingly, in one aspect the RNAs (e.g. mRNAs, gRNAs) described herein can contain one or more modified nucleosides or nucleotides, *e.g.*, to introduce stability toward intracellular or serum-based nucleases. In some embodiments, the modified RNA molecules described herein can exhibit a reduced innate immune response when introduced into a population of cells, both *in vivo* and *ex vivo*. The term "innate immune response" includes a cellular response to exogenous nucleic acids, including single stranded nucleic acids, which involves the induction of cytokine expression and release, particularly the interferons, and cell death.

Accordingly, in some embodiments, an RNA or nucleic acid comprises at least one modification which confers increased or enhanced stability to the nucleic acid, including, for example, improved resistance to nuclease digestion in vivo. As used herein, the terms "modification" and "modified" as such terms relate to the nucleic acids provided herein, include at least one alteration which preferably enhances stability and renders the RNA or nucleic acid

more stable (e.g., resistant to nuclease digestion) than the wild-type or naturally occurring version of the RNA or nucleic acid. As used herein, the terms "stable" and "stability" and such terms relate to the nucleic acids described herein, and particularly with respect to the RNA, refer to increased or enhanced resistance to degradation by, for example nucleases (i.e., endonucleases or exonucleases) which are normally capable of degrading such RNA. Increased stability can include, for example, less sensitivity to hydrolysis or other destruction by endogenous enzymes (e.g., endonucleases or exonucleases) or conditions within the target cell or tissue, thereby increasing or enhancing the residence of such RNA or nucleic acid in the target cell, tissue, subject and/or cytoplasm. The stabilized RNA or nucleic acid molecules provided herein demonstrate longer half-lives relative to their naturally occurring, unmodified counterparts (e.g. the wild-type version of the molecule). Also contemplated by the terms "modification" and "modified" as such terms related to the mRNA of the LNP compositions disclosed herein are alterations which improve or enhance translation of mRNA nucleic acids, including for example, the inclusion of sequences which function in the initiation of protein translation (e.g., the Kozak consensus sequence). (Kozak, M., Nucleic Acids Res 15 (20): 8125-48 (1987)).

In some embodiments, the RNA or nucleic acid has undergone a chemical or biological modification to render it more stable. Exemplary modifications to an RNA or nucleic acid include the depletion of a base (e.g., by deletion or by the substitution of one nucleotide for another) or modification of a base, for example, the chemical modification of a base. The phrase "chemical modifications" as used herein, includes modifications which introduce chemistries which differ from those seen in naturally occurring RNA or nucleic acids, for example, covalent modifications such as the introduction of modified nucleotides, (e.g., nucleotide analogs, or the inclusion of pendant groups which are not naturally found in such RNA, such as a deoxynucleoside, or nucleic acid molecules).

In some embodiments of a backbone modification, the phosphate group of a modified residue can be modified by replacing one or more of the oxygens with a different substituent. Further, the modified residue, *e.g.*, modified residue present in a modified nucleic acid, can include the wholesale replacement of an unmodified phosphate moiety with a modified phosphate group as described herein. In some embodiments, the backbone modification of the

phosphate backbone can include alterations that result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

In some embodiments, a composition or formulation disclosed herein comprises an mRNA comprising an open reading frame (ORF) encoding an RNA-guided DNA-binding agent, such as a Cas nuclease, or Class 2 Cas nuclease as described herein. In some embodiments, an mRNA comprising an ORF encoding an RNA-guided DNA-binding agent, such as a Cas nuclease or Class 2 Cas nuclease, is provided, used, or administered. An mRNA may comprise one or more of a 5' cap, a 5' untranslated region (UTR), a 3' UTRs, and a polyadenine tail. The mRNA may comprise a modified open reading frame, for example to encode a nuclear localization sequence or to use alternate codons to encode the protein.

The mRNA in the disclosed LNP compositions may encode a cell surface or intracellular polypeptide. The mRNA in the disclosed LNP compositions may encode, for example, a secreted hormone, enzyme, receptor, polypeptide, peptide or other protein of interest that is normally secreted. In some embodiments, the mRNA may optionally have chemical or biological modifications which, for example, improve the stability and/or half-life of such mRNA or which improve or otherwise facilitate protein production.

In addition, suitable modifications include alterations in one or more nucleotides of a codon such that the codon encodes the same amino acid but is more stable than the codon found in the wild-type version of the mRNA. For example, an inverse relationship between the stability of RNA and a higher number cytidines (C's) and/or uridines (U's) residues has been demonstrated, and RNA devoid of C and U residues have been found to be stable to most RNases (Heidenreich, et al. J Biol Chem 269, 2131-8 (1994)). In some embodiments, the number of C and/or U residues in an mRNA sequence is reduced. In another embodiment, the number of C and/or U residues is reduced by substitution of one codon encoding a particular amino acid for another codon encoding the same or a related amino acid. Contemplated modifications to the mRNA nucleic acids also include the incorporation of pseudouridines. The incorporation of pseudouridines into the mRNA nucleic acids may enhance stability and translational capacity, as well as diminishing immunogenicity in vivo. See, e.g., Karikó, K., et al., Molecular Therapy 16 (11): 1833-1840 (2008). Substitutions and modifications to the mRNA may be performed by methods readily known to one or ordinary skill in the art.

The constraints on reducing the number of C and U residues in a sequence will likely be greater within the coding region of an mRNA, compared to an untranslated region, (i.e., it will likely not be possible to eliminate all of the C and U residues present in the message while still retaining the ability of the message to encode the desired amino acid sequence). The degeneracy of the genetic code, however presents an opportunity to allow the number of C and/or U residues that are present in the sequence to be reduced, while maintaining the same coding capacity (i.e., depending on which amino acid is encoded by a codon, several different possibilities for modification of RNA sequences may be possible).

The term modification also includes, for example, the incorporation of non-nucleotide linkages or modified nucleotides into the mRNA sequences (e.g., modifications to one or both the 3′ and 5′ ends of an mRNA molecule encoding a functional secreted protein or enzyme). Such modifications include the addition of bases to an mRNA sequence (e.g., the inclusion of a poly A tail or a longer poly A tail), the alteration of the 3′ UTR or the 5′ UTR, complexing the mRNA with an agent (e.g., a protein or a complementary nucleic acid molecule), and inclusion of elements which change the structure of an mRNA molecule (e.g., which form secondary structures).

The poly A tail is thought to stabilize natural messengers. Therefore, a long poly A tail may be added to an mRNA molecule thus rendering the mRNA more stable. Poly A tails can be added using a variety of art-recognized techniques. For example, long poly A tails can be added to synthetic or in vitro transcribed mRNA using poly A polymerase (Yokoe, et al. Nature Biotechnology. 1996; 14: 1252-1256). A transcription vector can also encode long poly A tails. In addition, poly A tails can be added by transcription directly from PCR products. In some embodiments, the length of the poly A tail is at least about 90, 200, 300, 400 at least 500 nucleotides. In certain embodiments, the length of the poly A tail is adjusted to control the stability of a modified mRNA molecule and, thus, the transcription of protein. For example, since the length of the poly A tail can influence the half-life of an mRNA molecule, the length of the poly A tail can be adjusted to modify the level of resistance of the mRNA to nucleases and thereby control the time course of protein expression in a cell. In some embodiments, the stabilized mRNA molecules are sufficiently resistant to in vivo degradation (e.g., by nucleases), such that they may be delivered to the target cell without a transfer vehicle.

In certain embodiments, an mRNA can be modified by the incorporation 3′ and/or 5′ untranslated (UTR) sequences which are not naturally found in the wild-type mRNA. In some embodiments, 3′ and/or 5′ flanking sequence which naturally flanks an mRNA and encodes a second, unrelated protein can be incorporated into the nucleotide sequence of an mRNA molecule encoding a therapeutic or functional protein in order to modify it. For example, 3′ or 5′ sequences from mRNA molecules which are stable (e.g., globin, actin, GAPDH, tubulin, histone, or citric acid cycle enzymes) can be incorporated into the 3′ and/or 5′ region of a sense mRNA nucleic acid molecule to increase the stability of the sense mRNA molecule. See, e.g., US2003/0083272.

More detailed descriptions of the mRNA modifications can be found in US2017/0210698A1, at pages 57-68, the contents of which are incorporated herein.

In some embodiments, the nucleic acid is purified. In some embodiments, the nucleic acid is purified using a precipitation method (*e.g.*, LiCl precipitation, alcohol precipitation, or an equivalent method, *e.g.*, as described herein). In some embodiments, the nucleic acid is purified using a chromatography-based method, such as an HPLC-based method or an equivalent method (*e.g.*, as described herein). In some embodiments, the nucleic acid is purified using both a precipitation method (*e.g.*, LiCl precipitation) and an HPLC-based method. In some embodiments, the nucleic acid is purified by tangential flow filtration (TFF).

Pharmaceutical Compositions

The ionizable lipids prepared by the disclosed methods can be useful in pharmaceutical compositions or formulations used for delivery of biologically active agents. For pharmaceutical use, the lipid compositions of the invention may be administered by enteral or parenteral routes, including intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), oral, intranasal, rectal, vaginal, buccal, nasopharangeal, gastrointestinal or sublingual administration. The administration may be systemic (e.g., IV) or local (e.g., IM, SC, TD, intranasal, or topical). Topical administration may involve, e.g., catheterization, implantation, osmotic pumping, direct injection, dermal/transdermal application, stenting, ear/eye drops or portal vein administration. The compositions of the invention will generally, but not necessarily, be administered as a formulation in association with one or more pharmaceutically acceptable excipients. An

excipient may impart either a functional (e.g drug release rate controlling) and/or a non-functional (e.g. processing aid or diluent) characteristic to the formulations. The choice of excipient will to a large extent depend on factors such as the particular mode of administration, the effect of the excipient on solubility and stability, and the nature of the dosage form.

The lipid compositions of the invention can be formulated as pharmaceutical compositions suitable for delivery to a subject. The pharmaceutical compositions of the invention will often further comprise one or more buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose, dextrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

In some embodiments, one or more excipients or accessory ingredients may make up greater than 50% of the total mass or volume of a pharmaceutical composition including a nanoparticle composition. For example, the one or more excipients or accessory ingredients may make up 50%, 60%, 70%, 80%, 90%, or more of a pharmaceutical convention. In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

Relative amounts of the one or more lipid nanoparticles, the one or more pharmaceutically acceptable excipients, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, a pharmaceutical composition may comprise between 0.1 % and 100% (wt/wt) of one or more lipid nanoparticles. As another

example, a pharmaceutical composition may comprise between 0.1 % and 15% (wt/vol) of one or more amphiphilic polymers (e.g., 0.5%, 1 %, 2.5%, 5%, 10%, or 12.5% w/v).

In certain embodiments, the lipid nanoparticles and/or pharmaceutical compositions of the disclosure are refrigerated or frozen for storage and/or shipment (e.g., being stored at a temperature of 4 °C or lower, such as a temperature between about -150 °C and about 0 °C or 25 between about -80 °C and about -20 °C (e.g., about -5 °C, -10 °C, -15 °C, -20 °C, -25 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, -130 °C or -150 °C). For example, the pharmaceutical composition comprising one or more lipid nanoparticles is a solution or solid (e.g., via lyophilization) that is refrigerated for storage and/or shipment at, for example, about -20 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, or -80 °C. In certain embodiments, the disclosure 30 also relates to a method of increasing stability of the lipid nanoparticles and by storing the lipid nanoparticles and/or pharmaceutical compositions thereof at a temperature of 4 °C or lower, such as a temperature between about -150 °C and about 0 °C or between about -80 °C and about -20 °C, e.g., about -5 °C, -10 °C, -15 °C, -20 °C, -25 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, -130 °C or -150 °C).

Pharmaceutical compositions including one or more nanoparticle compositions may be administered to any patient or subject, including those patients or subjects that may benefit from a therapeutic effect provided by the delivery of a therapeutic and/or prophylactic to one or more particular cells, tissues, organs, or systems or groups thereof, such as the renal system. Although the descriptions provided herein of pharmaceutical compositions are principally directed to compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other mammal. Modification of compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the compositions is contemplated include, but are not limited to, humans, other primates, and other mammals, including commercially relevant mammals such as cattle, pigs, hoses, sheep, cats, dogs, mice, and/or rats.

A pharmaceutical composition including one or more lipid nanoparticles may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if desirable or necessary, dividing, shaping, and/or packaging the product into a desired single- or multi-dose unit.

A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient *(e.g.,* lipid nanoparticle). The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

Uses in Gene Editing

The LNP compositions disclosed herein may be used for gene editing *in vivo* and *in vitro*. In one embodiment, one or more LNP compositions described herein may be administered to a subject in need thereof. In one embodiment, one or more LNP compositions described herein may contact a cell. In one embodiment, a therapeutically effective amount of a composition described herein may contact a cell of a subject in need thereof. In one embodiment, a genetically engineered cell may be produced by contacting a cell with an LNP composition described herein. In various embodiments, the methods comprise introducing a template nucleic acid to a cell or subject, as set forth above.

In some embodiments, the methods involve administering the LNP composition to a cell associated with a liver disorder. In some embodiments, the methods involve treating a liver disorder. In certain embodiments, the methods involve contacting a hepatic cell with the LNP composition. In certain embodiments, the methods involve contacting a hepatocyte with the LNP composition. In some embodiments, the methods involve contacting an ApoE binding cell with the LNP composition.

In one embodiment, an LNP composition comprising an mRNA encoding a Class 2 Cas nuclease and a gRNA may be administered to a cell, such as an ApoE binding cell. In additional

embodiments, a template nucleic acid is also introduced to the cell. In certain instances, an LNP composition comprising a Class 2 Cas nuclease and an sgRNA may be administered to a cell, such as an ApoE binding cell. In one embodiment, an LNP composition comprising an mRNA encoding a Class 2 Cas nuclease, a gRNA, and a template may be administered to a cell. In certain instances, an LNP composition comprising a Cas nuclease and an sgRNA may be administered to a liver cell. In some cases, the liver cell is in a subject.

In certain embodiments, a subject may receive a single dose of an LNP composition. In other examples, a subject may receive multiple doses of an LNP composition. In some embodiments, the LNP composition is administered 2-5 times. Where more than one dose is administered, the doses may be administered about 1, 2, 3, 4, 5, 6, 7, 14, 21, or 28 days apart; about 2, 3, 4, 5, or 6 months apart; or about 1, 2, 3, 4, or 5 years apart. In certain embodiments, editing improves upon readministration of an LNP composition.

In one embodiment, an LNP composition comprising an mRNA encoding a Cas nuclease such as a Class 2 Cas nuclease, may be administered to a cell, separately from the administration of a composition comprising a gRNA. In one embodiment, an LNP composition comprising an mRNA encoding a Cas nuclease such as a Class 2 Cas nuclease and a gRNA may be administered to a cell, separately from the administration of a template nucleic acid to the cell. In one embodiment, an LNP composition comprising an mRNA encoding a Cas nuclease such as a Class 2 Cas nuclease may be administered to a cell, followed by the sequential administration of an LNP composition comprising a gRNA and then a template to the cell. In embodiments where an LNP composition comprising an mRNA encoding a Cas nuclease is administered before an LNP composition comprising a gRNA, the administrations may be separated by about 4, 6, 8, 12, or 24 hours; or 2, 3, 4, 5, 6, or 7 days.

In some embodiments, the cell is an immune cell. As used herein, "immune cell" refers to a cell of the immune system, including *e.g.*, a lymphocyte (*e.g.*, T cell, B cell, natural killer cell ("NK cell", and NKT cell, or iNKT cell)), monocyte, macrophage, mast cell, dendritic cell, or granulocyte (*e.g.*, neutrophil, eosinophil, and basophil). In some embodiments, the cell is a primary immune cell. In some embodiments, the immune system cell may be selected from CD3+, CD4+ and CD8+ T cells, regulatory T cells (Tregs), B cells, NK cells, and dendritic cells (DC). In some embodiments, the immune cell is allogeneic. In some embodiments, the cell is a

lymphocyte. In some embodiments, the cell is an adaptive immune cell. In some embodiments, the cell is a T cell. In some embodiments, the cell is a B cell. In some embodiments, the cell is a NK cell.

As used herein, a T cell can be defined as a cell that expresses a T cell receptor ("TCR" or "αβ TCR" or "γδ TCR"), however in some embodiments, the TCR of a T cell may be genetically modified to reduce its expression (*e.g.*, by genetic modification to the TRAC or TRBC genes), therefore expression of the protein CD3 may be used as a marker to identify a T cell by standard flow cytometry methods. CD3 is a multi-subunit signaling complex that associates with the TCR. Thus, a T cell may be referred to as CD3+. In some embodiments, a T cell is a cell that expresses a CD3+ marker and either a CD4+ or CD8+ marker.

In some embodiments, the T cell expresses the glycoprotein CD8 and therefore is CD8+ by standard flow cytometry methods and may be referred to as a "cytotoxic" T cell. In some embodiments, the T cell expresses the glycoprotein CD4 and therefore is CD4+ by standard flow cytometry methods and may be referred to as a "helper" T cell. CD4+ T cells can differentiate into subsets and may be referred to as a Th1 cell, Th2 cell, Th9 cell, Th17 cell, Th22 cell, T regulatory ("Treg") cell, or T follicular helper cells ("Tfh"). Each CD4+ subset releases specific cytokines that can have either proinflammatory or anti-inflammatory functions, survival or protective functions. A T cell may be isolated from a subject by CD4+ or CD8+ selection methods.

In some embodiments, the T cell is a memory T cell. In the body, a memory T cell has encountered antigen. A memory T cell can be located in the secondary lymphoid organs (central memory T cells) or in recently infected tissue (effector memory T cells). A memory T cell may be a CD8+ T cell. A memory T cell may be a CD4+ T cell. As used herein, a "central memory T cell" can be defined as an antigen-experienced T cell, and for example, may expresses CD62L and CD45RO. A central memory T cell may be detected as CD62L+ and CD45RO+ by Central memory T cells also express CCR7, therefore may be detected as CCR7+ by standard flow cytometry methods.

As used herein, an "early stem-cell memory T cell" (or "Tscm") can be defined as a T cell that expresses CD27 and CD45RA, and therefore is CD27+ and CD45RA+ by standard flow cytometry methods. A Tscm does not express the CD45 isoform CD45RO, therefore a Tscm will

further be CD45RO- if stained for this isoform by standard flow cytometry methods. A CD45RO- CD27+ cell is therefore also an early stem-cell memory T cell. Tscm cells further express CD62L and CCR7, therefore may be detected as CD62L+ and CCR7+ by standard flow cytometry methods. Early stem-cell memory T cells have been shown to correlate with increased persistence and therapeutic efficacy of cell therapy products.

In some embodiments, the cell is a B cell. As used herein, a "B cell" can be defined as a cell that expresses CD19 and/or CD20, and/or B cell mature antigen ("BCMA"), and therefore a B cell is CD19+, and/or CD20+, and/or BCMA+ by standard flow cytometry methods. A B cell is further negative for CD3 and CD56 by standard flow cytometry methods. The B cell may be a plasma cell. The B cell may be a memory B cell. The B cell may be a naïve B cell. The B cell may be IgM+ or has a class-switched B cell receptor (*e.g.*, IgG+, or IgA+).

Cells used in ACT therapy are included, such as mesenchymal stem cells (*e.g.*, isolated from bone marrow (BM), peripheral blood (PB), placenta, umbilical cord (UC) or adipose); hematopoietic stem cells (HSCs; *e.g.* isolated from BM); mononuclear cells (*e.g.*, isolated from BM or PB); endothelial progenitor cells (EPCs; isolated from BM, PB, and UC); neural stem cells (NSCs); limbal stem cells (LSCs); or tissue-specific primary cells or cells derived therefrom (TSCs). Cells used in ACT therapy further include induced pluripotent stem cells (iPSCs) that may be induced to differentiate into other cell types including *e.g.*, islet cells, neurons, and blood cells; ocular stem cells; pluripotent stem cells (PSCs); embryonic stem cells (ESCs); cells for organ or tissue transplantations such as islet cells, cardiomyocytes, thyroid cells, thymocytes, neuronal cells, skin cells, retinal cells, chondrocytes, myocytes, and keratinocytes.

In some embodiments, the cell is a human cell, such as a cell from a subject. In some embodiments, the cell is isolated from a human subject, such as a human donor. In some embodiments, the cell is isolated from human donor PBMCs or leukopaks. In some embodiments, the cell is from a subject with a condition, disorder, or disease. In some embodiments, the cell is from a human donor with Epstein Barr Virus ("EBV").

In some embodiments, the cell is a mononuclear cell, such as from bone marrow or peripheral blood. In some embodiments, the cell is a peripheral blood mononuclear cell ("PBMC"). In some embodiments, the cell is a PBMC, *e.g.* a lymphocyte or monocyte. In some embodiments, the cell is a peripheral blood lymphocyte ("PBL").

In some embodiments, the methods are carried out *ex vivo*. As used herein, "*ex vivo*" refers to an *in vitro* method wherein the cell is capable of being transferred into a subject, *e.g.*, as an ACT therapy. In some embodiments, an *ex vivo* method is an *in vitro* method involving an ACT therapy cell or cell population.

In some embodiments, the cell is maintained in culture. In some embodiments, the cell is transplanted into a patient. In some embodiments, the cell is removed from a subject, genetically modified *ex vivo*, and then administered back to the same patient. In some embodiments, the cell is removed from a subject, genetically modified *ex vivo*, and then administered to a subject other than the subject from which it was removed.

In some embodiments, the cell is from a cell line. In some embodiments, the cell line is derived from a human subject. In some embodiments, the cell line is a lymphoblastoid cell line ("LCL"). The cell may be cryopreserved and thawed. The cell may not have been previously cryopreserved.

In some embodiments, the cell is from a cell bank. In some embodiments, the cell is genetically modified and then transferred into a cell bank. In some embodiments the cell is removed from a subject, genetically modified *ex vivo*, and transferred into a cell bank. In some embodiments, a genetically modified population of cells is transferred into a cell bank. In some embodiments, a genetically modified population of immune cells is transferred into a cell bank. In some embodiments, a genetically modified population of immune cells comprising a first and second subpopulations, wherein the first and second sub-populations have at least one common genetic modification and at least one different genetic modification are transferred into a cell bank.

In some embodiments, the T cell is activated by polyclonal activation (or "polyclonal stimulation") (not antigen-specific stimulation). In some embodiments, the T cell is activated by CD3 stimulation (*e.g.*, providing an anti-CD3 antibody). In some embodiments, the T cell is activated by CD3 and CD28 stimulation (*e.g.*, providing an anti-CD3 antibody and an anti-CD28 antibody). In some embodiments, the T cell is activated using a ready-to-use reagent to activate the T cell (*e.g.*, via CD3/CD28 stimulation). In some embodiments, the T cell is activated by via CD3/CD28 stimulation provided by beads. In some embodiments, the T cell is activated by via CD3/CD28 stimulation wherein one or more components is soluble and/or one or more

components is bound to a solid surface (*e.g.*, plate or bead). In some embodiments, the T cell is activated by an antigen-independent mitogen (*e.g.*, a lectin, including *e.g.*, concanavalin A ("ConA"), or PHA).

In some embodiments, one or more cytokines are used for activation of T cells. IL-2 is provided for T cell activation and/or to promote T cell survival. In some embodiments, the cytokine(s) for activation of T cells is a cytokine that binds to the common gamma chain (γc) receptor. In some embodiments, IL-2 is provided for T cell activation. In some embodiments, IL-7 is provided for T cell activation. In some embodiments, IL-15 is provided for T cell activation. In some embodiments, IL-21 is provided for T cell activation. In some embodiments, a combination of cytokines is provided for T cell activation, including, *e.g.*, IL-2, IL-7, IL-15, and/or IL-21.

In some embodiments, the T cell is activated by exposing the cell to an antigen (antigen stimulation). A T cell is activated by antigen when the antigen is presented as a peptide in a major histocompatibility complex ("MHC") molecule (peptide-MHC complex). A cognate antigen may be presented to the T cell by co-culturing the T cell with an antigen-presenting cell (feeder cell) and antigen. In some embodiments, the T cell is activated by co-culture with an antigen-presenting cell that has been pulsed with antigen. In some embodiments, the antigen-presenting cell has been pulsed with a peptide of the antigen.

In some embodiments, the T cell may be activated for 12 to 72 hours. In some embodiments, the T cell may be activated for 12 to 48 hours. In some embodiments, the T cell may be activated for 12 to 24 hours. In some embodiments, the T cell may be activated for 24 to 48 hours. In some embodiments, the T cell may be activated for 24 to 72 hours. In some embodiments, the T cell may be activated for 12 hours. In some embodiments, the T cell may be activated for 48 hours. In some embodiments, the T cell may be activated for 72 hours.

In one embodiment, the LNP compositions may be used to edit a gene resulting in a gene knockout. In an embodiment, the LNP compositions may be used to edit a gene resulting in gene knockdown in a population of cells. In another embodiment, the LNP compositions may be used to edit a gene resulting in a gene correction. In a further embodiment, the LNP compositions may be used to edit a cell resulting in gene insertion.

In one embodiment, administration of the LNP compositions may result in gene editing which results in persistent response. For example, administration may result in a duration of response of a day, a month, a year, or longer. As used herein, “duration of response” means that, after cells have been edited using an LNP composition disclosed herein, the resulting modification is still present for a certain period of time after administration of the LNP composition. The modification may be detected by measuring target protein levels. The modification may be detected by detecting the target DNA. In some embodiments, the duration of response may be at least 1 week. In other embodiments, the duration of response may be at least 2 weeks. In one embodiment, the duration of response may be at least 1 month. In some embodiments, the duration of response may be at least 2 months. In one embodiment, the duration of response may be at least 4 months. In one embodiment, the duration of response may be at least 6 months. In certain embodiments, the duration of response may be about 26 weeks. In some embodiments, the duration of response may be at least 1 year. In some embodiments, the duration of response may be at least 5 years. In some embodiments, the duration of response may be at least 10 years. In some embodiments, a persistent response is detectable after at least 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, or 24 months, either by measuring target protein levels or by detection of the target DNA. In some embodiments, a persistent response is detectable after at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, or 20 years, either by measuring target protein levels or by detection of the target DNA.

The LNP compositions can be administered parenterally. The LNP compositions may be administered directly into the blood stream, into tissue, into muscle, or into an internal organ. Administration may be systemic, *e.g.*, to injection or infusion. Administration may be local. Suitable means for administration include intravenous, intraarterial, intrathecal, intraventricular, intraurethral, intrasternal, intracranial, subretinal, intravitreal, intra-anterior chamber, intramuscular, intrasynovial, intradermal, and subcutaneous. Suitable devices for administration include needle (including microneedle) injectors, needle-free injectors, osmotic pumps, and infusion techniques.

The LNP compositions will generally, but not necessarily, be administered as a formulation in association with one or more pharmaceutically acceptable excipients. The term "excipient" includes any ingredient other than the compound(s) of the disclosure, the other lipid

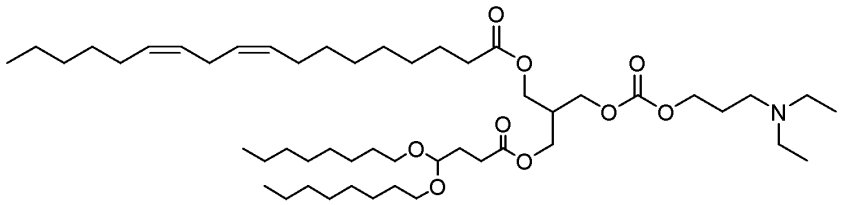
component(s) and the biologically active agent. An excipient may impart either a functional (*e.g.* drug release rate controlling) and/or a non-functional (*e.g.* processing aid or diluent) characteristic to the formulations. The choice of excipient will to a large extent depend on factors such as the particular mode of administration, the effect of the excipient on solubility and stability, and the nature of the dosage form.

Parenteral formulations are typically aqueous or oily solutions or suspensions. Where the formulation is aqueous, excipients such as sugars (including but not restricted to glucose, mannitol, sorbitol, *etc.)* salts, carbohydrates and buffering agents (preferably to a pH of from 3 to 9), but, for some applications, they may be more suitably formulated with a sterile non-aqueous solution or as a dried form to be used in conjunction with a suitable vehicle such as sterile, pyrogen-free water (WFI).

Embodiments

In certain aspects, provided herein, the disclosure relates to a composition, as determined by ultra-performance liquid chromatography (UPLC), comprising:

at least 90% of Lipid A



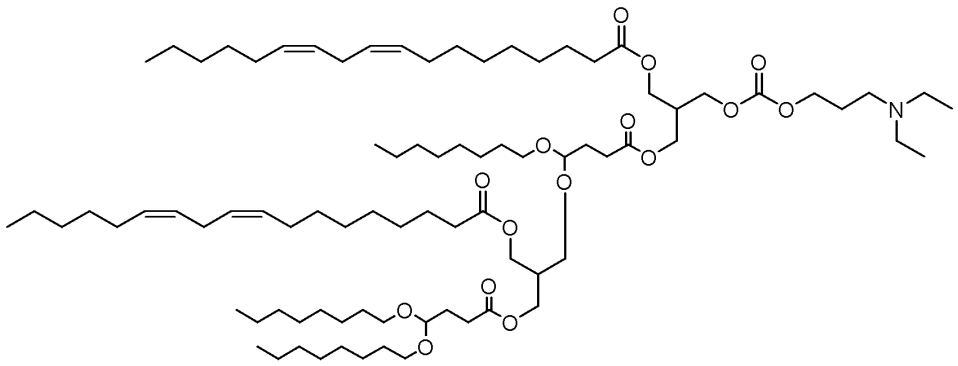
Lipid A.

In certain preferred embodiments, the composition comprises at least 91% of Lipid A. In certain preferred embodiments, the composition comprises at least 92% Lipid A. In certain preferred embodiments, the composition comprises at least 93% Lipid A. In certain preferred embodiments, the composition comprises at least 94% Lipid A. In certain preferred embodiments, the composition comprises at least 95% Lipid A. In certain preferred embodiments, the composition comprises at least 96% Lipid A. In certain preferred embodiments, the composition comprises at least 97% Lipid A. In certain preferred

embodiments, the composition comprises at least 98% Lipid A . In certain preferred embodiments, the composition comprises at least 99% Lipid A.

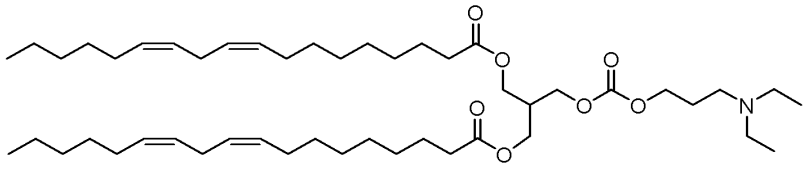
In one embodiment, the composition comprising Lipid A is substantially free of impurities. In certain embodiments, the disclosure relates to any one of the compositions described herein, wherein the composition comprises:

(A) less than about 1.1%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (V)



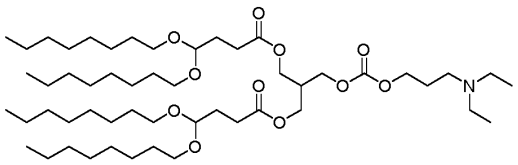
(V), or

(B) less than about 0.6% or less than about 0.5% of a compound having the structure of formula (VI)



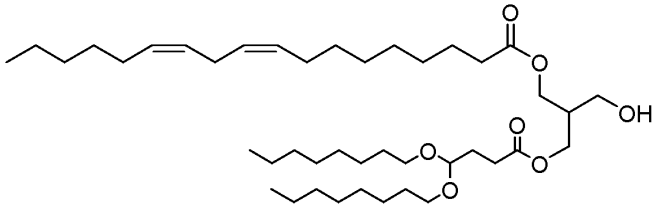
(VI), or

(C) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, or less than about 0.2%, of a compound having the structure of formula (VII)



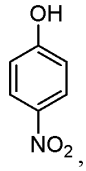
(VII), or

(D) less than about 0.3% or less than 0.2% of a compound having the structure of formula (III)



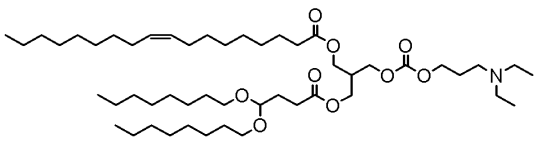
(III),

(E) less than about 190 ppm, less than about 180 ppm, less than about 170 ppm, less than about 160 ppm, less than about 150 ppm, less than about 140 ppm, less than about 130 ppm, less than about 120 ppm, less than about 110 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



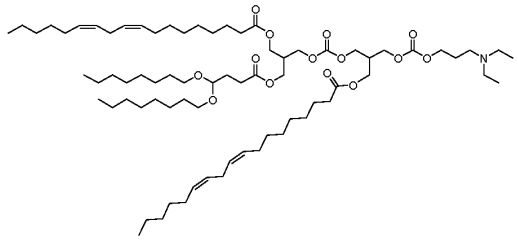
, or

(F) less than about 0.4% or less than about 0.3% of a compound having the structure of formula (VIII)



(VIII), and/or

(G) less than about 0.2% or less than 0.1% of a compound having the structure of formula (IX)



(IX).

In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (V), preferably less than about 0.2% of the compound of formula (V), preferably less than about 0.1% of the compound of formula (V), even more preferably wherein the compound of formula (V) is undetectable. In certain embodiments, the composition comprises less than about 1.0% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.9% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.8% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.7% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.6% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.5% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (V).

