

IONIZABLE CATIONIC LIPIDS FOR RNA DELIVERY

TECHNICAL FIELD

[0001] Embodiments herein relate generally to lipids. In particular, embodiments herein relate to new lipids and lipid compositions that facilitate the intracellular delivery of biologically active and therapeutic molecules.

BACKGROUND

[0002] The variety of nucleic acid-based therapeutics for targeted delivery creates a challenge for lipid-based delivery vehicles. For example, nucleic acids are structurally diverse in size and type. Examples include DNA used in gene therapy, plasmids, small interfering nucleic acids (siNA), and microRNA (miRNA) for use in RNA interference (RNAi), antisense molecules, ribozymes, antagomirs, and aptamers.

[0003] The design and use of cationic lipids and ionizable cationic lipids for inclusion in such lipid-based delivery vehicles has shown great advantages. However, use of these lipids can contribute to significant side effects when administered in vivo. One problem that has been observed includes low biodegradability and clearance from target tissues, thus creating an in vivo build up of the lipid. Another problem is that large amounts of the lipid may cause an adverse immunogenic effects, which can result in discomfort in the subject and a decrease in the therapeutic effect of the active ingredient. A third problem associated with many cationic lipids is a low percentage of effective delivery to the target, thus resulting in a relatively low therapeutic effect or low potency. Finally, it is not only important that the cationic lipid in the delivery vehicle have a specially tuned pKa so it can formulate with the nucleic acid-based therapeutic agent and protect it from degradation during administration, but be able to release the therapeutic agent once the vehicle has reached its target. Thus, there is a need in the art for the development of new lipids that can meet the special needs of lipid-nucleic acid delivery systems.

[0004] Each of the following references is hereby incorporated by reference in its entirety: international application number PCT/US2014/066242, published as WO2015074085A1, international application number PCT/US2015/030218, published as WO2016081029A1, US patent number US10227302, US patent number US10383952, and US patent number US10526284, each of which discloses ionizable cationic lipids for RNA delivery; international application number PCT/US2016/069493, published as WO2017117530A1, which discloses

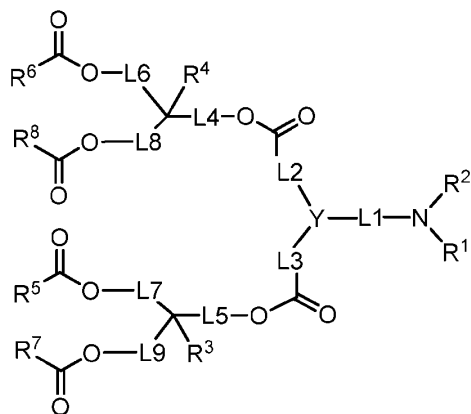
ionizable cationic lipids; international application number PCT/US2019/025246, published as WO2019191780A1, which discloses lipid particles for nucleic acid delivery; and US application number 16/823212, published as US2020/0297634, which discloses methods of making lipid-encapsulated RNA nanoparticles.

SUMMARY

[0005] The present disclosure provides lipids of Formula (I) as described herein useful for lipid-based delivery of nucleic acids and other therapeutic agents for treating diseases. These and other uses will be apparent to those skilled in the art. Additional features and advantages of the subject technology will be set forth in the description below, and in part will be apparent from the description, or may be learned by practice of the subject technology. The advantages of the subject technology will be realized and attained by the structures particularly pointed out in the written description and embodiments.

[0006] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the subject technology.

[0007] In some embodiments, the present disclosure provides a compound of Formula I, or a pharmaceutically acceptable salt thereof:



(I)

wherein:

R¹ and R² are each independently H or C₁₋₆ alkyl; or

R¹ and R² are joined to form a saturated heterocyclic ring, wherein:

R¹ is a linear C₁₋₄ alkylene; and

R^2 is $-(CH_2)_m(X)_n-$, wherein

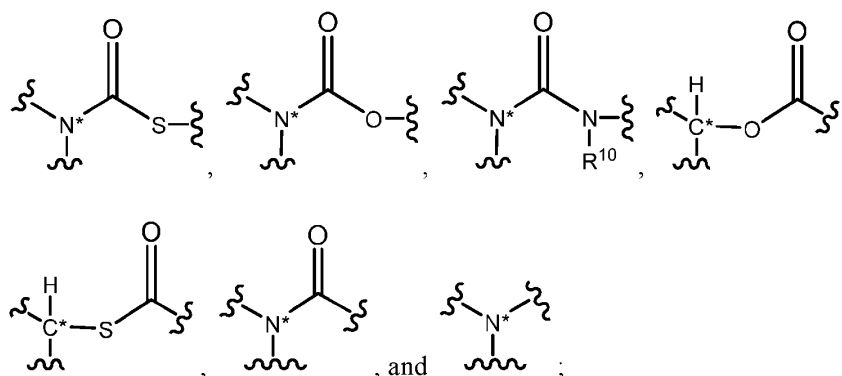
X is O, S, or NR^9 , wherein R^9 is H or C_{1-6} alkyl;

m is 1, 2, 3 or 4, and

n is 0 or 1;

L1 is a linear C_{1-6} alkylene optionally substituted with one to three methyl groups;

Y is selected from the group consisting of:



wherein:

each asterisk (*) indicates the atom attached to L2 and L3; and

R^{10} is H or C_{1-6} alkyl;

L2 and L3 are each independently a linear C_{1-8} alkylene;

L4, L5, L6, L7, L8 and L9 are each independently absent or $-CH_2-$, provided that:

at least two of L4, L6 and L8 are $-CH_2-$; and

at least two of L5, L7 and L9 are $-CH_2-$;

R^3 and R^4 are each independently H, methyl or ethyl; and

R^5 , R^6 , R^7 and R^8 are each independently selected from the group consisting of:

linear C_{1-20} alkyl, wherein each said linear C_{1-20} alkyl is optionally substituted

with one or more substituents selected from the group consisting of:

C_{6-10} aryl, wherein each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl;

6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl; C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl; and 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

[0008] In some embodiments, the present disclosure provides a lipid nanoparticle, comprising a plurality of ligands, wherein each ligand is independently a compound described herein, wherein the plurality of ligands self-assembles to form the lipid nanoparticle comprising an interior and exterior.

[0009] In some embodiments, the present disclosure provides a pharmaceutical composition comprising the compound described herein or the lipid nanoparticle described herein, and a pharmaceutically acceptable excipient.

[0010] In some embodiments, the present disclosure provides a method of treating a disease in a subject in need thereof, comprising administering a therapeutically effective amount to the subject the compound described herein, the lipid nanoparticle described herein, or the pharmaceutical composition described herein.

[0011] In some embodiments, the present disclosure provides a method of delivering a nucleic acid to a subject in need thereof, comprising encapsulating a therapeutically effective amount of the nucleic acid in the lipid nanoparticle described herein, and administering the lipid nanoparticle to the subject.

DETAILED DESCRIPTION

I. GENERAL

[0012] It is understood that various configurations of the subject technology will become readily apparent to those skilled in the art from the disclosure, wherein various configurations of the subject technology are shown and described by way of illustration. As will be realized, the subject technology is capable of other and different configurations and its several details are capable of modification in various other respects, all without departing from the scope of the

subject technology. Accordingly, the summary and detailed description are to be regarded as illustrative in nature and not as restrictive.

[0013] The detailed description set forth below is intended as a description of various configurations of the subject technology and is not intended to represent the only configurations in which the subject technology may be practiced. The detailed description includes specific details for the purpose of providing a thorough understanding of the subject technology. However, it will be apparent to those skilled in the art that the subject technology may be practiced without these specific details.

II. DEFINITIONS

[0014] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual subcombination of the members of such groups and ranges. For example, the term “C₁₋₆ alkyl” is specifically intended to individually disclose methyl, ethyl, C₃ alkyl, C₄ alkyl, C₅ alkyl, and C₆ alkyl.

[0015] The term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0016] In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0017] The term “alkoxy,” as used herein, alone or in combination, refers to an alkyl ether radical, wherein the term alkyl is as defined below. Alkoxy groups may have the general formula: alkyl-O-. As for alkyl group, alkoxy groups can have any suitable number of carbon atoms, such as C₁₋₆. Alkoxy groups include, for example, methoxy, ethoxy, propoxy, iso-propoxy, butoxy, 2-butoxy, iso-butoxy, sec-butoxy, tert-butoxy, pentoxy, hexoxy, and the like. The alkoxy groups can be further optionally substituted as defined herein.

[0018] As used herein, “alkyl” refers to a straight or branched hydrocarbon chain that is fully saturated (i.e., contains no double or triple bonds). The alkyl group may have 1 to 20 carbon atoms (whenever it appears herein, a numerical range such as “1 to 20” refers to each integer in the given range; e.g., “1 to 20 carbon atoms” means that the alkyl group may consist of 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 20 carbon atoms, although the present definition also covers the occurrence of the term “alkyl” where no numerical range is designated). The alkyl group may have 1 carbon, 2 carbons, 3 carbons, 4 carbons, 5 carbons, 6 carbons, 7 carbons, 8 carbons, 9 carbons, 10 carbons, 11 carbons, 12 carbons, 13 carbons, 14 carbons, 15 carbons, 16 carbons, 17 carbons, 18 carbons, 19 carbons, or 20 carbons. The alkyl group may be linear or branched. Alkyl can include any number of carbons, such as C₁₋₂, C₁₋₃, C₁₋₄, C₁₋₅, C₁₋₆, C₁₋₇, C₁₋₈, C₁₋₉, C₁₋₁₀, C₂₋₃, C₂₋₄, C₂₋₅, C₂₋₆, C₃₋₄, C₃₋₅, C₃₋₆, C₄₋₅, C₄₋₆ and C₅₋₆. The alkyl group may also be a medium size alkyl having 1 to 9 carbon atoms. The alkyl group could also be a lower alkyl having 1 to 6 carbon atoms. The alkyl group may be designated as “C₁₋₄ alkyl” or similar designations. By way of example only, “C₁₋₄ alkyl” indicates that there are one to four carbon atoms in the alkyl chain, i.e., the alkyl chain is selected from the group consisting of methyl, ethyl, propyl, isopropyl, n-butyl, iso-butyl, sec-butyl, and t-butyl. Typical alkyl groups include, but are in no way limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, pentyl, hexyl, and the like.

[0019] “Alkylene” refers to a straight or branched, saturated, aliphatic radical having the number of carbon atoms indicated, and linking at least two other groups, i.e., a divalent hydrocarbon radical. The two moieties linked to the alkylene can be linked to the same atom or different atoms of the alkylene group. For instance, a straight chain alkylene can be the bivalent radical of $-(CH_2)_n-$ where “n” is 1, 2, 3, 4, 5 or 6. Representative alkylene groups include, but are not limited to, methylene, ethylene, propylene, isopropylene, butylene, isobutylene, sec-butylene, pentylene and hexylene. Alkylene groups can be substituted or unsubstituted.

[0020] The term “lower alkyl” means a group having one to six carbons in the chain which chain may be straight or branched. Non-limiting examples of suitable alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, n-pentyl, and hexyl.

[0021] The term “amino,” as used herein, represents $-N(R^{N1})_2$, wherein each R^{N1} is, independently, H, OH, NO_2 , $N(R^{N2})_2$, SO_2OR^{N2} , SO_2R^{N2} , SOR^{N2} , an N-protecting group, alkyl, alkenyl, alkynyl, alkoxy, aryl, alkaryl, cycloalkyl, alkylcycloalkyl, carboxyalkyl (e.g., optionally substituted with an O-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., optionally substituted with an O-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), heterocyclyl (e.g., heteroaryl), or alkylheterocyclyl (e.g., alkylheteroaryl), wherein each of these recited R^{N1} groups can be optionally substituted, as defined herein for each group; or two R^{N1} combine to form a heterocyclyl or an N-protecting group, and wherein each R^{N2} is, independently, H, alkyl, or aryl. The amino groups of the disclosure can be an unsubstituted amino (i.e., $-NH_2$) or a substituted amino (i.e., $-N(R')_2$). In a preferred embodiment, amino is $-NH_2$ or $-NHR^{N1}$, wherein R^{N1} is, independently, OH, NO_2 , NH_2 , $N(R^{N2})_2$, SO_2OR^{N2} , SO_2R^{N2} , SOR^{N2} , alkyl, carboxyalkyl, sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., t-butoxycarbonylalkyl) or aryl, and each R^{N2} can be H, C_{1-20} alkyl (e.g., C_{1-6} alkyl), or C_{1-10} aryl.

[0022] The term “anionic lipid” means a lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0023] The term “aryl,” as used herein, alone or in combination, means a carbocyclic aromatic system containing one, two or three rings wherein such rings may be attached together in a pendent manner or may be fused. The term “aryl” embraces aromatic radicals such as benzyl, phenyl, naphthyl, anthracenyl, phenanthryl, indanyl, indenyl, annulenyl, azulenyl, tetrahydronaphthyl, and biphenyl. A C_{6-10} aryl of the present disclosure includes a C_6 aryl, a C_7 aryl, a C_8 aryl, C_9 aryl, or a C_{10} aryl. In embodiments, the C_{6-10} aryl is monocyclic, such as a

phenyl group. In embodiments, the C₆₋₁₀ aryl is bicyclic, such as biphenyl, naphthyl group, or an indanyl group. Substituents for the above noted aryl ring systems are selected from the group of acceptable substituents described below. An “arylene” alone or as part of another substituent, means a divalent radical derived from an aryl.

[0024] The phrase “at least one of” preceding a series of items, with the term “and” or “or” to separate any of the items, modifies the list as a whole, rather than each member of the list (i.e., each item). The phrase “at least one of” does not require selection of at least one of each item listed; rather, the phrase allows a meaning that includes at least one of any one of the items, and/or at least one of any combination of the items, and/or at least one of each of the items. By way of example, the phrases “at least one of A, B, and C” or “at least one of A, B, or C” each refer to only A, only B, or only C; any combination of A, B, and C; and/or at least one of each of A, B, and C.

[0025] The terms “include,” “have,” or the like is used in the description or the claims, such term is intended to be inclusive in a manner similar to the term “comprise” as “comprise” is interpreted when employed as a transitional word in a claim.

[0026] The term “cationic lipid” means amphiphilic lipids and salts thereof having a positive, hydrophilic head group; one, two, three, or more hydrophobic fatty acid or fatty alkyl chains; and a connector between these two domains. An ionizable or protonatable cationic lipid is typically protonated (i.e., positively charged) at a pH below its pKa and is substantially neutral at a pH above the pKa. Preferred ionizable cationic lipids are those having a pKa that is less than physiological pH, which is typically about 7.4. The cationic lipids of the disclosure may also be termed titratable cationic lipids. The cationic lipids can be an “amino lipid” having a protonatable tertiary amine (e.g., pH-titratable) head group. Some exemplary amino lipids can include C18 alkyl chains; and ether, ester, or ketal linkages between the head group and alkyl chains. Such cationic lipids include, but are not limited to, DSDMA, DODMA, DLinDMA, DLenDMA, γ -DLenDMA, DLin-K-DMA, DLin-K-C2-DMA (also known as DLin-C2K-DMA, XTC2, and C2K), DLin-K-C3 -DMA, DLin-K-C4-DMA, DLen-C2K-DMA, γ -DLen-C2K-DMA, DLin-M-C2-DMA (also known as MC2), DLin-M-C3 -DMA (also known as MC3) and (DLin-MP- DMA)(also known as 1-BI 1).

[0027] The term “comprising” is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term “comprising” is used herein, the terms “consisting of” and “consisting essentially of” are thus also encompassed and disclosed.

[0028] The term “commercially available chemicals” and the chemicals used in the Examples set forth herein may be obtained from standard commercial sources, where such sources include, for example, Acros Organics (Pittsburgh, Pa.), Sigma-Adrich Chemical (Milwaukee, Wis.), Avocado Research (Lancashire, U.K.), Bionet (Cornwall, U.K.), Boron Molecular (Research Triangle Park, N.C.), Combi-Blocks (San Diego, Calif.), Eastman Organic Chemicals, Eastman Kodak Company (Rochester, N.Y.), Fisher Scientific Co. (Pittsburgh, Pa.), Frontier Scientific (Logan, Utah), ICN Biomedicals, Inc. (Costa Mesa, Calif.), Lancaster Synthesis (Windham, N.H.), Maybridge Chemical Co. (Cornwall, U.K.), Pierce Chemical Co. (Rockford, Ill.), Riedel de Haen (Hannover, Germany), Spectrum Quality Product, Inc. (New Brunswick, N.J.), TCI America (Portland, Or.), and Wako Chemicals USA, Inc. (Richmond, Va.).

[0029] The phrase “compounds described in the chemical literature” may be identified through reference books and databases directed to chemical compounds and chemical reactions, as known to one of ordinary skill in the art. Suitable reference books and treatise that detail the synthesis of reactants useful in the preparation of compounds disclosed herein, or provide references to articles that describe the preparation of compounds disclosed herein, include for example, “Synthetic Organic Chemistry”, John Wiley and Sons, Inc. New York; S. R. Sandler et al, “Organic Functional Group Preparations,” 2nd Ed., Academic Press, New York, 1983; H. O. House, “Modern Synthetic Reactions,” 2nd Ed., W. A. Benjamin, Inc. Menlo Park, Calif., 1972; T. L. Glichrist, “Heterocyclic Chemistry,” 2nd Ed. John Wiley and Sons, New York, 1992; J. March, “Advanced Organic Chemistry: Reactions, Mechanisms and Structure,” 5th Ed., Wiley Interscience, New York, 2001; Specific and analogous reactants may also be identified through the indices of known chemicals prepared by the Chemical Abstract Service of the American Chemical Society, which are available in most public and university libraries, as well as through online databases (the American Chemical Society, Washington, D.C. may be contacted for more details). Chemicals that are known but not commercially available in catalogs may be prepared by custom chemical synthesis houses, where many of the standard chemical supply houses (such as those listed above) provide custom synthesis services.

[0030] The term “effective amount” of an agent, as used herein, is that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an “effective amount” depends upon the context in which it is being applied. For example, in the context of administering an agent that treats cancer, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of cancer, as compared to the response obtained without administration of the agent.

[0031] The term “fully encapsulated” means that the nucleic acid (e.g., mRNA) in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free RNA. When fully encapsulated, preferably less than 25% of the nucleic acid in the particle is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than 10%, and most preferably less than 5% of the nucleic acid in the particle is degraded. “Fully encapsulated” also means that the nucleic acid-lipid particles do not rapidly decompose into their component parts upon in vivo administration.

[0032] The term “compound,” is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[0033] The term “cycloalkyl,” or, alternatively, “carbocycle,” as used herein, alone or in combination, refers to a saturated or partially saturated monocyclic, or bicyclic alkyl radical wherein each cyclic moiety contains from 3 to 12 carbon atom ring members and which may optionally be a benzo fused ring system which is optionally substituted as defined herein. In some embodiments, a cycloalkyl may comprise from from 3 to 8 carbon atoms, or from 7 to 12 carbon atoms. Examples of such cycloalkyl radicals include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, octahydronaphthyl, 2,3-dihydro-1H-indenyl, adamantyl and the like. “Bicyclic” and “tricyclic” as used herein are intended to include both fused ring systems, such as decahydronaphthalene, octahydronaphthalene as well as the multicyclic (multicentered) saturated or partially unsaturated type. The latter type of isomer is exemplified in general by, bicyclo[1.1.1]pentane, camphor, adamantane, and bicyclo[3.2.1]octane. In embodiments, the cycloalkyl ring is a monocyclic ring from 3- to 8-carbons. In embodiments, the monocyclic ring has 3 carbons, 4 carbons, 5 carbons, 6 carbons, 7 carbons, or 8 carbons. In embodiments, the cycloalkyl ring is a bicyclic ring from 7- to 12-carbons. In embodiments, the bicyclic ring has 7 carbons, 8 carbons, 9 carbons, 10 carbons, 11 carbons, or 12 carbons.

[0034] The term “delivery” refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[0035] The term “fragment,” as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

[0036] The terms “heteroatom” or “ring heteroatom,” as used herein, are meant to include oxygen (O), nitrogen (N), sulfur (S), phosphorus (P), selenium (Se), and silicon (Si). In embodiments, the terms “heteroatom” or “ring heteroatom” are meant to include oxygen (O), nitrogen (N), sulfur (S), phosphorus (P), and silicon (Si).

[0037] The term “heteroaryl,” “heteroaromatic ring,” or “heteroaromatic group” refers to an aromatic group that contain at least one heteroatom such as N, O, or S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. Thus, the term “heteroaryl” includes fused ring heteroaryl groups (i.e., multiple rings fused together wherein at least one of the fused rings is a heteroaromatic ring and wherein the multiple rings are attached to the parent molecular moiety through any atom contained within a heteroaromatic ring of the multiple rings). A 5,6-fused ring heteroarylene refers to two rings fused together, wherein one ring has 5 members and the other ring has 6 members, and wherein at least one ring is a heteroaryl ring. Likewise, a 6,6-fused ring heteroarylene refers to two rings fused together, wherein one ring has 6 members and the other ring has 6 members, and wherein at least one ring is a heteroaryl ring. And a 6,5-fused ring heteroarylene refers to two rings fused together, wherein one ring has 6 members and the other ring has 5 members, and wherein at least one ring is a heteroaryl ring. A heteroaryl group can be attached to the remainder of the molecule through a carbon or heteroatom. Non-limiting examples of heteroaryl groups include pyrrolyl, pyrazolyl, pyridazinyl, triazinyl, pyrimidinyl, imidazolyl, pyrazinyl, purinyl, oxazolyl, isoxazolyl, thiazolyl, furyl, thienyl, pyridyl, pyrimidyl, benzothiazolyl, benzoxazolyl, benzimidazolyl, benzofuran, isobenzofuranyl, indolyl, isoindolyl, benzothiophenyl, isoquinolyl, quinoxalyl, quinolyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalyl, 5-quinoxalyl, 3-quinolyl, and 6-quinolyl.

Substituents for each of the above noted heteroaryl ring systems are selected from the group of acceptable substituents described below. A “heteroarylene,” alone or as part of another substituent, means a divalent radical derived from a heteroaryl. A heteroaryl group substituent may be -O- bonded to a ring heteroatom nitrogen.

[0038] The term “hydrophobic lipids” means compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0039] The term “lipid” means an organic compound that comprises an ester of fatty acid and is characterized by being insoluble in water, but soluble in many organic solvents. Lipids are usually divided into at least three classes: (1) “simple lipids,” which include fats and oils as well as waxes; (2) “compound lipids,” which include phospholipids and glycolipids; and (3) “derived lipids” such as steroids.