In certain embodiments, the composition comprises about 1.0% of the compound of formula (V). In certain embodiments, the composition comprises about 0.9% of the compound of formula (V). In certain embodiments, the composition comprises about 0.8% of the compound of formula (V). In certain embodiments, the composition comprises about 0.7% of the compound of formula (V). In certain embodiments, the composition comprises about 0.6% of the compound of formula (V). In certain embodiments, the composition comprises about 0.5% of the compound of

formula (V). In certain embodiments, the composition comprises about 0.4% of the compound of formula (V). In certain embodiments, the composition comprises about 0.3% of the compound of formula (V). In certain embodiments, the composition comprises about 0.2% of the compound of formula (V). In certain embodiments, the composition comprises about 0.1% of the compound of formula (V). In certain embodiments, the composition is essentially free of the compound of formula (V).

In certain embodiments, the composition comprises less than about 0.55% of the compound of formula (VI), even more preferably less than about 0.5% of the compound of formula (VI).In certain embodiments, the composition comprises less than about 0.6% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.5% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.45% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.35% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (VI).

In certain embodiments, the composition comprises about 0.6% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.5% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.45% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.4% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.35% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.3% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.25% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.2% of the compound of formula (VI). In certain embodiments, the

composition comprises about 0.15% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.1% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.05% of the compound of formula (VI). In certain embodiments, the composition is essentially free of the compound of formula (VI).

In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (VII), preferably less than about 0.3% of the compound of formula (VII), even more preferably less than about 0.2% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.35% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (VII).

In certain embodiments, the composition comprises about 0.4% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.35% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.3% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.25% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.2% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.15% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.1% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.05% of the compound of formula (VII). In certain embodiments, the composition is essentially free of the compound of formula (VII).

In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (III), preferably less than about 0.2% of the compound of formula (III), even more preferably less than about 0.2% of the compound of formula (III). In certain

embodiments, the composition comprises less than about 0.2% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (III).

In certain embodiments, the composition comprises about 0.2% of the compound of formula (III). In certain embodiments, the composition comprises about 0.15% of the compound of formula (III). In certain embodiments, the composition comprises about 0.1% of the compound of formula (III). In certain embodiments, the composition comprises about 0.05% of the compound of formula (III). In certain embodiments, the composition is essentially free of the compound of formula (III).

In certain embodiments, the composition comprises less than about 190 ppm pNP. In certain embodiments, the composition comprises less than about 180 ppm pNP. In certain embodiments, the composition comprises less than about 170 ppm pNP. In certain embodiments, the composition comprises less than about 160 ppm pNP. In certain embodiments, the composition comprises less than about 150 ppm pNP. In certain embodiments, the composition comprises less than about 140 ppm pNP. In certain embodiments, the composition comprises less than about 130 ppm pNP. In certain embodiments, the composition comprises less than about 120 ppm pNP. In certain embodiments, the composition comprises less than about 110 ppm pNP. In certain embodiments, the composition comprises less than about 100 ppm pNP. In certain embodiments, the composition comprises less than about 90 ppm pNP. In certain embodiments, the composition comprises less than about 80 ppm pNP. In certain embodiments, the composition comprises less than about 70 ppm pNP. In certain embodiments, the composition comprises less than about 60 ppm pNP. In certain embodiments, the composition comprises less than about 50 ppm pNP. In certain embodiments, the composition comprises less than about 45 ppm pNP. In certain embodiments, the composition comprises less than about 40 ppm pNP. In certain embodiments, the composition comprises less than about 35 ppm pNP. In certain embodiments, the composition comprises less than about 30 ppm pNP. In certain embodiments, the composition comprises less than about 25 ppm pNP. In certain embodiments, the composition comprises less than about 20 ppm pNP. In certain embodiments, the composition

comprises less than about 15 ppm pNP. In certain embodiments, the composition comprises less than about 10 ppm pNP. In certain embodiments, the composition comprises less than about 5 ppm pNP. In certain embodiments, the composition comprises less than about 2 ppm pNP.

In certain embodiments, the composition comprises about 190 ppm pNP. In certain embodiments, the composition comprises about 180 ppm pNP. In certain embodiments, the composition comprises about 170 ppm pNP. In certain embodiments, the composition comprises about 160 ppm pNP. In certain embodiments, the composition comprises about 150 ppm pNP. In certain embodiments, the composition comprises about 140 ppm pNP. In certain embodiments, the composition comprises about 130 ppm pNP. In certain embodiments, the composition comprises about 120 ppm pNP. In certain embodiments, the composition comprises about 110 ppm pNP. In certain embodiments, the composition comprises about 100 ppm pNP. In certain embodiments, the composition comprises about 90 ppm pNP. In certain embodiments, the composition comprises about 80 ppm pNP. In certain embodiments, the composition comprises about 70 ppm pNP. In certain embodiments, the composition comprises about 60 ppm pNP. In certain embodiments, the composition comprises about 50 ppm pNP. In certain embodiments, the composition comprises about 45 ppm pNP. In certain embodiments, the composition comprises about 40 ppm pNP. In certain embodiments, the composition comprises about 35 ppm pNP. In certain embodiments, the composition comprises about 30 ppm pNP. In certain embodiments, the composition comprises about 25 ppm pNP. In certain embodiments, the composition comprises about 20 ppm pNP. In certain embodiments, the composition comprises about 15 ppm pNP. In certain embodiments, the composition comprises about 10 ppm pNP. In certain embodiments, the composition comprises about 5 ppm pNP. In certain embodiments, the composition comprises about 2 ppm pNP. In certain embodiments, the composition comprises pNP is undetectable.

In certain embodiments, the composition comprises less than about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.15% of a compound of formula (VIII). In certain embodiments, the

composition comprises less than about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (VIII).

In certain embodiments, the composition comprises about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.15% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.05% of a compound of formula (VIII). In certain embodiments, the composition is essentially free of a compound of formula (VIII).

In certain embodiments, the composition comprises less than about 0.1% of a compound of formula (IX)preferably when a compound of formula (IX)is undetectable. In certain embodiments, the composition comprises less than less than about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.09% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.06% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.04% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.01% of a compound of formula (IX).

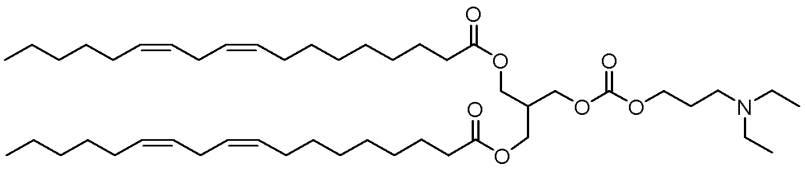
In certain embodiments, the composition comprises about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.09% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.06% of a compound of

formula (IX). In certain embodiments, the composition comprises about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.04% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.01% of a compound of formula (IX). In certain embodiments, the composition is essentially free of a compound of formula (IX).

In certain embodiments, the composition comprises any two of (A)-(G), as outlined above. In certain embodiments, the composition comprises any three of (A)-(G). In certain embodiments, the composition comprises any four of (A)-(G). In certain embodiments, the composition comprises any five of (A)-(G). In certain embodiments, the composition comprises any six of (A)-(G). In certain embodiments, the composition comprises all of (A)-(G).

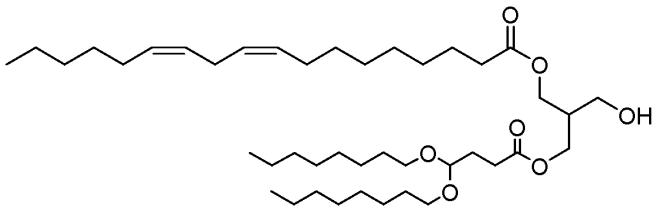
In certain embodiments, the composition comprises greater than 1 kg of Lipid A, and the composition comprises:

(A) less than about 2.7%, less than about 2.5%, less than about 2%, less than about 1.5%, less than 1%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



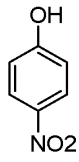
(VI), or

(B) less than about 1.3%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



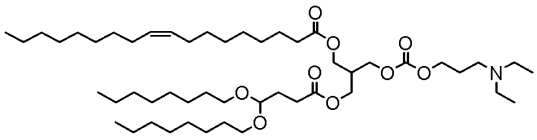
(III), or

(C) less than about 1850 ppm, less than about 1500 ppm, less than about 1250 ppm, less than about 1000 ppm, less than about 750 ppm, less than about 500 ppm, less than about 250 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



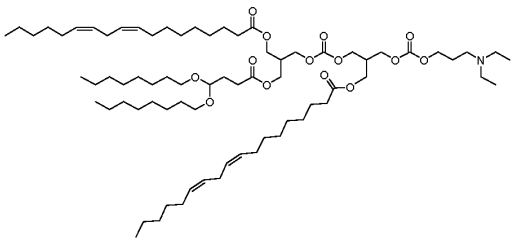
, or

(F) less than about 0.6%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (VIII)



(VIII), or

(G) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than 0.1% of a compound of formula (IX)



(IX).

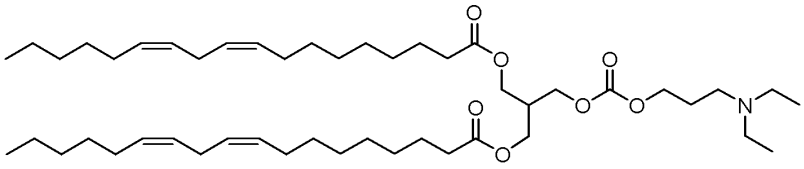
In certain embodiments, the composition comprises greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, or greater than about 5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 2 kg of Lipid A. In certain embodiments, the composition comprises greater than about 2.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 3 kg of Lipid A. In certain embodiments, the composition comprises greater than about 3.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 4 kg of Lipid A. In certain embodiments, the composition comprises greater than about 4.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 5.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 6 kg of Lipid A. In certain embodiments, the composition comprises greater than about 6.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 7 kg of Lipid A. In certain embodiments, the composition comprises greater than about 7.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 8 kg of Lipid A. In certain embodiments, the composition comprises greater than about 8.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 9 kg of Lipid A. In certain embodiments, the composition comprises greater than about 9.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 10 kg of Lipid A.

In certain embodiments, the composition comprises about 1 kg to about 10 kg, even more preferably about 1 to about 7 kg of a Lipid A. In certain embodiments, the composition comprises about 1 kg of a Lipid A. In certain embodiments, the composition comprises about 2 kg of Lipid A. In certain embodiments, the composition comprises about 3 kg of Lipid A. In certain embodiments, the composition comprises about 4 kg of Lipid A. In certain embodiments,

the composition comprises about 5 kg of Lipid A. In certain embodiments, the composition comprises about 6 kg of Lipid A. In certain embodiments, the composition comprises about 7 kg of Lipid A. In certain embodiments, the composition comprises about 8 kg of Lipid A. In certain embodiments, the composition comprises about 9 kg of Lipid A. In certain embodiments, the composition comprises about 10 kg of Lipid A.

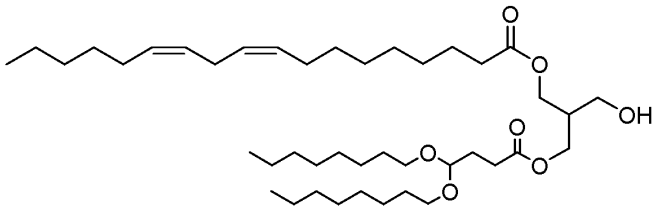
In certain embodiments, the composition comprises greater than about 2 kg, 3 kg, 4 kg, 5 kg, 6 kg, 7 kg, 8 kg, or more of Lipid A (*e.g.*, 2-8 kg Lipid A), and the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



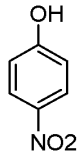
(VI), or

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



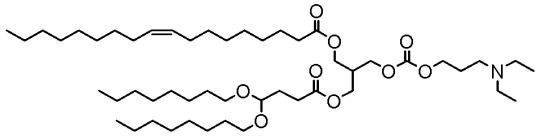
(III), or

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



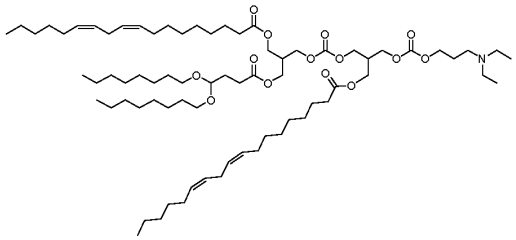
, or

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), or

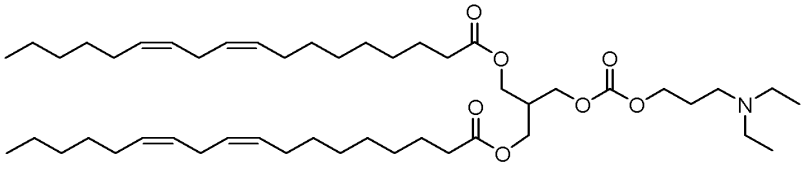
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

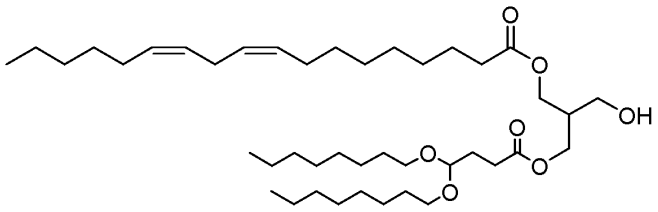
In certain embodiments, the composition comprises greater than about 6 kg of Lipid A, and the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



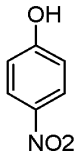
(VI), and

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



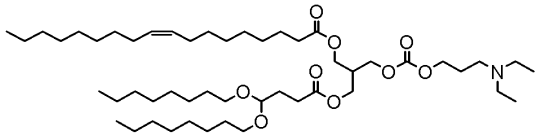
(III), and

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



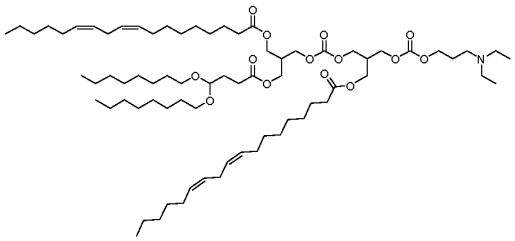
, and

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), and

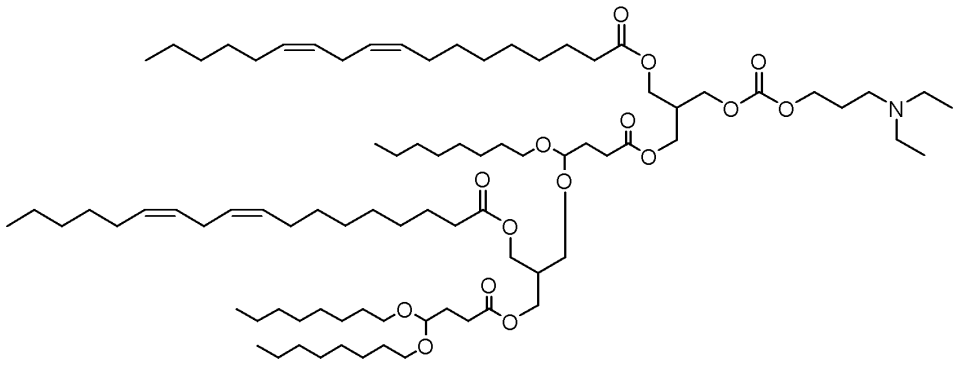
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

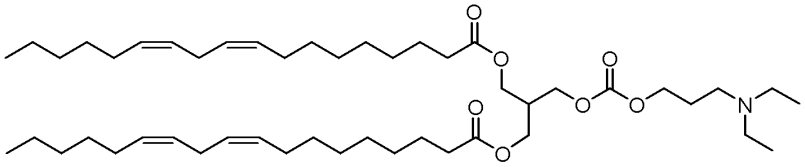
In certain embodiments, the composition comprises greater than about 6 kg of Lipid A, and the composition comprises:

(A) less than about 0.1% of a compound having the structure of formula (V)



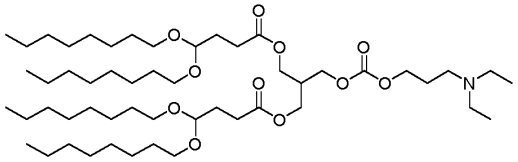
(V), and

(B) less than about 1.2% a compound having the structure of formula (VI)



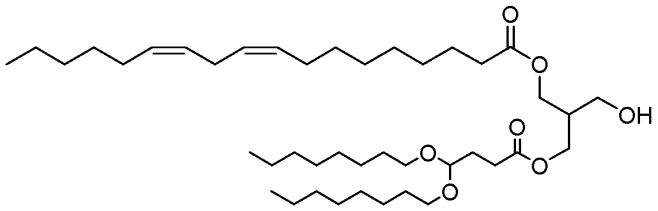
(VI), and

(C) less than about 0.3% of a compound having the structure of formula (VII)



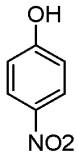
(VII), and

(C) less than about 1.0% of a compound having the structure of formula (III)



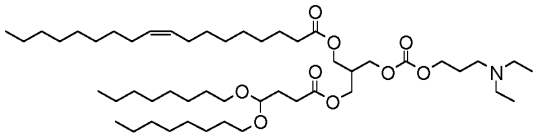
(III), and

(D) less than about 2 ppm p-nitrophenol (pNP)



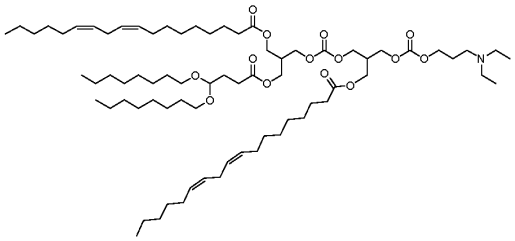
, and

(E) less than about 0.2% of a compound of formula (VIII)



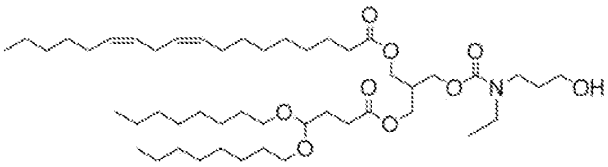
(VIII), and

(F) less than about 0.1% of a compound having the structure of formula (IX)



(IX).

In certain embodiments, the composition comprises less than about 2.5% of a compound of formula (X), preferably wherein a compound of formula (X) is undetectable



(X).

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-01 with Compound-02 at a temperature not exceeding 100°C to yield Compound-08;

b) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound 09;

c) treating Compound-09 with piperazine to yield Compound-10; and

d) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound-09;

b) treating Compound-09 with piperazine to yield Compound-10; and

c) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-09 with piperazine to yield Compound-10; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-09 with piperazine *p*-xylylenediamine to yield Compound-10; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-03 with Compound-04 and a coupling reagent to yield Compound-11;

b) treating Compound-11 with an ion exchange resin to yield Compound-12; and

c) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-11 with an ion exchange resin to yield Compound-12; and

b) generating Lipid A by combining Compound-10 and Compound-12.

In certain embodiments, the disclosure relates to a process for preparing any of the compositions described herein, comprising:

a) treating Compound-13 with *p*NPCF to generate Compound-14 *in situ*;

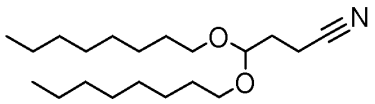
b) treating Compound-14 *in situ* with 3-dimethylamino-1-propanol;

c) extracting a reaction mixture with heptane; and

d) performing water and acetonitrile extractions to generate Lipid A.

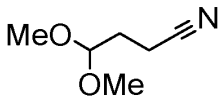
In certain embodiments, the disclosure relates to a process for preparing a composition comprising greater than about 1 kg, greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, greater than about 5 kg, or greater than about 6 kg of Lipid A. In some embodiments, the disclosure relates to a process for preparing a composition comprising greater than about 6 kg of Lipid A, for example, from about 6 kg to about 10 kg of Lipid A.

In certain embodiments, the disclosure relates to a process for preparing 4,4-bis(octyloxy)butanenitrile



,

comprising combining 4,4-dimethoxybutanenitrile



with 1-octanol to form the 4,4-bis(octyloxy)butanenitrile, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 100°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 95°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 90°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 85°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 80°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 75°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature not exceeding 70°C.

In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 40°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 45°C. In certain embodiments,

the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 50°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 55°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 60°C.

In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 40°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 45°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 50°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 55°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 60°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 65°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature of about 70°C.

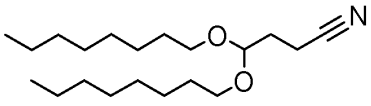
In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with an acid. In certain embodiments, the acid is pyridinium *p*-toluenesulfonate.

In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.01 to about 0.10 molar equivalents of the acid, wherein the molar equivalents are relative to the 4,4-dimethoxybutanenitrile. In certain preferred embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.05 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.01 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.02 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.03 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.04 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.05 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.06 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.07 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.08 molar equivalents of the acid. In certain embodiments, the 4,4-

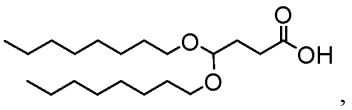
dimethoxybutanenitrile is combined with about 0.09 molar equivalents of the acid. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 0.10 molar equivalents of the acid.

In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with 2 to 2.5 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2.1 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2.2 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2.3 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2.4 molar equivalents of the 1-octanol. In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with about 2.5 molar equivalents of the 1-octanol.

In certain embodiments, the 4,4-dimethoxybutanenitrile is combined with a solvent. In certain embodiments, the solvent comprises toluene. In certain embodiments, the process further comprises purifying the 4,4-bis(octyloxy)butanenitrile by vacuum distillation. In certain embodiments, the process does not comprise purifying the 4,4-bis(octyloxy)butanenitrile by column chromatography.

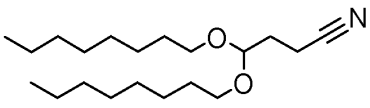
In certain embodiments, the disclosure relates to a composition comprising a compound 4,4-bis(octyloxy)butanenitrile,  wherein the composition is prepared according to the processes described herein.

In certain embodiments, the disclosure relates to a process for preparing 4,4-bis(octyloxy)butanoic acid



,

comprising hydrolyzing 4,4-bis(octyloxy)butanenitrile



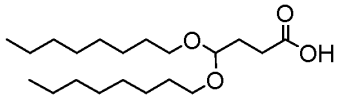
to form the 4,4-bis(octyloxy)butanoic acid, wherein the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 100°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 95°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 90°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 70°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 75°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 76°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 77°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 78°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 79°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 80°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 81°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 82°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 83°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 84°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 85°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 86°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 87°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 88°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 89°C. In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature of about 90°C.

In certain embodiments, the formation of the 4,4-bis(octyloxy)butanoic acid is carried out under basic conditions. In certain embodiments, the hydrolyzing step comprises combining the 4,4-bis(octyloxy)butanenitrile with potassium hydroxide, water, and ethanol. In certain

embodiments, the pH is adjusted to about 4 to 5 using an acid. In some embodiments, the acid is acetic acid or hydrochloric acid.

In certain embodiments, the 4,4-bis(octyloxy)butanenitrile is prepared by the processes described herein.

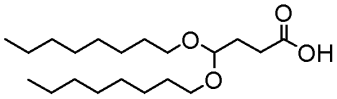
In certain embodiments, the disclosure relates to a composition comprising a compound 4,4-bis(octyloxy)butanoic acid



,

wherein the composition is prepared according to the processes described herein.

In certain embodiments, the disclosure relates to a process for preparing an amine salt of 4,4-bis(octyloxy)butanoic acid



comprising:

combining the 4,4-bis(octyloxy)butanoic acid with piperazine or *p*-xylylenediamine to form a reaction mixture, wherein the amine salt is formed in the reaction mixture; and

filtering the reaction mixture. In certain embodiments, the amine salt is a piperazinium salt. In certain embodiments, the amine salt is a *p*-xylylenediamine salt.

In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.5 to about 0.8 molar equivalents relative to the 4,4-bis(octyloxy)butanoic acid, preferably about 0.55 molar equivalents relative to the 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.5. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.55. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.6. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.65. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.7. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.75. In certain embodiments, the amount of the piperazine or *p*-xylylenediamine is about 0.8.

In certain embodiments, the combining step comprises combining the 4,4-bis(octyloxy)butanoic acid with a solvent. In certain embodiments, the solvent comprises isopropyl acetate.

In certain embodiments, the formation of the amine salt is carried out at a temperature not exceeding 70°C. In certain embodiments, the formation of the amine salt is carried out at a temperature not exceeding 65°C. In certain embodiments, the formation of the amine salt is carried out at a temperature not exceeding 60°C. In certain embodiments, the formation of the amine salt is carried out at a temperature not exceeding 55°C. In certain embodiments, the formation of the amine salt is carried out at a temperature of at least 0°C.

In certain embodiments, the formation of the amine salt is carried out at a temperature of about 0 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 5 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 10 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 15 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 20 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 25 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 30 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 35 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 40 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 45 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 50 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 55 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 60 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 65 °C. In certain embodiments, the formation of the amine salt is carried out at a temperature of about 70 °C.

In certain embodiments, the amine salt is the hemi(piperazinediium) salt of the 4,4-bis(octyloxy)butanoic acid.

In certain embodiments, the filtering step provides a solid comprising at least 80% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid

comprising at least 85% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising at least 90% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising at least 95% of the amine salt as determined by UPLC. In certain embodiments, the solid comprises at least 96% of the amine salt as determined by UPLC. In certain embodiments, the solid comprises at least 97% of the amine salt as determined by UPLC. In certain embodiments, the solid comprises at least 98% of the amine salt as determined by UPLC. In certain embodiments, the solid comprises at least 99% of the amine salt as determined by UPLC. In certain embodiments, the solid consists essentially of the amine salt as determined by UPLC.

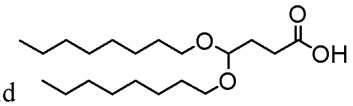
In certain embodiments, the filtering step provides a solid comprising about 80% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 81% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 82% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 83% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 84% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 85% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 86% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 87% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 88% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 89% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 90% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 91% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 92% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 93% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 94% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 95% of the amine

salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 96% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 97% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 98% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising about 99% of the amine salt as determined by UPLC. In certain embodiments, the filtering step provides a solid comprising consisting essentially of the amine salt as determined by UPLC.

In certain embodiments, the solid comprises less than about 5 wt.% octanol. In certain embodiments, the solid comprises less than about 4 wt.% octanol. In certain embodiments, the solid comprises less than about 3 wt.% octanol. In certain embodiments, the solid comprises less than about 2 wt.% octanol, preferably less than about 1 wt% octanol.

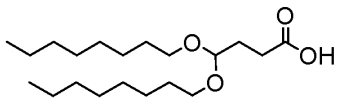
In certain embodiments, the solid comprises about 5 wt.% octanol. In certain embodiments, the solid comprises about 4 wt.% octanol. In certain embodiments, the solid comprises about 3 wt.% octanol. In certain embodiments, the solid comprises about 2 wt.% octanol. In certain embodiments, the solid comprises about 1 wt.% octanol. In certain embodiments, the solid is essentially free of octanol.

In certain embodiments, the 4,4-bis(octyloxy)butanoic acid is prepared by the processes described herein.

In certain embodiments, the disclosure relates to a composition comprising an amine salt of 4,4-bis(octyloxy)butanoic acid 

,wherein the composition is prepared according to the processes disclosed herein.

In certain embodiments, the disclosure relates to a compound that is a piperazinium salt or a *p*-xylylenediamine salt of 4,4-bis(octyloxy)butanoic acid



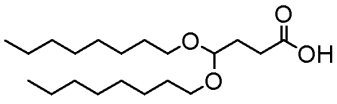
,

wherein the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.3 to about 0.7. In certain embodiments, the stoichiometric ratio of piperazine or *p*-

xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.1 to about 1.0. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.1. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.2. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.3. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.4. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.5. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.6. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.7. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.8. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.9. In certain embodiments, the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 1.0.

In certain embodiments, the compounds described herein are the hemi(piperazinediium) salt or the hemi(*p*-xylylenediamine) salt of 4,4-bis(octyloxy)butanoic acid.

In certain embodiments, the disclosure relates to a process for generating at least 90% free 4,4-bis(octyloxy)butanoic acid



comprising,

preparing an amine salt of the 4,4-bis(octyloxy)butanoic acid by the processes described herein; and

contacting the amine salt with an acid to form free 4,4-bis(octyloxy)butanoic acid.

In certain embodiments, the process generates at least 80% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates at least 85% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates at least 95% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates at least 96% of 4,4-bis(octyloxy)butanoic acid. In

certain embodiments, the process generates at least 97% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates at least 99% of 4,4-bis(octyloxy)butanoic acid.

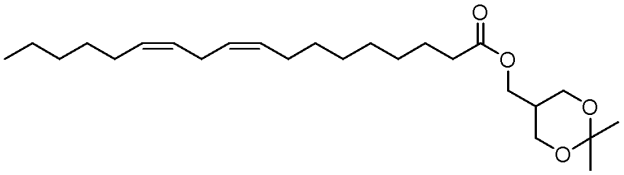
In certain embodiments, the process generates about 80% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 81% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 82% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 83% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 84% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 85% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 86% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 87% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 88% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 89% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 90% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 91% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 92% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 93% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 94% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 95% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 96% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 97% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 98% of 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the process generates about 99% of 4,4-bis(octyloxy)butanoic acid.

In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 1 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 2 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 3 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 4 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 5 wt.%

aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 6 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 7 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 8 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 9 wt.% aqueous solution of citric acid. In certain embodiments, the acid is citric acid. In certain embodiments, the citric acid is present in a 10 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 11 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 12 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 13 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 14 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 15 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 16 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 17 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 18 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 19 wt.% aqueous solution of citric acid. In certain embodiments, the citric acid is present in a 20 wt.% aqueous solution of citric acid.

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is contacted with an organic solvent. In certain embodiments, the organic solvent comprises cyclopentyl methyl ether.

In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (I):



(I),

comprising combining linoleic acid with (2,2-dimethyl-1,3-dioxan-5-yl)methanol and a coupling reagent.

In certain embodiments, the coupling reagent is *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC). In certain embodiments, the linoleic acid is combined with about 1.2 to about 3 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.7 to about 2 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.2 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.3 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.4 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.5 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.6 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.7 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.8 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 1.9 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.0 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.1 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.2 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.3 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.4 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.5 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.6 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.7 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.8 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 2.9 molar equivalents of the EDC. In certain embodiments, the linoleic acid is combined with about 3.0 molar equivalents of the EDC.

In certain embodiments, the linoleic acid is combined with 4-dimethylaminopyridine. In certain embodiments, linoleic acid is combined with about 0.1 to about 0.5 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.2 to about 0.3 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.1 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.2 molar equivalents of the 4-

dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.3 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.4 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the linoleic acid is combined with about 0.5 molar equivalents of the 4-dimethylaminopyridine.

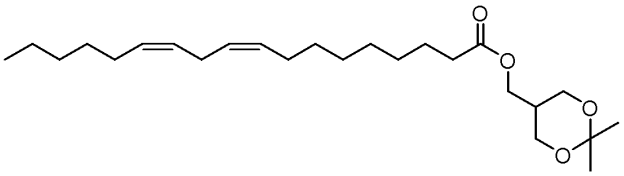
In certain embodiments, the linoleic acid is combined with an organic solvent. In certain embodiments, the organic solvent comprises acetonitrile.

In certain embodiments, the linoleic acid is combined with about 0.9 to about 2 molar equivalents of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol, wherein the molar equivalents are relative to the linoleic acid. In certain embodiments, the linoleic acid is combined with about 0.9 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.1 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.2 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.3 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.4 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.5 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.6 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.7 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.8 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 1.9 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol. In certain embodiments, the linoleic acid is combined with about 2 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol.

In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 20 °C to about 25 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 15 °C. In certain embodiments,

the formation of the compound of formula (I) is carried out at a temperature of about 16 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 17 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 18 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 19 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 20 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 21 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 22 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 23 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 24 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 25 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 26 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 27 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 28 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 29 °C. In certain embodiments, the formation of the compound of formula (I) is carried out at a temperature of about 30 °C.

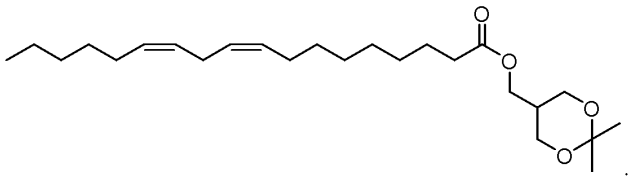
In certain embodiments, the disclosure relates to a compound having the structure of formula (I):



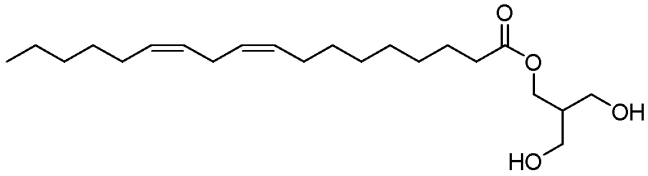
(I),

wherein the compound is prepared according to the processes described herein.

In certain embodiments, the disclosure relates to a compound that is a (2,2-dimethyl-1,3-dioxan-5-yl)methyl (9Z,12Z)-octadeca-9,12-dienoate

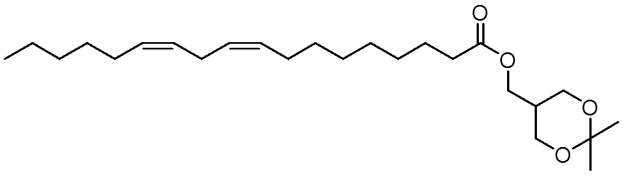


In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (II):



(II),

comprising contacting a compound having the structure of formula (I):



(I)

with an ion exchange resin. In certain embodiments, the ion exchange resin is a Dowex® resin.

In certain embodiments, the compound of formula (I) is contacted with a solvent. In certain embodiments, the solvent comprises methanol.

In certain embodiments, the contacting step provides a crude product comprising the compound of formula (II); and

the process further comprises purifying the compound of formula (II) by column chromatography.