[0040] The term “lipid delivery vehicle” means a lipid formulation that can be used to deliver a therapeutic nucleic acid (e.g., mRNA) to a target site of interest (e.g., cell, tissue, organ, and the like). The lipid delivery vehicle can be a nucleic acid-lipid particle, which can be formed from a cationic lipid, a non-cationic lipid (e.g., a phospholipid), a conjugated lipid that prevents aggregation of the particle (e.g., a PEG-lipid), and optionally cholesterol. Typically, the therapeutic nucleic acid (e.g., mRNA) may be encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation.

[0041] The term “lipid encapsulated” means a lipid particle that provides a therapeutic nucleic acid such as an mRNA with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid (e.g., mRNA) is fully encapsulated in the lipid particle.

[0042] The term “amphipathic lipid” or “amphiphilic lipid” means the material in which the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or

heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0043] The term “heteroalkyl,” as used herein, alone or in combination, refers to a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, fully saturated or containing from 1 to 3 degrees of unsaturation, consisting of the stated number of carbon atoms and from one to three heteroatoms selected from the group consisting of O, N, and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized (i.e. bond to 4 groups). The heteroatom(s) O, N and S may be placed at any interior position of the heteroalkyl group. Up to two heteroatoms may be consecutive, such as, for example, $-\text{CH}_2\text{NHOCH}_3$.

[0044] The term “linker” or “linking moiety” refers to a group of atoms, e.g., 10-100 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. The linker may be of sufficient length as to not interfere with incorporation into an amino acid sequence. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkyl, heteroalkyl, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers. Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond ($-\text{S}-\text{S}-$) or an azo bond ($-\text{N}=\text{N}-$), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bond include an amido bond, which can be cleaved for example by the use of tris(2-carboxyethyl)phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond, which can be cleaved for example by acidic or basic hydrolysis.

[0045] The term “mammal” means a human or other mammal or means a human being.

[0046] The term “messenger RNA” (mRNA) refers to any polynucleotide which encodes a protein or polypeptide of interest and which is capable of being translated to produce the encoded protein or polypeptide of interest in vitro, in vivo, in situ or ex vivo.

[0047] The term “modified” refers to a changed state or structure of a molecule of the disclosure. Molecules may be modified in many ways including chemically, structurally, and functionally. In one embodiment, nucleic acid active ingredients are modified by the introduction of non-natural nucleosides and/or nucleotides, e.g., as it relates to the natural ribonucleotides A, U, G, and C. Noncanonical nucleotides such as the cap structures are not considered “modified” although they may differ from the chemical structure of the A, C, G, U ribonucleotides.

[0048] The term “naturally occurring” means existing in nature without artificial aid.

[0049] The term “patient” refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.

[0050] The phrase “optionally substituted X” (e.g., optionally substituted alkyl) is intended to be equivalent to “X, wherein X is optionally substituted” (e.g., “alkyl, wherein said alkyl is optionally substituted.”) It is not intended to mean that the feature “X” (e.g. alkyl) per se is optional.

[0051] The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0052] The phrase “pharmaceutically acceptable excipient,” as used herein, refers any ingredient other than the compounds described herein (for example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluent), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben,

microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

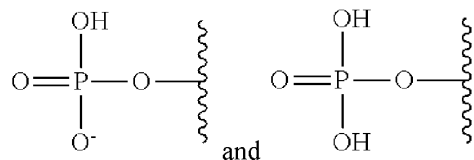
[0053] The phrase “pharmaceutically acceptable salts” refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically 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.

[0054] The term “pharmacokinetic” refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[0055] The term “pharmaceutically acceptable solvate,” as used herein, means a compound of the disclosure wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), N-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), N,N'-dimethylformamide (DMF), N,N'-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a “hydrate.”

[0056] The term “phosphate” is used in its ordinary sense as understood by those skilled in the art and includes its protonated forms, for example



[0057] As used herein, the terms “monophosphate,” “diphosphate,” and “triphosphate” are used in their ordinary sense as understood by those skilled in the art, and include protonated forms.

[0058] The term “preventing” refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[0059] The term “RNA” refers to a ribonucleic acid and means a molecule comprising at least one ribonucleotide residue. By “ribonucleotide” is meant a nucleotide with a hydroxyl group at the 2' position of a β -D-ribo-furanose moiety. The term includes double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution, and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of an interfering RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant disclosure can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA. As used herein, the terms “ribonucleic acid” and “RNA” refer to a molecule containing at least one ribonucleotide residue, including siRNA, antisense RNA, single stranded RNA, microRNA, mRNA, noncoding RNA, and multivalent RNA.

[0060] The term “sample” or “biological sample” refers to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). A sample further may include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the

external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[0061] The terms “significant” or “significantly” are used synonymously with the term “substantially.”

[0062] The phrase “single unit dose” is a dose of any therapeutic administered in one dose/at one time/single route/single point of contact, i.e., single administration event.

[0063] The term “siRNA” or small interfering RNA, sometimes known as short interfering RNA or silencing RNA, refers to a class of double-stranded RNA non-coding RNA molecules, typically 18-27 base pairs in length, similar to miRNA, and operating within the RNA interference (RNAi) pathway. It interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, thereby preventing translation.

[0064] The term “solvate” means a physical association of a compound of this disclosure with one or more solvent molecules. This physical association involves varying degrees of ionic bonding, including hydrogen bonding. In certain instances, the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of the crystalline solid. “Solvate” encompasses both solution-phase and isolatable solvates. Non-limiting examples of suitable solvates include ethanolates, methanolates, and the like.

[0065] The term “stable” refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[0066] The terms “stabilize”, “stabilized,” “stabilized region” means to make or become stable.

[0067] The term “substituted” means substitution with specified groups other than hydrogen, or with one or more groups, moieties, or radicals which can be the same or different, with each, for example, being independently selected.

[0068] The term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term

“substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0069] The phrase “substantially equal” relates to time differences between doses, the term means plus/minus 2%.

[0070] The phrase “substantially simultaneously” relates to plurality of doses, the term means within 2 seconds.

[0071] The phrase “suffering from” relates to an individual who is “suffering from” a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[0072] The phrase “susceptible to” relates to an individual who is “susceptible to” a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0073] The term “synthetic” means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present disclosure may be chemical or enzymatic.

[0074] The term “therapeutic agent” refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[0075] The term “therapeutically effective amount” means an amount of an agent to be delivered (e.g., nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, etc.) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0076] The term “therapeutically effective outcome” means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0077] The term “total daily dose” is an amount given or prescribed in 24 hour period. It may be administered as a single unit dose.

[0078] The term “treating” refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, “treating” cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0079] The term “unmodified” refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the “unmodified” starting molecule for a subsequent modification.

[0080] Compounds described herein can be asymmetric (e.g., having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically active starting materials are known in the art, such as by resolution of racemic mixtures or by enantio-selective and/or stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present

disclosure. *Cis* and *trans* geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[0081] Compounds of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Examples prototropic tautomers include ketone-enol pairs, amide-imidic acid pairs, lactam-lactim pairs, enamine-imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1*H*- and 3*H*-imidazole, 1*H*-, 2*H*- and 4*H*-1,2,4-triazole, 1*H*- and 2*H*-isoindole, and 1*H*- and 2*H*-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[0082] Compounds of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. “Isotopes” refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[0083] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[0084] The term “half-life” is the time required for a quantity such as nucleic acid or protein concentration or activity to fall to half of its value as measured at the beginning of a time period.

[0085] The term “in vitro” refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, in a Petri dish, etc., rather than within an organism (e.g., animal, plant, or microbe).

[0086] The term “in vivo” refers to events that occur within an organism (e.g., animal, plant, or microbe or cell or tissue thereof).

[0087] The term “monomer” refers to a single unit, e.g., a single nucleic acid, which may be joined with another molecule of the same or different type to form an oligomer. In some embodiments, a monomer may be an unlocked nucleic acid, i.e., a UNA monomer.

[0088] The term “neutral lipid” means a lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebroside, and diacylglycerols.

[0089] The term “non-cationic lipid” means an amphipathic lipid or a neutral lipid or anionic lipid and is described herein.

[0090] The terms “subject” or “patient” refers to any organism to which a composition in accordance with the disclosure may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[0091] The term “translatable” may be used interchangeably with the term “expressible” and refers to the ability of polynucleotide, or a portion thereof, to be converted to a polypeptide by a host cell. As is understood in the art, translation is the process in which ribosomes in a cell's cytoplasm create polypeptides. In translation, messenger RNA (mRNA) is decoded by tRNAs in a ribosome complex to produce a specific amino acid chain, or polypeptide. Furthermore, the term “translatable” when used in this specification in reference to an oligomer, means that at least a portion of the oligomer, e.g., the coding region of an oligomer sequence (also known as the coding sequence or CDS), is capable of being converted to a protein or a fragment thereof.

[0092] Abbreviations as used herein, are defined as follows: “1 x” for once, “2 x” for twice, “3 x” for thrice, “°C” for degrees Celsius, “eq” for equivalent or equivalents, “g” for gram or grams, “mg” for milligram or milligrams, “L” for liter or liters, “mL” for milliliter or milliliters, “μL” for microliter or microliters, “N” for normal, “M” for molar, “mmol” for millimole or millimoles, “min” for minute or minutes, “h” for hour or hours, “rt” for room temperature, “RT” for retention time, “RBF” for round bottom flask, “atm” for atmosphere, “psi” for pounds per square inch, “conc.” for concentrate, “RCM” for ring-closing metathesis, “sat” or “sat'd” for saturated, “SFC” for supercritical fluid chromatography “MW” for molecular weight, “mp” for melting point, “ee” for enantiomeric excess, “MS” or “Mass Spec” for mass spectrometry, “ESI” for electrospray ionization mass spectroscopy, “HR” for high resolution, “HRMS” for high resolution mass spectrometry, “LCMS” for liquid chromatography mass spectrometry, “HPLC” for high pressure liquid chromatography, “RP HPLC” for reverse phase HPLC, “TLC” or “tlc” for thin layer chromatography, “NMR” for nuclear magnetic resonance spectroscopy, “nOe” for nuclear Overhauser effect spectroscopy, “¹H” for proton, “δ” for delta, “s” for singlet, “d” for doublet, “t” for triplet, “q” for quartet, “m” for multiplet, “br” for broad, “Hz” for hertz, and “α”, “β”, “R”, “S”, “E”, and “Z” are stereochemical designations familiar to one skilled in the art.

Me	methyl
Et	ethyl
Pr	propyl
<i>i</i> -Pr	isopropyl
Bu	butyl
<i>i</i> -Bu	isobutyl
<i>t</i> -Bu	<i>tert</i> -butyl
Ph	phenyl
Bn	benzyl
Boc or BOC	<i>tert</i> -butoxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl dicarbonate
AcOH or HOAc	acetic acid
AlCl ₃	aluminum chloride
AIBN	azobisisobutyronitrile
aqueous	aq
BBr ₃	boron tribromide
BCl ₃	boron trichloride
BEMP	2- <i>tert</i> -butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine
BOC	<i>tert</i> -butoxycarbonyl
BOP reagent	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
Burgess reagent	1-methoxy-N-triethylammoniosulfonyl-methanimidate
Cbz	carbobenzyloxy
DCM or CH ₂ Cl ₂	dichloromethane
CH ₃ CN or ACN	acetonitrile
CDCl ₃	deutero-chloroform
CHCl ₃	chloroform
mCPBA or m-CPBA	<i>meta</i> -chloroperbenzoic acid
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cs ₂ CO ₃	cesium carbonate

Cu(OAc) ₂	copper (II) acetate
CuI	copper(I) iodide
CuSO ₄	copper(II) sulfate
Cy ₂ NMe	N-cyclohexyl-N-methylcyclohexanamine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCE	1,2-dichloroethane
DEA	diethylamine
Dess-Martin	1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1H)-one
DIC or DIPCDI	diisopropylcarbodiimide
DIEA, DIPEA or Hunig's base	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DME	1,2-dimethoxyethane
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
cDNA	complimentary DNA
Dppp	(<i>R</i>)-(+)-1,2-bis(diphenylphosphino)propane
DuPhos	(+)-1,2-bis((2 <i>S</i> ,5 <i>S</i>)-2,5-diethylphospholano)benzene
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide
EDCI	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
(<i>S,S</i>)-EtDuPhosRh(I)	(+)-1,2-bis((2 <i>S</i> ,5 <i>S</i>)-2,5-diethylphospholano)benzene(1,5-cyclooctadiene)rhodium(I) trifluoromethanesulfonate
Et ₃ N or TEA	triethylamine
EtOAc	ethyl acetate
Et ₂ O	diethyl ether
EtOH	ethanol
GMF	glass microfiber filter
Grubbs II	(1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)(tricyclohexylphosphine)ruthenium

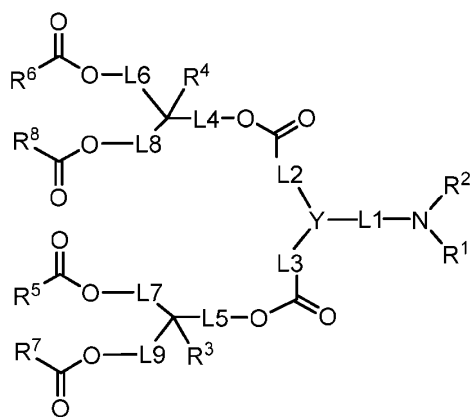
HCl	hydrochloric acid
HATU	O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
Hex	hexane
HOBt or HOBt	1-hydroxybenzotriazole
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulfuric acid
IBX	2-iodoxybenzoic acid
InCl ₃	Indium(III) chloride
Jones reagent	CrO ₃ in aqueous H ₂ SO ₄ , 2 M
K ₂ CO ₃	potassium carbonate
K ₂ HPO ₄	potassium phosphate dibasic
K ₃ PO ₄	potassium phosphate tribasic
KOAc	potassium acetate
K ₃ PO ₄	potassium phosphate
LAH	lithium aluminum hydride
LG	leaving group
LiOH	lithium hydroxide
MeOH	methanol
MgSO ₄	magnesium sulfate
MsOH or MSA	methylsulfonic acid
NaCl	sodium chloride
NaH	sodium hydride
NaHCO ₃	sodium bicarbonate
Na ₂ CO ₃	sodium carbonate
NaOH	sodium hydroxide
Na ₂ SO ₃	sodium sulfite
Na ₂ SO ₄	sodium sulfate
NBS	N-bromosuccinimide
NCS	N-chlorosuccinimide

NH ₃	ammonia
NH ₄ Cl	ammonium chloride
NH ₄ OH	ammonium hydroxide
NH ₄ COOH	ammonium formate
NMM	N-methylmorpholine
OTf	triflate or trifluoromethanesulfonate
PEG	polyethylene glycol
Pd ₂ (dba) ₃	tris(dibenzylideneacetone)dipalladium(0)
Pd(OAc) ₂	palladium(II) acetate
Pd/C	palladium on carbon
Pd(dppf)Cl ₂	[1,1'-bis(diphenylphosphino)-ferrocene]dichloropalladium(II)
Ph ₃ PCl ₂	triphenylphosphine dichloride
PG	protecting group
POCl ₃	phosphorus oxychloride
i-PrOH or IPA	isopropanol
PS	Polystyrene
rt	room temperature
SEM-Cl	2-(trimethylsilyl)ethoxymethyl chloride
SiO ₂	silica oxide
SnCl ₂	tin(II) chloride
TALEN	Transcription activator-like effector nucleases
TBAI	tetra- <i>n</i> -butylammonium iodide
TBN	<i>t</i> -butyl nitrite
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMSCHN ₂	trimethylsilyldiazomethane
T3P®	propane phosphonic acid anhydride
TRIS	tris (hydroxymethyl) aminomethane
pTsOH	p-toluenesulfonic acid

[0093] While this disclosure has been described in relation to certain embodiments, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that this disclosure includes additional embodiments, and that some of the details described herein may be varied considerably without departing from this disclosure. This disclosure includes such additional embodiments, modifications, and equivalents. In particular, this disclosure includes any combination of the features, terms, or elements of the various illustrative components and examples.

III. COMPOUNDS

[0094] In some embodiments, the present disclosure provides a compound of Formula I, or a pharmaceutically acceptable salt thereof:



(I)

wherein:

R¹ and R² are each independently H or C₁₋₆ alkyl; or

R¹ and R² are joined to form a saturated heterocyclic ring, wherein:

R¹ is a linear C₁₋₄ alkylene; and

R² is -(CH₂)_m(X)_n-, wherein

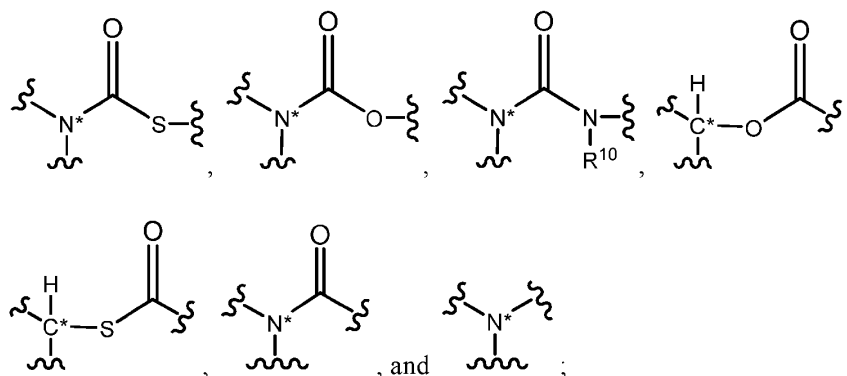
X is O, S, or NR⁹, wherein R⁹ is H or C₁₋₆ alkyl;

m is 1, 2, 3 or 4, and

n is 0 or 1;

L1 is a linear C₁₋₆ alkylene optionally substituted with one to three methyl groups;

Y is selected from the group consisting of:



wherein:

each asterisk (*) indicates the atom attached to L2 and L3; and

R¹⁰ is H or C₁₋₆ alkyl;

L2 and L3 are each independently a linear C₁₋₈ alkylene;

L4, L5, L6, L7, L8 and L9 are each independently absent or -CH₂-, provided that:

at least two of L4, L6 and L8 are -CH₂-; and

at least two of L5, L7 and L9 are -CH₂-;

R³ and R⁴ are each independently H, methyl or ethyl; and

R⁵, R⁶, R⁷ and R⁸ are each independently selected from the group consisting of:

linear C₁₋₂₀ alkyl, wherein each said linear C₁₋₂₀ alkyl is optionally substituted

with one or more substituents selected from the group consisting of:

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic

aromatic hydrocarbon optionally substituted with one or

more C₆₋₁₀ aryl that is optionally substituted with one or more

C₁₋₆ alkyl;

6-10 membered heteroaryl, wherein each said 6-10 membered

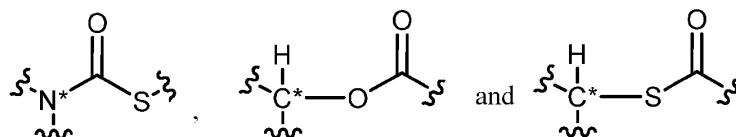
heteroaryl is a monocyclic or bicyclic aromatic system

optionally substituted with one or more C₁₋₆ alkyl;

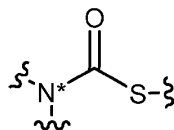
C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl; and 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

[0095] In some embodiments, R¹ is H or C₁₋₆ alkyl, and R² is C₁₋₆ alkyl, or R¹ and R² are joined to form said saturated heterocyclic ring. In some embodiments, R¹ is H or C₁₋₆ alkyl, and R² is C₁₋₆ alkyl. In some embodiments, R¹ and R² are joined to form said saturated heterocyclic ring.

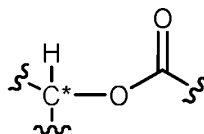
[0096] In some embodiments, Y is selected from the group consisting of:



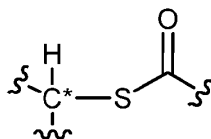
[0097] In some embodiments, Y is:



[0098] In some embodiments, Y is:



[0099] In some embodiments, Y is:



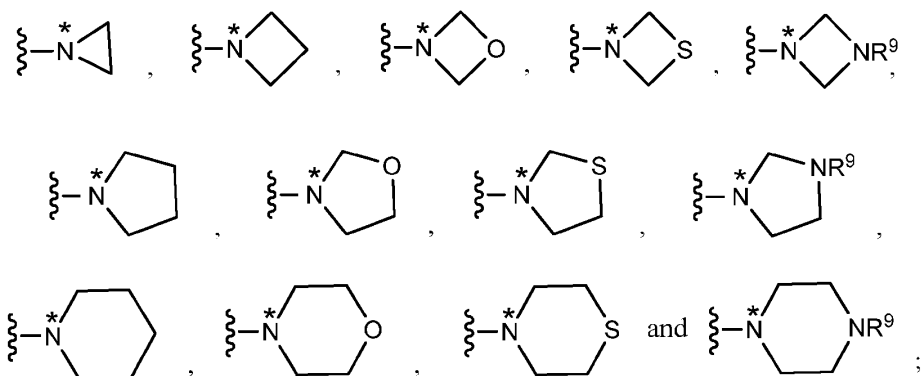
[0100] In some embodiments, at least one of R¹ and R² is H; and L₁ is -CH₂- or -CH₂CH₂-.

[0101] In some embodiments, R¹ and R² are each independently C₁₋₆ alkyl.

[0102] In some embodiments, R¹ and R² are each independently C₁₋₃ alkyl.

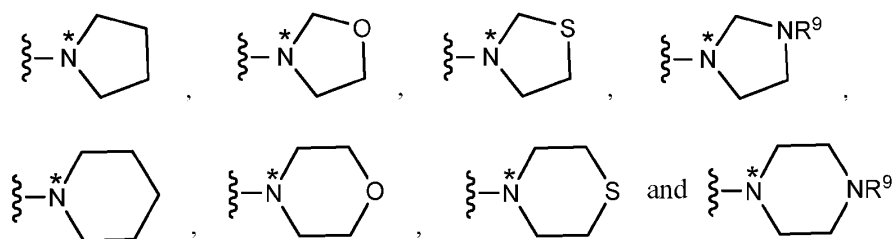
[0103] In some embodiments, R¹ and R² are each methyl.

[0104] In some embodiments, R^1 and R^2 are joined to form said heterocyclic ring. In some embodiments, the heterocyclic ring is selected from the group consisting of:

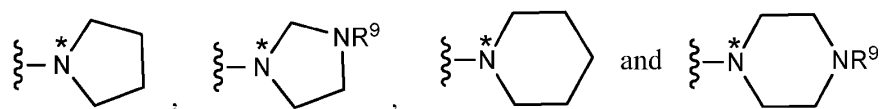


wherein each asterisk (*) indicates the atom attached to L1.

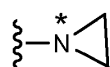
[0105] In some embodiments, the heterocyclic ring is selected from the group consisting of:



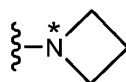
[0106] In some embodiments, the heterocyclic ring is selected from the group consisting of:



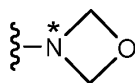
[0107] In some embodiments, the heterocyclic ring is:



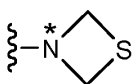
[0108] In some embodiments, the heterocyclic ring is:



[0109] In some embodiments, the heterocyclic ring is:



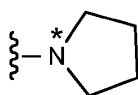
[0110] In some embodiments, the heterocyclic ring is:



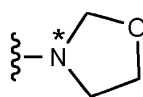
[0111] In some embodiments, the heterocyclic ring is:



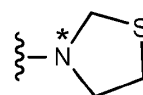
[0112] In some embodiments, the heterocyclic ring is:



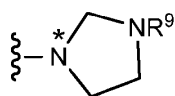
[0113] In some embodiments, the heterocyclic ring is:



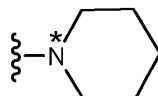
[0114] In some embodiments, the heterocyclic ring is:



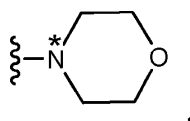
[0115] In some embodiments, the heterocyclic ring is:



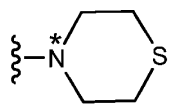
[0116] In some embodiments, the heterocyclic ring is:



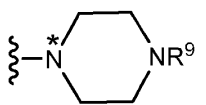
[0117] In some embodiments, the heterocyclic ring is:



[0118] In some embodiments, the heterocyclic ring is:



[0119] In some embodiments, the heterocyclic ring is:



[0120] In some embodiments, R^5 , R^6 , R^7 and R^8 are each independently linear C_{1-8} alkyl, wherein each said linear C_{1-8} alkyl is optionally substituted with one or more substituents selected from:

C_{6-10} aryl, wherein each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl; and

6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C_{1-6} alkyl. In some embodiments, R^5 , R^6 , R^7 and R^8 are each independently linear C_{1-8} alkyl. In some embodiments, each said linear C_{1-8} alkyl is substituted with one or more substituents selected from: C_{6-10} aryl, wherein each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl; and 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C_{1-6} alkyl.

[0121] In some embodiments, R^5 , R^6 , R^7 and R^8 are each independently C_{6-10} aryl, wherein each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon. In some embodiments, each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl. In some embodiments, each said C_{6-10} aryl is a monocyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl. In some

embodiments, each said C₆₋₁₀ aryl is a bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl.

[0122] In some embodiments, R⁵, R⁶, R⁷ and R⁸ are each independently a 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

[0123] In some embodiments, R⁵ and R⁶ are the same.

[0124] In some embodiments, R⁷ and R⁸ are the same.

[0125] In some embodiments, L1 is linear unsubstituted alkylene.

[0126] In some embodiments, L1 is propylene.

[0127] In some embodiments, L2 and L3 are each independently linear C₁₋₅ alkylene.

[0128] In some embodiments, L2 and L3 are the same.

[0129] In some embodiments, L4 and L5 are the same.

[0130] In some embodiments, L6 and L7 are the same.

[0131] In some embodiments, L8 and L9 are the same.

[0132] In some embodiments, L4, L5, L6, L7, L8 and L9 are each -CH₂-.

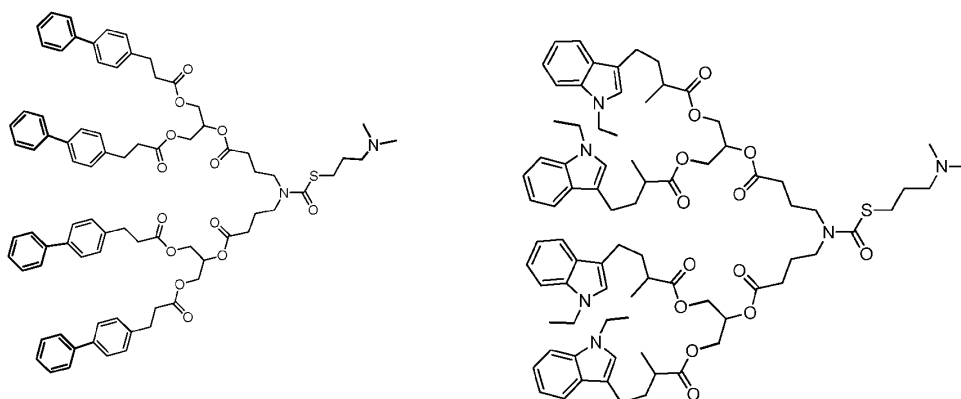
[0133] In some embodiments, L6, L7, L8 and L9 are each -CH₂-; and L4 and L5 are absent.

[0134] In some embodiments, L4, L5, L8 and L9 are each -CH₂-; and L6 and L7 are absent.

[0135] In some embodiments, L4, L5, L6 and L7 are each -CH₂-; and L8 and L9 are absent.

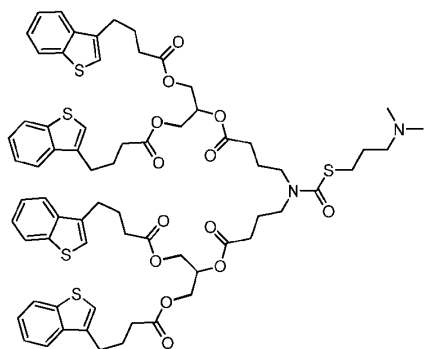
[0136] In some embodiments, R³ and R⁴ are each independently H or methyl. In some embodiments, R³ and R⁴ are each H. In some embodiments, R³ and R⁴ are each methyl.

[0137] In some embodiments, the compound is selected from the group consisting of:



Lipid 42

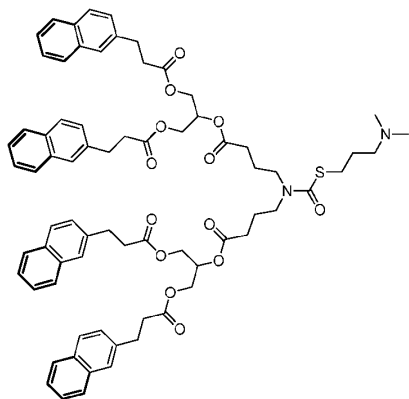
Lipid 43, and



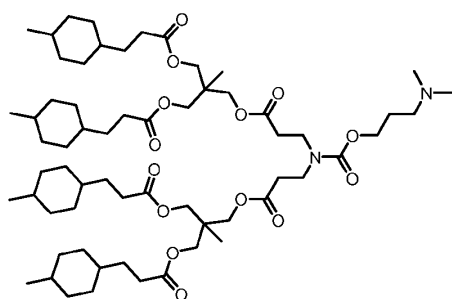
Lipid 45;

or pharmaceutically acceptable salts thereof. In some embodiments, the compound is Lipid 42 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 44 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 45 or pharmaceutically acceptable salt thereof.

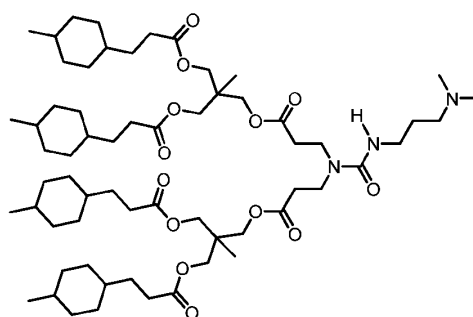
[0138] In some embodiments, the present disclosure provides a compound selected from the group consisting of:



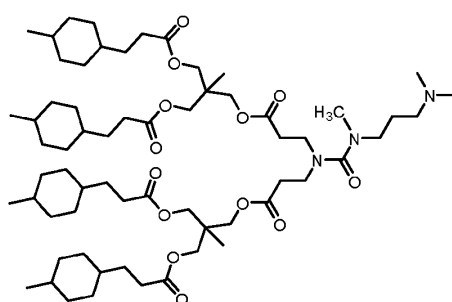
Lipid 44



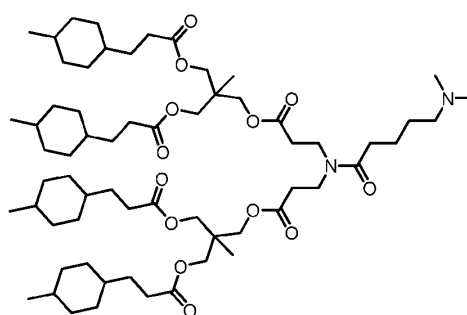
Lipid 46



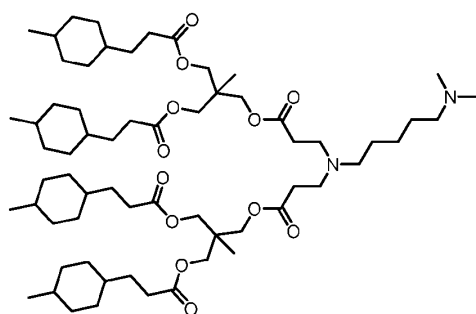
Lipid 47



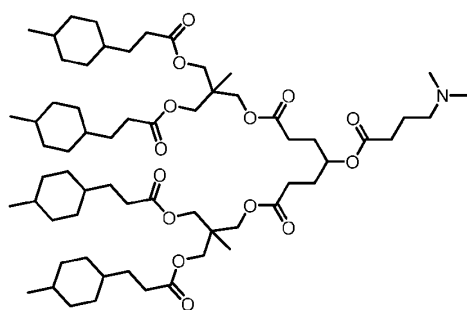
Lipid 48



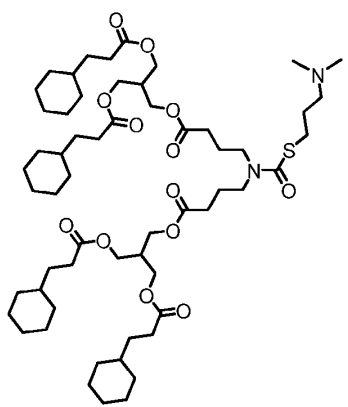
Lipid 49



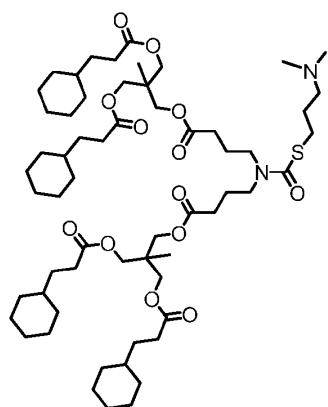
Lipid 50



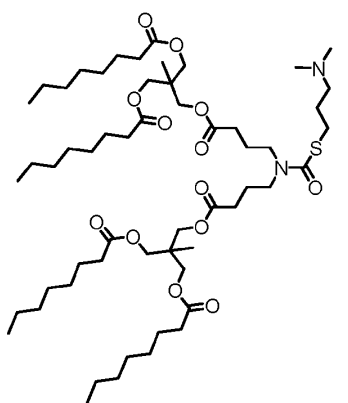
Lipid 51



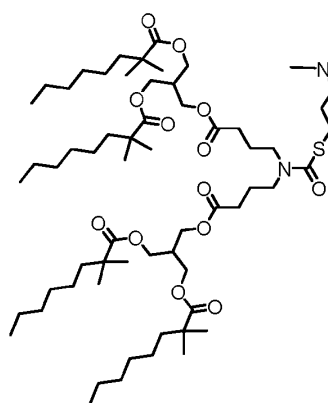
Lipid 52



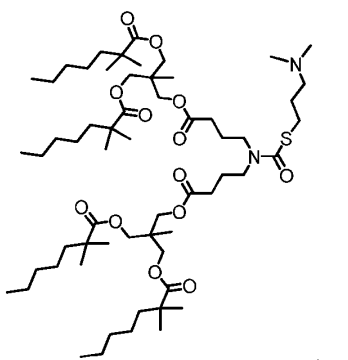
Lipid 53



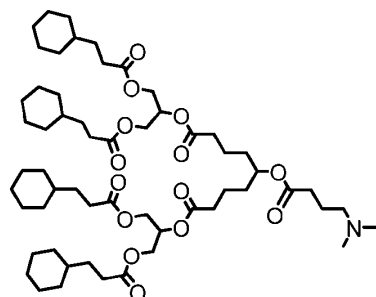
Lipid 54



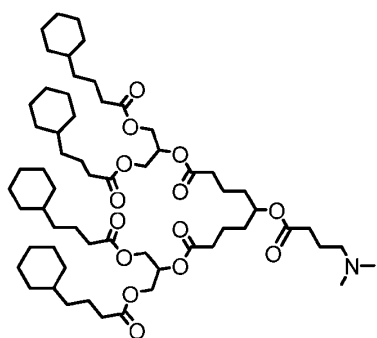
Lipid 55



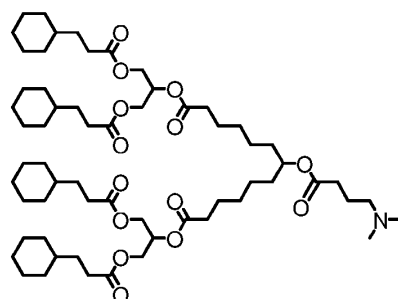
Lipid 56



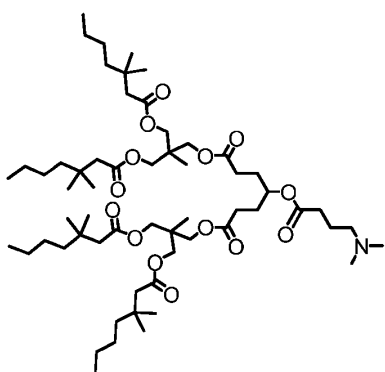
Lipid 57



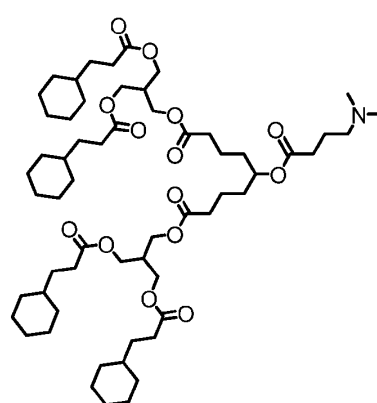
Lipid 58



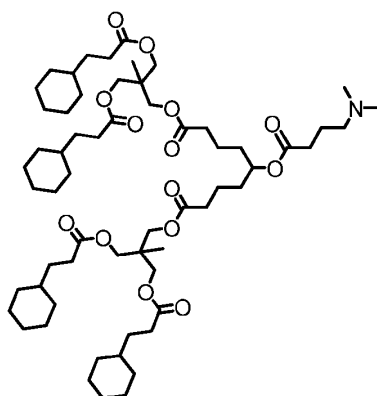
Lipid 59



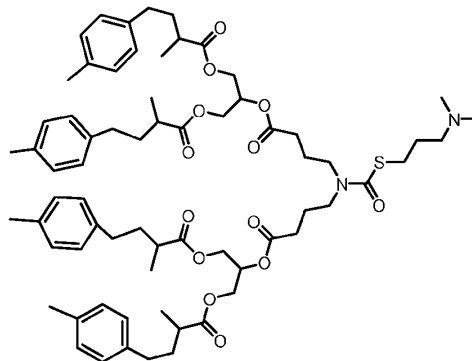
Lipid 60



Lipid 61



Lipid 62, and



Lipid 63;

or pharmaceutically acceptable salts thereof. In some embodiments, the compound is Lipid 44 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 46 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 47 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 48 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 49 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 50 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 51 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 52 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 53 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 54 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 55 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 56 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 57 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 58 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 59 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 60 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 61 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 62 or pharmaceutically acceptable salt thereof. In some embodiments, the compound is Lipid 63 or pharmaceutically acceptable salt thereof.

[0139] In some embodiments, the present disclosure provides a lipid composition comprising a nucleic acid and a compound of the present disclosure. In some embodiments, the nucleic acid is selected from an siRNA, an mRNA, a self-replicating RNA, a DNA plasmid, and an antisense oligonucleotide. In some embodiments, the nucleic acid is an siRNA. In some embodiments, the nucleic acid is an mRNA. In some embodiments, the nucleic acid is a self-replicating RNA. In some embodiments, the nucleic acid is a DNA plasmid. In some embodiments, the nucleic acid is an antisense oligonucleotide.

[0140] In some embodiments, the nucleic acid is an mRNA or a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest. In some embodiments, the nucleic acid is an mRNA comprising a coding region that encodes a therapeutic protein of interest. In some embodiments, the nucleic acid is a self-replicating RNA comprising a coding

region that encodes a therapeutic protein of interest. In some embodiments, the therapeutic protein of interest is an enzyme, an antibody, an antigen, a receptor, or a transporter. In some embodiments, the therapeutic protein of interest is an enzyme. In some embodiments, the therapeutic protein of interest is an antibody. In some embodiments, the therapeutic protein of interest is an antigen. In some embodiments, the therapeutic protein of interest is a receptor. In some embodiments, the therapeutic protein of interest is a transporter. In some embodiments, the therapeutic protein of interest is a gene-editing enzyme. In some embodiments, the gene-editing enzyme is selected from a TALEN, a CRISPR, a meganuclease, or a zinc finger nuclease. In some embodiments, the gene-editing enzyme is a TALEN. In some embodiments, the gene-editing enzyme is a CRISPR. In some embodiments, the gene-editing enzyme is a meganuclease. In some embodiments, the gene-editing enzyme is a zinc finger nuclease.

[0141] In some embodiments, the lipid composition comprises liposomes, lipoplexes, or lipid nanoparticles. In some embodiments, the lipid composition comprises liposomes. In some embodiments, the lipid composition comprises lipoplexes. In some embodiments, the lipid composition comprises lipid nanoparticles.

[0142] In some embodiments, the present disclosure provides a lipid nanoparticle comprising a plurality of ligands, wherein each ligand is independently a compound of the present disclosure. In some embodiments, the plurality of ligands self-assembles to form the lipid nanoparticle comprising an interior and exterior.

[0143] In some embodiments, the average particle size of the lipid nanoparticle is less than about 100 nm. In some embodiments, the average particle size of the lipid nanoparticle is about 55 nm to about 85 nm.

[0144] In some embodiments, the lipid nanoparticle further comprises a nucleic acid encapsulated in the interior. In some embodiments, the nucleic acid is selected from an siRNA, an mRNA, a self-replicating RNA, a DNA plasmid, and an antisense oligonucleotide. In some embodiments, the nucleic acid is an mRNA or a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest. In some embodiments, the nucleic acid is an siRNA. In some embodiments, the nucleic acid is an mRNA. In some embodiments, the nucleic acid is a self-replicating RNA. In some embodiments, the nucleic acid is a DNA plasmid. In some embodiments, the nucleic acid is an antisense oligonucleotide. In some embodiments, the nucleic acid is an mRNA comprising a coding region that encodes a therapeutic protein of

interest. In some embodiments, the nucleic acid is a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest. In some embodiments, the therapeutic protein of interest is an enzyme, an antibody, an antigen, a receptor, or a transporter. In some embodiments, the therapeutic protein of interest is an enzyme. In some embodiments, the therapeutic protein of interest is an antibody. In some embodiments, the therapeutic protein of interest is an antigen. In some embodiments, the therapeutic protein of interest is a receptor. In some embodiments, the therapeutic protein of interest is a transporter. In some embodiments, the therapeutic protein of interest is a gene-editing enzyme. In some embodiments, the gene-editing enzyme is selected from a TALEN, a CRISPR, a meganuclease, or a zinc finger nuclease. In some embodiments, the gene-editing enzyme is a TALEN. In some embodiments, the gene-editing enzyme is a CRISPR. In some embodiments, the gene-editing enzyme is a meganuclease. In some embodiments, the gene-editing enzyme is a zinc finger nuclease.

[0145] In some embodiments, the lipid nanoparticle further comprises a helper lipid selected from: dioleoylphosphatidyl ethanolamine (DOPE), dimyristoylphosphatidyl choline (DMPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidyl glycerol (DMPG), dipalmitoyl phosphatidylcholine (DPPC), and phosphatidylcholine (PC). In some embodiments, the helper lipid is DOPE. In some embodiments, the helper lipid is DMPC. In some embodiments, the helper lipid is DSPC. In some embodiments, the helper lipid is DMPG. In some embodiments, the helper lipid is DPPC. In some embodiments, the helper lipid is PC.

[0146] In some embodiments, the lipid nanoparticle further comprises cholesterol.

[0147] In some embodiments, the lipid nanoparticle further comprises a polyethylene glycol(PEG)-lipid conjugate. In some embodiments, the PEG-lipid conjugate is PEG-DMG. In some embodiments, the PEG-DMG is PEG2000-DMG.

[0148] In some embodiments, the lipid nanoparticle comprises about 45 mol% to 65 mol% of a compound of the present disclosure, about 2 mol% to about 15 mol% of a helper lipid, about 20 mol% to about 42 mol% of cholesterol, and about 0.5 mol% to about 3 mol% of a PEG-lipid conjugate.

[0149] In some embodiments, the lipid nanoparticle comprises about 50 mol% to about 61 mol% of a compound of the present disclosure, about 5 mol% to about 9 mol% of the helper lipid, about 29 mol% to about 38 mol% of cholesterol, and about 1 mol% to about 2 mol% of the PEG-lipid conjugate.

[0150] In some embodiments, the lipid nanoparticle comprises about 56 mol% to about 58 mol% of a compound of the present disclosure, about 6 mol% to about 8 mol% of DSPC, about 31 mol% to about 34 mol% of cholesterol, and about 1.25 mol% to about 1.75 mol% of the PEG-lipid conjugate.

[0151] In some embodiments, the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 50:1 to about 10:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 40:1 to about 20:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 35:1 to about 25:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 32:1 to about 28:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 31:1 to about 29:1.

[0152] In some embodiments, the present disclosure provides a pharmaceutical composition comprising a compound of the present disclosure or a lipid nanoparticle of the present disclosure, and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition is a lyophilized composition. In some embodiments, the pharmaceutical composition comprises a HEPES buffer at a pH of about 7.4. In some embodiments, the HEPES buffer is at a concentration of about 7 mg/mL to about 15 mg/mL. In some embodiments, the pharmaceutical composition further comprises about 2.0 mg/mL to about 4.0 mg/mL of NaCl. In some embodiments, the pharmaceutical composition further comprises one or more cryoprotectants. In some embodiments, the one or more cryoprotectants are selected from sucrose, glycerol, or a combination of sucrose and glycerol. In some embodiments, the one or more cryoprotectants is sucrose. In some embodiments, the one or more cryoprotectants is glycerol. In some embodiments, the one or more cryoprotectants is a combination of sucrose and glycerol. In some embodiments, the pharmaceutical composition comprises a combination of sucrose at a concentration of about 70 mg/mL to about 110 mg/mL and glycerol at a concentration of about 50 mg/mL to about 70 mg/mL.