In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 20 °C to about 25 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 15 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 16 °C. In

certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 17 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 18 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 19 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 20 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 21 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 22 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 23 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 24 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 25 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 26 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 27 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 28 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 29 °C. In certain embodiments, the formation of the compound of formula (II) is carried out at a temperature of about 30 °C.

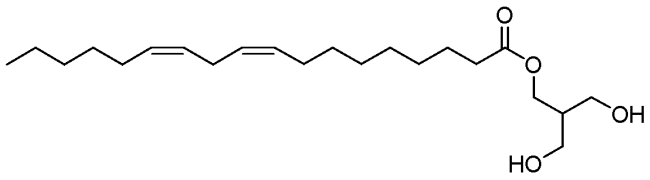
In certain embodiments, the compound of formula (II) is treated with a powdered activated carbon (PAC) material. In some embodiments, the PAC material is Norit KBEVN, Norit KBEV SUPRA, Norit BENTO NORIT CA1, Norit CGP Super, Norit CGSP, Cabot SX PLUS, Cabot A SUPRA EUR, Norit SX, Norit CASP, Norit SX PLUS LC, Norit CN1, Norit ROX 0.8, Norit C EXTRA USP, or Norit GAC 1240 PLUS. In certain embodiments, the PAC material is Cabot A SUPRA EUR.

In certain embodiments, the PAC material is used in various solvents and with various weight ratios of PAC to crude product. In some embodiments, the solvent is heptane or methanol. In some embodiments, the weight ratio of PAC material to crude product is 0%, 5%, 10%, 25%, 50%, or 100% by weight (wt%/wt%). In certain embodiments, the Cabot A SUPRA EUR is used with a 10% wt/wt PAC:crude product compound of formula (II) ratio. In certain embodiments, the Cabot A SUPRA EUR is used with a 25% wt/wt PAC:crude product

compound of formula (II) ratio. In certain embodiments, the Cabot A SUPRA EUR is used with a 50% wt/wt PAC:crude product compound of formula (II) ratio. In certain embodiments, the Cabot A SUPRA EUR is used with a 100% wt/wt PAC:crude product compound of formula (II) ratio.

In certain embodiments, described herein, are the process described herein, wherein a composition comprising the compound of formula (I) is prepared by the processes described herein.

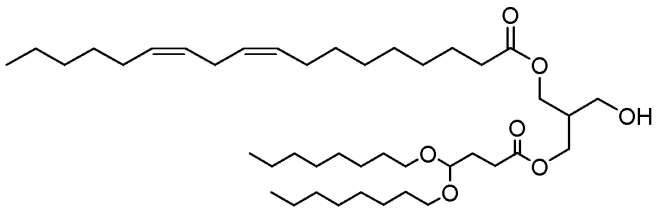
In certain embodiments, the disclosure relates to a composition comprising a compound having the structure of formula (II):



(II),

wherein the composition is prepared according to the processes described herein.

In certain embodiments, the disclosure relates to a process for preparing a compound having the structure of formula (III):

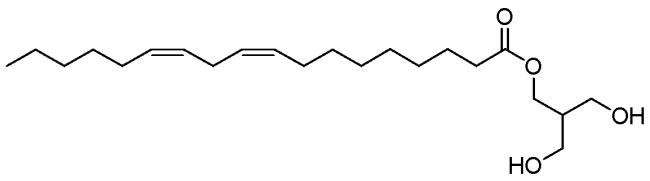


(III),

comprising:

generating free 4,4-bis(octyloxy)butanoic acid by the processes described herein; and

combining the purified 4,4-bis(octyloxy)butanoic acid with a compound having the structure of formula (II):



(II)

and a coupling reagent.

In certain embodiments, the coupling reagent is *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with an organic solvent. In certain embodiments, the organic solvent comprises cyclopentyl methyl ether.

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 to about 1.5 molar equivalents of the compound of formula (II) and about 1 to about 1.5 molar equivalents of the coupling reagent, wherein the molar equivalents are relative to the free 4,4-bis(octyloxy)butanoic acid. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.1 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.2 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.3 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.4 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.5 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.6 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.7 to molar equivalents of the compound of formula (II). In certain embodiments, the free

4,4-bis(octyloxy)butanoic acid is combined with about 1.8 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.9 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 2.0 to molar equivalents of the compound of formula (II).

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 to molar equivalents of the compound of formula (II). In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.1 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.2 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.3 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.4 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.5 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.6 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.7 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.8 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.9 to molar equivalents of the coupling reagent. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 2.0 to molar equivalents of the coupling reagent.

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.05 to about 0.5 molar equivalents of the 4-dimethylaminopyridine and 1 to 1.5 molar equivalents of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.01 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.05 molar equivalents of the 4-dimethylaminopyridine. In certain

embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.1 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.15 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.2 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.25 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.3 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.35 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.4 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.45 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.5 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.55 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.6 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.65 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.7 molar equivalents of the 4-dimethylaminopyridine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.75 molar equivalents of the 4-dimethylaminopyridine.

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.1 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.2 molar equivalents of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.3 molar

equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.4 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.5 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.6 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.7 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.8 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.9 molar equivalent of the *N,N*-diisopropylethylamine. In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 2.0 molar equivalent of the *N,N*-diisopropylethylamine.

In certain embodiments, the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.2 molar equivalents of the compound of formula (II), about 1.2 molar equivalents of the coupling reagent, about 0.2 molar equivalents of the 4-dimethylaminopyridine, and about 1.2 molar equivalents of the *N,N*-diisopropylethylamine.

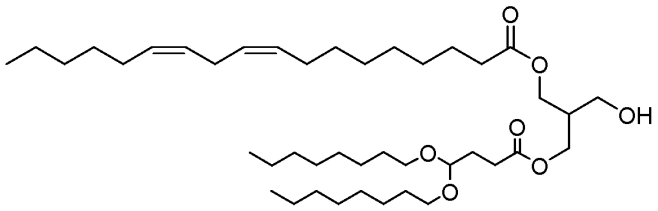
In certain embodiments, the process further comprises filtration over Celite. In certain embodiments, the process further comprises dilution of the reaction mixture in a solvent prior to filtration. In certain embodiments, the process further comprises dilution of the reaction mixture in heptane prior to filtration.

In certain embodiments, the process further comprises purifying the compound of formula (III) by column chromatography.

In certain embodiments, the process further comprises using a heptane/CPME solvent mixture for column chromatography.

In certain embodiments, a composition comprising the compound of formula (II) is prepared by the processes described herein.

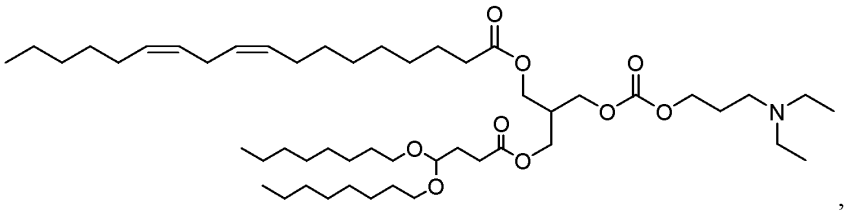
In certain embodiments, the disclosure relates to a composition comprising a compound having the structure of formula (III):



(III),

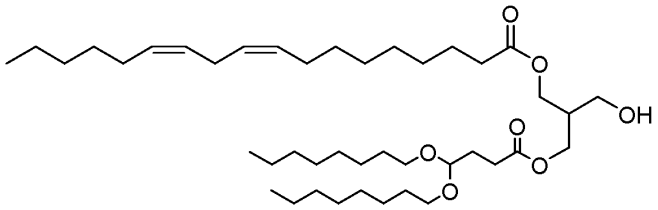
wherein the composition is prepared according to the processes described herein.

In certain embodiments, the disclosure relates to a process for preparing Lipid A:



,

comprising combining a compound having the structure of formula (III):



(III)

with 4-nitrophenyl chloroformate, about 2.2 to about 10 molar equivalents of pyridine, 4-dimethylaminopyridine, and 3-(diethylamino)propan-1-ol, wherein the molar equivalents are relative to the compound of formula (III). In certain embodiments, the compound of formula (III) is combined with about 1.5 to about 7.2 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.3 to about 5.2 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.3 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.4 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.5 molar equivalents of

the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.6 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.7 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.8 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 2.9 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.0 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.1 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.2 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.3 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.4 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.5 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.6 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.7 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.8 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 3.9 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.0 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.1 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.2 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.3 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.4 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.5 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.6 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.7 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 4.8 molar equivalents of the pyridine. In certain embodiments, the

compound of formula (III) is combined with about 4.9 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 5.0 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 5.1 molar equivalents of the pyridine. In certain embodiments, the compound of formula (III) is combined with about 5.2 molar equivalents of the pyridine.

In certain embodiments, the compound of formula (III) is combined with an organic solvent. In certain embodiments, the solvent comprises acetonitrile. In certain embodiments, the solvent does not comprise dichloromethane.

In certain embodiments, the compound of formula (III) is combined with about 1 to about 12 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 1 to about 10 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 2 to about 8 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 4 to about 8 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 1 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 2 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 3 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 4 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 5 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 6 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 7 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 8 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 9 molar equivalents of the 3-(diethylamino)propan-1-ol. In certain embodiments, the compound of formula (III) is combined with about 10 molar equivalents of the 3-(diethylamino)propan-1-ol.

In certain embodiments, the combining step forms a reaction mixture comprising Lipid A.

In certain embodiments, the process further comprises purifying Lipid A by column chromatography to provide a purified product comprising Lipid A. In some embodiments, the Lipid A is purified by HPLC. In some embodiments, the Lipid A is purified by prep HPLC.

In certain embodiments, the reaction mixture or the purified product, as determined by UPLC, comprises:

at least 95% Lipid A, and

(A) less than about 1.1% of a compound having the structure of formula (V), or

(B) less than about 0.6% of a compound having the structure of formula (VI), or

(C) less than about 0.6% of a compound having the structure of formula (VII), or

(D) less than about 0.3% of a compound having the structure of formula (III), or

(E) less than about 190 ppm p-nitrophenol (pNP)

(F) less than about 0.4% of a compound having the structure of formula (VIII), or

(G) less than about 0.2% of a compound having the structure of formula (IX).

In certain embodiments, the reaction mixture or the purified product comprises less than about 0.3% of the compound of formula (V), preferably 0.2% of the compound of formula (V), preferably less than about 0.1% of the compound of formula (V), even more preferably wherein the compound of formula (V) is undetectable. In certain embodiments, the reaction mixture or the purified product comprises about 1.0% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.9% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.8% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.7% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.6% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.5% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.4% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.3% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product

comprises about 0.2% of the compound of formula (V). In certain embodiments, the reaction mixture or the purified product comprises about 0.1% of the compound of formula (V).In certain embodiments, the reaction mixture or the purified product is essentially free of the compound of formula (V).

In certain embodiments, the reaction mixture or the purified product comprises less than about 0.55% of the compound of formula (VI), even more preferably less than about 0.5% of the compound of formula (VIIn certain embodiments, the reaction mixture or the purified product comprises about 0.55% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.5% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.45% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.4% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.35% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.3% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.25% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.2% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.15% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.1% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.05% of the compound of formula (VI).

In certain embodiments, the reaction mixture or the purified product comprises about 0.55% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.5% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.45% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.4% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.35% of the compound of formula (VI). In

certain embodiments, the reaction mixture or the purified product comprises about 0.3% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.25% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.2% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.15% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.1% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product comprises about 0.05% of the compound of formula (VI). In certain embodiments, the reaction mixture or the purified product is essentially free of the compound of formula (VI).

In certain embodiments, the reaction mixture or the purified product comprises less than about 0.4% of the compound of formula (VII), preferably less than about 0.3% of the compound of formula (VII), even more preferably less than about 0.2% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.4% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.35% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.3% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.25% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.2% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.15% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.1% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.05% of the compound of formula (VII).

In certain embodiments, the reaction mixture or the purified product comprises about 0.4% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.35% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.3% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product

comprises about 0.25% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.2% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.15% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.1% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises about 0.05% of the compound of formula (VII). In certain embodiments, the reaction mixture or the purified product comprises is essentially free of the compound of formula (VII).

In certain embodiments, the reaction mixture or the purified product comprises less than about 0.25% of the compound of formula (III), even more preferably less than about 0.2% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.25% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.2% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.15% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.1% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises less than about 0.05% of the compound of formula (III).

In certain embodiments, the reaction mixture or the purified product comprises about 0.25% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises about 0.2% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises about 0.15% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises about 0.1% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises about 0.05% of the compound of formula (III). In certain embodiments, the reaction mixture or the purified product comprises is essentially free of the compound of formula (III).

In certain embodiments, the composition comprises less than about 190 ppm pNP. In certain embodiments, the composition comprises less than about 180 ppm pNP. In certain embodiments, the composition comprises less than about 170 ppm pNP. In certain embodiments,

the composition comprises less than about 160 ppm pNP. In certain embodiments, the composition comprises less than about 150 ppm pNP. In certain embodiments, the composition comprises less than about 140 ppm pNP. In certain embodiments, the composition comprises less than about 130 ppm pNP. In certain embodiments, the composition comprises less than about 120 ppm pNP. In certain embodiments, the composition comprises less than about 110 ppm pNP. In certain embodiments, the composition comprises less than about 100 ppm pNP. In certain embodiments, the composition comprises less than about 90 ppm pNP. In certain embodiments, the composition comprises less than about 80 ppm pNP. In certain embodiments, the composition comprises less than about 70 ppm pNP. In certain embodiments, the composition comprises less than about 60 ppm pNP. In certain embodiments, the composition comprises less than about 50 ppm pNP. In certain embodiments, the composition comprises less than about 45 ppm pNP. In certain embodiments, the composition comprises less than about 40 ppm pNP. In certain embodiments, the composition comprises less than about 35 ppm pNP. In certain embodiments, the composition comprises less than about 30 ppm pNP. In certain embodiments, the composition comprises less than about 25 ppm pNP. In certain embodiments, the composition comprises less than about 20 ppm pNP. In certain embodiments, the composition comprises less than about 15 ppm pNP. In certain embodiments, the composition comprises less than about 10 ppm pNP. In certain embodiments, the composition comprises less than about 5 ppm pNP. In certain embodiments, the composition comprises less than about 2 ppm pNP. In certain embodiments, the composition comprises pNP is undetectable.

In certain embodiments, the composition comprises about 190 ppm pNP. In certain embodiments, the composition comprises about 180 ppm pNP. In certain embodiments, the composition comprises about 170 ppm pNP. In certain embodiments, the composition comprises about 160 ppm pNP. In certain embodiments, the composition comprises about 150 ppm pNP. In certain embodiments, the composition comprises about 140 ppm pNP. In certain embodiments, the composition comprises about 130 ppm pNP. In certain embodiments, the composition comprises about 120 ppm pNP. In certain embodiments, the composition comprises about 110 ppm pNP. In certain embodiments, the composition comprises about 100 ppm pNP. In certain embodiments, the composition comprises about 90 ppm pNP. In certain embodiments, the composition comprises about 80 ppm pNP. In certain embodiments, the composition comprises

about 70 ppm pNP. In certain embodiments, the composition comprises about 60 ppm pNP. In certain embodiments, the composition comprises about 50 ppm pNP. In certain embodiments, the composition comprises about 45 ppm pNP. In certain embodiments, the composition comprises about 40 ppm pNP. In certain embodiments, the composition comprises about 35 ppm pNP. In certain embodiments, the composition comprises about 30 ppm pNP. In certain embodiments, the composition comprises about 25 ppm pNP. In certain embodiments, the composition comprises about 20 ppm pNP. In certain embodiments, the composition comprises about 15 ppm pNP. In certain embodiments, the composition comprises about 10 ppm pNP. In certain embodiments, the composition comprises about 5 ppm pNP. In certain embodiments, the composition comprises about 2 ppm pNP.

In certain embodiments, the composition comprises less than about 0.4% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.35% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.15% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (VIII).

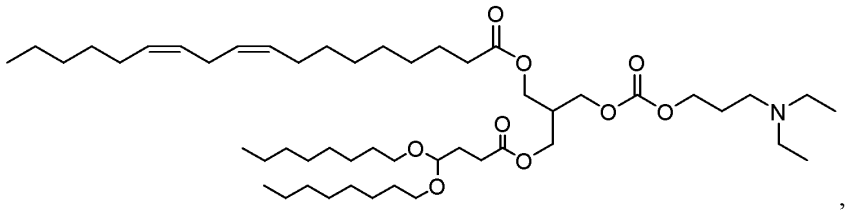
In certain embodiments, the composition comprises about 0.4% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.35% of a compound of formula (VIII). In In certain embodiments, the composition comprises about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.15% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.05% of a compound of formula (VIII). In certain embodiments, the composition is essentially free of a compound of formula (VIII).

In certain embodiments, the composition comprises less than about 0.1% of a compound of formula (IX), preferably wherein a compound of formula (IX)is undetectable. In certain embodiments, the composition comprises less than about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.9% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.06% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.04% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.01% of a compound of formula (IX).

In certain embodiments, the composition comprises about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.9% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.06% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.04% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.01% of a compound of formula (IX). In certain embodiments, the composition is essentially free of a compound of formula (IX).

In certain embodiments, the compound of formula (III) is prepared by the processes described herein.

In certain embodiments, the disclosure relates to a composition comprising Lipid A:



,

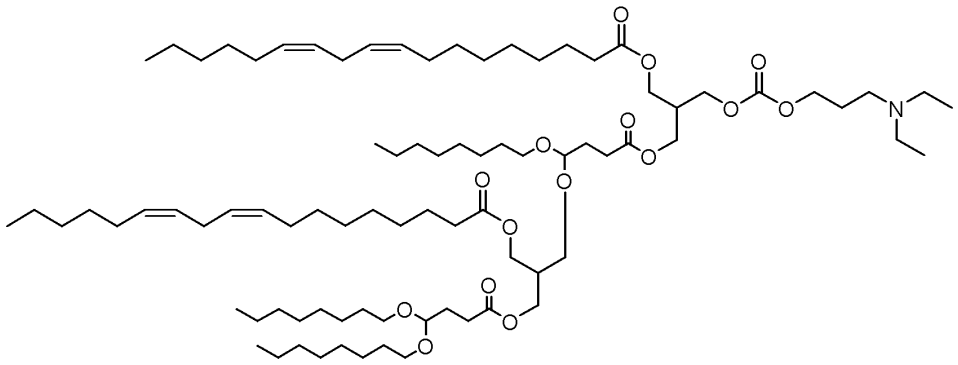
wherein the composition is prepared according to the processes described herein.

In certain embodiments, the composition comprises at least 90% Lipid A. In certain embodiments, the composition comprises at least 91% Lipid A. In certain embodiments, the composition comprises at least 92% Lipid A. In certain embodiments, the composition comprises at least 93% Lipid A. In certain embodiments, the composition comprises at least 94% Lipid A. In certain embodiments, the composition comprises at least 95% Lipid A. In certain embodiments, the composition comprises at least 96% Lipid A. In certain embodiments, the composition comprises at least 97% Lipid A. In certain embodiments, the composition comprises at least 98% Lipid A. In certain embodiments, the composition comprises greater than 99% Lipid A.

In certain embodiments, 95% of the composition is Lipid A. In certain embodiments, the composition comprises about 96% Lipid A. In certain embodiments, the composition comprises about 97% Lipid A. In certain embodiments, the composition comprises about 98% Lipid A. In certain embodiments, the composition comprises at least about 99% Lipid A.

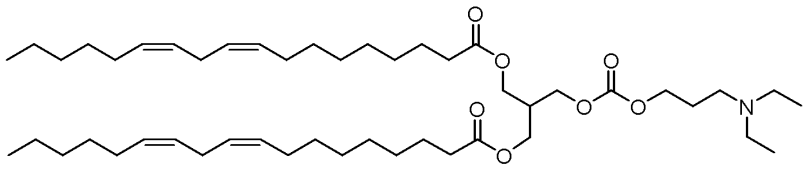
In certain embodiments, the composition comprises:

(A) less than about 1.1%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (V)



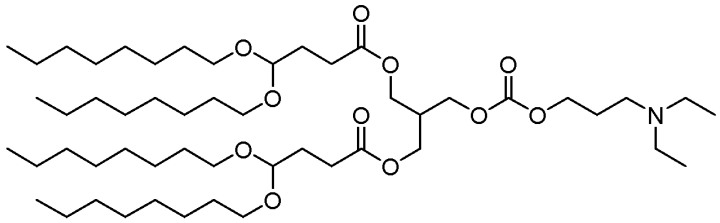
(V), or

(B) less than about 0.6% or less than about 0.5% of a compound having the structure of formula (VI)



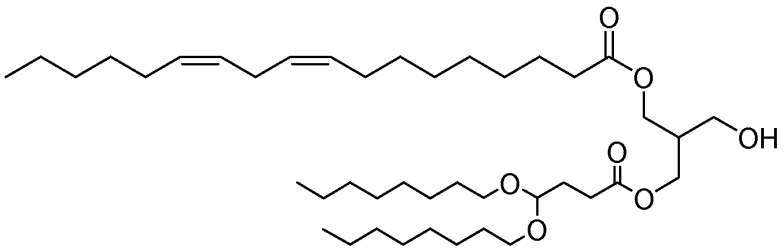
(VI), or

(C) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, or less than about 0.2%, of a compound having the structure of formula (VII)



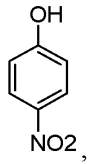
(VII), or

(D) less than about 0.3% or less than about 0.2% of a compound having the structure of formula (III)



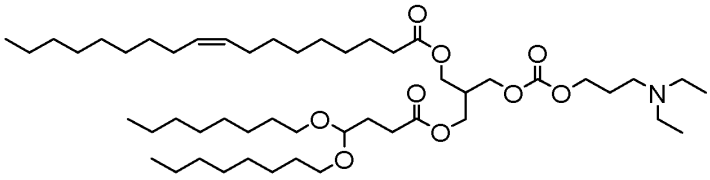
(III), or

(E) less than about 190 ppm, less than about 180 ppm, less than about 170 ppm, less than about 160 ppm, less than about 150 ppm, less than about 140 ppm, less than about 130 ppm, less than about 120 ppm, less than about 110 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



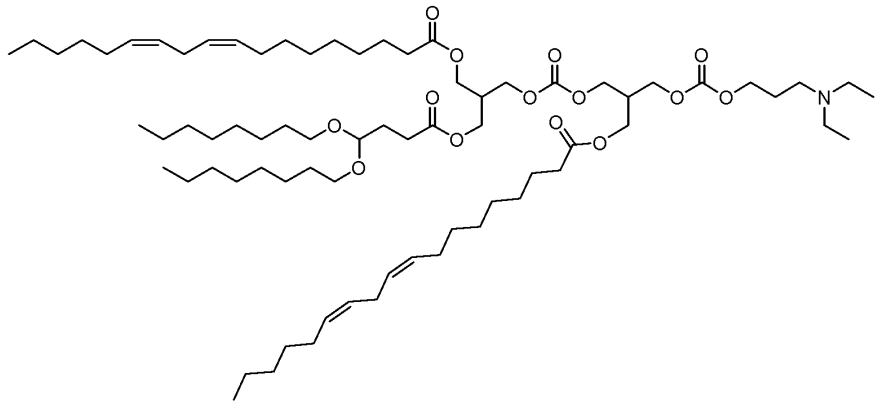
, or

(F) less than about 0.4% or less than about 0.3% of a compound having the structure of formula (VIII)



(VIII), or

(G) less than about 0.2% or less than 0.1% of a compound having the structure of formula (IX)



(IX).

In certain embodiments, the composition comprises less than about 1.1% of the compound of formula (V). In certain embodiments, the composition comprises less than about 1.0% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.9% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.8% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.7% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.6% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.5% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (V). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (V).

In certain embodiments, the composition comprises about 1.0% of the compound of formula (V). In certain embodiments, the composition comprises about 0.9% of the compound of formula (V). In certain embodiments, the composition comprises about 0.8% of the compound of formula (V). In certain embodiments, the composition comprises about 0.7% of the compound of formula (V). In certain embodiments, the composition comprises about 0.6% of the compound of formula (V). In certain embodiments, the composition comprises about 0.5% of the compound of formula (V). In certain embodiments, the composition comprises about 0.4% of the compound of

formula (V). In certain embodiments, the composition comprises about 0.3% of the compound of formula (V). In certain embodiments, the composition comprises about 0.2% of the compound of formula (V). In certain embodiments, the composition comprises about 0.1% of the compound of formula (V). In certain embodiments, the composition is essentially free of the compound of formula (V).

In certain embodiments, the composition comprises less than about 0.6% of the compound of formula (VI).In certain embodiments, the composition comprises less than about 0.55% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.5% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.45% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.35% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (VI). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (VI). In certain embodiments, the composition is essentially free of the compound of formula (VI).

In certain embodiments, the composition comprises about 0.6% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.55% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.5% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.45% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.4% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.35% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.3% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.25% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.2% of the compound of formula (VI). In certain embodiments,

the composition comprises about 0.15% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.1% of the compound of formula (VI). In certain embodiments, the composition comprises about 0.05% of the compound of formula (VI). In certain embodiments, the composition is essentially free of the compound of formula (VI).

In certain embodiments, the composition comprises less than about 0.6% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.55% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.5% of the compound of formula (VII).In certain embodiments, the composition comprises less than about 0.45% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.4% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.35% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (VII). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (VII).

In certain embodiments, the composition comprises about 0.6% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.55% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.5% of the compound of formula (VII).In certain embodiments, the composition comprises about 0.45% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.4% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.35% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.3% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.25% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.2% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.15% of the compound of formula (VII). In certain

embodiments, the composition comprises about 0.1% of the compound of formula (VII). In certain embodiments, the composition comprises about 0.05% of the compound of formula (VII). In certain embodiments, the composition is essentially free of the compound of formula (VII).

In certain embodiments, the composition comprises less than about 0.3% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.25% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.2% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.15% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.1% of the compound of formula (III). In certain embodiments, the composition comprises less than about 0.05% of the compound of formula (III).

In certain embodiments, the composition comprises about 0.3% of the compound of formula (III). In certain embodiments, the composition comprises about 0.25% of the compound of formula (III). In certain embodiments, the composition comprises about 0.2% of the compound of formula (III). In certain embodiments, the composition comprises about 0.15% of the compound of formula (III). In certain embodiments, the composition comprises about 0.1% of the compound of formula (III). In certain embodiments, the composition comprises about 0.05% of the compound of formula (III). In certain embodiments, the composition is essentially free of the compound of formula (III).

In certain embodiments, the composition comprises less than about 190 ppm pNP. In certain embodiments, the composition comprises less than about 180 ppm pNP. In certain embodiments, the composition comprises less than about 170 ppm pNP. In certain embodiments, the composition comprises less than about 160 ppm pNP. In certain embodiments, the composition comprises less than about 150 ppm pNP. In certain embodiments, the composition comprises less than about 140 ppm pNP. In certain embodiments, the composition comprises less than about 130 ppm pNP. In certain embodiments, the composition comprises less than about 120 ppm pNP. In certain embodiments, the composition comprises less than about 110 ppm pNP. In certain embodiments, the composition comprises less than about 100 ppm pNP. In certain embodiments, the composition comprises less than about 90 ppm pNP. In certain embodiments, the composition comprises less than about 80 ppm pNP. In certain embodiments, the

composition comprises less than about 70 ppm pNP. In certain embodiments, the composition comprises less than about 60 ppm pNP. In certain embodiments, the composition comprises less than about 50 ppm pNP. In certain embodiments, the composition comprises less than about 45 ppm pNP. In certain embodiments, the composition comprises less than about 40 ppm pNP. In certain embodiments, the composition comprises less than about 35 ppm pNP. In certain embodiments, the composition comprises less than about 30 ppm pNP. In certain embodiments, the composition comprises less than about 25 ppm pNP. In certain embodiments, the composition comprises less than about 20 ppm pNP. In certain embodiments, the composition comprises less than about 15 ppm pNP. In certain embodiments, the composition comprises less than about 10 ppm pNP. In certain embodiments, the composition comprises less than about 5 ppm pNP. In certain embodiments, the composition comprises less than about 2 ppm pNP.

In certain embodiments, the composition comprises about 190 ppm pNP. In certain embodiments, the composition comprises about 180 ppm pNP. In certain embodiments, the composition comprises about 170 ppm pNP. In certain embodiments, the composition comprises about 160 ppm pNP. In certain embodiments, the composition comprises about 150 ppm pNP. In certain embodiments, the composition comprises about 140 ppm pNP. In certain embodiments, the composition comprises about 130 ppm pNP. In certain embodiments, the composition comprises about 120 ppm pNP. In certain embodiments, the composition comprises about 110 ppm pNP. In certain embodiments, the composition comprises about 100 ppm pNP. In certain embodiments, the composition comprises about 90 ppm pNP. In certain embodiments, the composition comprises about 80 ppm pNP. In certain embodiments, the composition comprises about 70 ppm pNP. In certain embodiments, the composition comprises about 60 ppm pNP. In certain embodiments, the composition comprises about 50 ppm pNP. In certain embodiments, the composition comprises about 45 ppm pNP. In certain embodiments, the composition comprises about 40 ppm pNP. In certain embodiments, the composition comprises about 35 ppm pNP. In certain embodiments, the composition comprises about 30 ppm pNP. In certain embodiments, the composition comprises about 25 ppm pNP. In certain embodiments, the composition comprises about 20 ppm pNP. In certain embodiments, the composition comprises about 15 ppm pNP. In certain embodiments, the composition comprises about 10 ppm pNP. In certain embodiments, the composition comprises about 5 ppm pNP. In certain embodiments, the

composition comprises about 2 ppm pNP. In certain embodiments, the composition comprises pNP is undetectable.

In certain embodiments, the composition comprises less than about 0.4% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.35% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.15% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (VIII).

In certain embodiments, the composition comprises about 0.4% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.35% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.3% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.25% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.2% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.15% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.1% of a compound of formula (VIII). In certain embodiments, the composition comprises about 0.05% of a compound of formula (VIII). In certain embodiments, the composition is essentially free of a compound of formula (VIII).

In certain embodiments, the composition comprises less than about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.9% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.06% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.04% of a compound of formula (IX).

In certain embodiments, the composition comprises less than about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises less than about 0.01% of a compound of formula (IX).

In certain embodiments, the composition comprises about 0.1% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.9% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.08% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.07% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.06% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.05% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.04% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.03% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.02% of a compound of formula (IX). In certain embodiments, the composition comprises about 0.01% of a compound of formula (IX). In certain embodiments, the composition is essentially free of a compound of formula (IX).

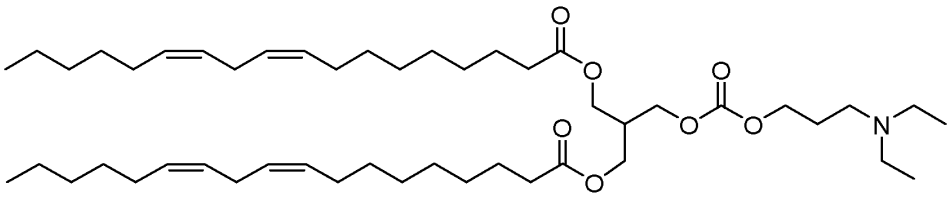
In certain embodiments, the composition comprises greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, or greater than about 5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 2 kg of Lipid A. In certain embodiments, the composition comprises greater than about 2.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 3 kg of Lipid A. In certain embodiments, the composition comprises greater than about 3.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 4 kg of Lipid A. In certain embodiments, the composition comprises greater than about 4.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 5.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 6 kg of Lipid A. In certain embodiments, the composition comprises greater than about 6.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 7 kg of Lipid A. In certain embodiments, the composition comprises greater than about 7.5 kg of Lipid A. In certain

embodiments, the composition comprises greater than about 8 kg of Lipid A. In certain embodiments, the composition comprises greater than about 8.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 9 kg of Lipid A. In certain embodiments, the composition comprises greater than about 9.5 kg of Lipid A. In certain embodiments, the composition comprises greater than about 10 kg of Lipid A.

In certain embodiments, the composition comprises about 1 kg to about 10 kg, even more preferably about 1 to about 7 kg of Lipid A. In certain embodiments, the composition comprises about 1 kg of Lipid A. In certain embodiments, the composition comprises about 2 kg of Lipid A. In certain embodiments, the composition comprises about 3 kg of Lipid A. In certain embodiments, the composition comprises about 4 kg of Lipid A. In certain embodiments, the composition comprises about 5 kg of Lipid A. In certain embodiments, the composition comprises about 6 kg of Lipid A. In certain embodiments, the composition comprises about 7 kg of Lipid A. In certain embodiments, the composition comprises about 8 kg of Lipid A. In certain embodiments, the composition comprises about 9 kg of Lipid A. In certain embodiments, the composition comprises about 10 kg of Lipid A.

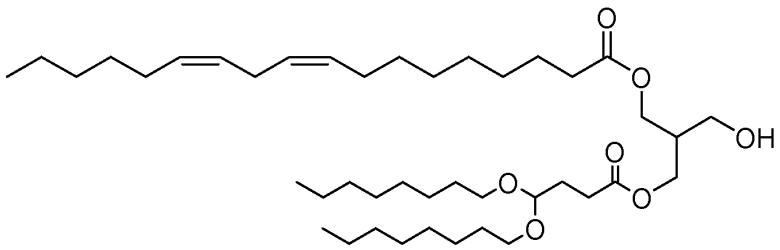
In certain embodiments, the composition comprises greater than about 6 kg of Lipid A, and the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



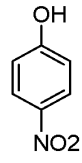
(VI), or

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



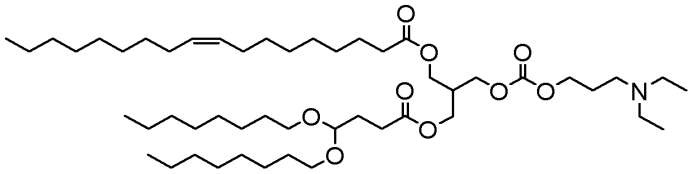
(III), or

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



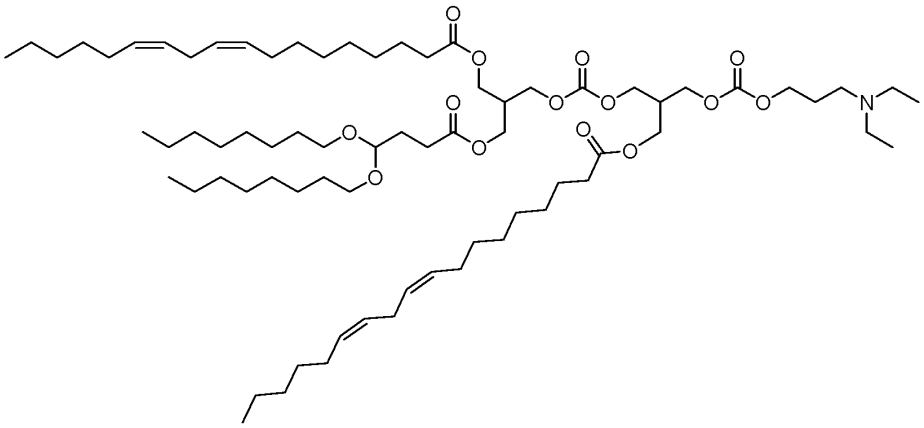
, or

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), or

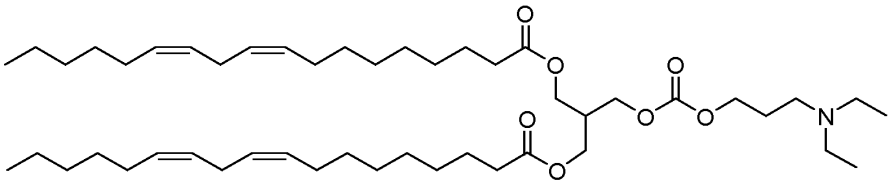
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

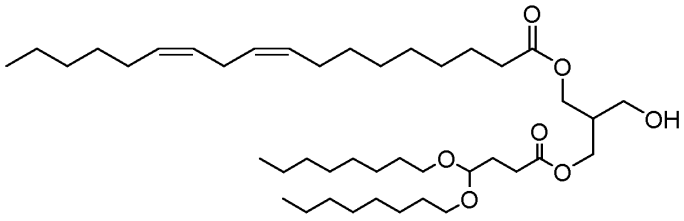
In certain embodiments, the composition comprises greater than about 6 kg of Lipid A, and the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



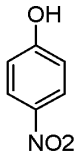
(VI), and

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



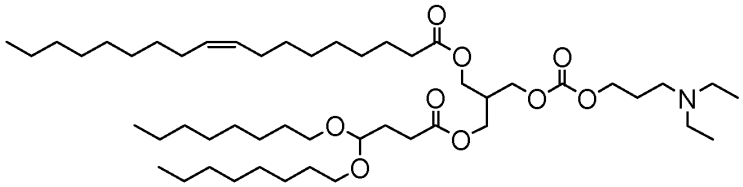
(III), and

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



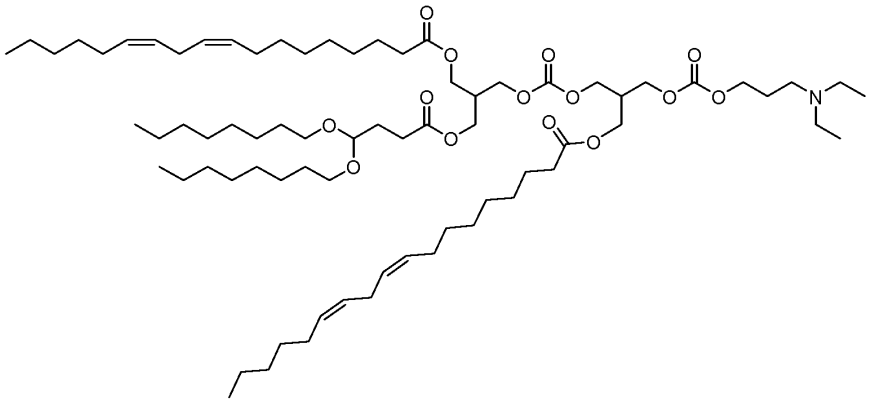
, and

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), and

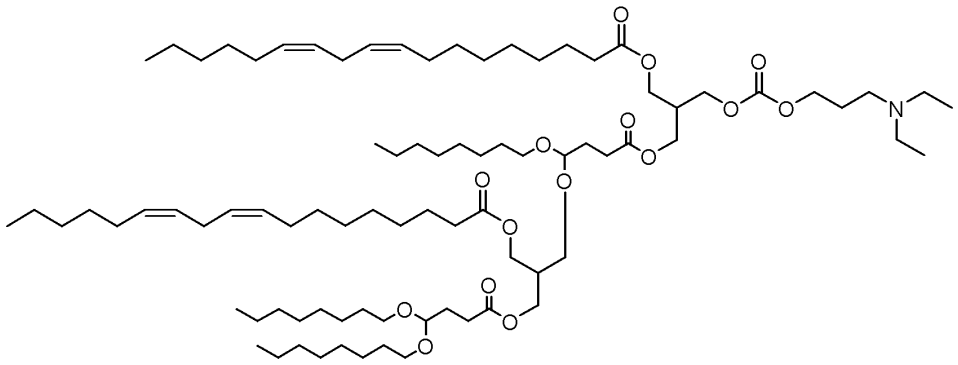
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

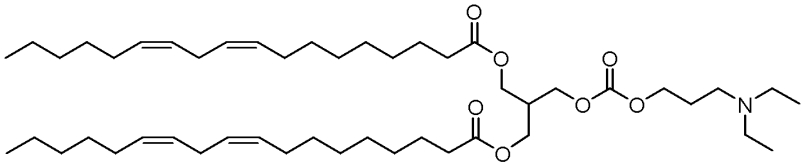
In certain embodiments, the composition comprises greater than about 6 kg of Lipid A, and the composition comprises:

(A) less than about 0.1% of a compound having the structure of formula (V)



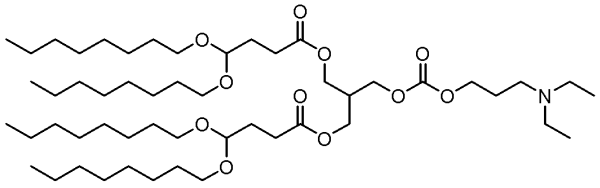
(V), and

(B) less than about 1.2% a compound having the structure of formula (VI)



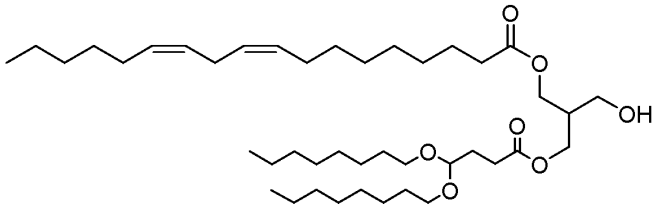
(VI), and

(C) less than about 0.3% of a compound having the structure of formula (VII)



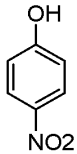
(VII), and

(C) less than about 1.0% of a compound having the structure of formula (III)



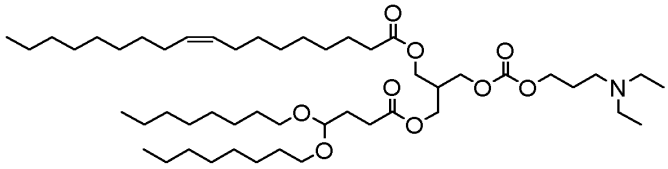
(III), and

(D) less than about 2 ppm p-nitrophenol (pNP)



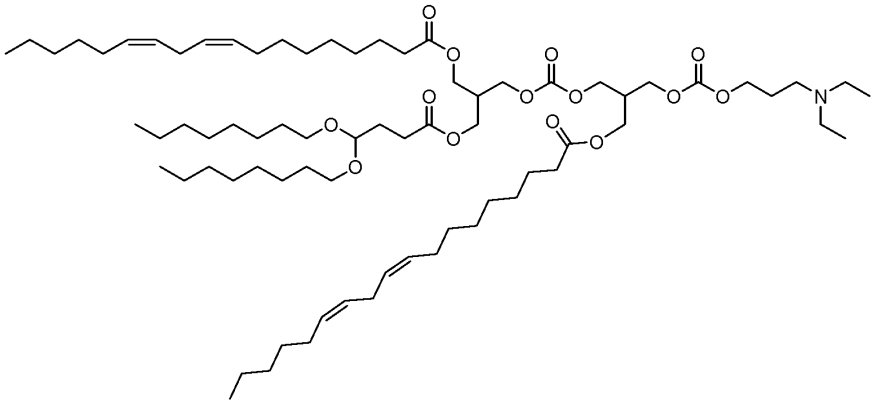
, and

(E) less than about 0.2% of a compound of formula (VIII)



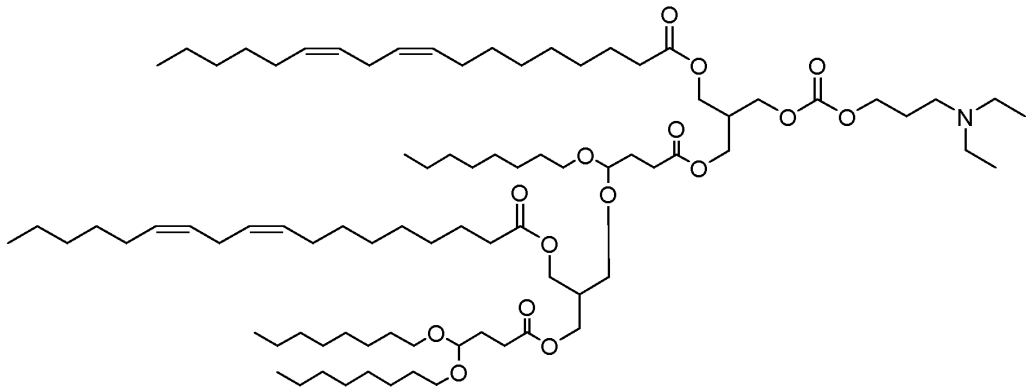
(VIII), and

(F) less than about 0.1% of a compound having the structure of formula (IX)



(IX).

In certain embodiments, the disclosure relates to a composition comprising at least 90% of a compound having the structure of formula (V)



(V).

In certain embodiments, the disclosure relates to a composition comprising at least 91% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 92% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 93% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 94% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 95% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 96% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 97% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 98% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition comprising at least 99% of a compound having the structure of formula (V). In certain embodiments, the disclosure relates to a composition consisting essentially of a compound having the structure of formula (V).

EXAMPLES

Example 1: General Methods

The appearance, purity and pNP content for Lipid A, synthesized according to any of the processes described herein, were determined using the following methods.

Appearance, including color, was determined by visual inspection.

Purity analysis was performed using Ultra-performance liquid chromatography (UPLC) with a Charged Aerosol Detector (CAD) on a Waters Acquity UPLC CSH C18 column (150x2.1 mm, 1.7 µm, & 130 Å). A gradient of 30% to 95% of Solvent B was employed over the time of 42 min with a flow rate of 0.32 mL/min. Solvent A (10 mM ammonium formate in acetonitrile (ACN)/H_2O 600/400 (v/v) + 0.1% HCOOH) and Solvent B (8.5 mM ammonium formate in isopropyl alcohol/ACN/H_2O (900/100/150 (v/v) + 0.085% HCOOH) were used as mobile phases. The purity of Lipid A and level of impurities were determined as the fraction of total peak area (%AUC, also referred to as a% or area%) attributed to the corresponding signal(s) in the UPLC-CAD chromatogram obtained from the Lipid A product. A mixture of synthesized reference impurities was analyzed with and without Lipid A to assign the impurity peaks in the Lipid A analysis.

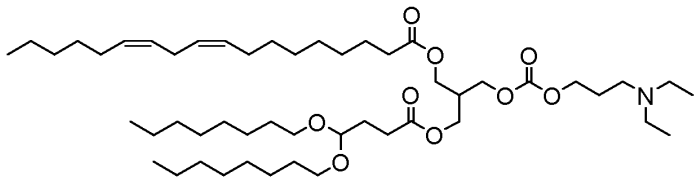
UPLC-UV was used to determine *p*-nitrophenol (pNP) content in Lipid A, based on chromatographic separation by Reversed Phase (RP) UPLC, using a Cyano column (Waters Acquity UPLC HSS 150x2.1 mm, 1.8 µm) with gradient elution 0% to 100% of mobile phase B over 21.25 minutes at a flow rate of 0.6 mL/min. The mobile phase A contained acetonitrile and 50 mM ammonium formate in water in a 3:7 volume ratio. The mobile phase B contained a mixture of acetonitrile:isopropyl alcohol (1:1 v:v). A UV detector at 317 nm was used to determine pNP content. pNP data was reported in parts per million (ppm).

Headspace-gas chromatography with flame ionizable detection (HS-GC(FID)) was used to determine the amount of 1-octanol present in Compound-09 and Compound-13. For the analysis, a VF-624MS 60m x 0.32mm x 1.8µm column was used. The carrier gas, helium, was set to a split flow of 4 mL/min. The limit of detection (LOD) and limit of quantification (LOQ) for octanol were 565 ppm and 1882 ppm, respectively.

Example 2: Initial Process for producing Lipid A (Process A)

Lipid A, corresponding to (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate, is composed of an unsaturated linoleic acid tail, branched acetal tail and a diethylaminopropyl headgroup. The molecular weight of Lipid A is

852.29 g/mol, and its molecular formula is $C_{50}H_{93}NO_9$. Lipid A is represented by the structural formula:



Lipid A is described in publication number WO2015/095340A1. Lipid A was produced generally following the process illustrated in Scheme 1, which generally follows that described in Example 14 of WO2015/095340A1. Briefly, the process involved the synthesis of two intermediates, namely Compound-09 and Compound-12, followed by the coupling of Compound-09 and Compound-12 to form intermediate Compound-13, and finally a carbodiimide ester bond formation with Compound-05 (3-diethylamino-1-propanol) to produce Lipid A, as shown in Scheme 1.

Compound-09 was formed in two steps. The first step involved the combination of 4,4-diethoxybutanenitrile (Compound-06) and 1-octanol (Compound-02) in toluene, using pyridinium-4-toluene sulfonate (PPTS) as a catalyst, at a temperature of 105 °C, resulting in the formation of intermediate Compound-08. In the second step, the resultant Compound-08, isolated by chromatography as an oil, was then subjected to hydrolysis in the presence of 3.0 equivalents of KOH in ethanol/water at a temperature of 110 °C. This reaction, run overnight, produced the corresponding carboxylic acid Compound-09 in oil form.

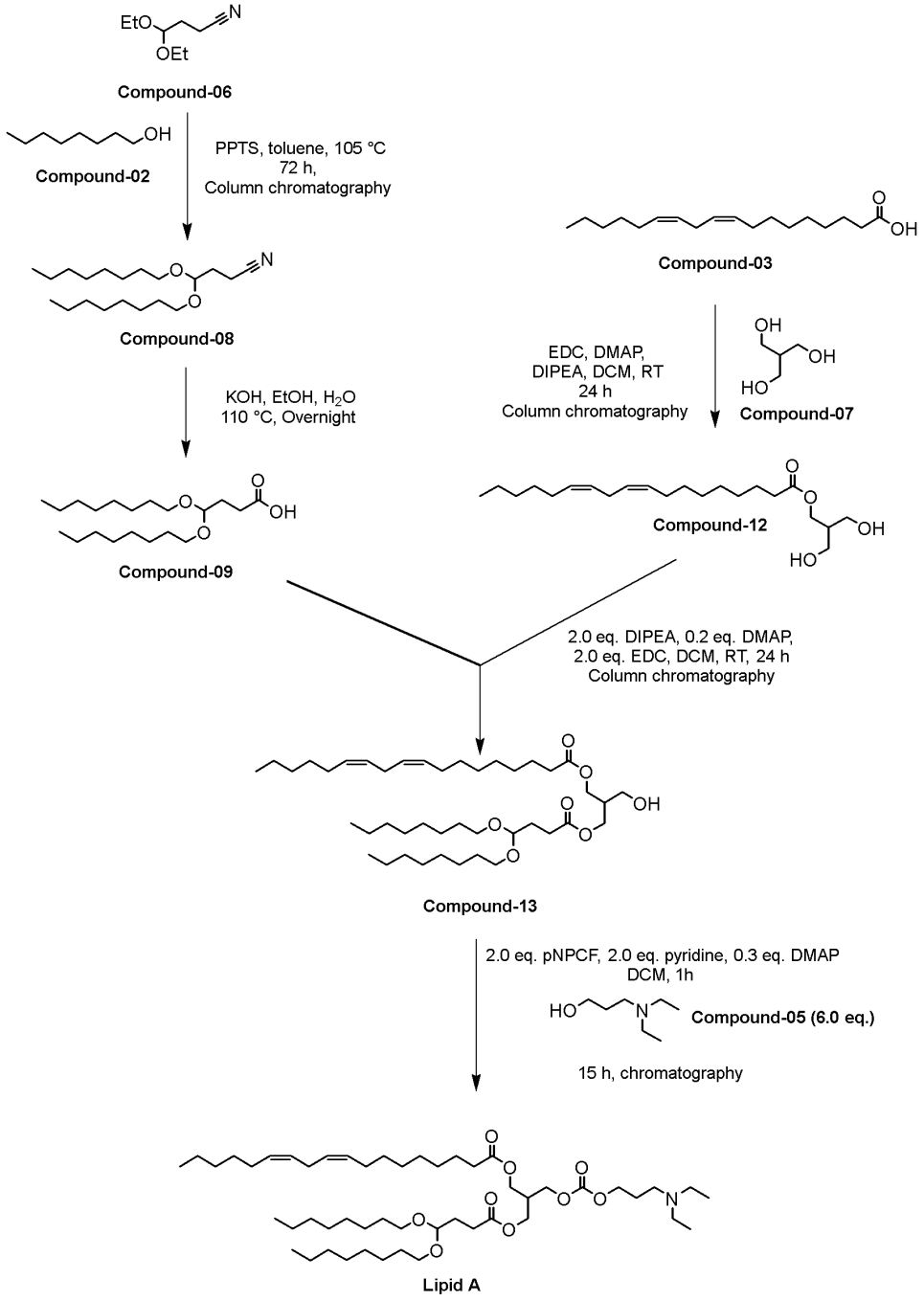
The synthesis of Compound-12 involved the combination of Compound-03 (linoleic acid) and Compound-07 (tris(hydroxymethyl)methane) with 1.5 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC, along with 1.5 equivalents of *N,N*-diisopropylethylamine (DIPEA) and a catalytic amount (0.2 equivalents) of 4-dimethylaminopyridine (DMAP) in dichloromethane (DCM), leading to the formation of the intermediate crude Compound-12, which was then purified via silica gel chromatography with an ethyl acetate/heptane step gradient, resulting in Compound-12.

The synthesis of Compound-13 was performed through carbodiimide-promoted monoester coupling of Compound-09 and 1.2 equivalents of Compound-12 with 2.0 equivalents of EDC, along with 2.0 equivalents of DIPEA and a catalytic amount (0.2 equivalents) of DMAP

(base) in DCM for 24 h, followed by purification of Compound-13 through silica gel chromatography with an ethyl acetate/heptane step gradient.

Compound-13 was further modified by incorporating an ionizable amine head group, which was achieved through carbonate coupling of Compound-13 with 2.0 equivalents of *p*NPCF, 2.0 equivalents of pyridine, and 0.3 equivalents of DMAP in DCM to generate Compound-14, followed by the addition of 6.0 equivalents of Compound-05. The resulting crude Lipid A product was then purified by silica gel chromatography with an ethyl acetate/heptane step gradient.

Scheme 1: Process flow diagram for Lipid A manufacturing by Process A



Example 3: Determination of purity of Lipid A produced by Process A, and levels of impurities

Lipid A was synthesized according to Process A, as shown in Scheme 1 and outlined in Example 2, at a 5 g-scale. The purity of Lipid A determined using the UPLC-CAD method of Example 1 was reported as 88.20%. The total level of other impurities was 11.80%. Data are reported in Table 1 and shown in FIG. 1.

Table 1: Results of Lipid A analysis for material generated using the procedure described in Example 2 (Process A), run at a 5 g-scale.

Attribute	**Test**	**Results corresponding to the product obtained from Process A**	
Appearance (color)	Visual inspection	Pale-yellow oil	
Purity of Lipid A	UPLC-CAD	88.20%	
Total Impurities*	UPLC-CAD	11.80%	
Identified Impurities**	UPLC-CAD	Compound-13	0.35%
		Impurity-22	0.25%
		Impurity-01	0.64%
		Impurity-10	0.62%
		Impurity-23	0.40%
		Impurity-24	0.25%
		Impurity-11	1.15%
		Impurity-20	0.84%
Additional Impurities**	UPLC-CAD	Impurity-12	2.07%
		Impurity-13	0.24%
		Impurity-14	1.98%
		Impurity-15	0.37%
		Impurity-16	0.40%
		Impurity-17	0.25%

		Impurity-18	0.51%
		Impurity-19	0.21%
		Impurity-21	0.36%
4-Nitrophenol (pNP)	UPLC-UV	192 ppm	

*Total impurities comprise both identified and additional impurities that were detected at a level ≥ 0.1%; **Identified and additional impurities listed in Table 1 were detected at a level ≥ 0.2%; identified impurities refer to impurities that were observed by the UPLC-CAD method and that have been structurally characterized by NMR techniques or confirmed by mass spectrometry via impurity synthesis and spiking, whereas additional impurities refer to impurities that were observed by the UPLC-CAD method and characterized by their UPLC-CAD chromatogram profile, but which have not been structurally characterized. .

In the following Examples 4-10, a series of process development steps were implemented to improve the Lipid A synthesis process in order to improve the purity of the Lipid A product through controlling the level of detected impurities, and to enable process scale-up for Lipid A. Unless otherwise indicated, all procedures described in Examples 4-10, and the corresponding impurity levels reported, were conducted at a process development scale (< 10 g).

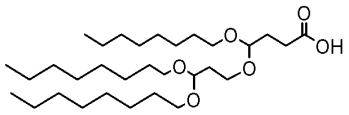
Example 4: Synthesis of Compound-10

As part of the Lipid A synthesis process improvement, Compound-01 (4,4-dimethoxybutanenitrile) was selected instead of Compound-06 (4,4-diethoxybutanenitrile) as a precursor for the synthesis of intermediate Compound-08. Compound-01 was shown to have several advantages relative to Compound-06, including a lower reaction temperature, lower cost of Compound-01, and a streamlined purification process for Compound-08 when produced from Compound-01.

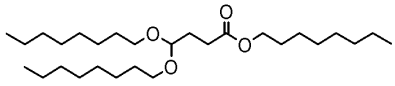
Compound-08 was synthesized as shown in Scheme 3 (left panel) using 4,4-dimethoxybutanenitrile (Compound-01, 1.0 equivalent), 1-octanol (Compound-02, 2.2 equivalents) and catalyst PPTS (0.05 equivalents) in toluene at 65 °C for 1 h. The toluene solution was then partially concentrated under vacuum. Toluene was added, and the solution was again partially concentrated under a vacuum. The steps of toluene addition and distillation were

repeated until the Compound-01 was consumed. The crude product containing Compound-08 in toluene was washed with water (1 X) and concentrated under reduced pressure at 40°C to obtain no more than 15 wt% of toluene solution. Compound-09 was synthesized with Compound-08 in toluene (1.0 equivalent) via 12.9 w/w% aqueous KOH (2.76 equivalents) hydrolysis in ethanol/water at a temperature of 80 °C and stirred for at least 36 h. The reaction was cooled to 50 °C and pH was adjusted to 4-5 using concentrated HCl while controlling the reaction mixture temperature at 0-10 °C. The crude Compound-09 mixture was extracted with heptane (3X). The combined heptane phases were extracted with 20% w/w% of aqueous sodium chloride solution. The organic product solution was dried over sodium sulfate and distilled to obtain Compound-09 as an oil.

The isolated Compound-09 oil generated in this manner at manufacturing scale (>1 kg) presented high levels of octanol (up to 7% w/w as measured by the HS-GC(FID) method of Example 1), Impurity-03 (~ 1%), Impurity-04 (~2.3%), and other impurities; some of which could not be removed by purification via acid/base extractions. Impurity-03 and Impurity-04 are represented in the below structural formulas.



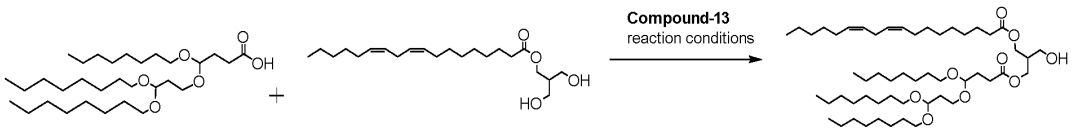
Impurity-03



Impurity-04

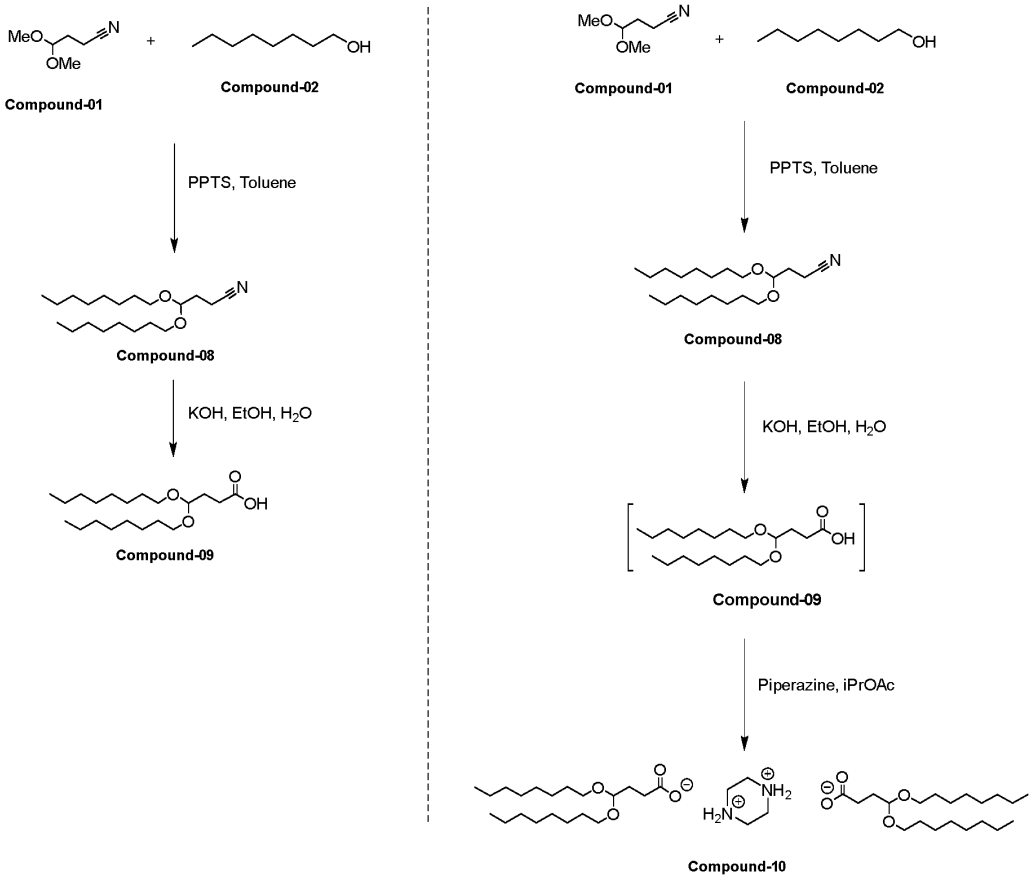
Such impurities can further react with reagents used in subsequent steps in the Lipid A synthesis, generating additional downstream impurities. For example, Impurity-03 was observed to carry through downstream operations yielding Impurity-05 after reaction with Compound-12 in the reaction towards Compound-13 (see Scheme 2 below).

Scheme 2: Proposed reaction for Impurity-05 formation from Impurity-03 reaction with Compound-12



Impurity-03 Compound-12 Impurity-05

Scheme 3: Left panel: Improved synthesis of Compound-09 (oil) using Compound-01; Right Panel: Synthesis of Compound-10 (salt).



In order to remove the described impurities, and to increase Compound-09 stability and enhance process scalability, isolation of Compound-09 as a salt via co-crystallization (Compound-10) was implemented. Compound-09 was synthesized as shown in Scheme 3 (right panel) from Compound-01 described above. Compound-09 was isolated as an oil at approximately 94% purity and subjected to co-crystallization, leading to Compound-10.

In order to develop the co-crystallization process, salt screening was performed using different inorganic bases (including NaOMe, KOMe, and $NaHCO_3$) or organic amine bases

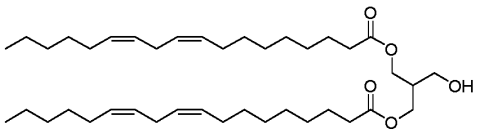
(including β-phenethylamine, dicyclohexylamine, cyclohexylamine, benzylamine, *tert*-butylamine, dibenzylamine, ethylenediamine, *tert*-octylamine, pyrrolidine, piperazine, 1,3-propylendiamine, and *p*-xylylendiamine). For this approach, briefly, 1.72 grams of Compound-09 oil was dissolved in 15 mL of methanol. The resulting Compound-09 stock solution was divided into fifteen 1 mL-vials. A base was added into each vial at a base:Compound-09 molar ratio of 1:1. Each vial (mixture) was stored at room temperature under air to form crystals. Two Compound-09 salts were isolated as solid crystals: Compound-09*piperazine (Compound-10 in Scheme 3 (right panel)) and Compound-09**p*-xylylenediamine, of which Compound-10 was preferred for further development as it had excellent solubility in water, even at neutral pH. This was beneficial for use of Compound-10 in downstream steps of the Lipid A synthesis process, to facilitate acidification in order to liberate the Compound-09 acid, making it available for chemical conversion in the subsequent step of the Lipid A synthesis.

As part of the development of the co-crystallization procedure, an extensive temperature-dependent solvent screen was performed to determine a suitable crystallization solvent. For this, Compound-10 was heated in organic solvents, including isopropyl alcohol, isopropyl acetate, methyl ethyl ketone (MEK) and methyl *tert*-butyl ether (MTBE) at 50 °C, and then left to cool at room temperature for an hour, to allow for crystallization/salt formation. The results of the solvent screen showed that Compound-10 had the highest solubility, resulting in the lowest recovery, in isopropyl alcohol, indicating that isopropyl alcohol was unsuitable for Compound-10 crystallization. On the other hand, solvents like isopropyl acetate, methyl ethyl ketone (MEK), and methyl tert-butyl ether (MTBE) showed comparable solubility profiles and recoveries, on the order of 85-90%. Moreover, the residual levels of 1-octanol in Compound-10 after co-crystallization were significantly reduced to ~1% (development scale), as measured by the HS-GC(FID) method of Example 1. Isopropyl acetate was selected as the solvent of choice for co-crystallization due to its higher boiling point (89 °C) and higher product recovery at high product purity. Compound-10 salt was generated in-situ in small quantity to obtain crystals for seeding. The seed crystals were added to the Compound-10 solution during co-crystallization to aid the crystallization process and support crystal formation . Compound-10 resulting from co-crystallization of Compound-09 with piperazine in isopropyl acetate had a purity of >99% as determined using the UPLC-CAD method of Example 1.

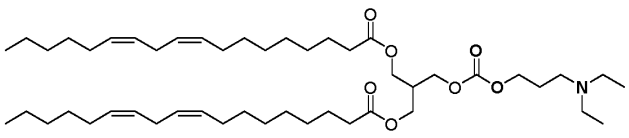
Compound-10 was acidified for salt splitting using 10% citric acid in CPME to obtain free Compound-09 acid for the synthesis of Compound-13. The purity of Compound-09 (free acid) (obtained after acidification of Compound-10) was maintained at >99%, measured using the UPLC-CAD method of Example 1, with reduced levels of 1-octanol (reduced to ~2.8% w/w), Impurity-03 (reduced to ~0.1%), and Impurity-04 (below level of detection), at manufacturing scale, relative to Compound-09 isolated as an oil (FIG. 2).

Example 5: Identification and control strategy for Impurity-06 and Impurity-07 during the synthesis of Compound-12

Impurity-01 observed in the impurity profile of Lipid A (Table 1) was identified as a follow-up impurity of Impurity-06. The structures of Impurity-01 and Impurity-06 are provided below.