IV. LIPID FORMULATIONS AND NANOPARTICLES

Lipid-Based Formulations

[0153] Therapies based on the intracellular delivery of nucleic acids to target cells face both extracellular and intracellular barriers. Indeed, naked nucleic acid materials cannot be easily systemically administered due to their toxicity, low stability in serum, rapid renal clearance, reduced uptake by target cells, phagocyte uptake and their ability in activating the immune response, all features that preclude their clinical development. When exogenous nucleic acid material (e.g., mRNA) enters the human biological system, it is recognized by the reticuloendothelial system (RES) as foreign pathogens and cleared from blood circulation before having the chance to encounter target cells within or outside the vascular system. It has been reported that the half-life of naked nucleic acid in the blood stream is around several minutes (Kawabata K, Takakura Y, Hashida M Pharm Res. 1995 Jun; 12(6):825-30). Chemical modification and a proper delivery method can reduce uptake by the RES and protect nucleic acids from degradation by ubiquitous nucleases, which increase stability and efficacy of nucleic acid-based therapies. In addition, RNAs or DNAs are anionic hydrophilic polymers that are not favorable for uptake by cells, which are also anionic at the surface. The success of nucleic acid-based therapies thus depends largely on the development of vehicles or vectors that can efficiently and effectively deliver genetic material to target cells and obtain sufficient levels of expression in vivo with minimal toxicity.

[0154] Moreover, upon internalization into a target cell, nucleic acid delivery vectors are challenged by intracellular barriers, including endosome entrapment, lysosomal degradation, nucleic acid unpacking from vectors, translocation across the nuclear membrane (for DNA), and release at the cytoplasm (for RNA). Successful nucleic acid-based therapy thus depends upon the ability of the vector to deliver the nucleic acids to the target sites inside of the cells in order to obtain sufficient levels of a desired activity such as expression of a gene.

[0155] While several gene therapies have been able to successfully utilize a viral delivery vector (e.g., AAV), lipid-based formulations have been increasingly recognized as one of the most promising delivery systems for RNA and other nucleic acid compounds due to their biocompatibility and their ease of large-scale production. One of the most significant advances in lipid-based nucleic acid therapies happened in August 2018 when Patisiran (ALN-TTR02) was

the first siRNA therapeutic approved by the Food and Drug Administration (FDA) and by the European Commission (EC). ALN-TTR02 is an siRNA formulation based upon the so-called Stable Nucleic Acid Lipid Particle (SNALP) transfecting technology. Despite the success of Patisiran, the delivery of nucleic acid therapeutics, including mRNA, via lipid formulations is still undergoing development. The use of mRNA in lipid delivery vehicles quickly rose to prominence as a result of the COVID-19 pandemic with several vaccines delivering mRNA encoding the spike protein of COVID-19 showing strong protective capabilities. Such lipid-based mRNA vaccines include Pfizer and BioNtech's BNT162b2 and Moderna's mRNA-1273, which have received emergency use authorization around the world.

[0156] Some art-recognized lipid-formulated delivery vehicles for nucleic acid therapeutics include, according to various embodiments, polymer based carriers, such as polyethyleneimine (PEI), lipid nanoparticles and liposomes, nanoliposomes, ceramide-containing nanoliposomes, multivesicular liposomes, proteoliposomes, both natural and synthetically-derived exosomes, natural, synthetic and semi-synthetic lamellar bodies, nanoparticulates, micelles, and emulsions. These lipid formulations can vary in their structure and composition, and as can be expected in a rapidly evolving field, several different terms have been used in the art to describe a single type of delivery vehicle. At the same time, the terms for lipid formulations have varied as to their intended meaning throughout the scientific literature, and this inconsistent use has caused confusion as to the exact meaning of several terms for lipid formulations. Among the several potential lipid formulations, liposomes, cationic liposomes, and lipid nanoparticles are specifically described in detail and defined herein for the purposes of the present disclosure.

Liposomes

[0157] Conventional liposomes are vesicles that consist of at least one bilayer and an internal aqueous compartment. Bilayer membranes of liposomes are typically formed by amphiphilic molecules, such as lipids of synthetic or natural origin that comprise spatially separated hydrophilic and hydrophobic domains (Lasic, Trends Biotechnol., 16: 307-321, 1998). Bilayer membranes of the liposomes can also be formed by amphiphilic polymers and surfactants (e.g., polymerosomes, niosomes, etc.). They generally present as spherical vesicles and can range in size from 20 nm to a few microns. Liposomal formulations can be prepared as a colloidal dispersion or they can be lyophilized to reduce stability risks and to improve the shelf-life for

liposome-based drugs. Methods of preparing liposomal compositions are known in the art and are within the skill of an ordinary artisan.

[0158] Liposomes that have only one bilayer are referred to as being unilamellar, and those having more than one bilayer are referred to as multilamellar. The most common types of liposomes are small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV). In contrast to liposomes, lysosomes, micelles, and reversed micelles are composed of monolayers of lipids. Generally, a liposome is thought of as having a single interior compartment, however some formulations can be multivesicular liposomes (MVL), which consist of numerous discontinuous internal aqueous compartments separated by several nonconcentric lipid bilayers.

[0159] Liposomes have long been perceived as drug delivery vehicles because of their superior biocompatibility, given that liposomes are basically analogs of biological membranes, and can be prepared from both natural and synthetic phospholipids (Int. J. Nanomedicine. 2014; 9:1833-1843). In their use as drug delivery vehicles, because a liposome has an aqueous solution core surrounded by a hydrophobic membrane, hydrophilic solutes dissolved in the core cannot readily pass through the bilayer, and hydrophobic compounds will associate with the bilayer. Thus, a liposome can be loaded with hydrophobic and/or hydrophilic molecules. When a liposome is used to carry a nucleic acid such as RNA, the nucleic acid is contained within the liposomal compartment in an aqueous phase.

Cationic Liposomes

[0160] Liposomes can be composed of cationic, anionic, and/or neutral lipids. As an important subclass of liposomes, cationic liposomes are liposomes that are made in whole or part from positively charged lipids, or more specifically a lipid that comprises both a cationic group and a lipophilic portion. In addition to the general characteristics profiled above for liposomes, the positively charged moieties of cationic lipids used in cationic liposomes provide several advantages and some unique structural features. For example, the lipophilic portion of the cationic lipid is hydrophobic and thus will direct itself away from the aqueous interior of the liposome and associate with other nonpolar and hydrophobic species. Conversely, the cationic moiety will associate with aqueous media and more importantly with polar molecules and species with which it can complex in the aqueous interior of the cationic liposome. For these

reasons, cationic liposomes are increasingly being researched for use in gene therapy due to their favorability towards negatively charged nucleic acids via electrostatic interactions, resulting in complexes that offer biocompatibility, low toxicity, and the possibility of the large-scale production required for in vivo clinical applications. Cationic lipids suitable for use in cationic liposomes are listed herein below.

Lipid Nanoparticles

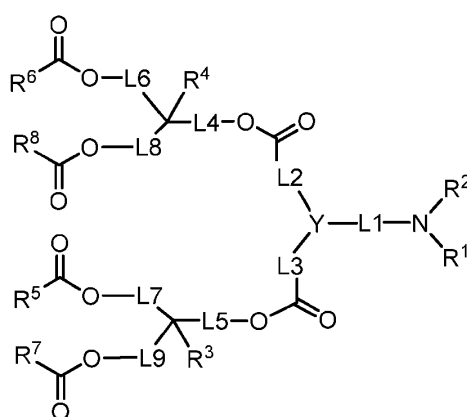
[0161] In contrast to liposomes and cationic liposomes, lipid nanoparticles (LNP) have a structure that includes a single monolayer or bilayer of lipids that encapsulates a compound in a solid phase. Thus, unlike liposomes, lipid nanoparticles do not have an aqueous phase or other liquid phase in its interior, but rather the lipids from the bilayer or monolayer shell are directly complexed to the internal compound thereby encapsulating it in a solid core. Lipid nanoparticles are typically spherical vesicles having a relatively uniform dispersion of shape and size. While sources vary on what size qualifies a lipid particle as being a nanoparticle, there is some overlap in agreement that a lipid nanoparticle can have a diameter in the range of from 10 nm to 1000 nm. However, more commonly they are considered to be smaller than 120 nm or even 100 nm.

[0162] For lipid nanoparticle nucleic acid delivery systems, the lipid shell can be formulated to include an ionizable cationic lipid which can complex to and associate with the negatively charged backbone of the nucleic acid core. Ionizable cationic lipids with apparent pKa values below about 7 have the benefit of providing a cationic lipid for complexing with the nucleic acid's negatively charged backbone and loading into the lipid nanoparticle at pH values below the pKa of the ionizable lipid where it is positively charged. Then, at physiological pH values, the lipid nanoparticle can adopt a relatively neutral exterior allowing for a significant increase in the circulation half-lives of the particles following i.v. administration. In the context of nucleic acid delivery, lipid nanoparticles offer many advantages over other lipid-based nucleic acid delivery systems including high nucleic acid encapsulation efficiency, potent transfection, improved penetration into tissues to deliver therapeutics, and low levels of cytotoxicity and immunogenicity.

[0163] Prior to the development of lipid nanoparticle delivery systems for nucleic acids, cationic lipids were widely studied as synthetic materials for delivery of nucleic acid medicines. In these early efforts, after mixing together at physiological pH, nucleic acids were condensed by

cationic lipids to form lipid-nucleic acid complexes known as lipoplexes. However, lipoplexes proved to be unstable and characterized by broad size distributions ranging from the submicron scale to a few microns. Lipoplexes, such as the LIPOFECTAMINE® reagent, have found considerable utility for in vitro transfection. However, these first-generation lipoplexes have not proven useful in vivo. The large particle size and positive charge (imparted by the cationic lipid) result in rapid plasma clearance, hemolytic and other toxicities, as well as immune system activation.

[0164] In some embodiments, the lipid nanoparticle comprises a lipid of Formula I:



(I)

wherein:

R^1 and R^2 are each independently H or C_{1-6} alkyl; or

R^1 and R^2 are joined to form a saturated heterocyclic ring, wherein:

R^1 is a linear C_{1-4} alkylene; and

R^2 is $-(CH_2)_m(X)_n-$, wherein

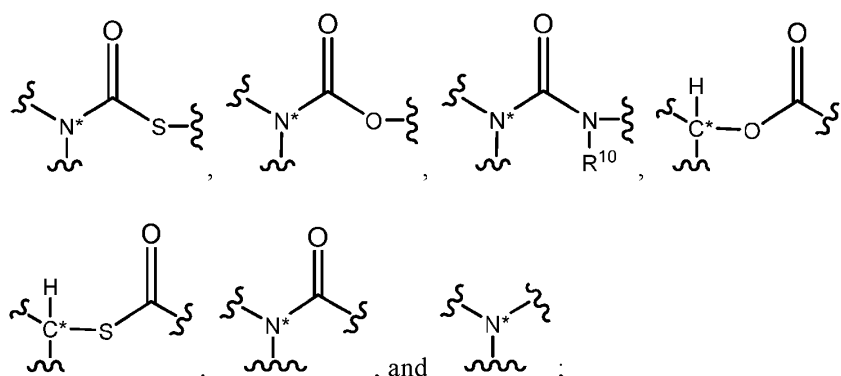
X is O, S, or NR^9 , wherein R^9 is H or C_{1-6} alkyl;

m is 1, 2, 3 or 4, and

n is 0 or 1;

L1 is a linear C_{1-6} alkylene optionally substituted with one to three methyl groups;

Y is selected from the group consisting of:



wherein:

each asterisk (*) indicates the atom attached to L2 and L3; and

R¹⁰ is H or C₁₋₆ alkyl;

L2 and L3 are each independently a linear C₁₋₈ alkylene;

L4, L5, L6, L7, L8 and L9 are each independently absent or -CH₂-, provided that:

at least two of L4, L6 and L8 are -CH₂-; and

at least two of L5, L7 and L9 are -CH₂-;

R³ and R⁴ are each independently H, methyl or ethyl; and

R⁵, R⁶, R⁷ and R⁸ are each independently selected from the group consisting of:

linear C₁₋₂₀ alkyl, wherein each said linear C₁₋₂₀ alkyl is optionally substituted with one or more substituents selected from the group consisting of:

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl;

6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl;

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl; and

6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

[0165] In some embodiments, any one or more lipids recited herein may be expressly excluded.

[0166] In some embodiments, the present disclosure provides a lipid nanoparticle, comprising a plurality of ligands, wherein each ligand is independently a compound described herein, wherein the plurality of ligands self-assembles to form the lipid nanoparticle comprising an interior and exterior.

[0167] In some embodiments, the average size of the lipid nanoparticle is about 100 nm. In some embodiments, the average size of the lipid nanoparticle is less than about 100 nm. In some embodiments, the average particle size of the lipid nanoparticle is about 40 nm to about 100 nm. In some embodiments, the average particle size of the lipid nanoparticle is about 50 nm to about 90 nm. In some embodiments, the average particle size of the lipid nanoparticle is about 55 nm to about 85 nm.

[0168] In some embodiments, the lipid nanoparticle further comprises nucleic acids in the interior. In some embodiments, the nucleic acid is selected from an siRNA, an mRNA, a self-replicating RNA, a DNA plasmid, and an antisense oligonucleotide. In some embodiments, the nucleic acid is a mRNA or a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest. In some embodiments, the therapeutic protein of interest is an enzyme, and antibody, an antigen, a receptor, or a transporter. In some embodiments, the therapeutic protein of interest is a gene-editing enzyme. In some embodiments, the gene-editing enzyme is selected from a TALEN, a CRISPR, a meganuclease, or a zinc finger nuclease.

[0169] In some embodiments, the lipid nanoparticle further comprises siRNA or mRNA in the interior. In some embodiments, the lipid nanoparticle further comprises mRNA in the interior.

[0170] In some embodiments, the lipid nanoparticle further comprises a helper lipid as described below. In some embodiments, the lipid nanoparticle further comprises PEG-lipid conjugates as described herein.

[0171] In some embodiments, the lipid nanoparticle comprises about 45 mol% to 65 mol% of the compound of the present disclosure, about 2 mol% to about 15 mol% of a helper lipid, about 20 mol% to about 42 mol% of cholesterol, and about 0.5 mol% to about 3 mol% of a PEG-lipid conjugate. In some embodiments, the lipid nanoparticle comprises about 50 mol% to about 61

mol% of the compound of the present disclosure, about 5 mol% to about 9 mol% of the helper lipid, about 29 mol% to about 38 mol% of cholesterol, and about 1 mol% to about 2 mol% of the PEG-lipid conjugate. In some embodiments, the lipid nanoparticle comprises about 56 mol% to about 58 mol% of the compound of the present disclosure, about 6 mol% to about 8 mol% of DSPC, about 31 mol% to about 34 mol% of cholesterol, and about 1.25 mol% to about 1.75 mol% of the PEG-lipid conjugate.

[0172] In some embodiments, the lipid nanoparticle comprises about 50 mol% to 61 mol% of the compound of the present disclosure, about 2 mol% to about 12 mol% of DSPC, about 25 mol% to about 42 mol% of cholesterol, and about 0.5 mol% to about 3 mol% of PEG2000-DMG. In some embodiments, the lipid nanoparticle comprises about 50 mol% to about 61 mol% of the compound of the present disclosure, about 5 mol% to about 9 mol% of DSPC, about 29 mol% to about 38 mol% of cholesterol, and about 1 mol% to about 2 mol% of PEG2000-DMG. In some embodiments, the lipid nanoparticle comprises about 56 mol% to about 58 mol% of the compound of the present disclosure, about 6 mol% to about 8 mol% of DSPC, about 31 mol% to about 34 mol% of cholesterol, and about 1.25 mol% to about 1.75 mol% of PEG2000-DMG.

[0173] In some embodiments, the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 50:1 to about 10:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 40:1 to about 20:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 35:1 to about 25:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 32:1 to about 28:1. In some embodiments, the lipid nanoparticle has a total lipid: nucleic acid weight ratio of about 31:1 to about 29:1.

[0174] In some embodiments, the lipid nanoparticle has a total lipid:mRNA weight ratio of about 50:1 to about 10:1. In some embodiments, the lipid nanoparticle has a total lipid:mRNA weight ratio of about 40:1 to about 20:1. In some embodiments, the lipid nanoparticle has a total lipid:mRNA weight ratio of about 35:1 to about 25:1. In some embodiments, the lipid nanoparticle has a total lipid:mRNA weight ratio of about 32:1 to about 28:1. In some embodiments, the lipid nanoparticle has a total lipid:mRNA weight ratio of about 31:1 to about 29:1.

[0175] In some embodiments, the lipid nanoparticle comprises a HEPES buffer at a pH of about 7.4. In some embodiments, the HEPES buffer is at a concentration of about 7

mg/mL to about 15 mg/mL. In some embodiments, the lipid nanoparticle further comprises about 2.0 mg/mL to about 4.0 mg/mL of NaCl.

[0176] In some embodiments, the lipid nanoparticle further comprises one or more cryoprotectants. In some embodiments, the one or more cryoprotectants are selected from sucrose, glycerol, or a combination of sucrose and glycerol. In some embodiments, the lipid nanoparticle comprises a combination of sucrose at a concentration of about 70 mg/mL to about 110 mg/mL and glycerol at a concentration of about 50 mg/mL to about 70 mg/mL.

Lipid-Nucleic Acid Formulations

[0177] A nucleic acid or a pharmaceutically acceptable salt thereof can be incorporated into a lipid formulation (i.e., a lipid-based delivery vehicle).

[0178] In the context of the present disclosure, a lipid-based delivery vehicle typically serves to transport a desired nucleic acid (siRNA, plasmid DNA, mRNA, self-replicating RNA, etc.) to a target cell or tissue. The lipid-based delivery vehicle can be any suitable lipid-based delivery vehicle known in the art. In some embodiments, the lipid-based delivery vehicle is a liposome, a cationic liposome, or a lipid nanoparticle containing a nucleic acid. In some embodiments, the lipid-based delivery vehicle comprises a nanoparticle or a bilayer of lipid molecules and a nucleic acid. In some embodiments, the lipid bilayer preferably further comprises a neutral lipid or a polymer. In some embodiments, the lipid formulation preferably comprises a liquid medium. In some embodiments, the formulation preferably further encapsulates a nucleic acid. In some embodiments, the lipid formulation preferably further comprises a nucleic acid and a neutral lipid or a polymer. In some embodiments, the lipid formulation preferably encapsulates the nucleic acid.

[0179] The description provides lipid formulations comprising one or more therapeutic nucleic acid molecules encapsulated within the lipid formulation. In some embodiments, the lipid formulation comprises liposomes. In some embodiments, the lipid formulation comprises cationic liposomes. In some embodiments, the lipid formulation comprises lipid nanoparticles.

[0180] In some embodiments, the nucleic acid is fully encapsulated within the lipid portion of the lipid formulation such that the nucleic acid in the lipid formulation is resistant in aqueous solution to nuclease degradation. In other embodiments, the lipid formulations described herein are substantially non-toxic to mammals such as humans.

[0181] The lipid formulations of the disclosure also typically have a total lipid: nucleic acid ratio (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 45:1, from about 3:1 to about 40:1, from about 5:1 to about 38:1, or from about 6:1 to about 40:1, or from about 7:1 to about 35:1, or from about 8:1 to about 30:1; or from about 10:1 to about 25:1; or from about 8:1 to about 12:1; or from about 13:1 to about 17:1; or from about 18:1 to about 24:1; or from about 20:1 to about 30:1. In some preferred embodiments, the total lipid: nucleic acid ratio (mass/mass ratio) is from about 10:1 to about 25:1. The ratio may be any value or subvalue within the recited ranges, including endpoints.

[0182] The lipid formulations of the present disclosure typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, about 35 nm, about 40 nm, about 45 nm, about 50 nm, about 55 nm, about 60 nm, about 65 nm, about 70 nm, about 75 nm, about 80 nm, about 85 nm, about 90 nm, about 95 nm, about 100 nm, about 105 nm, about 110 nm, about 115 nm, about 120 nm, about 125 nm, about 130 nm, about 135 nm, about 140 nm, about 145 nm, or about 150 nm, and are substantially non-toxic. The diameter may be any value or subvalue within the recited ranges, including endpoints. In addition, nucleic acids, when present in the lipid nanoparticles of the present disclosure, are resistant in aqueous solution to degradation with a nuclease.

[0183] In preferred embodiments, the lipid formulations comprise a nucleic acid, a cationic lipid (e.g., one or more cationic lipids or salts thereof described herein), a phospholipid, and a conjugated lipid that inhibits aggregation of the particles (e.g., one or more PEG-lipid conjugate and/or other lipid conjugate of the disclosure). The lipid formulations can also include cholesterol.

[0184] In some embodiments, the lipid nanoparticle further comprises a PEG-lipid conjugate. In some embodiments, the PEG-lipid conjugate is PEG-DMG. In some embodiments, the PEG-DMG is PEG2000-DMG. In embodiments, PEG2000-DMG is the addition of polyethylene glycol to myristoyl diglyceride.

[0185] In the nucleic acid-lipid formulations, the nucleic acid may be fully encapsulated within the lipid portion of the formulation, thereby protecting the nucleic acid from nuclease

degradation. In preferred embodiments, a lipid formulation comprising a nucleic acid is fully encapsulated within the lipid portion of the lipid formulation, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the lipid formulation is not substantially degraded after exposure of the particle to a nuclease at 37 °C for at least 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the lipid formulation is not substantially degraded after incubation of the formulation in serum at 37 °C for at least 30, 45, or 60 minutes or at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the nucleic acid is complexed with the lipid portion of the formulation.

[0186] In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Encapsulation is determined by adding the dye to a lipid formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the lipid layer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be calculated as $E = (I_0 - I)/I_0$, where I and I_0 refer to the fluorescence intensities before and after the addition of detergent.

[0187] In other embodiments, the present disclosure provides a nucleic acid-lipid composition comprising a plurality of nucleic acid-liposomes, nucleic acid-cationic liposomes, or nucleic acid-lipid nanoparticles. In some embodiments, the nucleic acid-lipid composition comprises a plurality of nucleic acid-liposomes. In some embodiments, the nucleic acid-lipid composition comprises a plurality of nucleic acid-cationic liposomes. In some embodiments, the nucleic acid-lipid composition comprises a plurality of nucleic acid-lipid nanoparticles.

[0188] In some embodiments, the lipid formulations comprise a nucleic acid that is fully encapsulated within the lipid portion of the formulation, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about

90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% (or any fraction thereof or range therein) of the particles have the nucleic acid encapsulated therein. The amount may be any value or subvalue within the recited ranges, including endpoints.

[0189] In some embodiments, the polydispersity index (PDI) ranges from 0.010 to 1.10. In embodiments, the PDI ranges from 0.010 to 1.05, from 0.010 to 1.00, from 0.010 to 0.95, from 0.010 to 0.90, from 0.010 to 0.85, from 0.010 to 0.80, from 0.010 to 0.75, from 0.010 to 0.70, from 0.010 to 0.65, from 0.010 to 0.60, from 0.010 to 0.55, from 0.010 to 0.50, from 0.010 to 0.45, from 0.010 to 0.40, from 0.010 to 0.35, from 0.010 to 0.30, from 0.010 to 0.25, from 0.010 to 0.20, from 0.010 to 0.15, from 0.010 to 0.10, from 0.010 to 0.09, from 0.010 to 0.08, from 0.010 to 0.07, from 0.010 to 0.06, from 0.010 to 0.05, from 0.010 to 0.04, from 0.010 to 0.03, from 0.010 to 0.02, from 0.010 to 0.019, from 0.010 to 0.018, from 0.010 to 0.017, from 0.010 to 0.016, from 0.010 to 0.015, from 0.010 to 0.014, from 0.010 to 0.013, from 0.010 to 0.012, from 0.010 to 0.011 (or any ranges therein.) The amount may be any value or subvalue within the recited ranges, including endpoints.

[0190] Depending on the intended use of the lipid formulation, the proportions of the components can be varied, and the delivery efficiency of a particular formulation can be measured using assays known in the art.

[0191] According to some embodiments, expressible polynucleotides, nucleic acid active agents, and mRNA constructs can be lipid formulated. The lipid formulation is preferably selected from, but not limited to, liposomes, cationic liposomes, and lipid nanoparticles. In one preferred embodiment, a lipid formulation is a cationic liposome or a lipid nanoparticle (LNP) comprising:

- (a) a nucleic acid (mRNA, siRNA, etc.),
- (b) a lipid of the present disclosure, which may be cationic
- (c) optionally a non-cationic lipid (such as a neutral lipid), and
- (d) optionally, a sterol.

Cationic Lipids

[0192] The lipid formulation preferably includes a cationic lipid suitable for forming a cationic liposome or lipid nanoparticle. Cationic lipids are widely studied for nucleic acid delivery because they can bind to negatively charged membranes and induce uptake. Generally, cationic lipids are amphiphiles containing a positive hydrophilic head group, two (or more) lipophilic tails, or a steroid portion and a connector between these two domains. Preferably, the cationic lipid carries a net positive charge at about physiological pH. Cationic liposomes have been traditionally the most commonly used non-viral delivery systems for oligonucleotides, including plasmid DNA, antisense oligos, and siRNA/small hairpin RNA-shRNA. Cationic lipids, such as DOTAP, (1,2-dioleoyl-3-trimethylammonium-propane) and DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate) can form complexes or lipoplexes with negatively charged nucleic acids by electrostatic interaction, providing high in vitro transfection efficiency.

[0193] In the presently disclosed lipid formulations, the cationic lipid may include, for example, N,N-dimethyl-N,N-di-9-cis-octadecenylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), 1,2-dioleoyltrimethylammoniumpropane chloride (DOTAP) (also known as N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride and 1,2-Dioleoyloxy-3-trimethylaminopropane chloride salt), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA), 1,2-Dilinoleylcarbamoxyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleoxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoleoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleoyloxy-3-dimethylaminopropane (DLin-2-DMA), 1,2-Dilinoleoyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleoxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanediol (DOAP), 1,2-Dilinoleoxyloxy-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-

octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine, (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediy)l)didodecan-2-ol (C12-200), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (MC3 Ether), 4-((6Z,9Z,28Z,31 Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylbutan-1-amine (MC4 Ether), or any combination thereof. Other cationic lipids include, but are not limited to, N,N-distearyl-N,N-dimethylammonium bromide (DDAB), 3P-(N-(N',N'-dimethylaminoethane)- carbamoyl)cholesterol (DC-Chol), N-(1-(2,3-dioleoyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate (DOSPA), dioctadecylamidoglycyl carboxyspermine (DOGS), 1,2-dioleoyl-sn-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-dimethylammonium propane (DODAP), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), and 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC). Additionally, commercial preparations of cationic lipids can be used, such as, e.g., LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and Lipofectamine (comprising DOSPA and DOPE, available from GIBCO/BRL).

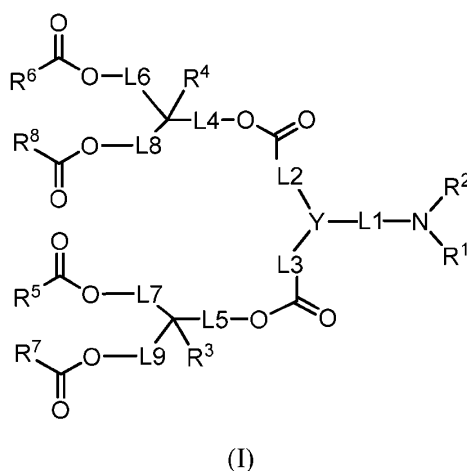
[0194] Other suitable cationic lipids are disclosed in International Publication Nos. WO 09/086558, WO 09/127060, WO 10/048536, WO 10/054406, WO 10/088537, WO 10/129709, and WO 2011/153493; U.S. Patent Publication Nos. 2011/0256175, 2012/0128760, and 2012/0027803; U.S. Patent No. 8,158,601; and Love et al., PNAS, 107(5), 1864-69, 2010, the contents of which are herein incorporated by reference.

[0195] Other suitable cationic lipids include those having alternative fatty acid groups and other dialkylamino groups, including those, in which the alkyl substituents are different (e.g., N-ethyl- N-methylamino-, and N-propyl-N-ethylamino-). These lipids are part of a subcategory of cationic lipids referred to as amino lipids. In some embodiments of the lipid formulations described herein, the cationic lipid is an amino lipid. In general, amino lipids having less saturated alkyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing unsaturated fatty acids with carbon chain lengths in the range of C₁₄ to C₂₂ may be used. Other scaffolds can

also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid.

[0196] In some embodiments, cationic lipids of the present disclosure are ionizable and have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g., pH 7.4), and neutral at a second pH, preferably at or above physiological pH. Of course, it will be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. Lipids that have more than one protonatable or deprotonatable group, or which are zwitterionic, are not excluded from use in the disclosure. In certain embodiments, the protonatable lipids have a pKa of the protonatable group in the range of about 4 to about 11. In some embodiments, the ionizable cationic lipid has a pKa of about 5 to about 7. In some embodiments, the pKa of an ionizable cationic lipid is about 6 to about 7.

[0197] In some embodiments, the lipid formulation comprises a lipid of Formula I:



wherein:

R^1 and R^2 are each independently H or C_{1-6} alkyl; or

R^1 and R^2 are joined to form a saturated heterocyclic ring, wherein:

R^1 is a linear C_{1-4} alkylene; and

R^2 is $-(CH_2)_m(X)_n-$, wherein

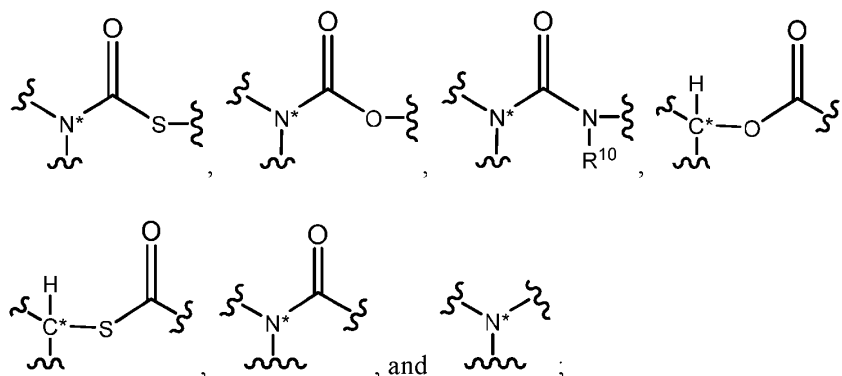
X is O, S, or NR^9 , wherein R^9 is H or C_{1-6} alkyl;

m is 1, 2, 3 or 4, and

n is 0 or 1;

L1 is a linear C₁₋₆ alkylene optionally substituted with one to three methyl groups;

Y is selected from the group consisting of:



wherein:

each asterisk (*) indicates the atom attached to L2 and L3; and

R¹⁰ is H or C₁₋₆ alkyl;

L2 and L3 are each independently a linear C₁₋₈ alkylene;

L4, L5, L6, L7, L8 and L9 are each independently absent or -CH₂-, provided that:

at least two of L4, L6 and L8 are -CH₂-; and

at least two of L5, L7 and L9 are -CH₂-;

R³ and R⁴ are each independently H, methyl or ethyl; and

R⁵, R⁶, R⁷ and R⁸ are each independently selected from the group consisting of:

linear C₁₋₂₀ alkyl, wherein each said linear C₁₋₂₀ alkyl is optionally substituted

with one or more substituents selected from the group consisting of:

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic

aromatic hydrocarbon optionally substituted with one or

more C₆₋₁₀ aryl that is optionally substituted with one or more

C₁₋₆ alkyl;

6-10 membered heteroaryl, wherein each said 6-10 membered

heteroaryl is a monocyclic or bicyclic aromatic system

optionally substituted with one or more C₁₋₆ alkyl;

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl; and
 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

[0198] In some embodiments, any one or more lipids recited herein may be expressly excluded.

Helper Lipids and Sterols

[0199] The mRNA-lipid formulations of the present disclosure can comprise a helper lipid, which can be referred to as a neutral lipid, a neutral helper lipid, non-cationic lipid, non-cationic helper lipid, anionic lipid, anionic helper lipid, or a zwitterionic lipid. It has been found that lipid formulations, particularly cationic liposomes and lipid nanoparticles have increased cellular uptake if helper lipids are present in the formulation. (Curr. Drug Metab. 2014; 15(9):882-92). For example, some studies have indicated that neutral and zwitterionic lipids such as 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), Di-Oleoyl-Phosphatidyl-Ethanolamine (DOPE) and 1,2-DiStearoyl-sn-glycero-3-PhosphoCholine (DSPC), being more fusogenic (i.e., facilitating fusion) than cationic lipids, can affect the polymorphic features of lipid-nucleic acid complexes, promoting the transition from a lamellar to a hexagonal phase, and thus inducing fusion and a disruption of the cellular membrane. (Nanomedicine (Lond). 2014 Jan; 9(1):105-20). In addition, the use of helper lipids can help to reduce any potential detrimental effects from using many prevalent cationic lipids such as toxicity and immunogenicity.

[0200] Non-limiting examples of non-cationic lipids suitable for lipid formulations of the present disclosure include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebroside, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyl-oleoyl-phosphatidylcholine (POPC),

palmitoyl-oleoyl-phosphatidylethanolamine (POPE), palmitoyl-oleoyl-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearyl-oleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C10-C24 carbon chains, e.g., lauroyl, myristoyl, palmitoyl, stearyl, or oleoyl.

[0201] In some embodiments, the helper lipid is selected from: dioleoylphosphatidylethanolamine (DOPE), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC), and phosphatidylcholine (PC). In some embodiments, the helper lipid is distearoylphosphatidylcholine (DSPC).

[0202] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof. One study concluded that as a helper lipid, cholesterol increases the spacing of the charges of the lipid layer interfacing with the nucleic acid making the charge distribution match that of the nucleic acid more closely. (J. R. Soc. Interface. 2012 Mar 7; 9(68): 548–561). Non-limiting examples of cholesterol derivatives include polar analogues such as 5 α -cholestanol, 5 α -coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5 α -cholestane, cholestenone, 5 α -cholestanone, 5 α -cholestanone, and cholesteryl decanoate; and mixtures thereof. In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether.

[0203] In some embodiments, the helper lipid present in the lipid formulation comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof. In other embodiments, the helper lipid present in the lipid formulation comprises or consists of one or more phospholipids, e.g., a cholesterol-free lipid formulation. In yet other embodiments, the helper lipid present in the lipid formulation comprises or consists of cholesterol or a derivative thereof, e.g., a phospholipid-free lipid formulation. In some embodiments, the lipid nanoparticle further comprises cholesterol.

[0204] Other examples of helper lipids include nonphosphorous containing lipids such as, e.g., stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyoxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, and sphingomyelin.

[0205] In some embodiments, the helper lipid comprises from about 1 mol% to about 50 mol%, from about 5 mol% to about 48 mol%, from about 5 mol% to about 46 mol%, about 25 mol% to about 44 mol%, from about 26 mol% to about 42 mol%, from about 27 mol% to about 41 mol%, from about 28 mol% to about 40 mol%, or about 29 mol%, about 30 mol%, about 31 mol%, about 32 mol%, about 33 mol%, about 34 mol%, about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, or about 39 mol% (or any fraction thereof or the range therein) of the total lipid present in the lipid formulation. In some embodiments, the helper lipid comprises from about 1 mol% to about 20 mol%, about 2 mol% to about 12mol%, about 5 mol% to about 9 mol% or about 6 mol% to about 8 mol%.

[0206] In some embodiments, the total of helper lipid in the formulation comprises two or more helper lipids and the total amount of helper lipid comprises from about 20 mol% to about 50 mol%, from about 22 mol% to about 48 mol%, from about 24 mol% to about 46 mol%, about 25 mol% to about 44 mol%, from about 26 mol% to about 42 mol%, from about 27 mol% to about 41 mol%, from about 28 mol% to about 40 mol%, or about 29 mol%, about 30 mol%, about 31 mol%, about 32 mol%, about 33 mol%, about 34 mol%, about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, or about 39 mol% (or any fraction thereof or the range therein) of the total lipid present in the lipid formulation. In some embodiments, the helper lipids are a combination of DSPC and DOTAP. In some embodiments, the helper lipids are a combination of DSPC and DOTMA.

[0207] The cholesterol or cholesterol derivative in the lipid formulation may comprise up to about 40 mol%, about 45 mol%, about 50 mol%, about 55 mol%, or about 60 mol% of the total lipid present in the lipid formulation. In some embodiments, the cholesterol or cholesterol derivative comprises about 15 mol% to about 45 mol%, about 20 mol% to about 40 mol%, about 30 mol% to about 40 mol%, or about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, about 39 mol%, or about 40 mol% of the total lipid present in the lipid formulation.

[0208] The percentage of helper lipid present in the lipid formulation is a target amount, and the actual amount of helper lipid present in the formulation may vary, for example, by ± 5 mol%.

Mechanism of Action for Cellular Uptake of Lipid Formulations

[0209] Lipid formulations for the intracellular delivery of nucleic acids, particularly liposomes, cationic liposomes, and lipid nanoparticles, are designed for cellular uptake by penetrating target cells through exploitation of the target cells' endocytic mechanisms where the contents of the lipid delivery vehicle are delivered to the cytosol of the target cell. (Nucleic Acid Therapeutics, 28(3):146-157, 2018). Specifically, in the case of a nucleic acid-lipid formulations described herein, the lipid formulation enters cells through receptor mediated endocytosis. Prior to endocytosis, functionalized ligands such as a the lipid conjugate of the disclosure at the surface of the lipid delivery vehicle can be shed from the surface, which triggers internalization into the target cell. During endocytosis, some part of the plasma membrane of the cell surrounds the vector and engulfs it into a vesicle that then pinches off from the cell membrane, enters the cytosol and ultimately undergoes the endolysosomal pathway. For ionizable cationic lipid-containing delivery vehicles, the increased acidity as the endosome ages results in a vehicle with a strong positive charge on the surface. Interactions between the delivery vehicle and the endosomal membrane then result in a membrane fusion event that leads to cytosolic delivery of the payload. For mRNA or self-replicating RNA payloads, the cell's own internal translation processes will then translate the RNA into the encoded protein. The encoded protein can further undergo post-translational processing, including transportation to a targeted organelle or location within the cell.

[0210] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the lipid formulation and, in turn, the rate at which the lipid formulation becomes fusogenic. In addition, other variables including, e.g., pH, temperature, or ionic strength, can be used to vary and/or control the rate at which the lipid formulation becomes fusogenic. Other methods which can be used to control the rate at which the lipid formulation becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the liposomal or lipid particle size.

Lipid Formulation Manufacture

[0211] There are many different methods for the preparation of lipid formulations comprising a nucleic acid. (Curr. Drug Metabol. 2014, 15, 882–892; Chem. Phys. Lipids 2014, 177, 8–18; Int. J. Pharm. Stud. Res. 2012, 3, 14–20). The techniques of thin film hydration, double emulsion, reverse phase evaporation, microfluidic preparation, dual asymmetric centrifugation, ethanol injection, detergent dialysis, spontaneous vesicle formation by ethanol dilution, and encapsulation in preformed liposomes are briefly described herein.

Thin Film Hydration

[0212] In Thin Film Hydration (TFH) or the Bangham method, the lipids are dissolved in an organic solvent, then evaporated through the use of a rotary evaporator leading to a thin lipid layer formation. After the layer hydration by an aqueous buffer solution containing the compound to be loaded, Multilamellar Vesicles (MLVs) are formed, which can be reduced in size to produce Small or Large Unilamellar vesicles (LUV and SUV) by extrusion through membranes or by the sonication of the starting MLV.

Double Emulsion

[0213] Lipid formulations can also be prepared through the Double Emulsion technique, which involves lipids dissolution in a water/organic solvent mixture. The organic solution, containing water droplets, is mixed with an excess of aqueous medium, leading to a water-in-oil-in-water (W/O/W) double emulsion formation. After mechanical vigorous shaking, part of the water droplets collapse, giving Large Unilamellar Vesicles (LUVs).

Reverse Phase Evaporation

[0214] The Reverse Phase Evaporation (REV) method also allows one to achieve LUVs loaded with nucleic acid. In this technique a two-phase system is formed by phospholipids dissolution in organic solvents and aqueous buffer. The resulting suspension is then sonicated briefly until the mixture becomes a clear one-phase dispersion. The lipid formulation is achieved after the organic solvent evaporation under reduced pressure. This technique has been used to encapsulate different large and small hydrophilic molecules including nucleic acids.

Microfluidic Preparation

[0215] The Microfluidic method, unlike other bulk techniques, gives the possibility of controlling the lipid hydration process. The method can be classified in continuous-flow microfluidic and droplet-based microfluidic, according to the way in which the flow is manipulated. In the microfluidic hydrodynamic focusing (MHF) method, which operates in a continuous flow mode, lipids are dissolved in isopropyl alcohol which is hydrodynamically focused in a microchannel cross junction between two aqueous buffer streams. Vesicles size can be controlled by modulating the flow rates, thus controlling the lipids solution/buffer dilution process. The method can be used for producing oligonucleotide (ON) lipid formulations by using a microfluidic device consisting of three-inlet and one-outlet ports.

Dual Asymmetric Centrifugation

[0216] Dual Asymmetric Centrifugation (DAC) differs from more common centrifugation as it uses an additional rotation around its own vertical axis. An efficient homogenization is achieved due to the two overlaying movements generated: the sample is pushed outwards, as in a normal centrifuge, and then it is pushed towards the center of the vial due to the additional rotation. By mixing lipids and an NaCl-solution a viscous vesicular phospholipid gel (VPC) is achieved, which is then diluted to obtain a lipid formulation dispersion. The lipid formulation size can be regulated by optimizing DAC speed, lipid concentration and homogenization time.

Ethanol Injection

[0217] The Ethanol Injection (EI) method can be used for nucleic acid encapsulation. This method provides the rapid injection of an ethanolic solution, in which lipids are dissolved, into an aqueous medium containing nucleic acids to be encapsulated, through the use of a needle. Vesicles are spontaneously formed when the phospholipids are dispersed throughout the medium.

Detergent Dialysis

[0218] The Detergent dialysis method can be used to encapsulate nucleic acids. Briefly lipid and plasmid are solubilized in a detergent solution of appropriate ionic strength, after removing

the detergent by dialysis, a stabilized lipid formulation is formed. Unencapsulated nucleic acid is then removed by ion-exchange chromatography and empty vesicles by sucrose density gradient centrifugation. The technique is highly sensitive to the cationic lipid content and to the salt concentration of the dialysis buffer, and the method is also difficult to scale.

Spontaneous Vesicle Formation by Ethanol Dilution

[0219] Stable lipid formulations can also be produced through the Spontaneous Vesicle Formation by Ethanol Dilution method in which a stepwise or dropwise ethanol dilution provides the instantaneous formation of vesicles loaded with nucleic acid by the controlled addition of lipid dissolved in ethanol to a rapidly mixing aqueous buffer containing the nucleic acid.

V. PHARMACEUTICAL COMPOSITIONS AND DELIVERY METHODS

[0220] To facilitate nucleic acid activity (e.g., mRNA expression, or knockdown by an ASO or siRNA) in vivo, the lipid formulation delivery vehicles described herein can be combined with one or more additional nucleic acids, carriers, targeting ligands or stabilizing reagents, or in pharmacological compositions where it is mixed with suitable excipients. Techniques for formulation and administration of drugs may be found in “Remington's Pharmaceutical Sciences,” Mack Publishing Co., Easton, Pa., latest edition.

[0221] The lipid formulations and pharmaceutical compositions of the present disclosure may be administered and dosed in accordance with current medical practice, taking into account the clinical condition of the subject, the site and method of administration, the scheduling of administration, the subject's age, sex, body weight and other factors relevant to clinicians of ordinary skill in the art. The “effective amount” for the purposes herein may be determined by such relevant considerations as are known to those of ordinary skill in experimental clinical research, pharmacological, clinical and medical arts. In some embodiments, the amount administered is effective to achieve at least some stabilization, improvement or elimination of symptoms and other indicators as are selected as appropriate measures of disease progress, regression or improvement by those of skill in the art. For example, a suitable amount and dosing regimen is one that causes at least transient protein (e.g., enzyme) production.

[0222] The pharmaceutical compositions disclosed herein can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit a sustained or delayed release (e.g., from a depot formulation of the nucleic acid); (4) alter the biodistribution (e.g., target the nucleic acid to specific tissues or cell types); (5) increase the activity of the nucleic acid or a protein expressed therefrom *in vivo*; and/or (6) alter the release profile of the nucleic acid or an encoded protein *in vivo*.

[0223] Preferably, the lipid formulations may be administered in a local rather than systemic manner. Local delivery can be affected in various ways, depending on the tissue to be targeted. For example, aerosols containing compositions of the present disclosure can be inhaled (for nasal, tracheal, or bronchial delivery).

[0224] Pharmaceutical compositions may be administered to any desired tissue. In some embodiments, the nucleic acid delivered by a lipid formulation or composition of the present disclosure is active in the tissue in which the lipid formulation and/or composition was administered. In some embodiments, the nucleic acid is active in a tissue different from the tissue in which the lipid formulation and/or composition was administered. Example tissues in which the nucleic acid may be delivered include, but are not limited to the lung, trachea, and/or nasal passages, muscle, liver, eye, or the central nervous system.

[0225] The pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient (i.e., nucleic acid) with an excipient and/or one or more other accessory ingredients. 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.

[0226] Pharmaceutical compositions may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired.