Impurity-06



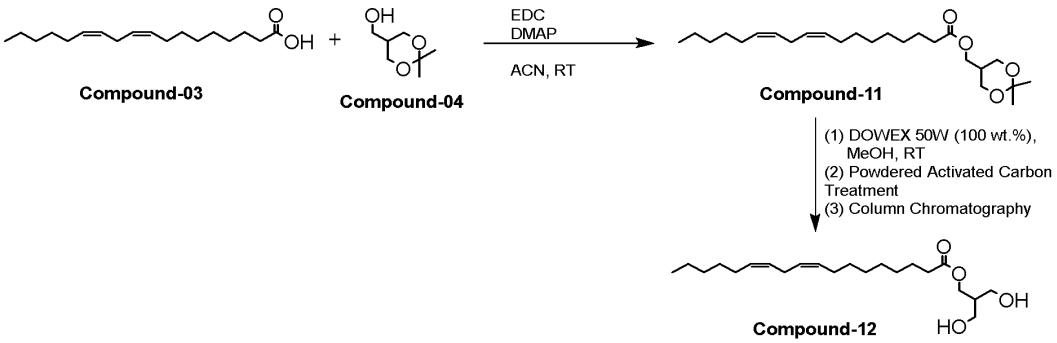
Impurity-01

Impurity-06 was postulated to form during the Compound-12 synthesis process via bis-esterification of Compound-07 with linoleic acid. Hence, a protecting group strategy was devised, and the synthesis of Compound-12 was modified in order to avoid the undesired bis-esterification of the Compound-07 product (resulting in the formation of Impurity-06). A dimethyl acetyl protecting group was introduced to protect the free alcohol in Compound-07 to obtain Compound-04 (2,2-dimethyl-1,3-dioxane-5-methanol), which was used to synthesize Compound-11, as shown in Scheme 4, by combining Compound-03 (linoleic acid) and Compound-04 with 1.86 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and a catalytic amount of 0.26 equivalents of 4-dimethylaminopyridine (DMAP) in acetonitrile. The reaction led to the formation of the crude intermediate Compound-11. Compound-11 was

then treated with a Dowex 50W ion exchange resin using methanol as the eluent with a flow rate of 2 mL/min to form Compound-12 as shown in Scheme 4. While the protecting group strategy was suited to avoid the formation of Impurity-06 during the synthesis of Compound-12, it was later observed that Impurity-06 also formed during conversion from Compound-11 to Compound-12 (Scheme 4), most likely due to trans-esterification (self-esterification) of Compound-12. The crude Compound-12 obtained from the above-described method at development scale contained ~25% Impurity-06 as measured by the UPLC-CAD method of Example 1.

An extensive screen of 14 powdered activated carbon (PAC) materials was performed in order to develop a process that leverages the selective adsorbance of Impurity-06 from crude Compound-12 to increase the purity of the Compound-12 crude product (containing ~25% of Impurity-06). The different PAC materials tested (including Norit KBEVN, Norit KBEV SUPRA, Norit BENTO NORIT CA1, Norit CGP Super, Norit CGSP, Cabot SX PLUS, Cabot A SUPRA EUR, Norit SX, Norit CASP, Norit SX PLUS LC, Norit CN1, Norit ROX 0.8, Norit C EXTRA USP, Norit GAC 1240 PLUS) featured different surface properties, including different surface areas, different bulk densities and different pore sizes. The PAC materials were tested in different solvents (e.g., heptane, methanol), and with different weight ratios of PAC to crude Compound-12 (e.g., PAC loading rates in the crude Compound-12 of 0%, 5%, 10%, 25%, 50%, or 100% by weight) to assess the impact of the PAC material, solvent, and PAC:crude weight ratio on the levels of Impurity-06 in the crude Compound-12 after PAC treatment. Each mixture was stirred for 1 hour in 1 mL of either heptane or methanol. The PAC was filtered off and the resulting solution was evaporated to dryness to measure Compound-12 purity and impurity levels using the UPLC-CAD method of Example 1. It was observed that treatment of the crude Compound-12 with the Cabot A SUPRA EUR PAC in methanol with a 25% wt/wt PAC:crude ratio reduced Impurity-06 levels to 15% without any loss of Compound-12. Therefore, this PAC at this ratio in methanol was selected for treating the crude Compound-12 as part of an improved Lipid A synthesis process. After treatment of the crude Compound-12 with the Cabot A SUPRA EUR PAC, the mixture was purified via silica gel chromatography with an ethyl acetate/heptane step gradient to completely purge Impurity-06 and obtain Compound-12 with a purity of 98.72% at development scale.

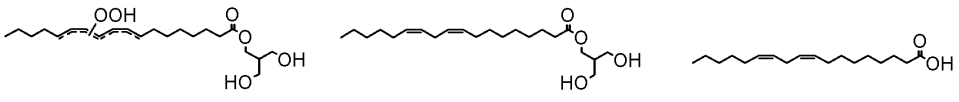
Scheme 4: Synthesis of Compound-12 using Compound-03 (linoleic acid) and Compound-04 (2,2-Dimethyl-1,3-dioxane-5-methanol)



Another impurity, Impurity-07, was detected, using the UPLC-CAD method of Example 1, in purified Compound-12 batches. The Impurity-07 amounts measured at different timepoints ranged from 2.5% to 6%, at manufacturing scale.

The molecular weight of Impurity-07 was determined using LC-MS and found to be 400.2 g/mol, equal to that of Compound-12 + 32 g/mol. It was proposed that Impurity-07 corresponded to a Compound-12 hydroperoxide impurity. Compound-12 is prone to undergoing lipid oxidative degradation due to the presence of double bonds with an active methylene group. Compound-03 and Compound-11, both used in the synthesis of Compound-12 as shown in Scheme 4, are also prone to oxidation, resulting in the formation of the corresponding hydroperoxide impurities, suggesting that hydroperoxide impurities could be introduced in the process with Coumpound-03 or Compound-11, or during the synthesis of Compound-12.

Structures of Compound-12 and Compound-03 and proposed structure of Impurity-07 are shown below.



Chemical Formula: $C_{22}H_{40}O_6$
Exact Mass: 400.28
Proposed Structure for Impurity-07

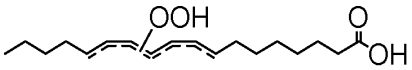
Chemical Formula: $C_{22}H_{40}O_4$
Exact Mass: 368.29
Compound-12

Chemical Formula: $C_{18}H_{32}O_2$
Exact Mass: 280.24
Compound-03

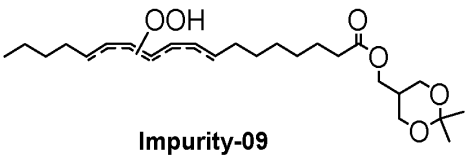
Compound-11 obtained as described above, and linoleic acid (Compound-03) were analyzed using the UPLC-CAD method of Example 1 with MS detection to test for the

corresponding hydroperoxide impurities (FIG. 3). The presence of the hydroperoxide impurities was confirmed by detection of the respective intact masses. Impurity-08 and Impurity-09 (see proposed regioisomers below) correspond to the hydroperoxide impurities from Compound-03 and Compound-11, respectively. With this observation, it was confirmed that Impurity-07 was also formed by its precursor impurities Impurity-08 (linoleic acid/Compound-03 stage) and Impurity-09 (Compound-11 stage).

Proposed structures for possible regioisomers of Impurity-08 and Impurity-09 are shown below.



Impurity-08



Impurity-09

Potential drivers for hydroperoxide formation are heat, light, and air. Multiple experiments, as shown in Table 2 and FIG. 4, were carried out to see the effect of heat, light and air on the rate of formation of Impurity-07 in the synthesized Compound-12 product over time. It was observed that the rate of formation for Impurity-07 increased at the highest temperature condition tested (40 °C). In addition, the formation of Impurity-07 increased under air exposure (higher rate of formation than that observed under argon), even at lower temperatures. Exposure to light had a significantly lesser impact on the rate of peroxide formation. The specification to store Compound-12 was modified with added controls in place to protect Compound-12 from heat, light and air and to store it at -20 °C.

Table 2: Data from stress study of Compound-12 produced at development scale (UPLC-CAD readout), demonstrating the effects of air, temperature, and light exposure (at different timepoints) on the formation of Impurity-07.

Experiment #	Temperature [°C]	Storage Conditions		t = 0 h*		t = 24 h (1 d)		t = 120 h (5 d)	
		Stored under	Vial type	Impurity-07, %	Compound-12, %	Impurity-07, %	Compound-12, %	Impurity-07, %	Compound-12, %
1	0	Air	Clear	5.39	88.19	7.27	85.14	12.07	80.32
2	0	Air	Amber			9.12	83.09	11.31	80.98
3	0	Argon	Clear			6.38	86.30	7.66	84.34
4	0	Argon	Amber			7.52	86.11	7.74	83.47
5	20	Air	Clear			9.16	81.74	16.27	70.38
6	20	Air	Amber			8.87	82.97	16.06	70.57
7	20	Argon	Clear			8.51	83.72	13.21	75.24
8	20	Argon	Amber			7.03	85.28	7.9	83.68
9	40	Air	Clear			12.13	74.91	18.83	40.03
10	40	Air	Amber			12.43	74.46	19.82	41.47
11	40	Argon	Clear			7.26	85.07	10.22	79.57
12	40	Argon	Amber			6.25	86.85	7.2	83.72

* t = 0 h corresponded to the start of the stress study (after ~6 weeks of sample being stored at -20°C without inert gas overlay).

Example 6: Purging of hydroperoxide impurities and use of Compound-10 at the Compound-13 synthesis stage

In the following example, an improved process for the synthesis of Compound-13 is described. Here, Compound-10 and Compound-12, produced according to the improved processes described in Example 4 and Example 5 respectively, are used to synthesize Compound-13. The effect of using these improved processes for the synthesis of the precursors, Compound-10 and Compound-12, is assessed by measuring the purity of Compound-13 as well as the impurity profile at Compound-13 stage.

Compound-13 synthesis

The Compound-10 salt, synthesized as described in Example 4, was acidified with 10% citric acid in CPME solution to release the Compound-09 free acid. The resulting Compound-09 as a solution of CPME was treated with 1.2 equivalents of Compound-12 synthesized as described in Example 5 (Scheme 4) in the presence of 1.2 equivalents of EDC, along with 1.2 equivalents of DIPEA and a catalytic amount of 0.2 equivalents of DMAP (base), using CPME as a solvent at room temperature. The reaction mixture was filtered through Celite®545 and purified via silica gel chromatography using a heptane/ethyl acetate step gradient to yield Compound-13.

Impact of the use of Compound-10 on the levels of Compound-09 related impurities in Compound-13

1-octanol levels measured by the HS-GC(FID) method of Example 1 in the purified Compound-13 product after completion of the reaction as described above, using purified Compound-10, were reproducibly reduced to 1% (development scale) compared to 7% (manufacturing scale) in Compound-09 isolated as an oil (i.e., without co-crystallization). Other impurities, such as Impurity-04, were purged during the purification of Compound-13 and reduced to non-detectable levels in the purified Compound-13 product based on UPLC-CAD data measured with the method of Example 1. Impurity-05, introduced by the reaction of Impurity-03 with Compound-12 at the Compound-13 stage (see Example 4 and Scheme 2) was not detectable as well in the purified Compound-13 product.

As described above, the use of Compound-10 for the generation of Compound-13 has proven to significantly improve the Compound-13 manufacturing outcome. Appropriate controls for piperazine content ≤ 0.15% (HS-GC(FID) method of Example 1) and citrate ≤ 0.1% (UPLC-CAD method of Example 1) (introduced during acidification to release the Compound-09 free acid) were introduced and both reagents were efficiently purged from the process.

Fate of hydroperoxide impurities during Compound-13 synthesis

The UPLC-CAD method of Example 1 was employed to observe the effect of the presence of hydroperoxide impurities on the synthesis of Compound-13, and to evaluate if the hydroperoxide impurity (Impurity-07) or it's follow-up impurity that can be generated during Compound-13 synthesis, can be purged at the Compound-13 stage, to avoid carry-over into Lipid A synthesis. This was done by comparing the UPLC-CAD chromatogram of a crude Compound-13 product obtained via the method described above (using Compound-10 as described in Example 4 and Compound-12 as described in Example 5), to the UPLC-CAD chromatogram of the same Compound-13 material after purification by silica gel chromatography with an ethyl acetate/heptane step gradient.

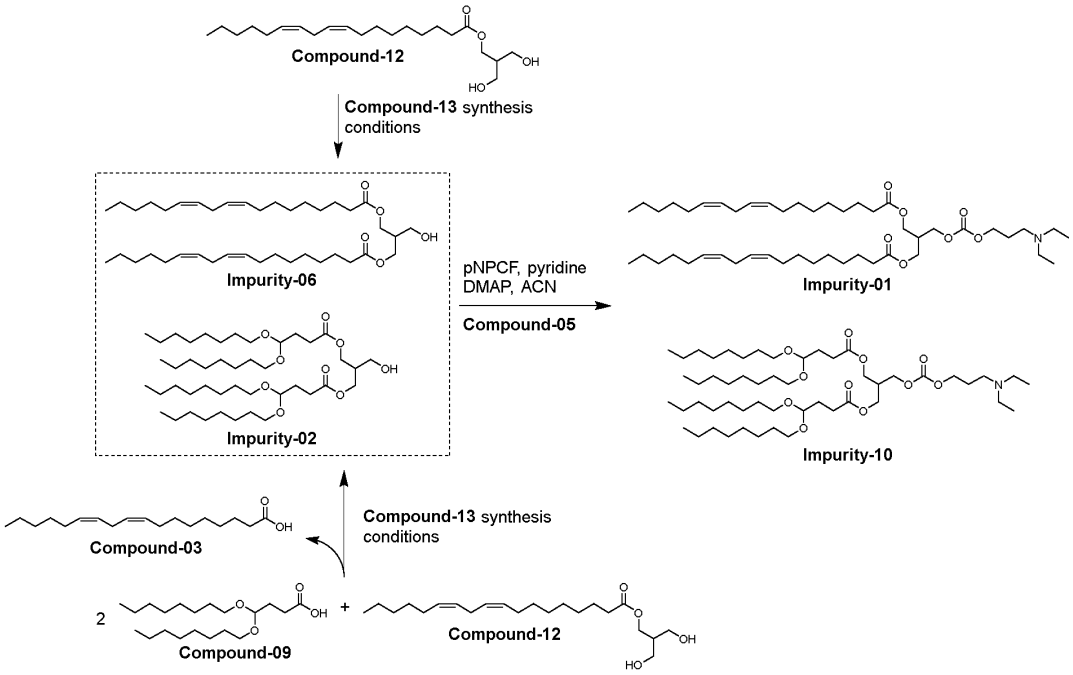
FIG. 5 shows the UPLC-CAD overlays of i) the crude Compound-13 material containing significant levels of Impurity-07 and related impurities as well as ii) the purified Compound-13 material, where hydroperoxide impurities are absent. Therefore, it was demonstrated at development scale that hydroperoxide levels of up to 6% in the crude Compound-13 product (carried over from Compound-12) could be purged to undetectable levels (<LOD) at the Compound-13 level through chromatographic purification using silica gel chromatography with an ethyl acetate/heptane step gradient.

Example 7: Identification and Control Strategy for Impurity-06 and Impurity-02 (and related downstream impurities)

Impurity-06 and Impurity-02, proposed precursors of Impurity-01 and Impurity-10 detected in the impurity profile of Lipid A (Table 1), were postulated to be esterification byproducts of the Compound-13 synthesis. Impurity-06 was hypothesized to form via self-esterification of Compound-12 at the Compound-12 synthesis step, where it is fully purged as

described in Example 5, but it can also form during Compound-13 synthesis (Scheme 5). If not controlled at this step, this impurity could further react in the Lipid A synthesis step to form Impurity-01, as shown in Scheme 5. Similarly, Impurity-02 was postulated to form via a esterification/transesterification reaction between Compound-09 and Compound-12, releasing linoleic acid. Impurity-02, if not controlled, would be converted to Impurity-10 during Lipid A formation, as shown in Scheme 5. It is critical to control these byproducts (i.e., Impurity-01, Impurity-10, Impurity-06, and Impurity-02) as they are not purged through purification of Compound-13 with column chromatography and co-elute with the product at the Compound-13 and Lipid A synthesis stages.

Scheme 5: Proposed formation of Impurity-06, Impurity-02, and their downstream impurities (Impurity-01 and Impurity-10 respectively).



Compound-13 was synthesized according to Process A, as described in Example 2 at development scale (5g), with modifications of Compound-09 and Compound-12 synthesis. Compound-13 was generated by carbodiimide-promoted monoester coupling of Compound-09

(obtained as shown in Scheme 3, left panel) and 1.2 equivalents of Compound-12 (obtained as described in Example 5) with 2.0 equivalents of EDC, along with 2.0 equivalents of DIPEA and a catalytic amount (0.2 equivalents) of DMAP (base) in DCM. The reaction mixture was stirred at a temperature of 20-22°C for approximately an hour or until the reaction was completed, as confirmed using the UPLC-CAD method of Example 1. The mixture was then diluted with DCM and washed twice with 6.3% aqueous NaCl solution. The resulting DCM layer was then distilled under reduced pressure at 40°C while being shielded from light. The isolated Compound-13 crude purity was measured by UPLC-CAD.

Stress studies were performed on crude Compound-13 demonstrating that under different reaction and storage conditions, Compound-13 was essentially stable. However, it was observed that Impurity-06 and Impurity-02 would form under thermal stress in concentrated solutions as experienced during Compound-13 synthesis via distillation/evaporation. For stress studies mimicking the in-process conditions of distillations, the concentrated crude Compound-13 material was stored for 1, 4 or 8 days at 40 °C or 60 °C. Even after one day of storage at 40 °C, the esterification byproducts developed in considerable amounts as shown in FIGs. 6-7 and Tables 3-4, with Impurity-06 detected at levels ranging from about 9% to about 13%, and Impurity-02 detected at levels ranging from about 2% to 7%. Impurity-06 and Impurity-02 can not be purged by silica gel column chromatography.

As a result of the study outcomes, the Compound-13 synthesis process was modified. In a first iteration of process updates, instead of distilling the crude reaction mixture to dryness, a solvent chase from DCM to heptane was employed via azeotropic distillation, to yield about 10 wt% to 50 wt% crude Compound-13 in DCM/heptanes.

Table 3: Stress study data of crude Compound-13 at 40 °C

	Purity (area %) measured by UPLC-CAD for different storage times		
Compound/Impurity	**1 d**	**4 d**	**8 d**
Compound-13	25.38%	14.26%	14.48%
Impurity-06	8.78%	10.57%	10.70%
Impurity-02	1.35%	6.61%	6.80%

Table 4: Stress study data of crude Compound-13 at 60 °C

	Purity (area %) measured by UPLC-CAD for different storage times		
Compound/Impurity	**1 d**	**4 d**	**8 d**
Compound-13	14.08%	13.57%	14.41%
Impurity-06	12.90%	9.58%	11.03%
Impurity-02	6.61%	6.96%	7.04%

In addition to the thermal stress study on concentrated crude Compound-13, a reaction solvent screen was performed. For this purpose, Compound-13 was synthesized as described above in different solvents (including DCM, CPME, tetrahydrofuran, 2-methyltetrahydrofuran, ACN, acetone, and toluene). The different reaction mixtures were subjected to NaCl extractions and co-evaporated multiple times with heptane. The resulting Compound-13 crude product in mixtures of heptane and the respective reaction solvents was subjected to different thermal stress conditions. The purity of Compound-13 and the levels of Impurity-06 and Impurity-02 were monitored at different time points using the UPLC-CAD method of Example 1.

It was concluded that changing the reaction solvent from DCM to CPME had a positive impact on Compound-13 purity and reduced impurity formation. FIG. 8 shows increased amounts of impurities in the concentrated crude Compound-13 when using DCM (reaction solvent used in Process A) compared to when CPME was used as a reaction solvent, as shown in FIG. 9. The thermal stress studies also supported the use of CPME over DCM for higher Compound-13 purity, even at temperatures as high as 50 °C.

The Compound-13 synthesis reaction was further investigated by screening reagent ratios and equivalents in a matrixed (one-factor-at-a-time) approach using CPME as solvent. Examples of bases screened include, but are not limited to, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,4-diazabicyclo[2.2.2]octane (DABCO), in amounts ranging from 0.01 equivalents to 4.0 equivalents. 4-pyrrolidinyl pyridine and *N*-Me-imidazole were screened as catalysts, in amounts ranging from 0.01 equivalents to 1.0 equivalents. It was demonstrated that changing the reaction conditions by reducing EDC and DIPEA equivalents from 2.0 and 2.0, to 1.2 and 1.2

respectively, produced similar Compound-13 yields with improved Compound-13 purity (>96%) and reduced impurity levels (Impurity-06 ~1%, and Impurity-02 <LOD) at development scale after purification using silica gel chromatography with heptane/ethyl acetate step gradient.

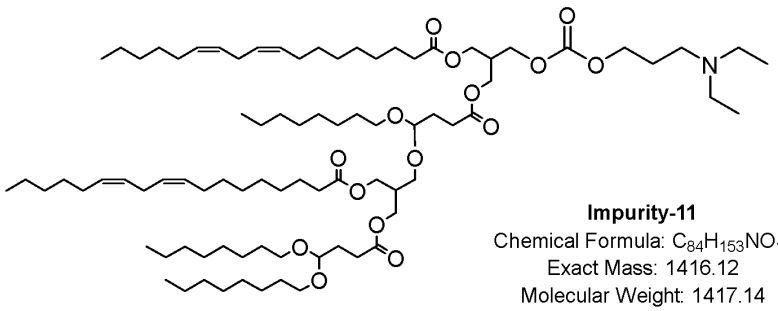
As discussed above, concentrated crude Compound-13 under thermal stress of a distillation is prone to forming Impurity-06 and Impurity-02. These impurities also form during storage at room temperature when crude Compound-13 is stored as a concentrated solution. It was further observed that Compound-13 esterification leading to the described by-products was faster when DCM was used as a reaction solvent. In order to completely avoid thermal stress on the Compound-13 crude product and reduce hold times, both to limit Impurity-06 and Impurity-02 byproduct formation, loading of the reaction mixture in CPME directly to the chromatography column was explored. For this, and to avoid concentrating the crude mixture, the NaCl extractions following the reaction were replaced by Celite®545 filtrations. Ultimately, the crude Compound-13 solution in CPME was diluted with heptanes up to a heptane:CPME volume ratio of 75:25, then filtered through Celite®545 before loading onto the purification column. This process allowed to control the levels of Impurity-06 and Impurity-02. The levels of Impurity-06 and Impurity-02 were 0.25% and 0.12% (at development scale), respectively.

Example 8: Identification of Impurity-11 and Impurity Control Strategy

As described in Example 3, Impurity-11 was observed during purity analysis of the Lipid A product obtained from Process A. It corresponded to a relative retention time of RRT=1.51 (relative to Lipid A) in the UPLC-CAD chromatogram obtained using the UPLC-CAD method of Example 1 (FIG. 1). A control strategy was developed to reduce the level of this impurity. Lipid A was synthesized according to Process A as described in Example 2 with minor modifications, wherein the carbonate coupling reaction of Compound-13 was performed in ACN instead of DCM. The resulting reaction mixture was diluted with heptane followed by a series of water and ACN extractions and chromatographic purification. Impurity-11 was detected in the final product at levels of 0.86% at a scale of 1kg.

Impurity-11 was isolated using preparative reverse-phase ultra-performance liquid chromatography (RP-UPLC) and identified using Liquid Chromatography-Mass Spectrometry (LC-MS). The initial structure was identified as a “Lipid A dimer” (Observed mass: 1417.21

g/mol) based on the preliminary LC-MS analysis. However, further structural analysis using ^{1}H-NMR (FIG. 10), ^{13}C-NMR and 2D-NMR techniques confirmed the structure shown below based on the chemical shifts and integrals.



Impurity-11
Chemical Formula: $C_{84}H_{153}NO_{15}$
Exact Mass: 1416.12
Molecular Weight: 1417.14

Comparison of Impurity-11 with Lipid A showed that Impurity-11 had twice the number of protons between the olefins (5.35 ppm, m, 8H) and the acetals (4.8, t, 2H) as compared to Lipid A (olefins: 5.28 ppm, m, 4H; acetals: 4.41 ppm, t, 1H). The presence of two terminal methyl groups (1.04 ppm, t, 6H) along with three methylene groups (2.58 ppm, m, 6H) further confirmed the structure of Impurity-11.

A mechanism was hypothesized for the formation of Impurity-11 as shown in FIG. 11. If the amount of pyridine is insufficient for quenching the HCl generated (1 equivalent) during the Compound-13 activation reaction, the remaining HCl may catalyze the formation of an Impurity-11 precursor. As shown in FIG. 11, Compound-13 would react with *p*NPCF to form Compound-14 and HCl. Instead of forming the desired Lipid A product, in a side reaction, a Compound-13 "dimer" intermediate would be generated that would be transformed to Impurity-11 via addition of the amino-headgroup through coupling with 3-diethylamino-1-propanol (Compound-05 in FIG. 11). It is also possible that Lipid A could react with any unreacted Compound-13 and HCl (generated in situ) to form Impurity-11.

In the process, without added process controls, it was possible to partially remove Impurity-11 from the final product via re-purification and by varying the pooling strategy, which was associated with a significant loss of Lipid A yield (~50% loss of yield). Design of experiments (DoE) was used to evaluate the reaction conditions, including testing the impact of varying amounts of pyridine. For the DoE execution, equivalents (eq.) for reagents such as

*p*NPCF (e.g., 1-3 eq.), pyridine (e.g., 1-6 eq.), 4-dimethylaminopyridine (DMAP) (e.g., 0-1 eq.), and Compound-05 (e.g., 1-12 eq.) were varied. In addition, the temperature upon addition of *p*NPCF (e.g., 0-20 °C) and the temperature after addition of Compound-05 (e.g., 0-40 °C) were adjusted alongside the volume additions of acetonitrile (e.g., 3-25 mL) following the Compound-13 reaction. The responses monitored using the UPLC-CAD method of Example 1 included but were not limited to the total peak area percentage (a%) for Lipid A, Compound-13, Compound-14, Impurity-11, and the total amount of other impurities. The statistical DoE analysis showed that there was a strong negative correlation between pyridine equivalents and the detected amounts of Impurity-11 suggesting that lower pyridine levels led to an increase in Impurity-11 levels. FIG. 12 shows the effects of levels of pyridine equivalents and Compound-05 equivalents on the overall purity of Lipid A. Data is reported in Table 5. The contour plots show a positive correlation between the equivalents of pyridine and Compound-05 on one hand and the purity of Lipid A on the other hand. However, it was also found that Compound-05 had a positive correlation with Impurity-11 suggesting that increasing Compound-05 equivalents may also increase Impurity-11 levels in the Lipid A product (FIG. 12).

Table 5: Effects of pyridine equivalents and Compound-05 equivalents on the overall purity of Lipid A and the levels of Impurity-11 in the Lipid A product obtained as described in Example 8.

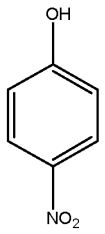
Exp No	**Pyridine eq.**	**Compound-05 eq.**	**Lipid A (%)**	**Impurity-11**
1	0.5	1	19.2	5.71
2	0.5	1	0.00	0.00
3	0.5	12	2.06	7.07
4	0.5	12	15.26	4.36
5	5.2	12	76.16	0.16
6	5.2	12	54.65	0.00
7	5.2	1	0.19	0.00
8	5.2	1	0.00	0.00
9	0.5	12	55.33	23.58
10	0.5	12	39.77	10.56
11	0.5	1	24.58	0.00

12	0.5	1	0.09	0.00
13	5.2	1	80.18	0.00
14	5.2	1	34.87	0.00
15	5.2	12	94.22	0.00
16	5.2	12	92.56	0.00
17	2.85	6.5	95.62	0.00
18	2.85	6.5	95.35	0.00
19	2.85	6.5	95.63	0.00

Verification experiments were carried out to validate the observed DoE correlations. The experiments suggested that a change in Compound-05 equivalents, from 1 equivalent to 12 equivalents, did not affect the formation of Impurity-11 if at least 3.5 equivalents of pyridine were used. After a detailed analysis of the experimental data, the amount of pyridine to be added to Compound-13 in the Lipid A synthesis process was set to 5.0 equivalents; the added amount of Compound-05 remained unchanged at 6.0 equivalents.

Example 9: Identification of pNP and Impurity Control Strategy

As evidenced from the impurity profile of Lipid A as described in Example 3, para-nitrophenol (pNP; also referred to herein as 4-nitrophenol) was observed as a byproduct of the synthesis of Lipid A. pNP is a bright yellow process-related impurity with structure shown below:

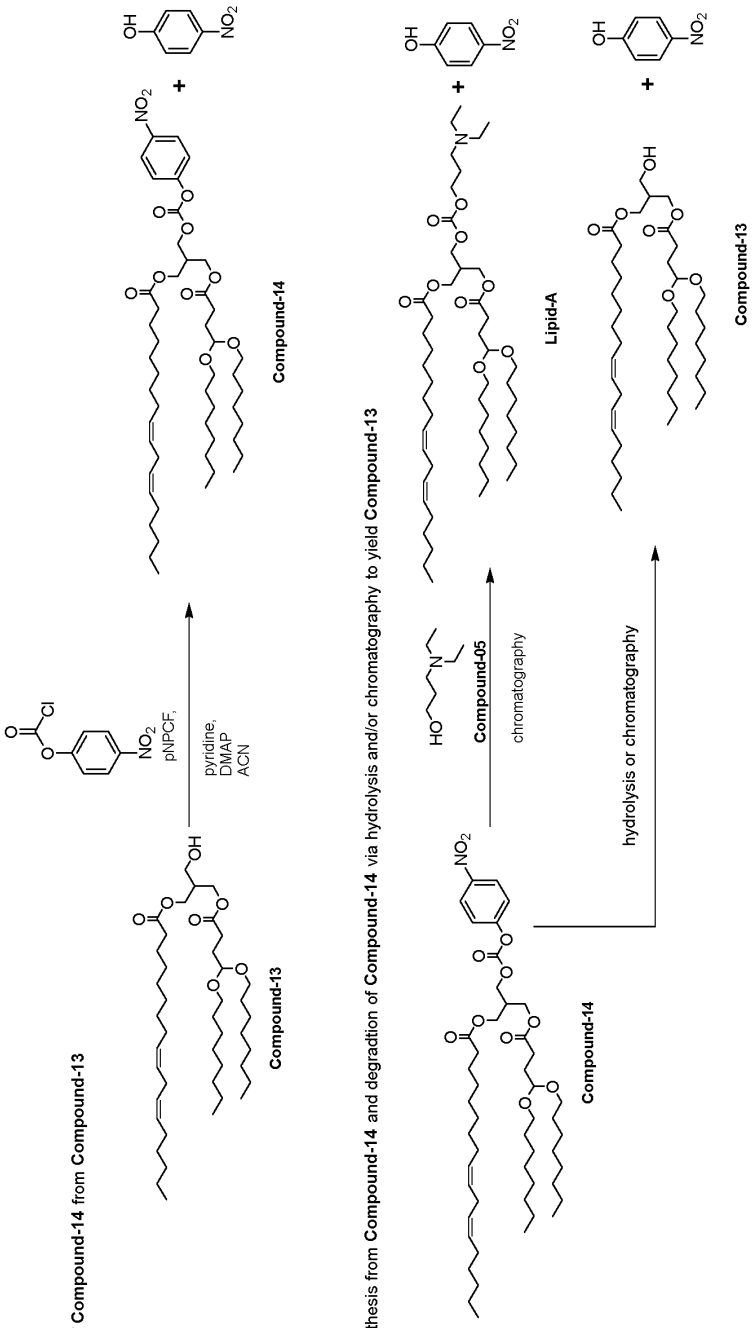


pNP
Chemical Formula: $C_6H_5NO_3$
Exact Mass: 139.03
Molecular Weight: 139.11

In order to track pNP levels in the process and in the final product, a UPLC-UV assay for pNP was developed and is described in Example 1. This assay was employed for process control and monitoring.

pNP results as a byproduct from the reaction of Compound-13 with *p*NPCF, as shown in Scheme 6, and the resulting Compound-14 releases pNP when coupled to the Compound-05 amino-alcohol headgroup. Usually, the pNP byproduct can be removed via water and ACN extractions of the crude Lipid A product, followed by chromatographic purification. However, it was also postulated that traces of unreacted Compound-14 could remain in the Lipid A product and could then release pNP during water extractions and/or chromatographic purification, as shown in Scheme 6. This hypothesis was corroborated by the detection of pNP at 192 ppm using the UPLC-UV method of Example 1 in the final purified Lipid A product obtained by Process A at development scale, which led to implementation of process improvements to remove trace amounts of pNP.

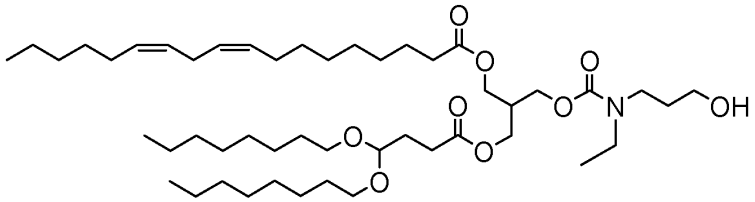
Scheme 6: Postulated reactions leading to the formation of pNP



It was postulated that incomplete conversion of Compound-14 to Lipid A significantly increased the amount of pNP in the final product (Lipid A). Hence, the amino alcohol Compound-05 (3-dimethylamino-1-propanol) is added to the reaction in excess to drive the synthesis of Lipid A to completion. Additionally, water and ACN extractions were used for crude Lipid A to deplete pNP levels. Since pNP was still detectable with UPLC-UV in trace amounts in the final Lipid A product, additional process improvements were introduced to remove trace pNP. It was determined that implementation of additional ACN extractions post chromatographic purification could reduce pNP levels from 24 ppm to 2 ppm at a process-development scale and to undetectable levels at scale (1kg-6 kg).

Example 10: Identification of Impurity-20 and Impurity Control Strategy

Impurity-20 was observed when producing Lipid A through Process A (0.84%). The LC-MS data was characteristic of a sodium adduct at m/z = 846.6428 ($M+Na^+$). The structure of Impurity-20 is shown below. The structural analysis was supported by ^{1}H-NMR (FIG. 13), ^{13}C-NMR and 2D-NMR techniques based on the chemical shifts and integrals.



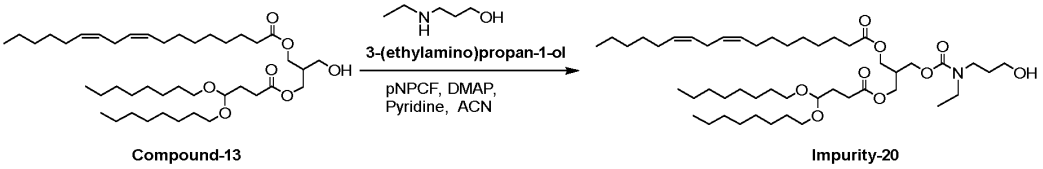
Impurity-20

Comparison of Impurity-20 with Lipid A showed that Impurity-20 contains only one methyl residue in the lipid head group at 1.03 ppm (br, 3H) whereas the head group methyl residues of Lipid A are observed at 1.04 ppm (t, 6H). Further, Impurity-20 ^{1}H-NMR confirms the presence of only two N-methylene groups at 3.20 ppm (m, 4H) as opposed to three N-methylene groups for Lipid A at 2.58 ppm (m, 6H). The molecular connectivity is further confirmed by 2D-NMR techniques.

A mechanism was hypothesized for the formation of Impurity-20 as shown in Scheme 7 below. 3-(ethylamino)propan-1-ol is an impurity present in commercially available Compound-

05 that is used in Lipid A synthesis. As shown in Scheme 7, Compound-13 is hypothesized to react with 3-(ethylamino)propan-1-ol (from Compound-05) to yield Impurity-20.

Scheme 7: Postulated formation for Impurity-20 from Compound-13 and 3-(ethylamino)propan-1-ol



Impurity-20 was present at 0.84% in the final Lipid A composition when Lipid A was produced using Process A (g-scale). Based on process improvements as described in Examples 4-9, Process B (as described in Example 11), and Process D (as described in Example 13) were performed at kg-scale. When Lipid A was produced using Process D, Impurity-20 was present at 0.53% before purification and was reduced to <LOQ (LOQ = Limit of Quantification) after purification (as described in Example 13). When Lipid A was produced using Process B (kg-scale, as described in Example 11), Impurity-20 was present at 0.65% before purification and was reduced to <LOQ after purification (as described in Example 11).

Further controls to the fraction selection process can be implemented to limit the amount of Impurity-20 in the final Lipid A product. Controls to limit the amount of 3-(ethylamino)propan-1-ol in Compound-05 are being implemented as part of the Impurity-20 control strategy.

Example 11: Synthesis of Lipid A using Process B

An improved Lipid A synthesis method, herein referred to as Process B, was developed featuring impurity control and improved process robustness, informed by the learnings obtained from Examples 4-9, which also enabled scale-up compared to Process A described in Example 2. Briefly, Process B involved the synthesis of two compounds, namely Compound-10 and Compound-12, followed by the coupling of in-situ generated Compound-09 (from salt splitting of Compound-10) and Compound-12 to form Compound-13. Finally, carbodiimide ester bond formation with Compound-05 (3-dimethylamino-1-propanol) produced Lipid A, as shown in Scheme 8.

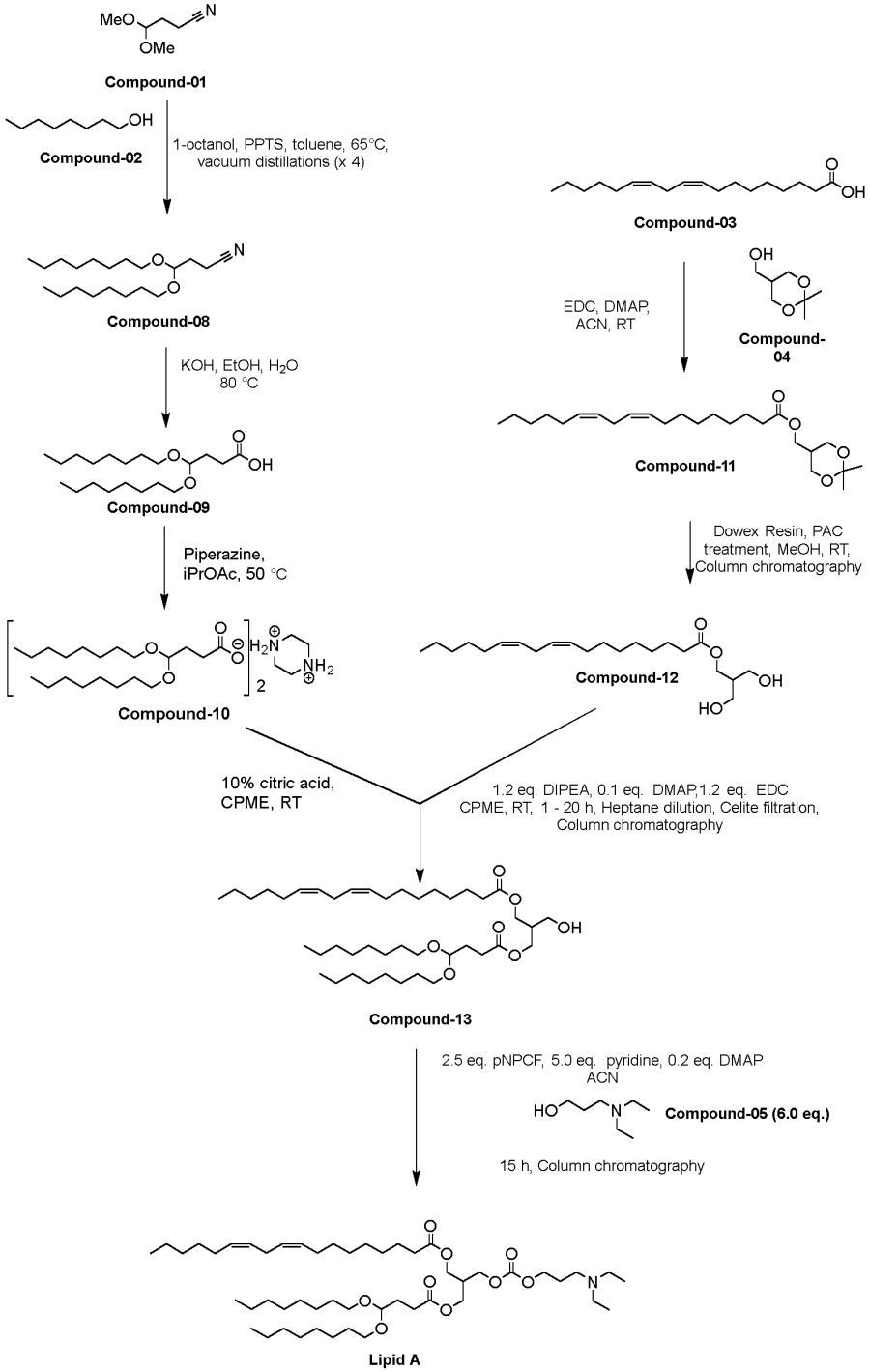
Compound-10 was formed as follows. First, 4,4-dimethoxybutanenitrile (Compound-01) was combined with 1-octanol (Compound-02) in toluene, using PPTS as a catalyst at a temperature of 65 °C for 1 h, with a partial distillation of toluene resulting in the formation of intermediate Compound-08. The resulting Compound-08 solution in toluene was subjected to hydrolysis in ethanol/water via addition of 2.76 equivalents of KOH at an internal temperature 80 °C for 40 h, producing the corresponding carboxylic acid Compound-09. Isolated Compound-09 was further purified via co-crystallization as a piperazine salt from a solution of isopropyl acetate. This was facilitated by treatment of Compound-09 with 0.55 equivalents of piperazine at 50 °C to ultimately produce the high purity Compound-10 salt form in large quantities via inoculation from small quantities of Compound-10 crystals.

Compound-12 was formed in two steps. The first step involved the combination of Compound-03 (Linoleic acid) and Compound-04 (2,2-Dimethyl-1,3-dioxane-5-methanol) with 1.86 equivalents of EDC and a catalytic amount of 0.26 equivalents of DMAP in ACN, leading to the formation of crude Compound-11. The reaction mixture was then quenched with 5% citric acid and extracted with heptane (3 X). In the second step, Compound-11 was treated with a Dowex 50W ion exchange resin using methanol as the eluent with a flow rate of 2 mL/min. Following the Dowex treatment, the crude mixture was concentrated twice with methanol, and the process was repeated until Compound-11 was consumed. The reaction mixture was subjected to PAC treatment (Cabot A SUPRA EUR PAC). The filtrate was then solvent-exchanged for heptane under reduced pressure. The crude product was purified via silica gel chromatography by elution with heptane/ethyl acetate mixtures (step gradient), resulting in Compound-12.

The synthesis of Compound-13 involved carbodiimide-promoted monoester coupling of Compound-09 free acid and Compound-12. First, Compound-10 salt solution in CPME was acidified with 10% citric acid to generate the free acid form, Compound-09. The resulting Compound-09 free acid solution in CPME was treated with 1.2 equivalents of Compound-12 in the presence of 1.2 equivalents of EDC, along with 1.2 equivalents of DIPEA and a catalytic amount of 0.2 equivalents of DMAP (base), using CPME as a solvent. The reaction mixture was diluted with heptane and filtered through Celite®545. Distillation was performed to obtain a 3:1 volume ratio of heptanes:CPME that was loaded directly on the column for purification of Compound-13 via silica gel chromatography with an ethyl acetate/heptane step gradient. Compound-13 was further modified by incorporating an ionizable amine head group, which was

achieved through carbonate coupling of Compound-13 using 2.5 equivalents of *p*NPCF, 5.0 equivalents of pyridine, and 0.2 equivalents of DMAP in ACN to generate Compound-14, followed by addition of 6.0 equivalents of Compound-05 at 22 ± 2 °C. The reaction mixture was extracted with heptane (2 X), and the combined heptane phases containing the Lipid A product were extracted with acetonitrile (5 X) and with water (1 X). The crude Lipid A mixture was then purified by silica gel chromatography with an ethyl acetate/heptane step gradient. Additional ACN extractions post-chromatography were performed to further reduce pNP levels, if pNP content >20 ppm. Process B was optimized at development scale (50g) and then used to produce Lipid A at large scale (6 kg).

Scheme 8: Process flow diagram for manufacturing of Lipid A (Process B)



Example 12: Synthesis of Lipid A using Process C

Lipid A synthesis as described in Process A (Example 2) was improved as follows. The improved process, Process C, illustrated in Scheme 9, was targeted to improve process robustness to enable scale-up to 1 kg-scale. Briefly, Compound-08 was synthesized using 4,4-dimethoxybutanenitrile (Compound-01, 1.0 equivalent), 1-octanol (Compound-02, 2.2 equivalents) and catalyst PPTS (0.05 equivalents) in toluene at 65 °C for 1 h. The toluene solution was then partially concentrated under vacuum. Toluene was added and the solution was again partially concentrated under vacuum. The steps of toluene addition, and distillation were repeated until Compound-08 was consumed. The crude product containing Compound-08 was dissolved with heptane and washed with water (1 X) and acetonitrile (2 X). The two combined acetonitrile phases were extracted with heptane (2 X). The three heptane phases were combined and concentrated under reduced pressure at 40°C. Compound-09 was synthesized using Compound-08 (1.0 equivalent) and 2.76 equivalents of ethanol/aqueous KOH (12.9 w/w%) at 80 °C with stirring for at least 36 h. The reaction was cooled to 50 °C and pH was adjusted to 4-5 using concentrated HCl while controlling the reaction mixture temperature at 0-10 °C. The crude Compound-09 mixture was extracted with heptane (3X). The combined heptane phases were extracted with 20% w/w% of aqueous sodium chloride solution. The organic product solution was dried over sodium sulfate and distilled to obtain Compound-09 as an oil.

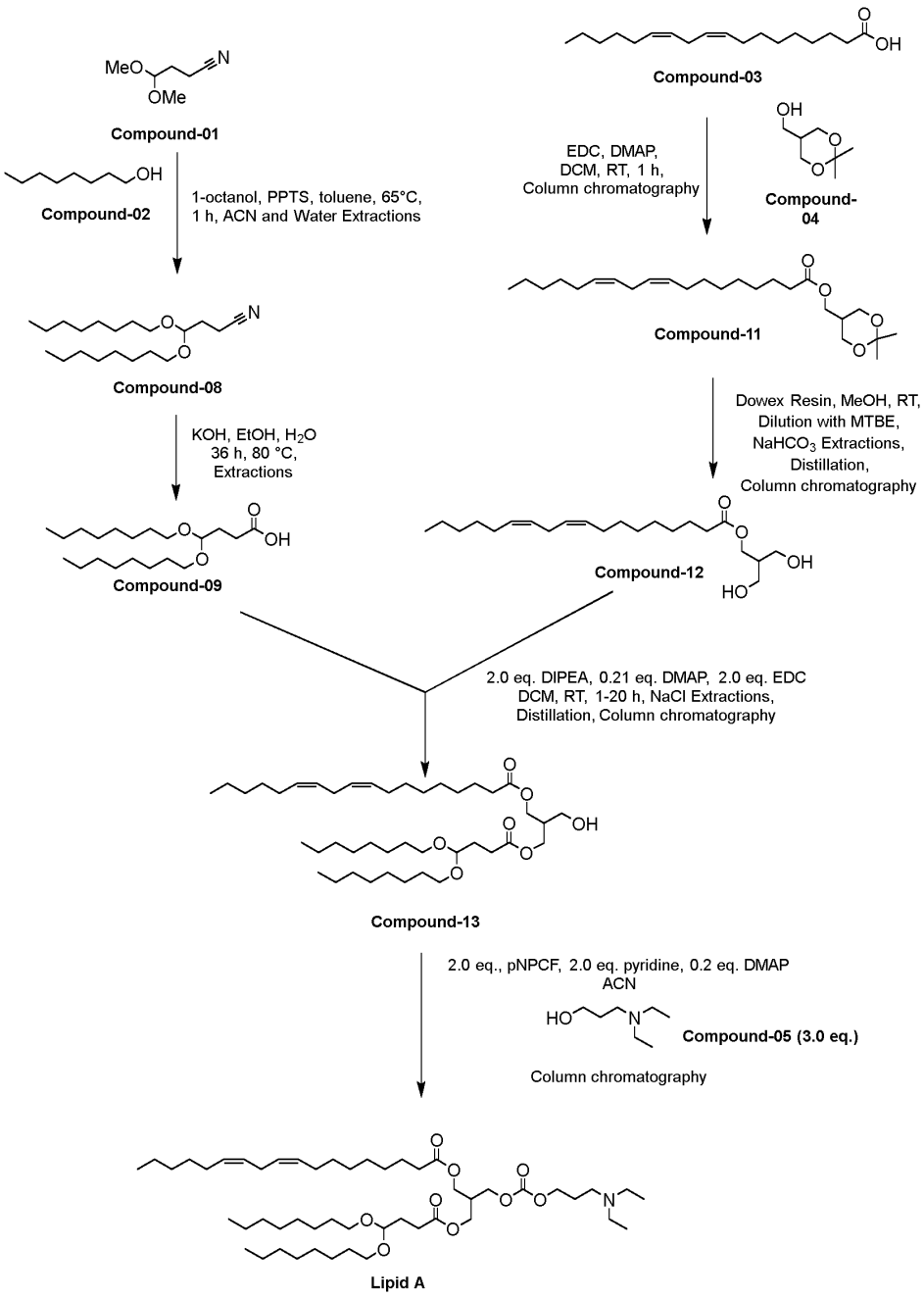
Compound-12 was synthesized in two steps using a protecting group strategy. In the first step, Compound-03 (Linoleic acid, 1.0 equivalents) was treated with Compound-04 (2,2-Dimethyl-1,3-dioxane-5-methanol, 1.0 equivalents) and 1.5 equivalents of EDC and a catalytic amount of 0.2 equivalents of DMAP in DCM. The resulting solution was stirred for one hour at 22 °C. The solution was partially concentrated under reduced pressure at 25°C while protecting from light. The crude product solution was then mixed with ethyl acetate and washed with an aqueous 20% NaCl solution (1X), 1 M HCl solution (1X), 20% w/w NaCl solution (1X), and 5% w/w sodium bicarbonate ($NaHCO_3$) solution (1X). The organic phase was then concentrated under reduced pressure at 40°C to distill DCM, while protecting from light. Finally, the crude product was purified through silica gel chromatography with an ethyl acetate/heptane gradient to obtain Compound-11. In the second step, Compound-11 (1.0 equivalents) was dissolved in methanol (MeOH) and treated with a Dowex 50W ion exchange resin using MeOH as an eluent with a flow rate of 2 mL/min. The mixture was stirred for at least 1 hour at 22 °C, then

concentrated under reduced pressure at 40 °C to 20% of the volume. MeOH was added again and stirred for 2 hours at 22 °C. This was repeated until Compound-11 was consumed. The mixture was filtered, and the filtrate was concentrated under reduced pressure at 40°C. The concentrated solution was diluted with MTBE and extracted with 5.3 w/w% aqueous sodium bicarbonate (1X). The aqueous layer was back extracted with MTBE. MTBE layers were combined and concentrated under reduced pressure. The crude product was purified via silica gel column chromatography by elution with heptane/ethyl acetate mixtures in a step gradient to obtain Compound-12.

Compound-12 (1.2 equivalents), DMAP (0.21 equivalents), DIPEA (2.0 equivalents), and EDC (2.0 equivalents) were dissolved in DCM. Then, Compound-09 (1.0 equivalents) was added in portions over 5 hours at 20-22 °C. After stirring for 1 hour, DCM was added, and dual extraction was performed using 6.3% w/w NaCl solution (2 X). The organic phase was fully evaporated under reduced pressure (40 °C). The product was purified via silica gel chromatography using an ethyl acetate/heptane step gradient, and concentrated under reduced pressure at 40°C, resulting in Compound-13.

*p*NPCF (2.0 equivalents) was added to Compound-13 (1.0 equivalents), pyridine (2.0 equivalents), DMAP (0.2 equivalents) in acetonitrile at 7-11°C to generate Compound-14, which was then reacted with Compound-05 (3.0 equivalents) at 22 ± 2 °C. The reaction mixture was extracted with heptane (2 X), and the combined heptane phases containing the Lipid A product were extracted with acetonitrile (5 X) and with water (1 X). Crude Lipid A was purified by silica gel chromatography using an ethyl acetate/heptane step gradient.

Scheme 9: Process flow diagram for manufacturing of Lipid A (Process C)



Example 13: Synthesis of Lipid A using Process D

Lipid A synthesis as described in Process C (Example 12) was improved further, using the learnings gathered from Examples 4-9, and implemented. The improved process, Process D, illustrated in Scheme 10, was used for large-scale production of Lipid A (up to 6 kg), and is suitable for a scale of up to 10 kg. Briefly, Compound-08 was synthesized using 4,4-dimethoxybutanenitrile (Compound-01, 1.0 equivalent), 1-octanol (Compound-02, 2.2 equivalents) and catalyst PPTS (0.05 equivalents) in toluene at 65 °C for 1 h. The toluene solution was then partially concentrated under vacuum. Toluene was added, and the solution was again partially concentrated under a vacuum. The steps of toluene addition and distillation were repeated until Compound-08 was consumed. The crude product containing Compound-08 in toluene was washed with water (1 X) and concentrated under reduced pressure at 40°C to obtain no more than 15 wt% of toluene solution. Compound-09 was synthesized with Compound-08 in toluene (1 equivalent) via 12.9 w/w% aqueous KOH (2.76 equivalents) hydrolysis in ethanol/water at a temperature of 80 °C with stirring for at least 36 h. The reaction was cooled to 50 °C and pH was adjusted to 4-5 using AcOH while controlling the reaction mixture temperature at 0-10 °C. The resulting mixture was co-evaporated with heptane, followed by a solvent exchange to isopropyl acetate. Piperazine (0.55 equivalents) was added to the Compound-09 solution in isopropyl acetate at 50°C. Compound-09 was crystallized following inoculation with the viscous suspension taken out from the reaction mixture, and Compound-10 product was filtered off.

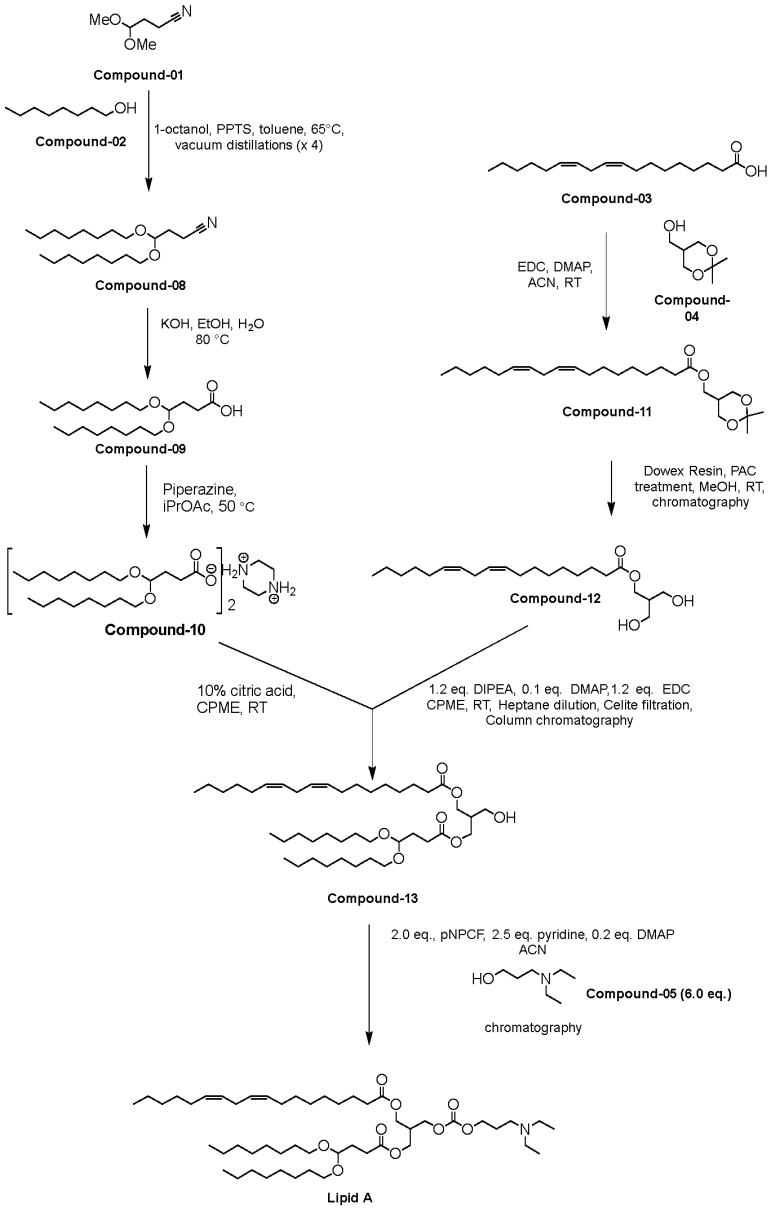
Compound-12 was synthesized in two steps. In the first step, Compound-03 (Linoleic acid, 1.0 equivalent) was treated with Compound-04 (2,2-Dimethyl-1,3-dioxane-5-methanol, 1.0 equivalent), EDC (1.5 equivalents), and a catalytic amount of DMAP (0.2 equivalents) in acetonitrile. The resulting solution was stirred for 3-4 hours at 22°C. The reaction mixture was then quenched with 5% citric acid and extracted with heptane (3 X). The organic phase was concentrated under reduced pressure at 40°C while protecting from light, resulting in the isolation of Compound-11. In the second step, Compound-11 (1.0 equivalent) was dissolved in methanol and passed through a column bed containing Dowex 50W ion exchange resin using methanol as the eluent with a flow rate of 2 mL/min, followed by a methanol wash. The reaction mixture was concentrated to half and refilled with methanol, and the process was repeated until Compound-11 was consumed. The methanol solution was treated with activated charcoal and

filtered. The filtrate was then solvent exchanged for heptane under reduced pressure. The crude product was purified via silica column chromatography by elution with heptane/ethyl acetate mixtures (step gradient), resulting in Compound-12.

The synthesis of Compound-13 involved carbodiimide-promoted monoester coupling of Compound-09 free acid and Compound-12. First, Compound-10 salt solution in CPME was acidified with 10% citric acid to generate the free acid form, Compound-09. The resulting Compound-09 free acid solution in CPME was treated with 1.2 equivalents of Compound-12 in the presence of 1.2 equivalents of EDC, along with 1.2 equivalents of DIPEA and a catalytic amount of 0.2 equivalents of DMAP (base), using CPME as a solvent. The reaction mixture was diluted with heptane and filtered through Celite®545. Distillation was performed to obtain a 3:1 volume ratio of heptanes:CPME that was loaded directly on the column for purification of Compound-13.

*p*NPCF (2.0 equivalents) was added to Compound-13 (1.0 equivalents), pyridine (2.5 equivalents), and DMAP (0.2 equivalents) in acetonitrile at 7-11 °C to generate Compound-14, which was then reacted with Compound-05 (3.0 equivalents) at 22 ± 2 °C. The reaction mixture was extracted with heptane (2 X), and the combined heptane phases containing the Lipid A product were extracted with acetonitrile (5 X) and with water (1 X). The crude Lipid A was purified by HPLC using an ethyl acetate/heptane step gradient. The purified Lipid A was further extracted with acetonitrile and distilled under reduced pressure to obtain pure Lipid A.

Scheme 10: Process flow diagram for manufacturing of Lipid A (Process D)



Example 14: Purity of Lipid A product obtained from Process A, Process B, Process C, and Process D performed at different scales

Table 6 depicts key differences between Processes A, B, C and D as described in Examples 2, 11, 12 and 13, respectively.

Table 6: Comparison of experimental conditions used at different stages of the processes described in Example 2 (Process A), Example 11 (Process B), Example 12 (Process C) and Example 13 (Process D).

Process	Compound-08	Compound-09	Compound-11	Compound-12	Compound-13	Lipid A
Process A	• Produced from Compound-06 as starting material • Reaction temperature: 105°C • Reaction duration: 72h • Reaction solvent: toluene • Column chromatography	• Produced from Compound-08 isolated as an oil and hydrolyzed with KOH in EtOH/H_2O • Reaction temperature: 110°C • Reaction duration: Overnight • pH adjustment with concentrated HCl	N/A	• Produced from Compound-03 and Compound-07 • Reaction solvent: DCM • Column chromatography	• Produced from Compound-09 and Compound-12 in DCM • Reagent equivalents: Compound-09 (1.0 eq.), Compound-12 (1.2 eq.), DMAP (0.2 eq.), EDC (2.0 eq.), DIPEA (2.0 eq.) • Reaction temperature: 20-22 °C • Reaction duration: 24h • Column chromatography	• Reagent equivalents: Compound-13 (1.0 eq.), DMAP (0.3 eq.), Pyridine (2.0 eq.), pNPCF (2.0 eq.), Compound-05 (6.0 eq.), • Reaction duration: 15h • Reaction solvent: DCM • Column chromatography
Process B	• Produced from Compound-01 as starting material • Reaction temperature: 65°C • Reaction duration: 1h • Reaction solvent: toluene • Partial distillation of toluene	• Use of Compound-08 isolated as solution of toluene and hydrolyzed with KOH in EtOH/H_2O • Reaction temperature: 80 °C • Reaction duration: 40h • Solvent chase to iPrOAc • pH adjustment to 4-5 with AcOH • Base addition (piperazine), 50 °C • Isolation of Compound-10 by filtration	• Introduced through a protecting group strategy by using Compound-03 and Compound-04 in ACN • Reaction duration: 3-4h • Quench with Citric acid • Extractions with Heptane (3X)	• Produced from Compound-11 treated with Dowex 50W ion exchange resin, MeOH • Distillation to 50% of initial volume of MeOH • PAC treatment, 0.5 h • Solvent chase to heptane. • Column chromatography	• Produced from Compound-10 salt split with citric acid and Compound-12 in CPME • Reagent equivalents: Compound-10 (1.0 eq.), Compound-12 (1.2 eq.), DMAP (0.1 eq.), EDC (1.2 eq.), DIPEA (1.2 eq.) • Reaction temperature: 20-22 °C • Reaction duration: 1-20h • Dilution with heptane • Celite filtration • Heptane/CPME (3:1 v/v) ratio adjustment for column chromatography • Column chromatography	• Reagent equivalents: Compound-13 (1.0 eq.), DMAP (0.2 eq.), Pyridine (5.0 eq.), pNPCF (2.5 eq.), Compound-05 (6.0 eq.) • Reaction duration: 15 h • Reaction solvent: ACN • Column chromatography • Optional ACN extractions if pNP content >20ppm
Process C	• Produced from Compound-01 as starting material	• Produced from Compound-08 isolated as an oil and	• Introduced through a protecting	• Produced from Compound-11 treated with	• Produced from Compound-09 and Compound-12 in DCM	• Reagent equivalents: Compound-13 (1.0 eq.), DMAP (0.2 eq.),

	• Reaction temperature: 65°C • Reaction duration: 1h • Reaction solvent: toluene • ACN and water extractions	hydrolyzed with KOH in EtOH/H_2O • Reaction temperature: 80°C • Reaction duration: 36h • pH adjustment with concentrated HCl • Extractions (heptanes, 20% NaCl)	group strategy by using Compound-03 and Compound-04 in DCM • Reaction duration: 1h • Solvent chase to EtOAc • Extractions • Column chromatography	Dowex 50W ion exchange resin, MeOH • Distillation of MeOH and dilution with MTBE • Extractions with 5.3% $NaHCO_3$ • Distillations of MTBE • Column chromatography	• Reagent equivalents: Compound-09 (1.0 eq.), Compound-12 (1.2 eq.), DMAP (0.21 eq.), EDC (2.0 eq.), DIPEA (2.0 eq.) • Reaction temperature: 20-22 °C • Reaction duration: 1-20h • Extractions (6.3% NaCl) • Distillation of DCM • Column chromatography	Pyridine (2.0 eq.), pNPCF (2.0 eq.), Compound-05 (3.0 eq.) • Reaction duration: 15 h • Reaction solvent: ACN • Extractions (Heptane, Acetonitrile, water) • Column chromatography
Process D	• Produced from Compound-01 as starting material • Reaction temperature: 65°C • Reaction duration: 1h • Reaction solvent: toluene • Partial distillation of toluene	• Use of Compound-08 isolated as solution of toluene and hydrolyzed with KOH in EtOH/H2O • Reaction temperature: 80 °C • Reaction duration: 36h • Solvent chase to iPrOAc • pH adjustment to 4-5 with AcOH • Base addition (piperazine), 50 °C. • Isolation of Compound-10 by filtration	• Introduced through a protecting group strategy by using Compound-03 and Compound-04 in ACN • Reaction duration: 3-4h • Quench with Citric acid • Extractions with Heptane (3X) •	• Produced from Compound-11 treated with Dowex 50W ion exchange resin, MeOH • Distillation to 50% of initial volume of MeOH • PAC treatment, 0.5 h • Solvent chase to heptane. • Column chromatography	• Produced from Compound-10 salt split with citric acid and Compound-12 in CPME • Reagent equivalents: Compound-10 (1.0 eq.), Compound-12 (1.2 eq.), DMAP (0.1 eq.), EDC (1.2 eq.), DIPEA (1.2 eq.) • Reaction temperature: 20-22 °C • Reaction duration: 1-20h • Dilution with heptane • Celite filtration • Heptane/CPME (3:1 v/v) ratio adjustment for column chromatography • Column chromatography	• Reagent equivalents: Compound-13 (1.0 eq.), DMAP (0.2 eq.), Pyridine (2.5 eq.), pNPCF (2.0 eq.), Compound-05 (6.0 eq.) • Reaction duration: 15 h • Reaction solvent: ACN • Extractions (Heptane, Acetonitrile, water) • Column chromatography/HPLC • Extractions (ACN)

Data from the purity and impurity analysis of Lipid A manufactured using Process A (5 g-scale), Process B (50 g-scale and 6 kg-scale), Process C (1 kg-scale) and Process D (6 kg-scale) are shown in Table 7. FIG. 14 shows an overlay of the UPLC-CAD

chromatograms for the Lipid A product obtained from Process B at the 50 g scale and the Lipid A product obtained from Process A at the 5 g-scale; the inset corresponds to the UPLC-CAD chromatogram obtained from Process B.

Table 7: Lipid A product data generated for material synthesized via Process A (5 g-scale, Example 2), Process B (50 g scale, Example 11), Process C (1 kg scale, Example 12) Process D (6 kg scale, Example 13), and Process B (6 kg scale).

Attribute	**Test**	**Compound/ Impurity tested**	**Process A (Example 2, scale: *5 g*)**	**Process B (Example 11, scale: *50 g*)**	**Process C (Example 12, scale: *1 kg*)**	**Process D (Example 13, scale: *6 kg*)**	**Process B (Example 11, scale: *6 kg*)**
Appearance (color)	Visual inspection	Lipid A	Pale-yellow oil	Colorless oil	Pale-yellow oil	Colorless oil	Clear oil
Purity of Lipid A	UPLC-CAD	Lipid A	88.20%	97.68%	93.23%	96.45%	97.1%
Total Impurities*	UPLC-CAD	Total Impurities	11.80%	2.32%	6.77%	3.55%	2.9%
Identified Impurities**	UPLC-CAD	Compound-13	0.35%	0.18%	1.25%	0.34%	1.0%
		Impurity-22	0.25%	0.27%	0.28%	0.23%	0.25%
		Impurity-01	0.64%	0.46%	2.67%	0.72%	1.2%
		Impurity-10	0.62%	0.12%	0.32%	0.87%	0.31%
		Impurity-23	0.40%	0.34%	0.55%	0.29%	0.17%
		Impurity-24	0.25%	<LOQ	0.61%	<LOQ	<LOQ
		Impurity-11	1.15%	<LOQ	<LOQ	1.10%	<LOQ
		Impurity-20[#]	0.84%	<LOQ	<LOQ	<LOQ	<LOQ
	UPLC-CAD	Impurity-12	2.07%	<LOQ	<LOQ	<LOQ	<LOQ

Additional Impurities**		Impurity-13	0.24%	<LOQ	<LOQ	<LOQ	<LOQ
		Impurity-14	1.98%	<LOQ	0.24%	<LOQ	<LOQ
		Impurity-15	0.37%	<LOQ	0.23%	<LOQ	<LOQ
		Impurity-16	0.40%	<LOQ	0.22%	<LOQ	<LOQ
		Impurity-17	0.25%	<LOQ	<LOQ	<LOQ	<LOQ
		Impurity-18	0.51%	<LOQ	<LOQ	<LOQ	<LOQ
		Impurity-19	0.21%	<LOQ	<LOQ	<LOQ	<LOQ
		Impurity-21	0.36%	<LOQ	<LOQ	<LOQ	<LOQ
		Impurity-25	<LOQ	<LOQ	0.26%	<LOQ	<LOQ
		Impurity-26	<LOQ	<LOQ	0.16%	<LOQ	<LOQ
4-Nitrophenol (pNP) content	UPLC-UV	pNP	192 ppm	n.d.	1840 ppm	n.d.	n.d.

n.d. = not detected; LOQ = Limit of Quantification; *Total impurities comprise both identified and additional impurities that were detected at a level ≥ 0.1%; **Identified and additional impurities listed in Table 7 were detected at a level ≥ 0.2%; identified impurities refer to impurities that were observed using the UPLC-CAD method of Example 1 and that have been characterized by mass spectrometry (confirmed by spiking comparison using synthesized impurities), whereas additional impurities refer to impurities that were observed by the UPLC-CAD method and characterized by their UPLC-CAD chromatogram profile, but which have not been identified. #Impurity-20 is now an identified impurity, controlled as such since Process B implementation (kg-scale).