[0227] In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients of the present disclosure can

include, without limitation, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with a primary DNA construct, or mRNA (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof.

[0228] Accordingly, the formulations described herein can include one or more excipients, each in an amount that together increases the stability of the nucleic acid in the lipid formulation, increases cell transfection by the nucleic acid (e.g., mRNA or siRNA), increases the expression of an encoded protein, and/or alters the release profile of the encoded protein, or increases knockdown of a target native nucleic acid. Further, a nucleic acid may be formulated using self-assembled nucleic acid nanoparticles.

[0229] Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, Md., 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the embodiments of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[0230] A dosage form of the composition of this disclosure can be solid, which can be reconstituted in a liquid prior to administration. The solid can be administered as a powder. In some embodiments, the pharmaceutical composition comprises a nucleic acid lipid formulation that has been lyophilized.

[0231] In a preferred embodiment, the dosage form of the pharmaceutical compositions described herein can be a liquid suspension of nucleic acid-lipid nanoparticles described herein. In some embodiments, the liquid suspension is in a buffered solution. In some embodiments, the buffered solution comprises a buffer selected from the group consisting of HEPES, MOPS, TES, and TRIS. In some embodiments, the buffer has a pH of about 7.4. In some preferred embodiments, the buffer is HEPES. In some further embodiments, the buffered solution further comprises a cryoprotectant. In some embodiments, the cryoprotectant is selected from a sugar and glycerol or a combination of a sugar and glycerol. In some embodiments, the sugar is a dimeric sugar. In some embodiments, the sugar is sucrose. In some preferred embodiments, the

buffer comprises HEPES, sucrose, and glycerol at a pH of 7.4. In some embodiments, the suspension is frozen during storage and thawed prior to administration. In some embodiments, the suspension is frozen at a temperature below about -70 °C. In some embodiments, the suspension is diluted with sterile water prior to inhalable administration. In some embodiments, an inhalable administration comprises diluting the suspension with about 1 volume to about 4 volumes of sterile water. In some embodiments, a lyophilized nucleic acid-lipid nanoparticle formulation can be resuspended in a buffer as described herein.

[0232] A dosage form of the composition of this disclosure can be solid, which can be reconstituted in a liquid prior to administration. The solid can be administered as a powder. The solid can be in the form of a capsule, tablet, or gel.

[0233] To formulate compositions for pulmonary delivery within the present disclosure, the nucleic acid-lipid formulation can be combined with various pharmaceutically acceptable additives, as well as a base or carrier for dispersion of the nucleic acid-lipid formulation(s). Examples of additives include pH control agents such as arginine, sodium hydroxide, glycine, hydrochloric acid, citric acid, and mixtures thereof. Other additives include local anesthetics (e.g., benzyl alcohol), isotonicizing agents (e.g., sodium chloride, mannitol, sorbitol), adsorption inhibitors (e.g., Tween 80), solubility enhancing agents (e.g., cyclodextrins and derivatives thereof), stabilizers (e.g., serum albumin), and reducing agents (e.g., glutathione). When the composition for mucosal delivery is a liquid, the tonicity of the formulation, as measured with reference to the tonicity of 0.9% (w/v) physiological saline solution taken as unity, is typically adjusted to a value at which no substantial, irreversible tissue damage will be induced in the mucosa at the site of administration. Generally, the tonicity of the solution is adjusted to a value of 1/3 to 3, more typically 1/2 to 2, and most often 3/4 to 1.7.

[0234] The nucleic acid-lipid formulation may be dispersed in a base or vehicle, which may comprise a hydrophilic compound having a capacity to disperse the nucleic acid-lipid formulation and any desired additives. The base may be selected from a wide range of suitable carriers, including but not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (e.g., maleic anhydride) with other monomers (e.g., methyl(meth)acrylate, acrylic acid, etc.), hydrophilic vinyl polymers such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives such as hydroxymethylcellulose, hydroxypropylcellulose, etc., and natural polymers such as chitosan, collagen, sodium alginate,

gelatin, hyaluronic acid, and nontoxic metal salts thereof. Often, a biodegradable polymer is selected as a base or carrier, for example, polylactic acid, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer, and mixtures thereof. Alternatively or additionally, synthetic fatty acid esters such as polyglycerin fatty acid esters, sucrose fatty acid esters, etc., can be employed as carriers. Hydrophilic polymers and other carriers can be used alone or in combination and enhanced structural integrity can be imparted to the carrier by partial crystallization, ionic bonding, crosslinking, and the like. The carrier can be provided in a variety of forms, including fluid or viscous solutions, gels, pastes, powders, microspheres, and films for direct application to the nasal mucosa. The use of a selected carrier in this context may result in promotion of absorption of the nucleic acid-lipid formulation.

[0235] The compositions of this disclosure may alternatively contain as pharmaceutically acceptable carriers substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, and wetting agents, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and mixtures thereof. For solid compositions, conventional nontoxic pharmaceutically acceptable carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

[0236] According to the present disclosure, a therapeutically effective dose of the provided composition, when administered regularly, results in an increased nucleic acid activity level in a subject as compared to a baseline activity level before treatment. Typically, the activity level is measured in a biological sample obtained from the subject such as blood, plasma or serum, urine, or solid tissue extracts. The baseline level can be measured immediately before treatment. In some embodiments, administering a pharmaceutical composition described herein results in an increased nucleic acid activity level in a biological sample (e.g., plasma/serum or lung epithelial swab) by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% as compared to a baseline level before treatment. In some embodiments, administering the provided composition results in an increased nucleic acid activity level in a biological sample (e.g., plasma/serum or lung epithelial swab) by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% as compared to a baseline level before treatment for at least about 24 hours,

at least about 48 hours, at least about 72 hours, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11 days, at least about 12 days, at least about 13 days, at least about 14 days, or at least about 15 days.

[0237] In some embodiments, the present disclosure provides a pharmaceutical composition comprising the compounds described herein, or the lipid nanoparticle described herein, and a pharmaceutically acceptable excipient.

[0238] In some embodiments, the present disclosure provides a method of delivering a nucleic acid to a subject in need thereof, comprising encapsulating a therapeutically effective amount of the a nucleic acid in the lipid nanoparticle described herein, and administering the lipid nanoparticle to the subject.

[0239] In some embodiments, the present disclosure provides a method of delivering mRNA to a subject in need thereof, comprising encapsulating a therapeutically effective amount of the mRNA in the lipid nanoparticle described herein, and administering the lipid nanoparticle to the subject.

VI. METHOD OF TREATMENT

[0240] In some embodiments, the present disclosure provides a method of treating a disease in a subject in need thereof, comprising administering a therapeutically effective amount to the subject of the compound described herein, the lipid nanoparticle described herein, or the pharmaceutical composition described herein. In some embodiments, the compound, composition, or lipid nanoparticle is administered intravenously or intramuscularly. In some embodiments, the compound, composition, or lipid nanoparticle is administered intravenously. In some embodiments, the compound, composition, or lipid nanoparticle is administered intramuscularly.

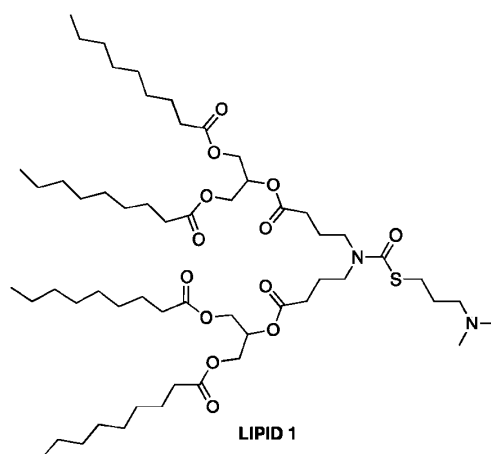
[0241] In some embodiments, a method of treating a disease in a subject in need thereof is provided comprising administering to the subject a lipid composition described herein. In some embodiments, the lipid composition is administered intravenously or intramuscularly. In some embodiments, the lipid composition is administered intravenously. In some embodiments, the lipid composition is administered intramuscularly.

[0242] In some embodiments, there are provided a methods of treating a disease or disorder in a mammalian subject. A therapeutically effective amount of a composition comprising a lipid, as disclosed herein, specifically a cationic lipid, a nucleic, an amphiphile, a phospholipid, cholesterol, and a PEG-linked cholesterol may be administered to a subject having a disease or disorder associated with expression or overexpression of a gene that can be reduced, decreased, downregulated, or silenced by the composition. The compositions described herein can be used in a methods for treating cancer or inflammatory disease. The disease may be one selected from the group consisting of central nervous system disorders, peripheral nervous system disorders, muscle atrophies, muscle dystrophies, immune disorder, cancer, renal disease, fibrotic disease, genetic abnormality, inflammation, and cardiovascular disorder.

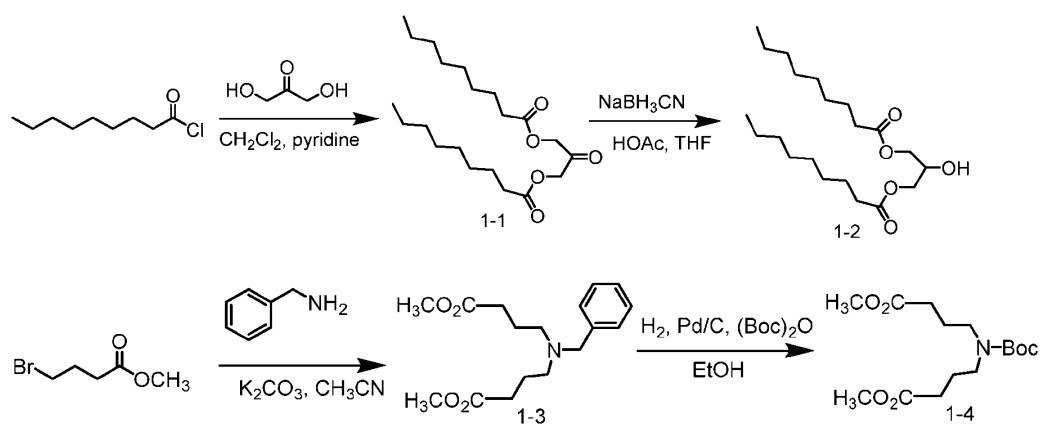
[0243] In some embodiments, the present disclosure provides a method of delivering a nucleic acid to a subject in needed thereof, comprising encapsulating a therapeutically effective amount of a nucleic acid in a lipid nanoparticle as described herein, and administering the lipid nanoparticle to the subject.

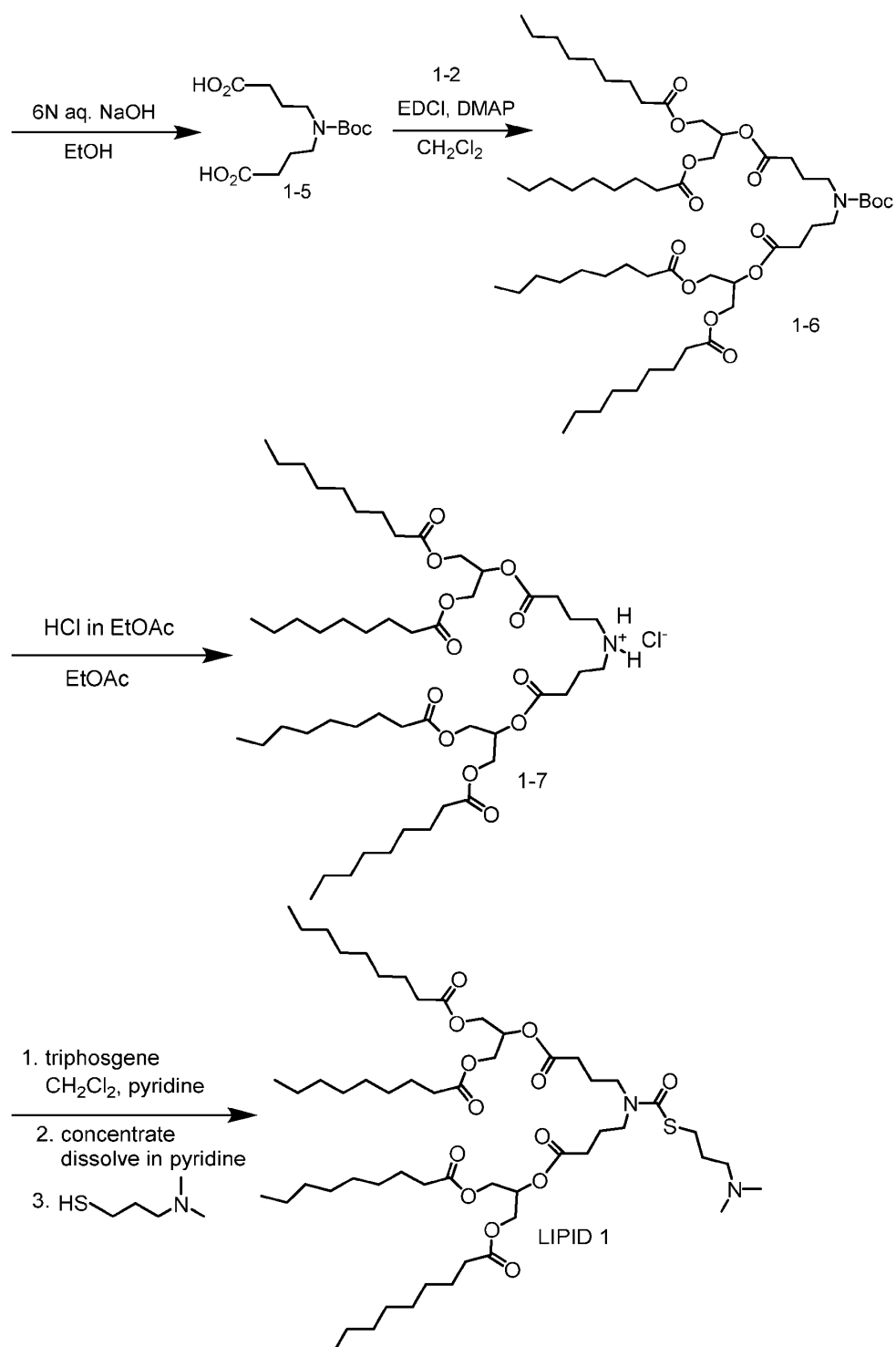
VII. EXAMPLES

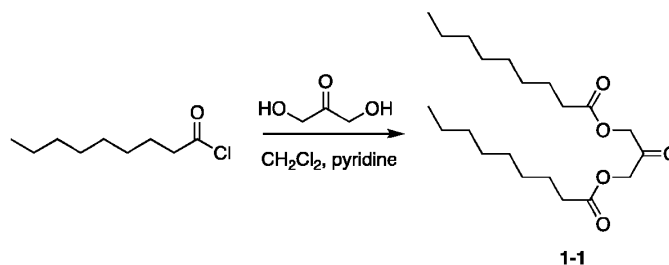
Example 1. Synthesis of LIPID 1: (((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



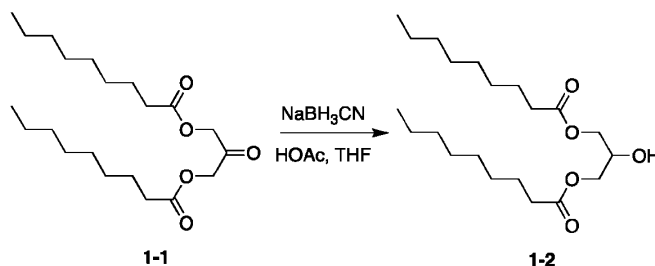
[0244] General Scheme:





[0245] Synthesis of 1-1: 2-Oxopropane-1,3-diyl dinonanoate

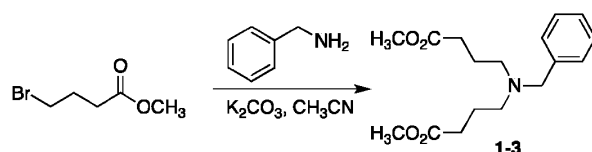
[0246] Into a 500 mL 3-necked round-bottom flask reactor under an atmosphere of N₂, was added a solution of 1,3-dihydroxyacetone (6.8 g, 1 eq) in CH₂Cl₂. Started agitation and adjusted temp to r.t.. Charged pyridine (17.9 g, 3 eq) to the reactor while maintain temperature at 25 ±5 °C. Charged 4-dimethylaminopyridine (DMAP) (0.276 g, 0.03 eq) to the reactor while maintain temperature at 25 ±5 °C. Charged nonanoyl chloride (20 g, 1.5 eq) dropwise to the reactor dropwise at 0°-5 °C. After charging kept the temperature at r.t. and stirred for 6 hours. Charged another 6.66 g of nonanoyl chloride (0.5 eq) to the reactor dropwise at 0-5 °C. The temperature of the reaction was raised to room temperature and stirred under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with CH₂Cl₂. The combined filtrate and washings were then washed with 200 mL each of 5% aq. NaHCO₃, 0.1N HCl and brine. The solution was then dried over Na₂SO₄ and concentrated under vacuum. The residue was then crystallized from methanol (50 mL) to give a white solid. This resulted in 16 g (59.7%) of white product. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min); RT 2.26 min, m/z (Calcd.) 370.27, (found) 371.00 (M+H⁺).

[0247] Synthesis of 1-2: 2-Hydroxypropane-1,3-diyl dinonanoate

[0248] Into a 250 mL three-necked round-bottom flask was placed a solution of Lipid-1-1 (9.6 g, 1.0 eq) in 100 mL THF. To the solution was added AcOH (2.02 g, 1.3 eq) at 0 °C. And then to

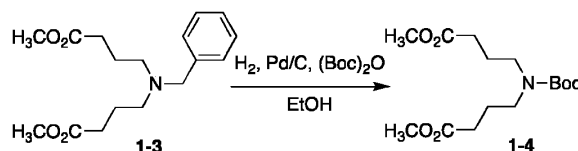
the mixture was added NaBH_3CN (1.96 g, 1.2 eq) at 0 °C. The mixture was Stirred 16 hs at r.t. under an atmosphere of nitrogen. The reaction mixture was quenched with 100 mL water. The mixture was extracted with 100 mL of EtOAc 3 times. The organic layers were combined, washed with brine (300 mL) and concentrated under vacuum. This resulted in 9.5 g (crude) of **1-2** which is used in the next step without purification.

[0249] Synthesis of 1-3: Dimethyl 4,4'-(benzylazanediy) dibutyrate



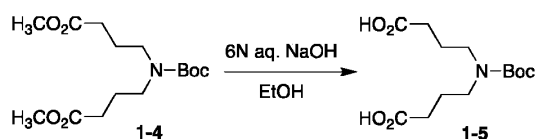
[0250] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was charged acetonitrile (208 mL), K_2CO_3 (31 g, 2.3 eq), benzyl amine (10.3 g, 1.0 eq) and methyl 4-bromobutyrate (40 g, 2.3 eq) at 25 °C. The mixture was heated to 80 °C and stirred for 15 h. The reaction was cooled to 25 °C. Charged water (200 mL, 20 V) into the flask and extracted with EtOAc (2 x 200 mL). The organic phase was dried (Na_2SO_4) and concentrated at 35 °C under reduced pressure. This resulted in **1-3** (23 g, crude) as a crude product which was carried forward without additional purification. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.26 min, m/z (Calcd.) 370.27, (found) 371.00 (M+H).

[0251] Synthesis of 1-4: Dimethyl 4,4'-((tert-butoxycarbonyl)azanediy) dibutyrate



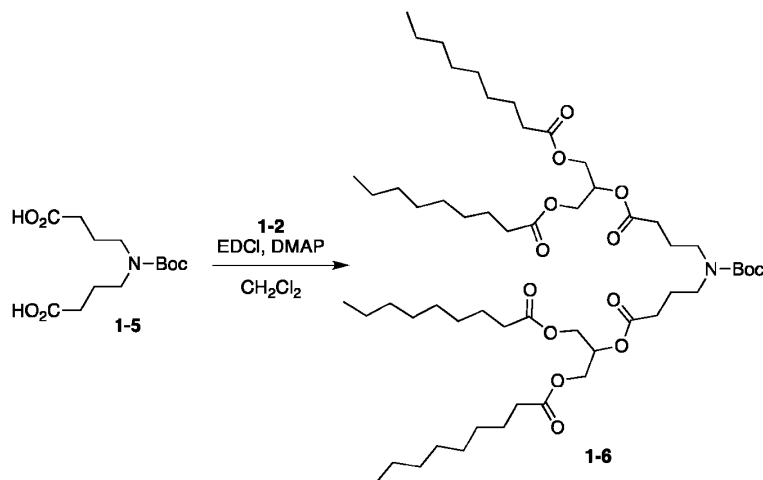
[0252] Charged EtOH (230 mL, 10 V), **1-3** (23.0 g, 1.0 eq), $(\text{Boc})_2\text{O}$ (18.0 g, 1.1 eq) and Pd/C (2.3 g, 10%w/w) into a 1 L hydrogenation autoclave at ambient temperature. Stirred for 16 hrs at room temperature under 5 atm hydrogen atmosphere. TLC observation indicated that **1-3** was completely consumed. The reaction mixture was filtered and concentrated under vacuum at 40 °C to get 22 g of crude **1-4**.

[0253] Synthesis of 1-5: 4,4'-((tert-Butoxycarbonyl)azanediy) dibutyric acid



[0254] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was charged a solution of **1-4** (22 g, 1.0 eq) in ethanol (110 mL). An aqueous solution of NaOH (6M, 110 mL, 5 V) was added at room temperature. The reaction mixture was stirred at 60 °C for 2 hours. The reaction was then diluted with brine (220 mL, 10 V), and was extracted with t-BuOH/n-heptane (2:1, 220 mL, 2x) to remove organic impurities. The aqueous phase was acidified by the addition of 3M aqueous HCl solution to about pH=3 and then extracted with t-BuOH : n-heptane (2:1) (220 mL). The organic layers were concentrated under reduced pressure. The residue was slurried with diethyl ether (44 mL, 2 V) and filtered. Collected the filter cake to give **1-5** (14 g, 43% yield in three steps) as white solid.