Scale up considerations related to lipid manufacturing and impurity control strategy

The scale at which a process is performed can have a significant impact on the formation of impurities and the resulting impurity profile. This is especially true for lipid products where certain side-reactions may be prominent and dependent on process parameters where scale can have a significant impact. For instance, the speed of heating/cooling or mixing may be significantly slower at larger scale due to the use of larger volumes, which could mean more stress on the product and an increase in side-products from transesterification and other side reactions (see below).

As described in this Example 14, different Lipid A synthesis processes were developed and conducted at different batch scales, including 5 g (Process A), 50 g (Process B), 1 kg (Process C) and 6 kg (Process B, and Process D). In order to discuss the effects of scale on impurity formation and impurity profiles, the respective processes and outcomes are compared in this Example 14.

Process C was initially developed as a scaled-up and improved process from Process A, incorporating process development learnings and significant process improvements, such as changes implemented in the synthesis of Compound 12, according to Example 12. The improvement of the process is demonstrated by the fact that despite a significantly increased scale, which often leads to a decrease of purity in the absence of additional process controls, Process C conducted at a 1kg scale shows superior process performance compared to Process A conducted as a lab-scale/benchtop process (5 g).

Additional process development efforts, as informed by the learnings of Examples 4-9 provided herein led to the development of Process B (50 g-scale, and 6 kg-scale, as described in Example 11), and Process D (6 kg-scale, as described in Example 13). From the comparison in Table 6, it is to be noted that Process D and Process B are largely similar. Process B was also run at a 6 kg-scale, for direct comparison of scale effect on each of Process B and Process D.

The impurity profile comparison for the processes as reported in Table 7 demonstrates how scale may affect the final Lipid A product purity as well as the impurity levels during lipid manufacturing. The control of process parameters at the kilogram scale is critical to control impurity formation, especially for a product like Lipid A that can undergo side reactions leading to lipid tail variation (for instance as described in Example 7).

The impact of scale can be highlighted via the following representative examples:

1) At development scales, Impurity-11 was present at a level of 1.15% in Process A (5 g-scale). In Process D (6 kg), Impurity-11 was also observed, at 1.10%, despite advanced in-process controls compared to Process A, possibly due to the larger scale at which Process D was run. When including further improvements to the process through the development of Process B (as described in Example 11), Impurity-11 was <LOQ at both g-scale (50 g) and kg-scale (6 kg).
2) Impurity-23 was observed at 0.40% in the small-scale Process A (5g). However, at scale in Process C (Example 12), Impurity-23 was observed at 0.55%. Further improvements to the process that were implemented in Process D (Example 13) allowed the reduction of Impurity-23 levels to 0.29% at large scale (6 kg). Finally, Impurity-23 levels were further reduced to 0.17% when Process B was scaled up to 6 kg, highlighting the efficacy of the improvements incorporated in this process, and the robustness of the impurity reduction despite scaling up.
3) Impurity-01 was observed at 0.64% in Process A (5g-scale), and at 2.67% in Process C (after scaling up to 1kg). Further development work and ultimately performance at large scale allowed for the reduction of Impurity-01 levels to 0.46% with Process B (50 g-scale) and 0.72% with Process D (6 kg-scale). Scaling Process B up to 6 kg, yields 1.2% Impurity-01, which is comparable to the Impurity-01 levels obtained in Process D (6 kg).
4) The measured pNP content was 192 ppm in a 5 g batch of Lipid A product (Process A). However, pNP was observed at 1840 ppm at the 1 kg-scale using Process C. When Lipid A was produced using Process B at small scale (50 g), or Process D at large scale (6 kg), pNP levels were reduced to levels below the limit of detection.
5) Compound-13 was observed at 0.35% in the Lipid A product for Process A (5 g-scale). Upon scale-up to 1 kg using Process C, Compound-13 levels increased to 1.25%. After further process modifications, Process D performed at large scale (6 kg) reduced the levels of Compound-13 to 0.34%. Process B reduced Compound-13 levels to 0.18% at small scale (50 g). However, scaling up Process B to 6 kg produced 1.0% of Compound-13. The increase in Compound-13 is attributed to degradation of Lipid A that occurred due to a process excursion during purification of Lipid A at large-scale (Process B).

The total level of impurities as shown in Table 7 comprises identified and additional impurities that were detected at a level ≥ 0.1%. Process A produced a composition comprising 88.20% Lipid A and 11.80% of total impurities at small scale (5 g). After process modifications, the level of total impurities obtained with Process C at intermediate scale (1 kg) was significantly reduced to 6.77%, despite certain individual impurities increasing (as discussed above) that can be attributed to the scaling of the process. Further improvements as described in Example 13 for Process D at large scale (6 kg) produced a composition comprising 96.45% Lipid A and 3.55% of total impurities, a significant reduction from g-scale Process A and kg-scale Process C. Process B, both at g-scale and kg-scale, produced the highest purity of Lipid A (97.68%, 50 g-scale and 97.1%, 6 kg-scale) and the lowest composition of total impurities (2.32%, 50 g-scale and 2.9%, 6 kg-scale).

INCORPORATION BY REFERENCE

The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicant reserves the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

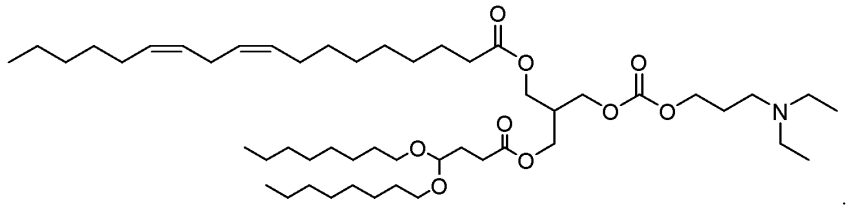
EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

CLAIMS

1. A composition, as determined by ultra-performance liquid chromatography (UPLC), comprising:

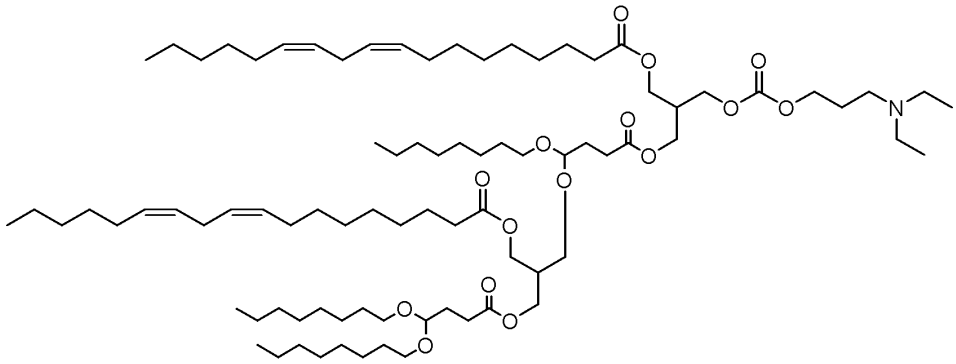
at least 90% of Lipid A



2. The composition of claim 1, wherein the composition comprises at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% Lipid A.

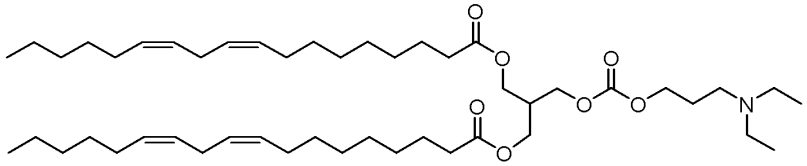
3. The composition of claim 1 or claim 2, wherein the composition comprises:

(A) less than about 1.1%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (V)



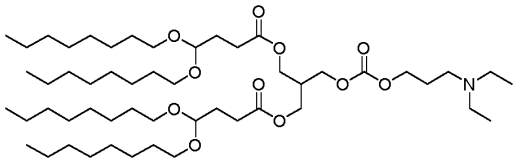
(V), or

(B) less than about 0.6% or less than about 0.5% of a compound having the structure of formula (VI)



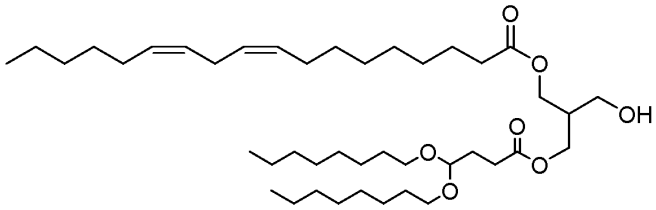
(VI), or

(C) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, or less than about 0.2%, of a compound having the structure of formula (VII)



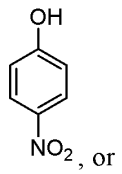
(VII), or

(D) less than about 0.3% or less than about 0.2% of a compound having the structure of formula (III)



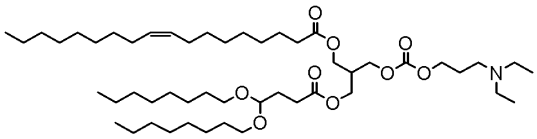
(III), or

(E) less than about 190 ppm, less than about 180 ppm, less than about 170 ppm, less than about 160 ppm, less than about 150 ppm, less than about 140 ppm, less than about 130 ppm, less than about 120 ppm, less than about 110 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



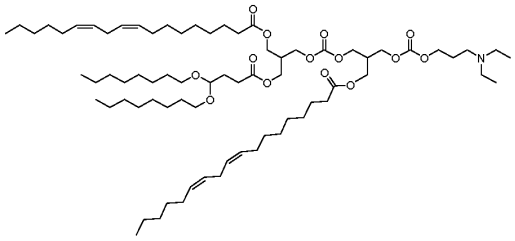
, or

(F) less than about 0.4% or less than about 0.3% of a compound having the structure of formula (VIII)



(VIII), and/or

(G) less than about 0.2% or less than 0.1% of a compound having the structure of formula (IX)



(IX).

4. The composition of claim 3, comprising less than about 0.3% of the compound of formula (V), preferably less than about 0.2% of the compound of formula (V), preferably less than about 0.1% of the compound of formula (V), even more preferably wherein the compound of formula (V) is undetectable.

5. The composition of any one of claims 3-4, comprising less than about 0.55% of the compound of formula (VI), even more preferably less than 0.5% of the compound of formula (VI).

6. The composition of any one of claims 3-5, comprising less than about 0.4% of the compound of formula (VII), preferably less than about 0.3% of the compound of formula (VII), even more preferably less than about 0.2% of the compound of formula (VII).

7. The composition of any one of claims 3-6 comprising less than about 0.25% of the compound of formula (III), even more preferably less than about 0.2% of the compound of formula (III).

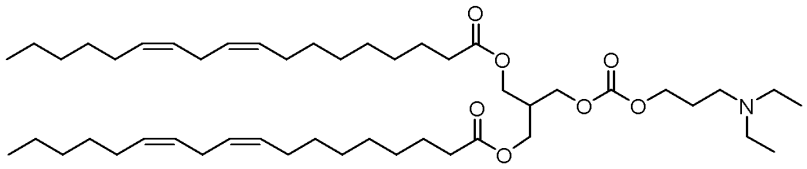
8. The composition of any one of claims 3-7, comprising less than about 50 ppm pNP, preferably less than about 40 ppm pNP, preferably less than about 30 ppm pNP, preferably less than about 20 ppm pNP, preferably less than about 10 ppm pNP, preferably less than about 5 ppm pNP, even more preferably wherein the pNP is undetectable.

9. The composition of any one of claims 3-8, comprising less than about 0.3% of a compound of formula (VIII).

10. The composition of any one of claims 3-9, comprising less than about 0.1% of a compound of formula (IX), preferably wherein a compound of formula (IX) is undetectable.

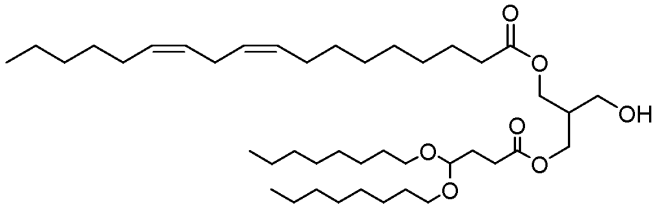
11. The composition of claim 1 or 2, wherein the composition comprises greater than 1 kg of Lipid A, and wherein the composition comprises:

(A) less than about 2.7%, less than about 2.5%, less than about 2%, less than about 1.5%, less than 1%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



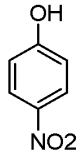
(VI), or

(B) less than about 1.3%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



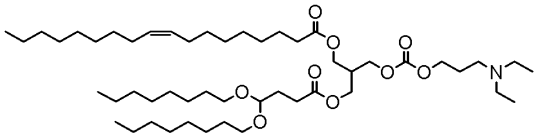
(III), or

(C) less than about 1850 ppm, less than about 1500 ppm, less than about 1250 ppm, less than about 1000 ppm, less than about 750 ppm, less than about 500 ppm, less than about 250 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



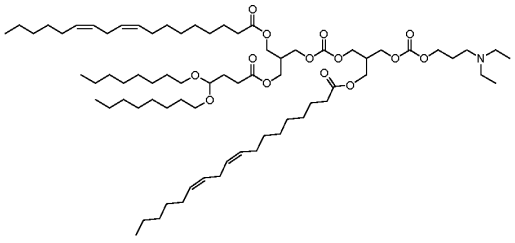
, or

(D) less than about 0.6%, less than about 0.4%, or less than about 0.3% of a compound of formula (VIII)



(VIII), or

(E) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than 0.1% of a compound having the structure of formula (IX)



(IX).

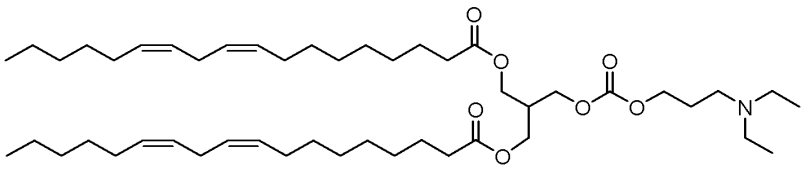
12. The composition of claim 11, wherein the composition comprises greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, or greater than about 5 kg of Lipid A.

13. The composition of claim 11, wherein the composition comprises about 1 kg to about 10 kg, even more preferably about 1 to about 7 kg of Lipid A.

14. The composition of claim 11, wherein the composition comprises about 2 to about 7 kg of Lipid A.

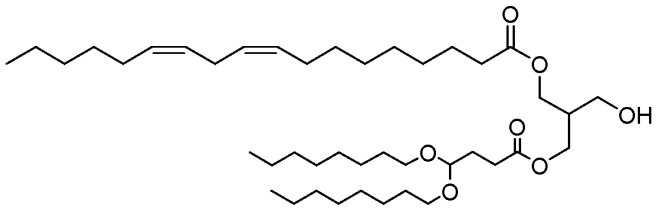
15. The composition of claim 11, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



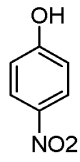
(VI), or

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



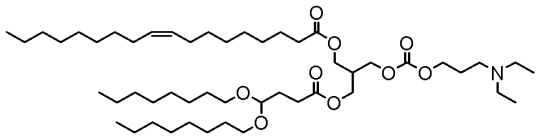
(III), or

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



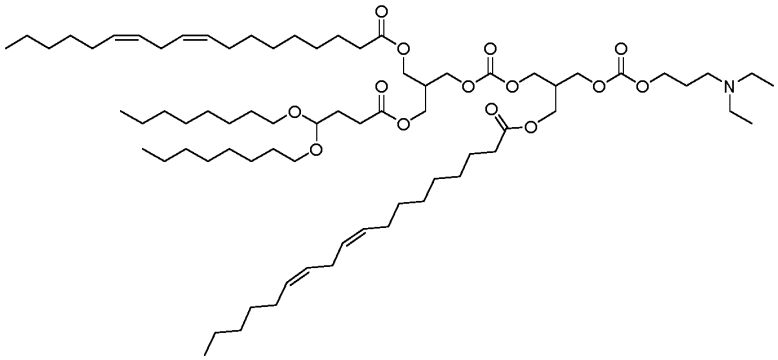
, or

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), or

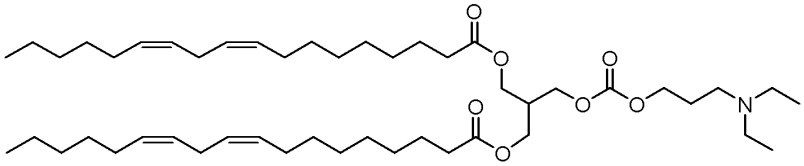
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

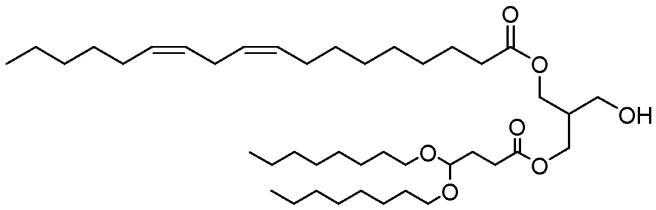
16. The composition of claim 11, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



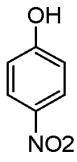
(VI), and

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



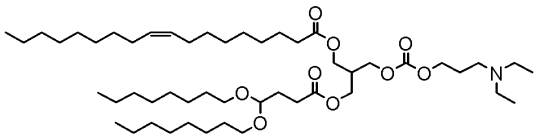
(III), and

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



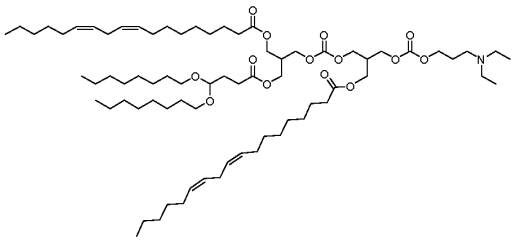
, and

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), and

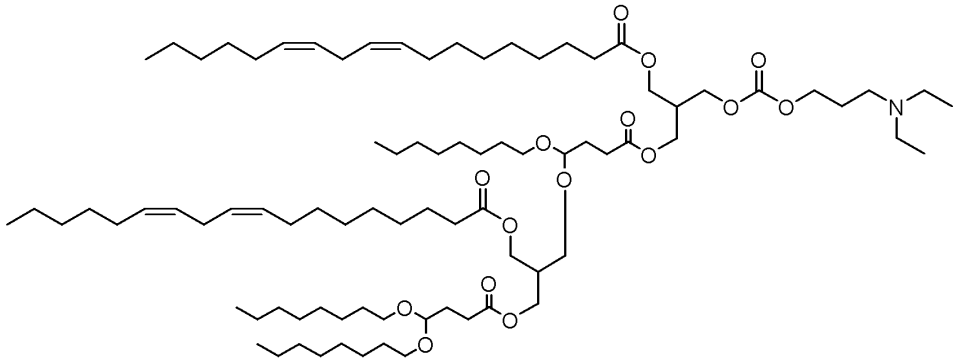
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

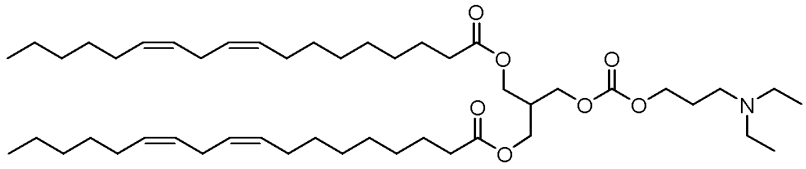
17. The composition of claim 11, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 0.1% of a compound having the structure of formula (V)



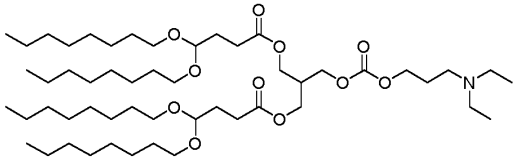
(V), and

(B) less than about 1.2% a compound having the structure of formula (VI)



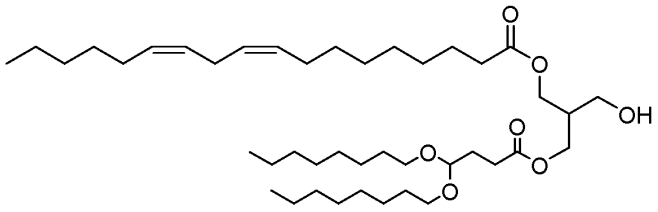
(VI), and

(C) less than about 0.3% of a compound having the structure of formula (VII)



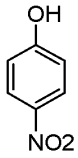
(VII), and

(C) less than about 1.0% of a compound having the structure of formula (III)



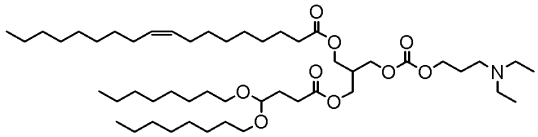
(III), and

(D) less than about 2 ppm p-nitrophenol (pNP)



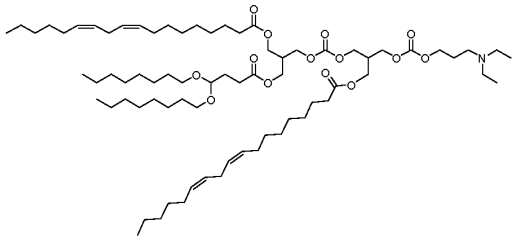
, and

(E) less than about 0.2% of a compound of formula (VIII)



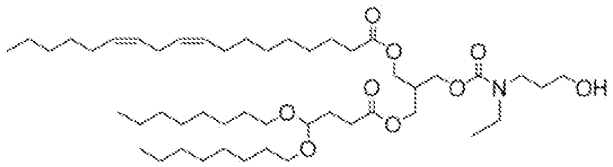
(VIII), and

(F) less than about 0.1% of a compound having the structure of formula (IX)



(IX).

18. The composition of any one of claims 1-17, comprising less than about 2.5% of a compound of formula (X), preferably wherein a compound of formula (X) is undetectable



(X).

19. A process for preparing a composition of any one of claims 1-18, comprising:
a) treating Compound-01 with Compound-02 at a temperature not exceeding 100°C to yield Compound-08;
b) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound 09;
c) treating Compound-09 with piperazine to yield Compound-10; and
d) generating Lipid A by combining Compound-10 and Compound-12.

20. A process for preparing a composition of any one of claims 1-18, comprising:
a) hydrolyzing Compound-08 at a temperature not exceeding 100 °C to yield Compound-09;
b) treating Compound-09 with piperazine to yield Compound-10; and
c) generating Lipid A by combining Compound-10 and Compound-12.

21. A process for preparing a composition of any one of claims 1-18, comprising:
a) treating Compound-09 with piperazine to yield Compound-10; and
b) generating Lipid A by combining Compound-10 and Compound-12.

22. A process for preparing a composition of any one of claims 1-18, comprising:
a) treating Compound-09 with piperazine *p*-xylylenediamine to yield Compound-10; and
b) generating Lipid A by combining Compound-10 and Compound-12.

23. A process for preparing a composition of any one of claims 1-18, comprising:

a) treating Compound-03 with Compound-04 and a coupling reagent to yield Compound-19;

b) treating Compound-11 with an ion exchange resin to yield Compound-12; and

c) generating Lipid A by combining Compound-10 and Compound-12.

24. A process for preparing a composition of any one of claims 1-18, comprising:

a) treating Compound-11 with an ion exchange resin to yield Compound-12; and

b) generating Lipid A by combining Compound-10 and Compound-12.

25. A process for preparing a composition of any one of claims 1-18, comprising:

a) treating Compound-13 with *p*NPCF to generate Compound-14 *in situ*;

b) treating Compound-14 *in situ* with 3-dimethylamino-1-propanol;

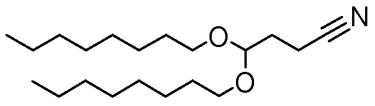
c) extracting a reaction mixture with heptane; and

d) performing water and acetonitrile extractions to generate Lipid A.

26. The process of any one of claims 19-25, wherein the composition comprises greater than about 1 kg, greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, greater than about 5 kg, or greater than about 6 kg of Lipid A.

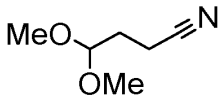
27. The process of any one of claims 19-25, wherein the composition comprises 1-10kg of Lipid A.

28. A process for preparing 4,4-bis(octyloxy)butanenitrile



,

comprising combining 4,4-dimethoxybutanenitrile



with 1-octanol to form the 4,4-bis(octyloxy)butanenitrile, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 100°C.

29. The process of claim 28, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at a temperature not exceeding 80°C.

30. The process of claim 29, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature not exceeding 70°C.

31. The process of any one of claims 28-30, wherein the formation of the 4,4-bis(octyloxy)butanenitrile is carried out at temperature of at least 40°C.

32. The process of any one of claims 28-31, wherein the 4,4-dimethoxybutanenitrile is combined with an acid.

33. The process of claim 32, wherein the acid is pyridinium *p*-toluenesulfonate.

34. The process of claim 32 or 33, wherein the 4,4-dimethoxybutanenitrile is combined with about 0.01 to about 0.10 molar equivalents of the acid, wherein the molar equivalents are relative to the 4,4-dimethoxybutanenitrile.

35. The process of claim 34, wherein the 4,4-dimethoxybutanenitrile is combined with about 0.05 molar equivalents of the acid.

36. The process of any one of claim 28-35, wherein the 4,4-dimethoxybutanenitrile is combined with 2 to 2.5 molar equivalents of the 1-octanol.

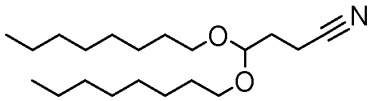
37. The process of any one of claims 28–36, wherein the 4,4-dimethoxybutanenitrile is combined with a solvent.

38. The process of claim 37, wherein the solvent comprises toluene.

39. The process of any one of claims 28-38, wherein the process further comprises purifying the 4,4-bis(octyloxy)butanenitrile by vacuum distillation.

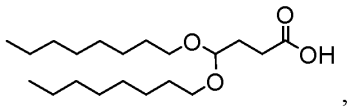
40. The process of any one of claims 28-39, wherein the process does not comprise purifying the 4,4-bis(octyloxy)butanenitrile by column chromatography.

41. A composition comprising a compound 4,4-bis(octyloxy)butanenitrile,



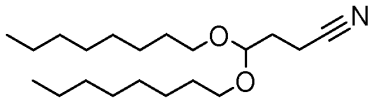
wherein the composition is prepared according to the process of any one of claims 26-38.

42. A process for preparing 4,4-bis(octyloxy)butanoic acid



,

comprising hydrolyzing 4,4-bis(octyloxy)butanenitrile



to form the 4,4-bis(octyloxy)butanoic acid, wherein the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 100°C.

43. The process of claim 42, wherein the formation of the 4,4-bis(octyloxy)butanoic acid is carried out at a temperature not exceeding 95°C.

44. The process of claim 42 or 43, wherein the formation of the 4,4-bis(octyloxy)butanoic acid is carried out under basic conditions.

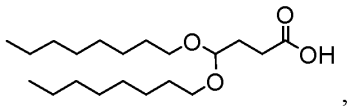
45. The process of any one of claims 42-44, wherein the hydrolyzing step comprises combining the 4,4-bis(octyloxy)butanenitrile with potassium hydroxide, water, and ethanol.

46. The process of any one of claims 42-45, wherein the pH is adjusted to about 4 to 5 using an acid.

47. The process of claim 46, wherein the acid is acetic acid or hydrochloric acid.

48. The process of any one of claims 42-45, wherein the 4,4-bis(octyloxy)butanenitrile is prepared by the process of any one of claims 28-40.

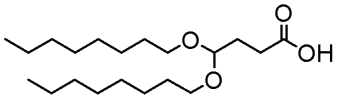
49. A composition comprising a compound 4,4-bis(octyloxy)butanoic acid



,

wherein the composition is prepared according to the process of any one of claims 42-48.

50. A process for preparing an amine salt of 4,4-bis(octyloxy)butanoic acid



comprising:

combining the 4,4-bis(octyloxy)butanoic acid with piperazine or *p*-xylylenediamine to form a reaction mixture, wherein the amine salt is formed in the reaction mixture; and

filtering the reaction mixture.

51. The process of claim 50, wherein the amine salt is a piperazinium salt.

52. The process of claim 50, wherein the amine salt is a *p*-xylylenediamine salt.

53. The process of any one of claims 50-52, wherein the amount of the piperazine or *p*-xylylenediamine is about 0.5 to about 0.8 molar equivalents relative to the 4,4-bis(octyloxy)butanoic acid, preferably about 0.55 molar equivalents relative to the 4,4-bis(octyloxy)butanoic acid.

54. The process of any one of claims 50-53, wherein the combining step comprises combining the 4,4-bis(octyloxy)butanoic acid with a solvent.

55. The process of claim 54, wherein the solvent comprises isopropyl acetate.

56. The process of any one of claims 50-55, wherein the formation of the amine salt is carried out at a temperature not exceeding 70°C.

57. The process of claim 56, wherein the formation of the amine salt is carried out at a temperature not exceeding 55°C.

58. The process of any one of claims 50-57, wherein the formation of the amine salt is carried out at a temperature of at least 0°C.

59. The process of any one of claims 50-51 and 53-58, wherein the amine salt is the hemi(piperazinediium) salt of the 4,4-bis(octyloxy)butanoic acid.

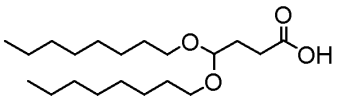
60. The process of any one of claims 50-59, wherein the filtering step provides a solid comprising at least 95% of the amine salt as determined by UPLC.

61. The process of claim 60, wherein the solid comprises at least 99% of the amine salt as determined by UPLC.

62. The process of any one of claims 50-61, wherein the solid comprises less than 2 wt.% octanol, preferably less than 1 wt% octanol.

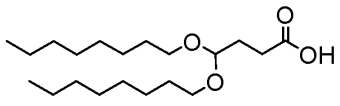
63. The process of any one of claims 50-62, wherein the 4,4-bis(octyloxy)butanoic acid is prepared by the process of any one of claims 42-48.

64. A composition comprising an amine salt of 4,4-bis(octyloxy)butanoic acid



,wherein the composition is prepared according to the process of any one of claims 50-63.

65. A compound that is a piperazinium salt or a *p*-xylylenediamine salt of 4,4-bis(octyloxy)butanoic acid



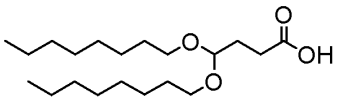
,

wherein the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.3 to about 0.7.

66. The compound of claim 65, wherein the stoichiometric ratio of piperazine or *p*-xylylenediamine to 4,4-bis(octyloxy)butanoic acid is about 0.5.

67. The compound of any one of claims 65-66 that is the hemi(piperazinediium) salt or the hemi(*p*-xylylenediamine) salt of 4,4-bis(octyloxy)butanoic acid.

68. A process for generating at least 90% free 4,4-bis(octyloxy)butanoic acid



comprising,

preparing an amine salt of the 4,4-bis(octyloxy)butanoic acid by the process of any one of claims 50–63; and

contacting the amine salt with an acid to form free 4,4-bis(octyloxy)butanoic acid.

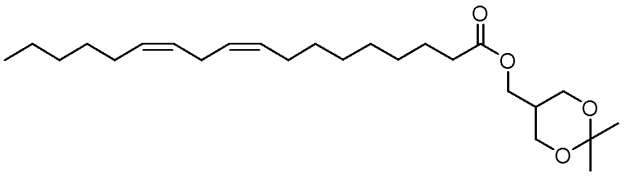
69. The process of claim 68, wherein the acid is citric acid.

70. The process of claim 69, wherein the citric acid is present in a 10 wt.% aqueous solution of citric acid.

71. The process of any one of claims 68-70, wherein the free 4,4-bis(octyloxy)butanoic acid is contacted with an organic solvent.

72. The process of claim 71, wherein the organic solvent comprises cyclopentyl methyl ether.

73. A process for preparing a compound having the structure of formula (I):



(I),

comprising combining linoleic acid with (2,2-dimethyl-1,3-dioxan-5-yl)methanol and a coupling reagent.

74. The process of claim 73, wherein the coupling reagent is *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC).

75. The process of claim 74, wherein the linoleic acid is combined with about 1.2 to about 3 molar equivalents of the EDC.

76. The process of claim 74, wherein the linoleic acid is combined with about 1.7 to about 2 molar equivalents of the EDC.

77. The process of any one of claims 73-76, wherein the linoleic acid is combined with 4-dimethylaminopyridine.

78. The process of claim 77, wherein linoleic acid is combined with about 0.1 to about 0.5 molar equivalents of the 4-dimethylaminopyridine.

79. The process of claim 77, wherein the linoleic acid is combined with about 0.2 to about 0.3 molar equivalents of the 4-dimethylaminopyridine.

80. The process of any one of claims 73-79, wherein the linoleic acid is combined with an organic solvent.

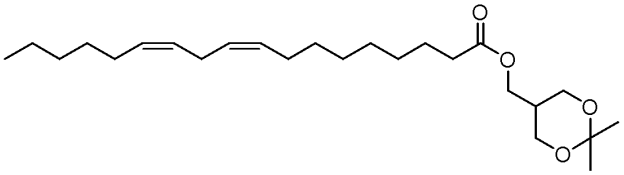
81. The process of claim 80, wherein the organic solvent comprises acetonitrile.

82. The process of any one of claims 73-81, wherein the linoleic acid is combined with about 0.9 to about 2 molar equivalents of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol, wherein the molar equivalents are relative to the linoleic acid.

83. The process of claim 82, wherein the linoleic acid is combined with about 1 molar equivalent of the (2,2-dimethyl-1,3-dioxan-5-yl)methanol.

84. The process of any one of claims 73-83, wherein the formation of the compound of formula (I) is carried out at a temperature of about 20°C to about 25°C.

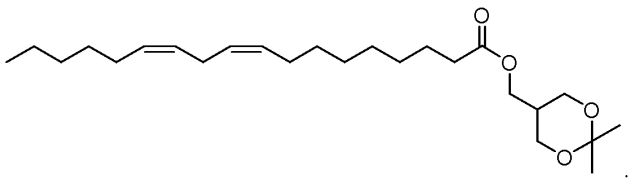
85. A compound having the structure of formula (I):



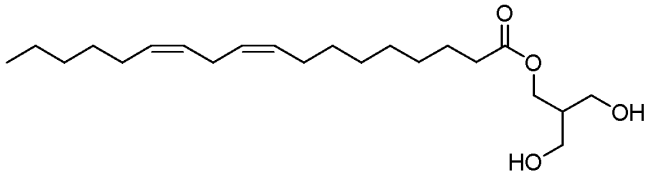
(I),

wherein the compound is prepared according to the process of any one of claims 73-84.

86. A compound that is a (2,2-dimethyl-1,3-dioxan-5-yl)methyl (9Z,12Z)-octadeca-9,12-dienoate

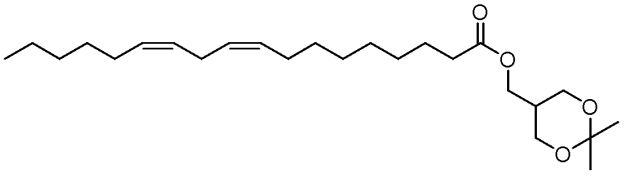


87. A process for preparing a compound having the structure of formula (II):



(II),

comprising contacting a compound having the structure of formula (I):



(I)

with an ion exchange resin.

88. The process of claim 87, wherein the ion exchange resin is a Dowex® resin.

89. The process of claim 87 or 88, wherein the compound of formula (I) is contacted with a solvent.

90. The process of claim 89, wherein the solvent comprises methanol.

91. The process of any one of claims 87-90, wherein the contacting step provides a crude product comprising the compound of formula (II); and

the process further comprises purifying the compound of formula (II) by column chromatography.

92. The process of any one of claims 87-91, wherein the formation of the compound of formula (II) is carried out at a temperature of about 20°C to about 25°C.

93. The process of any one of claims 87-92, wherein the compound of formula (II) is treated with a powdered activated carbon (PAC) material.

94. The process of claim 93, wherein the PAC material is used in heptane.

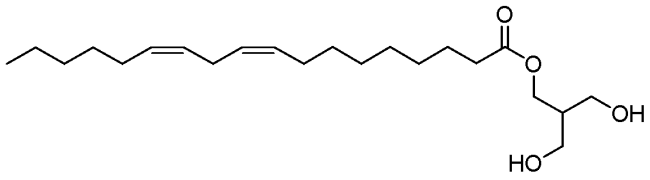
95. The process of claim 93, wherein the PAC material is used in methanol.

96. The process of claim 95, wherein the PAC material is Cabot A SUPRA EUR.

97. The process of claim 96, wherein the Cabot A SUPRA EUR is used with a 25% wt/wt PAC:crude product compound of formula (II) ratio.

98. The process of any one of claims 87-97, wherein a composition comprising the compound of formula (I) is prepared by the process of any one of claims 73-84.

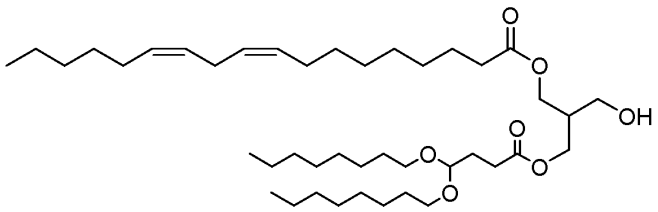
99. A composition comprising a compound having the structure of formula (II):



(II),

wherein the composition is prepared according to the process of any one of claims 87-98.

100. A process for preparing a compound having the structure of formula (III):

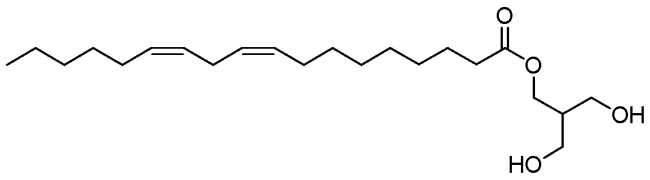


(III),

comprising:

generating free 4,4-bis(octyloxy)butanoic acid by the process of any one of claims 64-68; and

combining the purified 4,4-bis(octyloxy)butanoic acid with a compound having the structure of formula (II):



(II)

and a coupling reagent.

101. The process of claim 100, wherein the coupling reagent is *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC).

102. The process of claim 100 or 101, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with 4-dimethylaminopyridine.

103. The process of any one of claims 100-102, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with *N,N*-diisopropylethylamine.

104. The process of any one of claims 100-103, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with an organic solvent.

105. The process of claim 104, wherein the organic solvent comprises cyclopentyl methyl ether.

106. The process of any one of claims 100-105, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with about 1 to about 1.5 molar equivalents of the compound of formula (II) and about 1 to about 1.5 molar equivalents of the coupling reagent, wherein the molar equivalents are relative to the free 4,4-bis(octyloxy)butanoic acid.

107. The process of claim 106, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with about 0.05 to about 0.5 molar equivalents of the 4-dimethylaminopyridine and 1 to 1.5 molar equivalents of the *N,N*-diisopropylethylamine.

108. The process of claim 107, wherein the free 4,4-bis(octyloxy)butanoic acid is combined with about 1.2 molar equivalents of the compound of formula (II), about 1.2 molar equivalents of the coupling reagent, about 0.2 molar equivalents of the 4-dimethylaminopyridine, and about 1.2 molar equivalents of the *N,N*-diisopropylethylamine.

109. The process of any one of claims 100-108, wherein the process further comprises filtration over Celite.

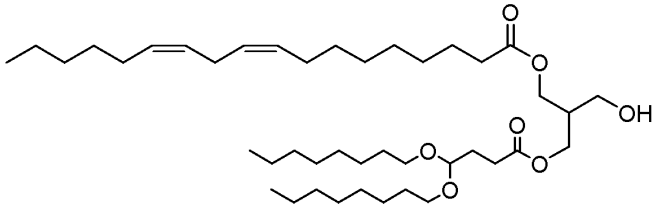
110. The process of any one of claims 100-109, wherein the process further comprises dilution of the reaction mixture in heptane prior to filtration.

111. The process of any one of claims 100-110, wherein the process further comprises purifying the compound of formula (III) by column chromatography.

112. The process of any one of claims 100-111, wherein the process further comprises using a heptane/CPME solvent mixture for column chromatography.

113. The process of any one of claims 100-112, wherein a composition comprising the compound of formula (II) is prepared by the process of any one of claims 87–98.

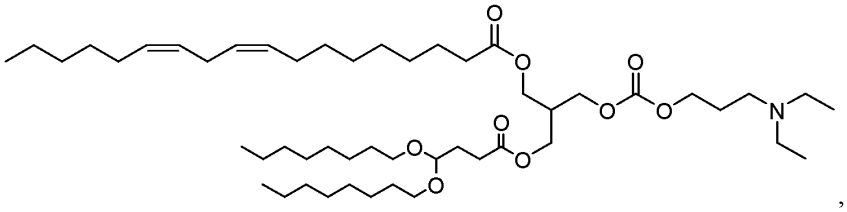
114. A composition comprising a compound having the structure of formula (III):



(III),

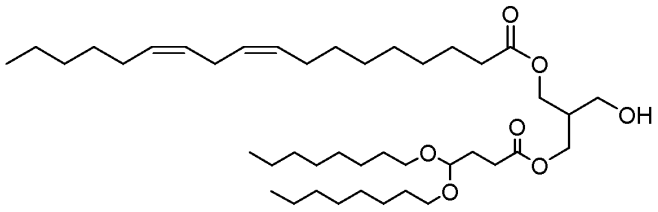
wherein the composition is prepared according to the process of any one of claims 100-113.

115. A process for preparing Lipid A:



,

comprising combining a compound having the structure of formula (III):



(III)

with 4-nitrophenyl chloroformate, about 2.2 to about 10 molar equivalents of pyridine, 4-dimethylaminopyridine, and 3-(diethylamino)propan-1-ol, wherein the molar equivalents are relative to the compound of formula (III).

116. The process of claim 115, wherein the compound of formula (III) is combined with about 1.5 to about 7.2 molar equivalents of the pyridine.

117. The process of claim 116, wherein the compound of formula (III) is combined with about 2.3 to about 5.2 molar equivalents of the pyridine.

118. The process of claim 117, wherein the compound of formula (III) is combined with about 2.5 molar equivalents of the pyridine.

119. The process of claim 117, wherein the compound of formula (III) is combined with about 3.5 molar equivalents of the pyridine.

120. The process of any one of claims 115-119, wherein the compound of formula (III) is combined with an organic solvent.

121. The process of claim 120, wherein the solvent comprises acetonitrile.

122. The process of claim 120 or 121, wherein the solvent does not comprise dichloromethane.

123. The process of any one of claims 115-122, wherein the compound of formula (III) is combined with about 2 to about 8 molar equivalents of the 3-(diethylamino)propan-1-ol.

124. The process of claim 123, wherein the compound of formula (III) is combined with about 5 molar equivalents of the 3-(diethylamino)propan-1-ol.

125. The process of claim 123, wherein the compound of formula (III) is combined with about 6 molar equivalents of the 3-(diethylamino)propan-1-ol.

126. The process of any one of claims 115-125, wherein the combining step forms a reaction mixture comprising Lipid A.

127. The process of claim 126, further comprising purifying Lipid A by column chromatography to provide a purified product comprising Lipid A.

128. The process of claim 127, wherein Lipid A is purified by preparative high performance liquid chromatography.

129. The process of any one of claims 126-128, wherein the reaction mixture or the purified product, as determined by UPLC, comprises:

at least 95% Lipid A, and

(A) less than about 1.1% of a compound having the structure of formula (V), or

(B) less than about 0.6% of a compound having the structure of formula (VI), or

(C) less than about 0.6% of a compound having the structure of formula (VII), or

(D) less than about 0.3% of a compound having the structure of formula (III), or

(E) less than about 190 ppm p-nitrophenol (pNP), or

(F) less than about 0.4% of a compound having the structure of formula (VIII), or

(G) less than about 0.2% of a compound having the structure of formula (IX).

130. The process of claim 129, wherein the reaction mixture or the purified product comprises less than about 0.3% of the compound of formula (V), preferably less than about 0.2% of the compound of formula (V), preferably less than about 0.1% of the compound of formula (V), even more preferably wherein the compound of formula (V) is undetectable.

131. The process of claim 129 or claim 130, wherein the reaction mixture or the purified product comprises less than less than 0.55% of the compound of formula (VI), even more preferably less than 0.5% of the compound of formula (VI).

132. The process of any one of claims 129-131, wherein the reaction mixture or the purified product comprises less than about 0.4% of the compound of formula (VII), preferably less than about 0.3% of the compound of formula (VII), even more preferably less than about 0.2% of the compound of formula (VII).

133. The process of any one of claims 129-132, wherein the reaction mixture or the purified product comprises less than about 0.25% of the compound of formula (III), even more preferably less than 0.2% of the compound of formula (III).

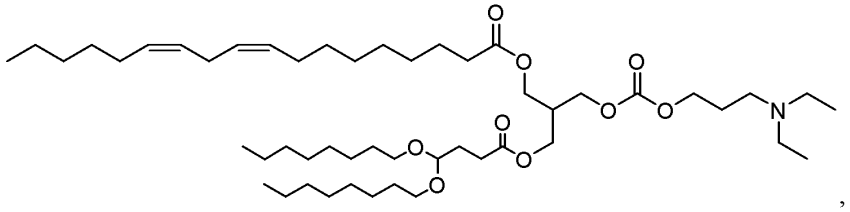
134. The process of any one of claims 129-133, wherein the reaction mixture or the purified product comprises less than about 50 ppm pNP, preferably less than about 40 ppm pNP, preferably less than about 30 ppm pNP, preferably less than about 20 ppm pNP, preferably less than about 10 ppm pNP, preferably less than about 5 ppm pNP, even more preferably wherein the pNP is undetectable.

135. The process of any one of claims 129-134, wherein the reaction mixture or the purified product comprises less than about 0.3% of a compound of formula (VIII).

136. The process of any one of claims 129-135, wherein the reaction mixture or the purified product comprises less than about 0.1% of a compound of formula (IX), preferably wherein a compound of formula (IX)is undetectable.

137. The process of any one of claims 115-136, wherein the compound of formula (III) is prepared by the process of any one of claims 100-113.

138. A composition comprising Lipid A:



,

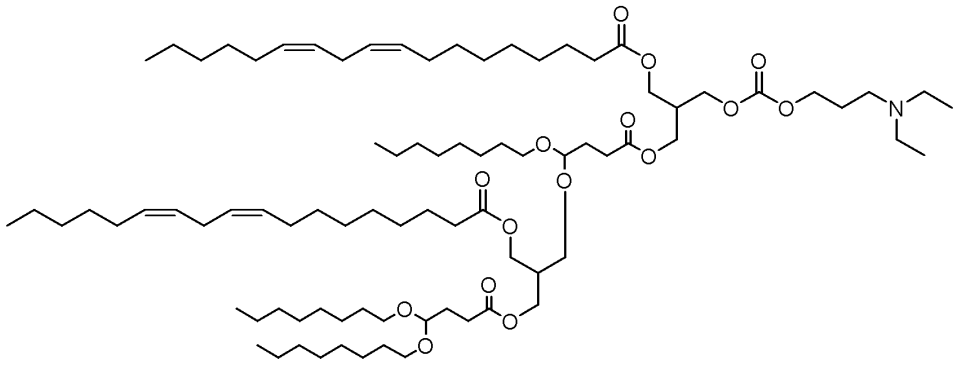
wherein the composition is prepared according to the process of any one of claims 115-137.

139. The composition of claim 138, wherein the composition comprises at least 90% of Lipid A.

140. The composition of any one of claims 138-139, wherein the composition comprises at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% Lipid A.

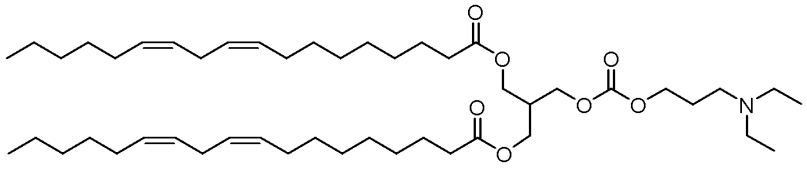
141. The composition of any one of claims 138-140, wherein the composition comprises:

(A) less than about 1.1%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (V)



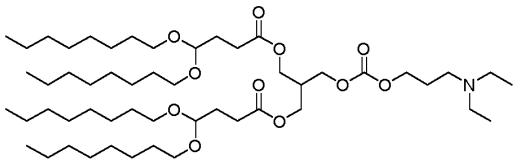
(V), or

(B) less than about 0.6% or less than about 0.5% of a compound having the structure of formula (VI)



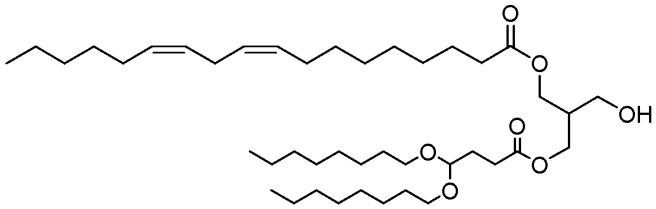
(VI), or

(C) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, or less than about 0.2%, of a compound having the structure of formula (VII)



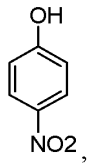
(VII), or

(D) less than about 0.3% or less than about 0.2% of a compound having the structure of formula (III)



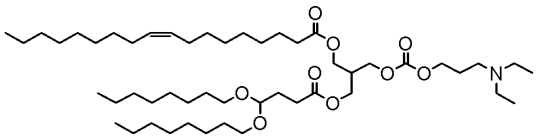
(III), or

(E) less than about 190 ppm, less than about 180 ppm, less than about 170 ppm, less than about 160 ppm, less than about 150 ppm, less than about 140 ppm, less than about 130 ppm, less than about 120 ppm, less than about 110 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



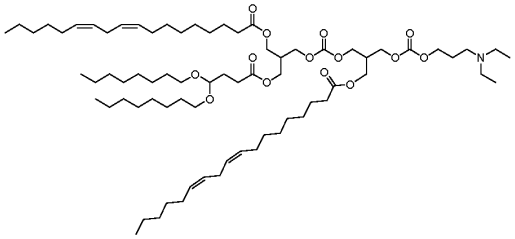
, or

(F) less than about 0.4% or less than about 0.3% of a compound having the structure of formula (VIII)



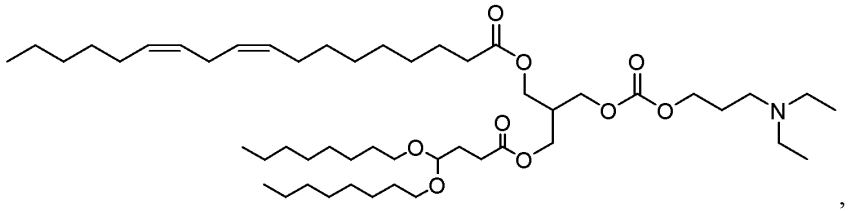
(VIII), or

(G) less than about 0.2% or less than 0.1% of a compound having the structure of formula (IX)



(IX).

142. A composition comprising Lipid A:

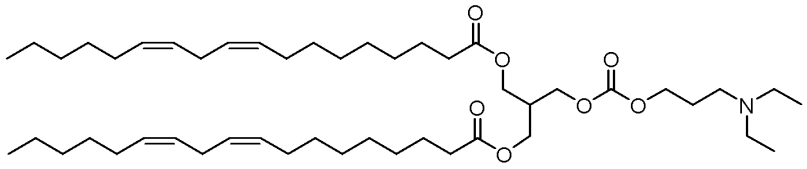


,

wherein the composition is prepared according to the process of any one of claims 115-137, and wherein the composition comprises greater than 1 kg of Lipid A.

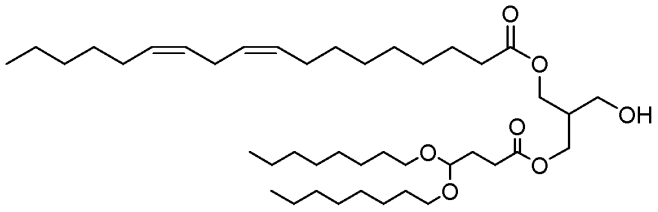
143. The composition of claim 142, wherein the composition comprises:

(A) less than about 2.7%, less than about 2.5%, less than about 2%, less than about 1.5%, less than 1%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



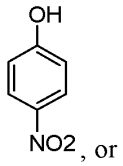
(VI), or

(B) less than about 1.3%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



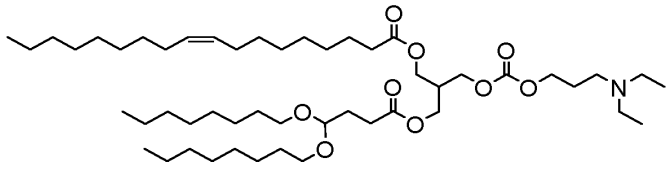
(III), or

(C) less than about 1800 ppm, less than about 1500 ppm, less than about 1250 ppm, less than about 1000 ppm, less than about 750 ppm, less than about 500 ppm, less than about 250 ppm, less than about 100 ppm, less than about 90 ppm, less than about 80 ppm, less than about 70 ppm, less than about 60 ppm, less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



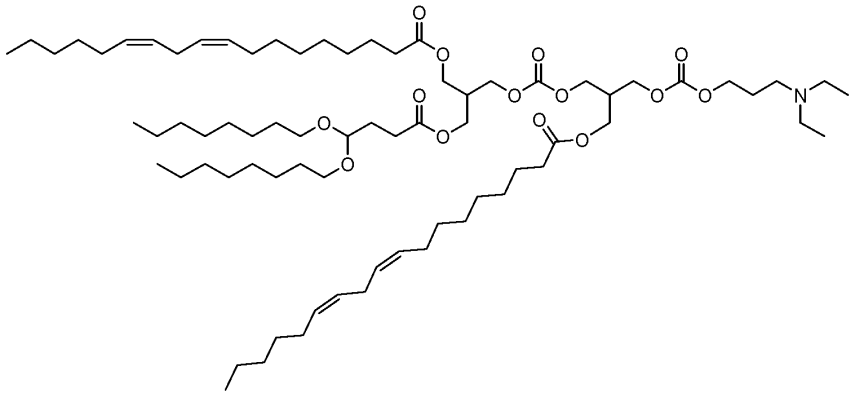
, or

(F) less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (VIII)



(VIII), or

(G) less than about 0.6%, less than about 0.5%, less than about 0.4%, less than about 0.3%, less than about 0.2%, or less than 0.1% of a compound having the structure of formula (IX)



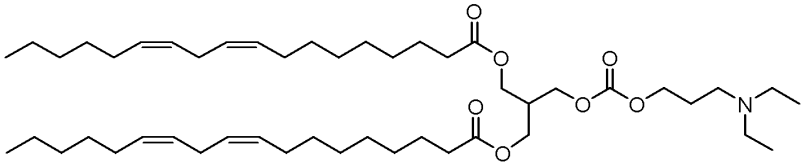
(IX).

144. The composition of claim 142 or 143, wherein the composition comprises greater than about 2 kg, greater than about 3 kg, greater than about 4 kg, or greater than about 5 kg of Lipid A.

145. The composition of claim 142 or 143, wherein the composition comprises about 1 kg to about 10 kg, even more preferably about 1 to about 7 kg of Lipid A.

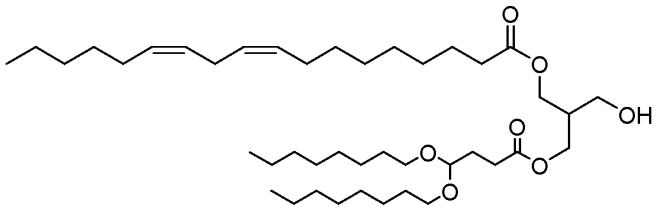
146. The composition of claim 142 or 143, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



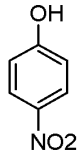
(VI), or

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



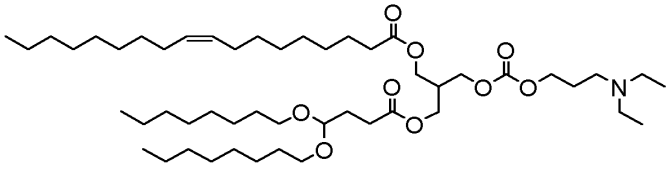
(III), or

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



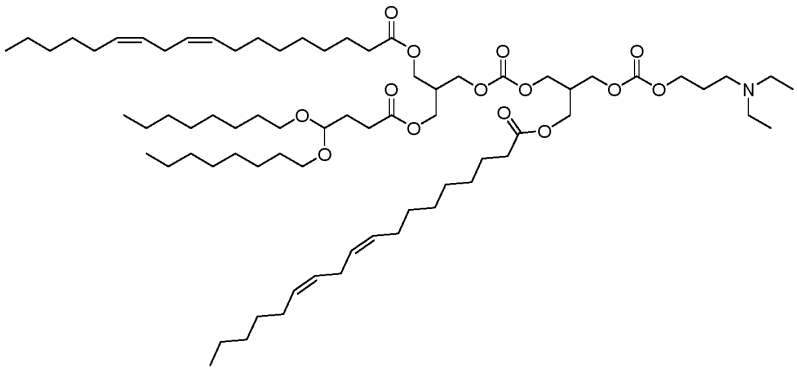
, or

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), or

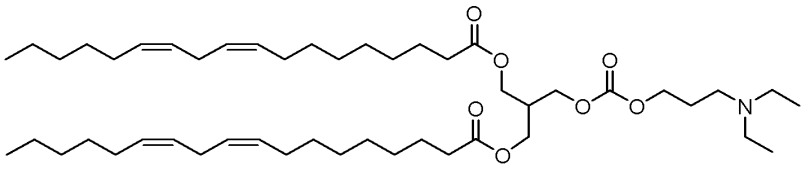
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

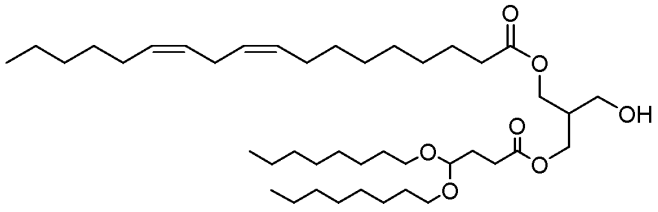
147. The composition of claim 142 or 143, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 1.2%, less than about 1.1%, less than about 1.0%, less than about 0.9%, less than about 0.8%, or less than about 0.7% of a compound having the structure of formula (VI)



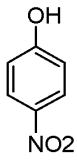
(VI), and

(B) less than about 1.0%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, less than about 0.5%, less than about 0.4%, or less than about 0.3% of a compound having the structure of formula (III)



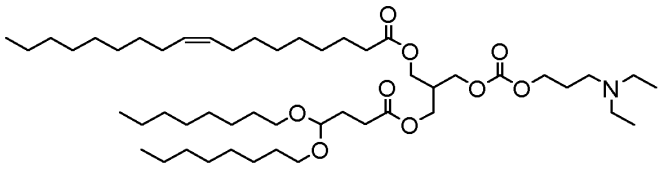
(III), and

(C) less than about 50 ppm, less than about 40 ppm, less than about 30 ppm, less than about 20 ppm, less than about 10 ppm, less than about 5 ppm, or less than about 2 ppm p-nitrophenol (pNP)



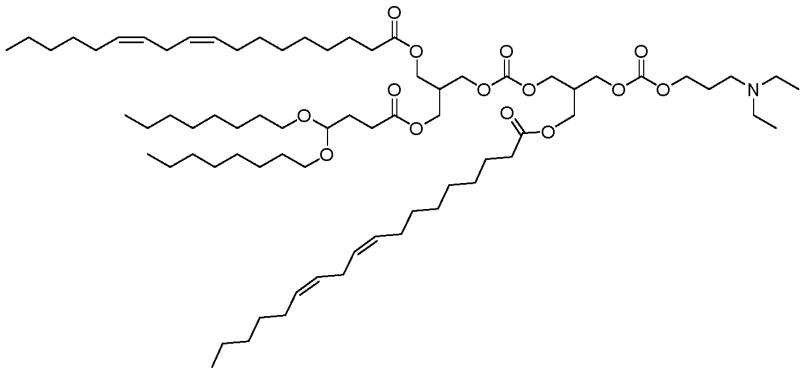
, and

(D) less than about 0.3% or less than about 0.2% of a compound of formula (VIII)



(VIII), and

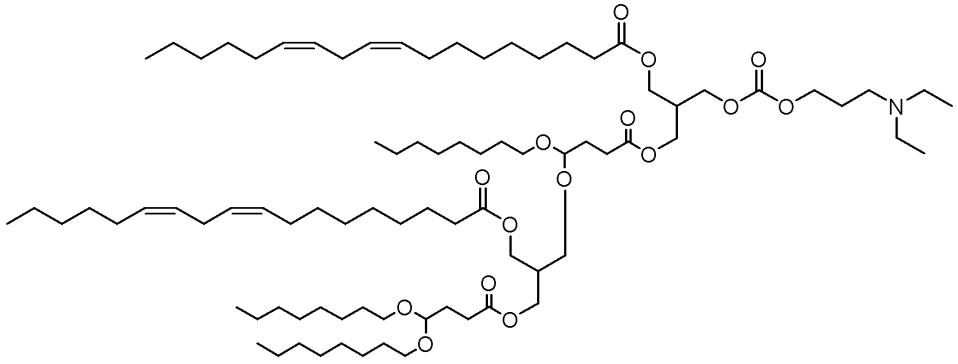
(E) less than about less than about 0.2%, or less than about 0.1% of a compound having the structure of formula (IX)



(IX).

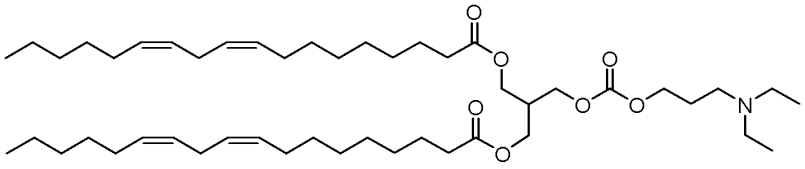
148. The composition of claim 142 or 143, wherein the composition comprises greater than about 6 kg of Lipid A, and wherein the composition comprises:

(A) less than about 0.1% of a compound having the structure of formula (V)



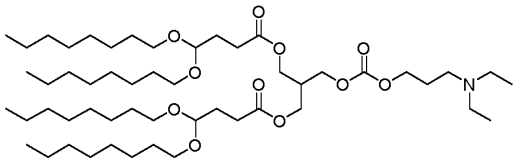
(V), and

(B) less than about 1.2% a compound having the structure of formula (VI)



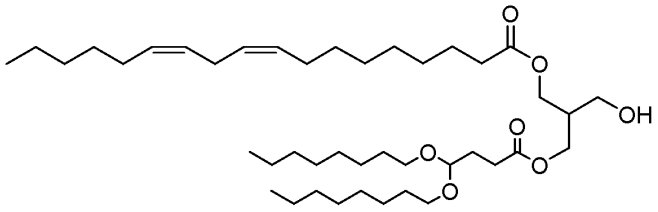
(VI), and

(C) less than about 0.3% of a compound having the structure of formula (VII)



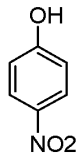
(VII), and

(C) less than about 1.0% of a compound having the structure of formula (III)



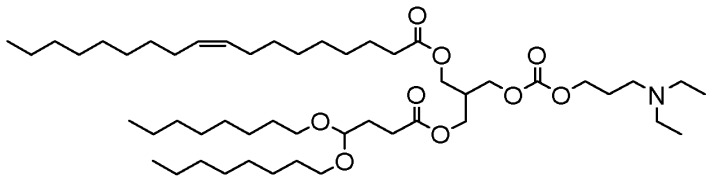
(III), and

(D) less than about 2 ppm p-nitrophenol (pNP)



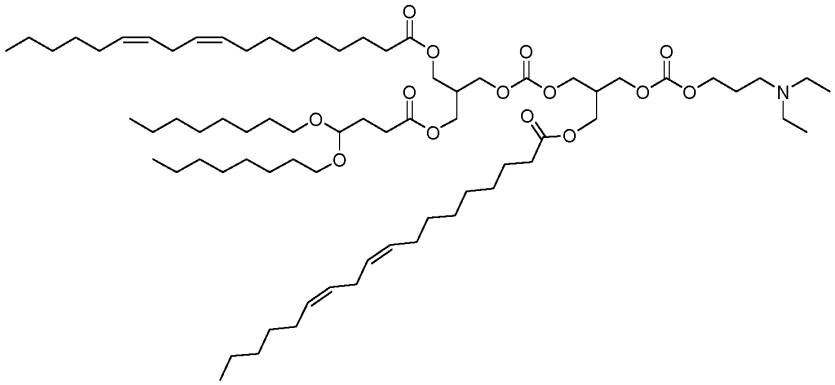
, and

(E) less than about 0.2% of a compound of formula (VIII)



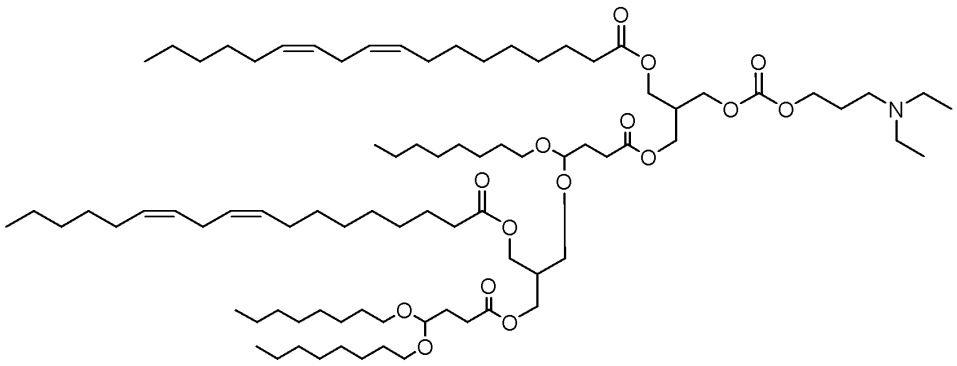
(VIII), and

(F) less than about 0.1% of a compound having the structure of formula (IX)



(IX).

149. A composition comprising at least 90% of a compound having the structure of formula (V)



(V).