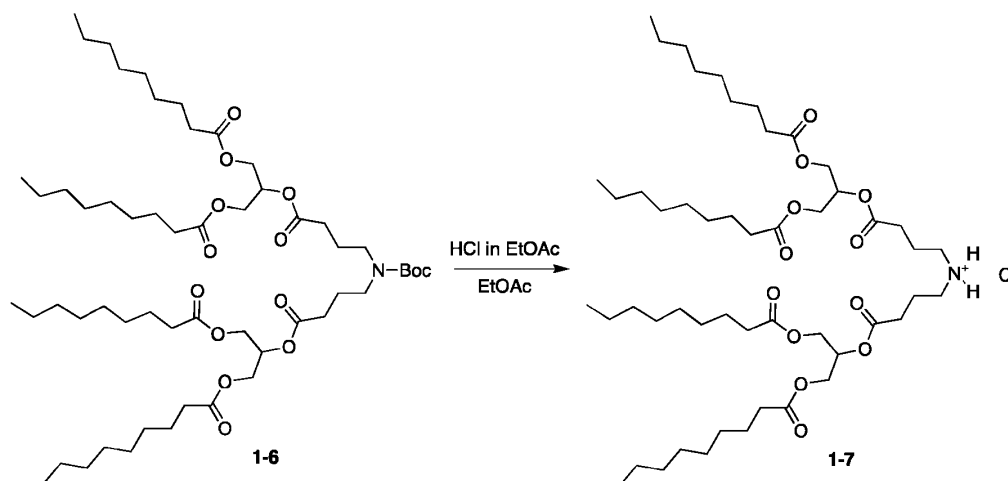
[0255] Synthesis of **1-6**: ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propene-2,1,3-triyl) tetranonanoate



[0256] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **1-5** (3.7 g, 1.00 equiv) in 100 mL of CH_2Cl_2 . To the solution was added **1-2** (9.5 g, 2.00 equiv), DMAP (4.69 g, 3 equiv) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (7.35 g, 3 equiv) at 0 °C. The reaction mixture was stirred overnight at 25 °C. The reaction was then quenched with 200 mL of 10% aq. citric acid. The organic layer was washed with 200 mL of brine. The mixture was dried over

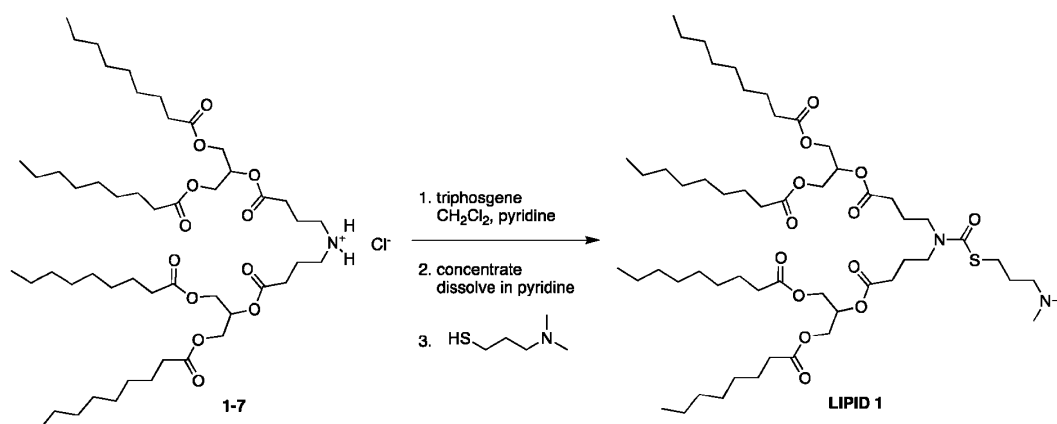
anhydrous sodium sulfate and concentrated under vacuum. This resulted in 12 g of **1-6** and used to the next step without additional purification. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.46 min, m/z (Calcd.) 997.71, (found) 1020.6 (M+Na).

[0257] Synthesis of 1-7: bis(4-((1,3-bis(nonanoyloxy)propan-2-yl)oxy)-4-oxobutyl)ammonium chloride



[0258] Into a 500 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **1-6** (12 g, 1.00 equiv) in EtOAc (68 mL). To the solution was added EtOAc/HCl (15 mL, 5.00 equiv, 4M) dropwise at 0-10 °C. The resulting solution was stirred for 5 h at room temperature. The resulting mixture was concentrated under vacuum. The residue was diluted with CH₂Cl₂ (100 mL) and 25 g of silica gel (type: ZCX-2, 100-200 mesh) was added (Note: ZCX-2 is sold by Sincem Silica Gel Co. Ltd.) The mixture was concentrated under vacuum and then applied onto atmospheric silica gel column with CH₂Cl₂/MeOH gradient from 1:0 to 15:1. The product eluent was collected from 20:1 to 15:1 and concentrated under vacuum. This resulted in 4.2 g of **1-7** as light yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.50 min, m/z (Calcd.) 897.65, (found) 898.6 (M+H).

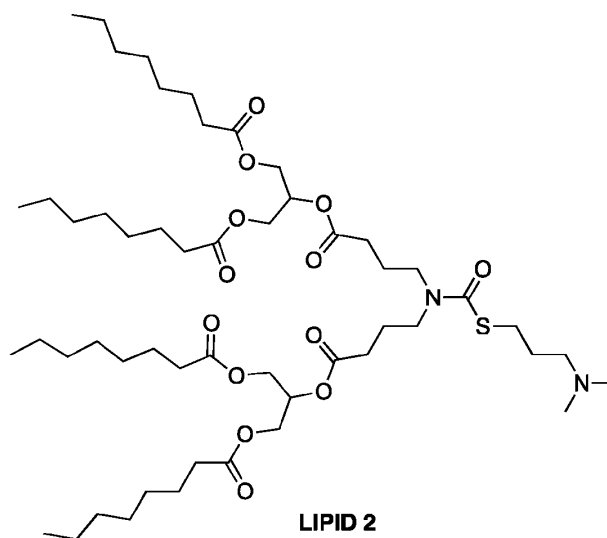
[0259] Synthesis of LIPID 1: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediy)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



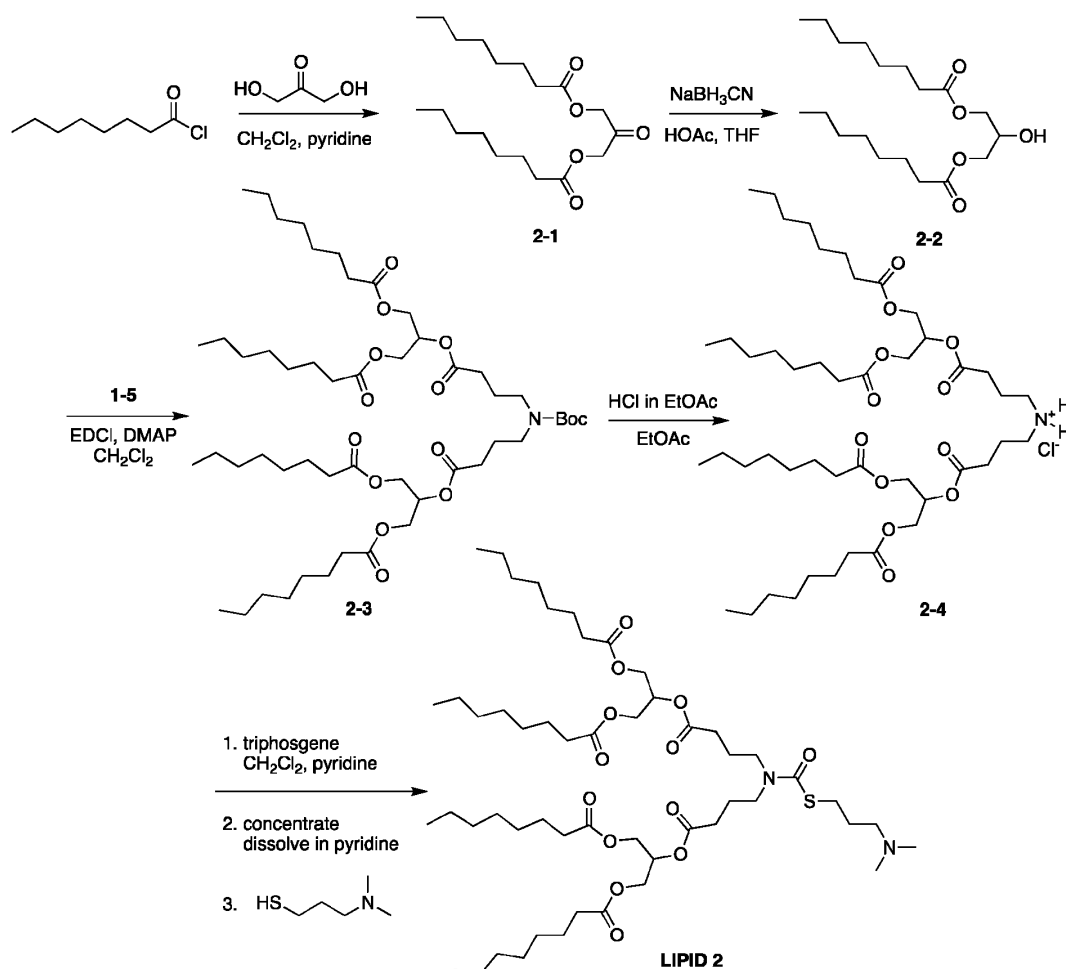
[0260] Into a 250-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **1-7** (4.2 g, 1 eq) in CH₂Cl₂ (150 mL). To the mixture was added triphosgene (1.33 g, 1 equiv) at r.t. This was followed by the addition of pyridine (1.78 g, 5.00 equiv) dropwise with stirring at 0 °C. The mixture was stirred for 4 h at r.t and then concentrated under vacuum. The residue was dissolved with pyridine (600 mL). To this solution was added 3-(dimethylamino)propane-1-thiol (0.92 g, 1.20 equiv) dropwise with stirring at 0 °C in 10 min. The resulting solution was stirred overnight at room temperature. The resulting mixture was concentrated under vacuum. The residue was diluted with 100 mL of EtOAc. The mixture was washed with 2 x 100 mL of 10% citric acid and 2 x 100 mL of NaHCO₃. The mixture was washed with 100 mL of brine and dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was dissolved in 100 mL CH₂Cl₂ and 12 g of Silica gel (type: ZCX-2, 100-200 mesh) was added. The mixture was concentrated under vacuum and then applied onto atmospheric silica gel column with CH₂Cl₂/MeOH gradient from 1:0 to 15:1. The product eluent was collected from 20:1 to 15:1 and concentrated under vacuum. The product was dissolved in 36 mL n-heptane (20V) and 0.09 g activated charcoal powder was added. The mixture was stirred for 4 h at r.t and then filtered. 0.09 g activated charcoal powder was added to the filtrate and the mixture was stirred for another 4 h at r.t. The mixture was filtered. 0.045 g activated charcoal powder was added to the filtrate and the mixture was stirred overnight at r.t. The mixture was filtered. A mixture of methanol (60 mL) and water (20 mL) was charged into the filtrate. The mixture was stirred for 30 min at r.t., kept for phase separation and collected the upper phase. A mixture of methanol (60 mL) and water (20 mL) was charged into the n-heptane phase. The mixture was stirred for 30 min at r.t., kept for phase separation and collected the

upper phase that was concentrated to afford 1.5 g (31.9%) of **LIPID 1** (HPLC Purity: 96.7%, 205 nm). ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 8 min, hold 0.7 min): RT 7.07 min, m/z (Calcd.) 1042.71, (found) 1043.8 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.27 (m, 2H), 4.33 (dd, *J* = 11.9, 4.4 Hz, 4H), 4.17 (dd, *J* = 11.9, 5.7 Hz, 4H), 3.41 (brm, 4H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.10-2.35 (20H), 1.92 (s, 6H), 1.65 (m, 8H), 1.10-1.57 (40H), 0.96 – 0.85 (12H).

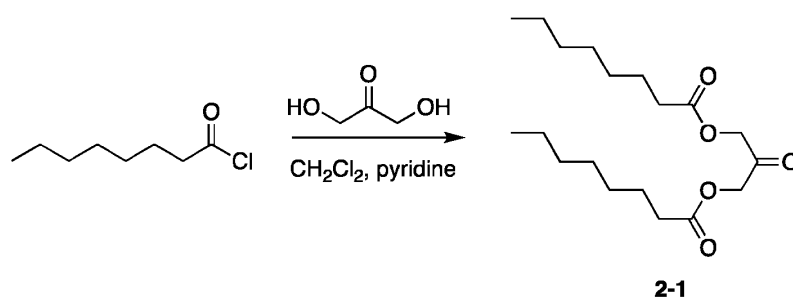
Example 2. Synthesis of LIPID 2: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetraoctanoate



[0261] General Scheme:



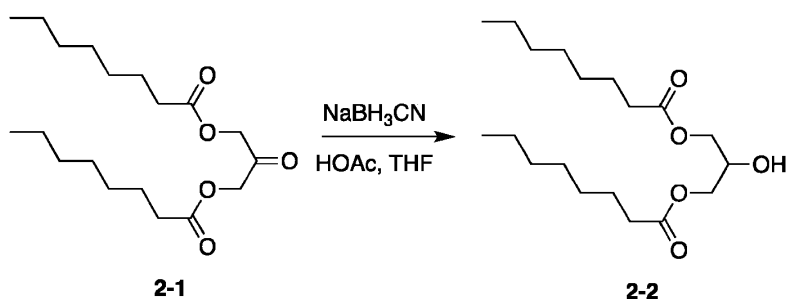
[0262] Synthesis of 2-1: 2-Oxopropane-1,3-diyl dioctanoate



[0263] Into a 2 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of 1,3-dihydroxyacetone (50 g, 1.0 eq) and octanoyl chloride (225.8 g, 2.5 eq) in CHCl_3 (900 mL). Added pyridine (175.8 g, 4.0 eq) to the

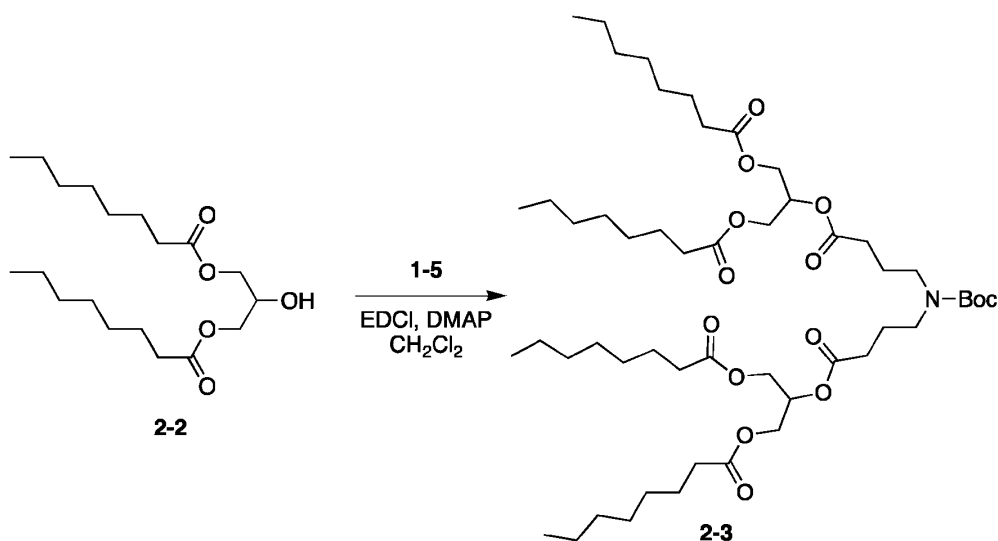
reactor while maintaining the temperature at 0 °C for 40 minutes. The mixture was stirred at room temperature under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with CH₂Cl₂. The combined filtrate and washings were then washed with 200 mL 5% aqueous NaHCO₃ and brine (200mL). The solution was then dried over Na₂SO₄ and concentrated under vacuum. The crude was slurred in n-heptane (125 mL) for 30 minutes and filtered. This resulted in 98 g (52% yield) **2-1** as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.09 min, m/z (Calcd.) 342.24, (found) 343.45 (M+H⁺).

[0264] Synthesis of 2-2: 2-Hydroxypropane-1,3-diyl dioctanoate



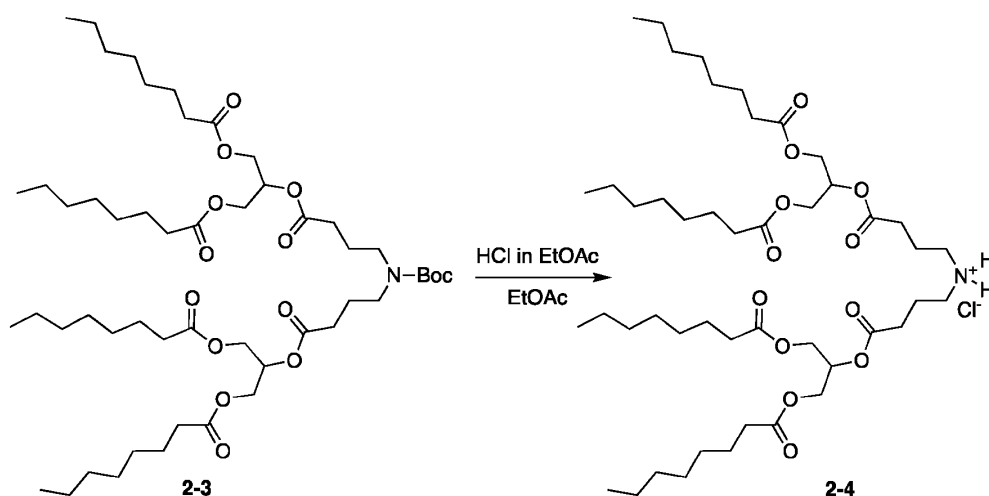
[0265] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **2-1** (20 g, 1.0 eq) in 200 mL of THF. To the solution was added AcOH (4.56 g, 1.3 eq) at 0 °C, and then to the mixture was added NaBH₃CN (4.3 g, 1.2 eq) at 0 °C. The mixture was stirred for 4 h at room temperature. The reaction mixture was quenched with 200 mL water. The mixture was extracted with CH₂Cl₂ (3 x 200 mL) and the combined organic phases were washed with 5% aq. NaHCO₃ (500 mL), brine (500 mL), then dried with Na₂SO₄. Filtration and concentration under vacuum gave crude **2-2** (19.5g) which was used in the next step without purification.

[0266] **Synthesis of 2-3:** ((4,4'-((*tert*-Butoxycarbonyl)azanediy)bis(butanoyl))bis(oxy))bis (propane-2,1,3-triyl) tetraoctanoate



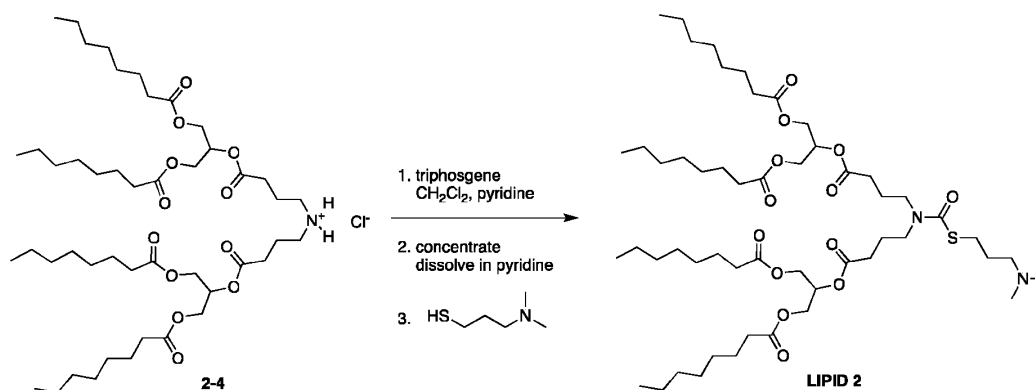
[0267] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **1-5** (8.4 g, 1.0 eq) flowed by a solution of **2-2** in CH_2Cl_2 (175 mL) and the solution was cooled in an ice-water bath. To the solution was added DMAP (3.55 g, 1.0 equiv) and EDCI (22.3 g, 4.0 equiv) at 0 °C. The reaction mixture was stirred overnight at 25 °C. The reaction was then quenched with 200 mL of 10% aqueous citric acid solution. The organic layer was separated, washed with brine (200 mL), and dried over anhydrous sodium sulfate. Filtration and concentration in vacuum provided crude **2-3** which was dissolved in CH_2Cl_2 and the solution adsorbed on 54 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (270 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using a petroleum ether/ethyl acetate gradient from 100:0 to 90:10. Product containing fractions were pooled, combined and concentrated under reduced pressure to obtain 15 g (55% for 2 steps) of **2-3** as light yellow oil.

[0268] **Synthesis of 2-4: bis(4-((1,3-bis(Octanoyloxy)propan-2-yl)oxy)-4-oxobutyl)ammonium chloride**



[0269] Into a 500 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **2-3** (15 g, 1.0 equiv) in EtOAc (85.5 mL), the mixture was cooled in an ice-water bath. To the solution was added HCl in EtOAc (80 mL, 10.0 equiv, 2 mol/L) dropwise at 0-10 °C. The resulting solution was stirred overnight at room temperature. The mixture was concentrated under vacuum. This resulted in 13 g (93% yield) of **2-4** as light-yellow oil that was used in the next reaction without further purification. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.746 min, m/z (Calcd.) 842.60, (found) 842.71 (M+H⁺).

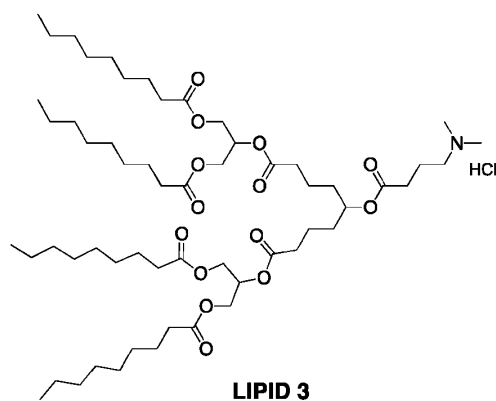
[0270] **Synthesis of LIPID 2: (((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetraoctanoate**



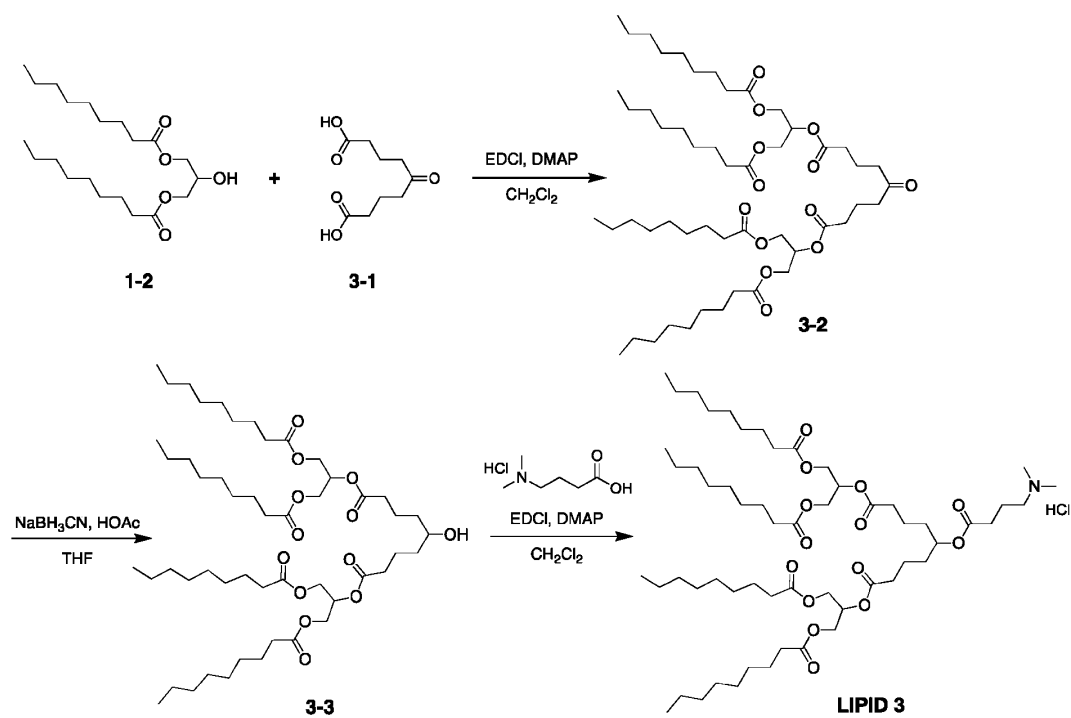
[0271] Into a 500-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **2-4** (8 g, 1 eq) dissolved in CH₂Cl₂ (280 mL), and the

solution was cooled in an ice-water bath. To the mixture was added triphosgene (2.82 g, 1 equiv) and this was followed by the addition of pyridine (3.76 g, 5.00 equiv) dropwise with stirring at 0 °C. The mixture was stirred for 4 h at r.t and then concentrated under vacuum. The residue was dissolved with pyridine (160 mL) and the solution was cooled in an ice-water bath under nitrogen. To this solution was added 3-(dimethylamino)propane-1-thiol (1.356 g, 1.20 equiv) dropwise with stirring at 0 °C over 10 min. The resulting solution was stirred overnight at room temperature. The mixture was concentrated under vacuum and the residue was dissolved in CH₂Cl₂ (200 mL). The mixture was washed with 10% aq. citric acid (2 x 100 mL), 5% aq. NaHCO₃ (2 x 100 mL), and brine (100 mL). The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was dissolved in CH₂Cl₂ (25 mL) and adsorbed on 12 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (80 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using CH₂Cl₂/MeOH gradient from 100:0 to 97:3. Fractions containing pure products were analyzed, pooled, combined and concentrated under reduced pressure. The product thus obtained was dissolved in 36 mL n-heptane and 0.22 g activated charcoal powder was added. The mixture was stirred for 4 h at r.t and then filtered. The filtrate was concentrated under vacuum. This resulted in 3.8 g (42%) of **2** as a viscous, pale yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.48 min, m/z (Calcd.) 986.65, (found) 987.4 (M+H); ¹H NMR (300 MHz, CDCl₃) δ 5.26 (m, 2H), 4.32 (dd, *J* = 11.9, 4.4 Hz, 4H), 4.16 (dd, *J* = 11.9, 5.7 Hz, 4H), 3.40 (brm, 4H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.39 – 2.25 (18H), 1.91-1.59 (16H), 1.06-1.45 (32H), 0.913 – 0.869 (12H).

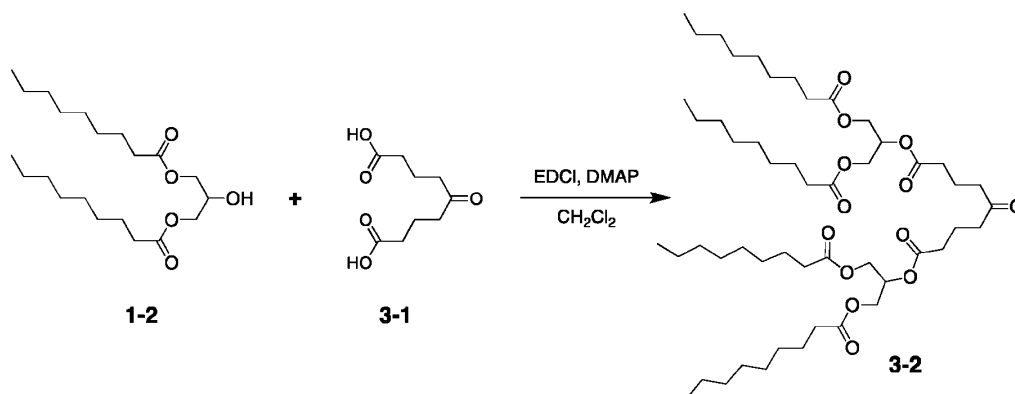
Example 3. Synthesis LIPID 3: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 5-((4-(dimethylamino)butanoyl)oxy)nonanedioate HCl salt



[0272] General Scheme:

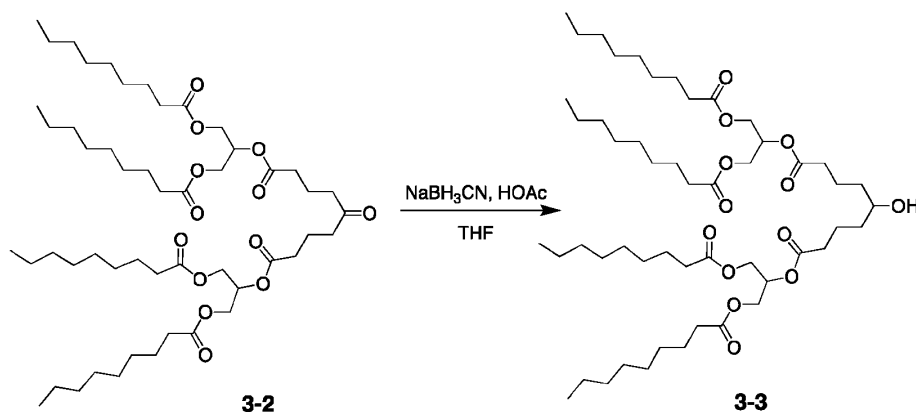


[0273] Synthesis of 3-2: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 5-oxononanedioate



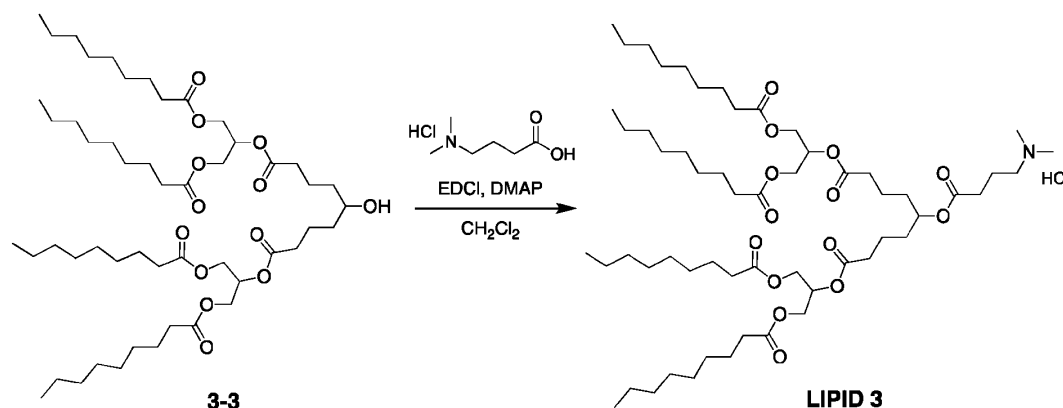
[0274] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **3-1** (7.04 g, 1.0 eq, *Chemistry – A European Journal* **2017**, 23, 12744-12748) into CH_2Cl_2 (100 mL), cooled in an ice-water bath under nitrogen. This was followed by the addition of **1-2** (25.95 g, 2.0 eq), DMAP (4.26 g, 1.0 eq), and EDCI (20.09 g, 3.0 eq) at 0 °C. The resulting solution was stirred for 16 h at room temperature. The reaction mixture was adsorbed on 90 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w/w.), and purified on a silica gel column (900 g, type: ZCX-2, 100-200 mesh) with PE/EA, gradient from 100:0 to 90:10. The fractions containing pure product were pooled, concentrated under vacuum and dried over P_2O_5 to get 19.7 g (62.2 %) of **3-2** as a yellow oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.87 min, m/z (Calcd.) 910.63, (found) 933.35 (M+Na).

[0275] **Synthesis of 3-3: bis(1,3-bis(nonanoyloxy)propan-2-yl) 5-hydroxynonanedioate**



[0276] Into a 250-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **3-2** (10.7 g, 1.0 eq) into THF (100 mL, 10 V), cooled in an ice-water bath. This was followed by the addition of HOAc (7.96 g, 11.3 eq), NaBH₃CN (8.88 g, 12.0 eq) at 0 °C. The resulting solution was stirred for 16 h at room temperature. The reaction was then quenched by the addition of water (100 mL, 10 V). The resulting solution was extracted with ethyl acetate (3 x100 mL) and the organic layers were combined. The resulting mixture was washed with brine (2 x100 mL). The mixture was dried over anhydrous sodium sulfate and the organic layers was concentrated under vacuum. The reaction mixture was adsorbed on 40 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (400 g, type: ZCX-2, 100-200 mesh) with PE/EA, gradient from 100:0 to 80:20. The fractions containing pure product was pooled, concentrated under vacuum and dried over P₂O₅ to get 7.42 g (69.2 %) of **3-3** as a yellow oil ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.87 min, m/z (Calcd.) 913.27, (found) 935.35 (M+Na).

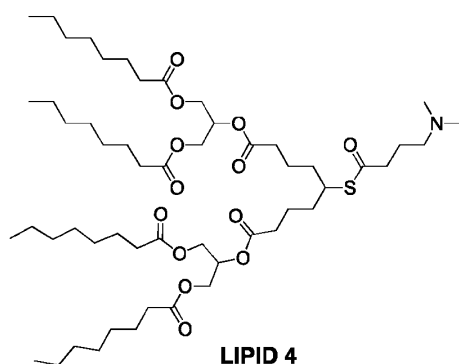
[0277] **Synthesis of LIPID 3: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 5-((4-(dimethylamino)butanoyl)oxy)nonanedioate HCl salt**



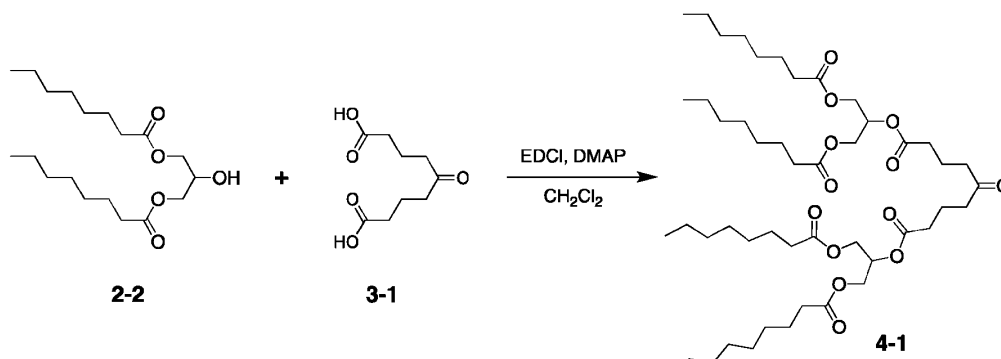
[0278] Into a 250-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **3-3** (7.42 g, 1.0 eq) in CH₂Cl₂ (110 mL), cooled in an ice-water bath. 4-(dimethylamino)butanoic acid-HCl salt (1.63 g, 1.2 eq), DMAP (0.4 g, 0.4 eq) were added, followed by the addition of EDCI (3.74 g, 2.4 eq) in portions at 0 °C. The resulting solution was stirred for 16 h at room temperature. Silica gel (40 g, type: ZCX-2, 100-200 mesh, 6.43 w./w.) was added to the reaction and adsorbed it on the silica gel. It was purified on a silica gel column (300 g, type: ZCX-2, 100-200 mesh) with EtOAc/THF, gradient from

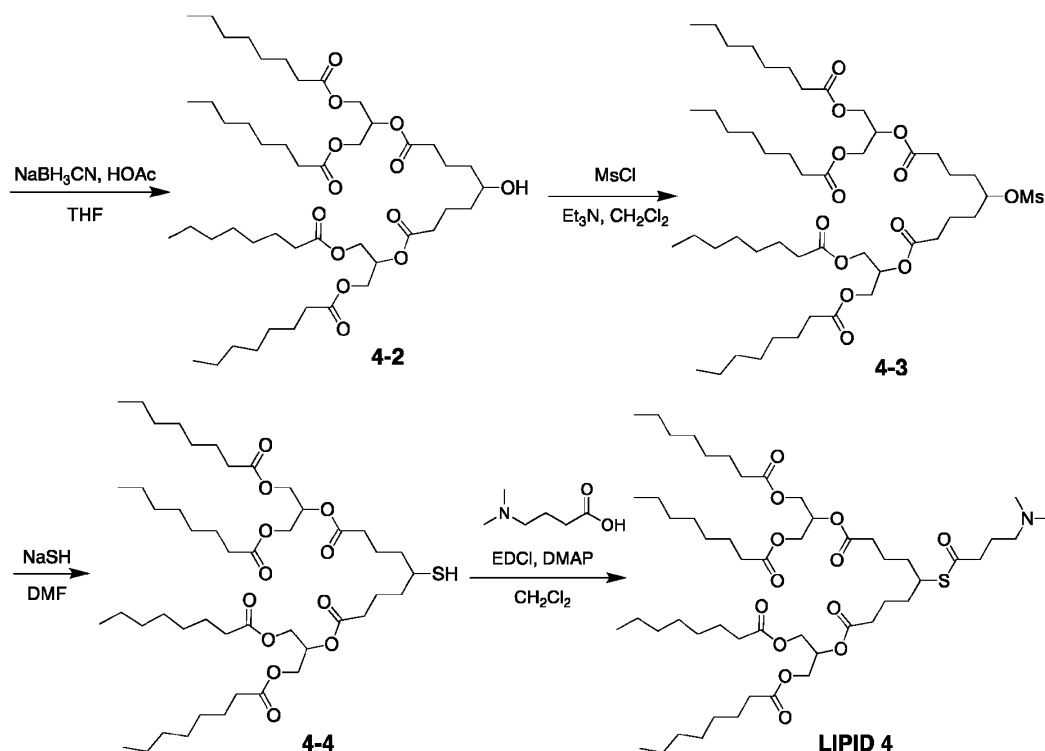
100:0 to 75:25. The fractions containing pure product were pooled and concentrated under vacuum to get 1.9 g (26.3%) of **3** as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 1.89 min, m/z (Calcd.) 1025.74, (found) 1026.55 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.21 (m, 2H), 4.89 (brs, 1H), 4.34 (m, 4H), 4.13 (m, 4H), 2.27-2.37 (16H), 2.22 (s, 6H), 2.04 (brm, 2H), 1.80 (m, 2H), 1.59-1.74 (14H), 1.15-1.28 (40H), 0.85-0.96 (12H).

Example 4. Synthesis of LIPID 4: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-((4-(dimethylamino)butanoyl)thio)nonanedioate

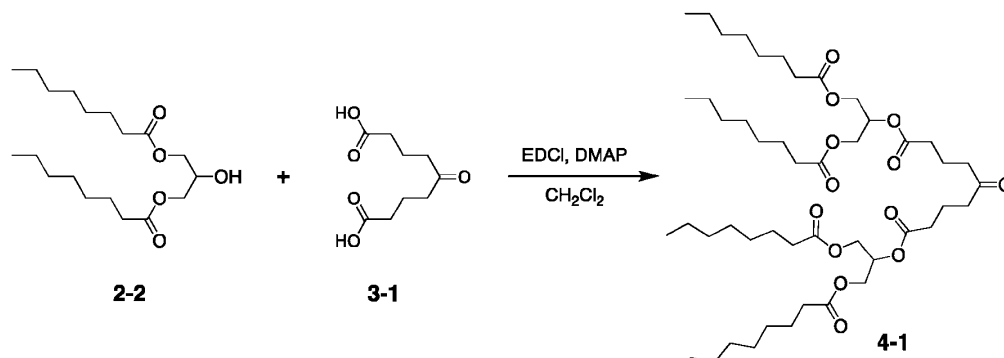


[0279] General Scheme:





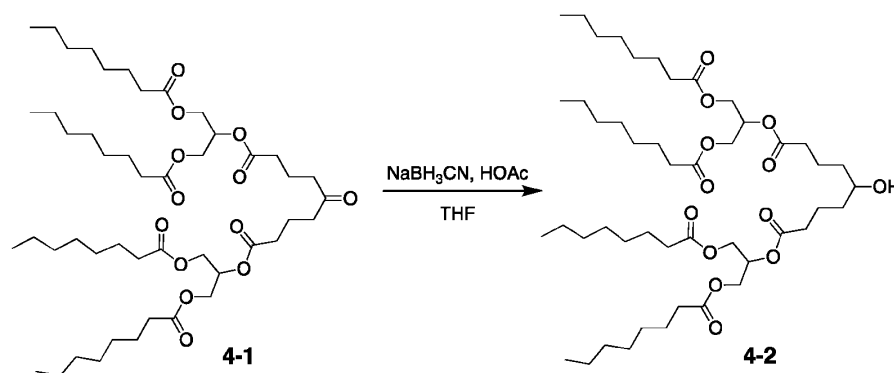
[0280] Synthesis of 4-1: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-oxononanedioate



[0281] Into a 1-L 3-necked round-bottom flask, was placed **3-1** (20.0 g, 98.909 mmol, 1.00 equiv), **2-2** (68.2 g, 197.818 mmol, 2 equiv) and DMAP (36.3 g, 296.727 mmol, 3 equiv) in CH_2Cl_2 (600 mL), cooled in an ice-water bath under nitrogen. This was followed by the addition of EDCI (56.9 g, 296.727 mmol, 3 equiv) in several batches at 0 °C. The resulting solution was stirred for 16 hours at room temperature. The reaction was then quenched by the addition of

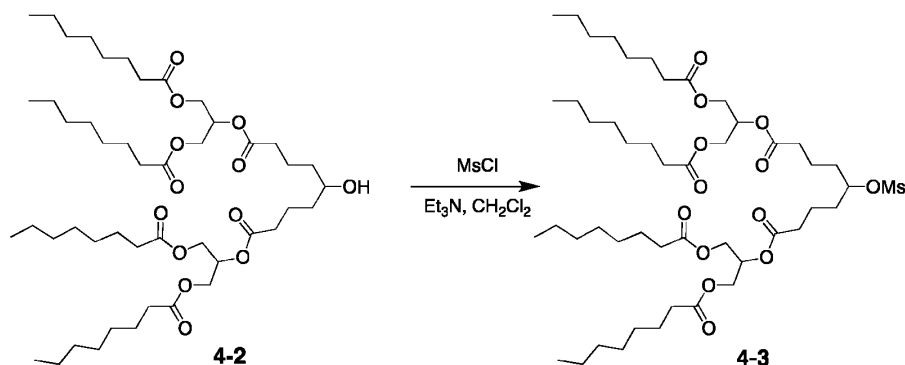
aqueous HCl (1 mol/L, 75 mL). The resulting solution was extracted with CH₂Cl₂ (200 mL). The combined organic phases were washed with brine (2 x 200 mL). The mixture was dried over anhydrous sodium sulfate and concentrated. The crude material dissolved in CH₂Cl₂ (200 mL) and was adsorbed on the silica gel (108 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (720 g, type: ZCX-2, 100-200 mesh) with PE/EA gradient from 100:0 to 90:10. The fractions containing pure product was pooled and concentrated under vacuum to get 50 g (53.2%) **4-1** as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.74 min, m/z 854.58 (Calcd.), (found) 877.75 (M+Na).

[0282] Synthesis of 4-2: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-hydroxynonanedioate



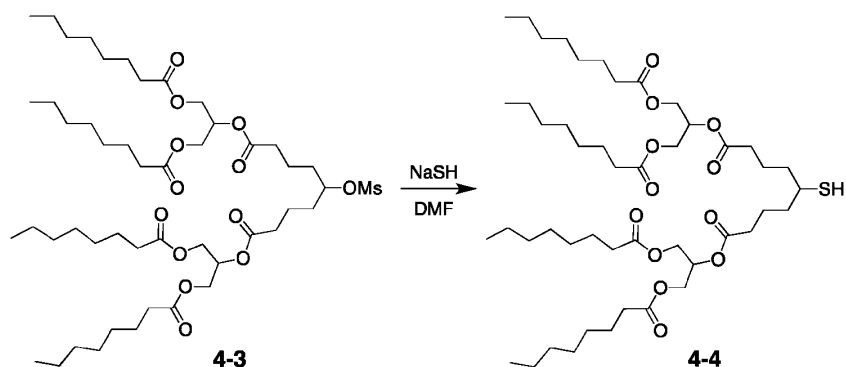
[0283] Into a 1-L 3-necked round-bottom flask, was placed **4-1** (50.0 g, 58.469 mmol, 1.00 equiv) in THF (500 mL). This solution was cooled in an ice-water bath under nitrogen and HOAc(35.1 g, 584.686 mmol, 10 equiv) was added at 0 °C. To this was added NaBH₃CN (36.7 g, 584.686 mmol, 10 equiv) in several portions at 0 °C. The resulting solution was stirred for 3 hours at room temperature. The reaction was then quenched by the addition of water (200 ml). The resulting solution was extracted with dichloromethane (1000 ml). the organic phase was dried over anhydrous Na₂SO₄ and concentrated. The crude material was adsorbed on the silica gel (160 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (400 g, type: ZCX-2, 100-200 mesh) with PE/EA gradient from 100:0 to 90:10. The fractions containing pure product was pooled and concentrated under vacuum to get 20 g (40%) **4-2** as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.00 min, m/z 856.59 (Calcd.), (found) 879.70 (M+Na).

[0284] Synthesis of 4-3: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-((methylsulfonyl)oxy)nonanedioate



[0285] Into a 500-mL 3-necked round-bottom flask, was placed **4-2** (25.0 g, 29.166 mmol, 1.00 equiv) and Et₃N (5.9 g, 58.331 mmol, 2 equiv) in CH₂Cl₂ (250 mL), cooled in an ice-water bath under nitrogen. This was followed by the addition of MsCl (5.0 g, 43.748 mmol, 1.5 equiv) dropwise with stirring at 0 °C over 20 min. The resulting solution was stirred for 3 hours at room temperature. The reaction was then quenched by the addition of water/ice (100 mL). The resulting solution was extracted with CH₂Cl₂ (2 x 100 mL). The combined organic phases were washed with brine (100 mL) and dried over Na₂SO₄. After concentration, this resulted in 24 g (crude) **4-3** as light-yellow oil that was used as such in the next reaction. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.71 min, m/z 934.57 (Calcd.), (found) 957.65 (M+Na).

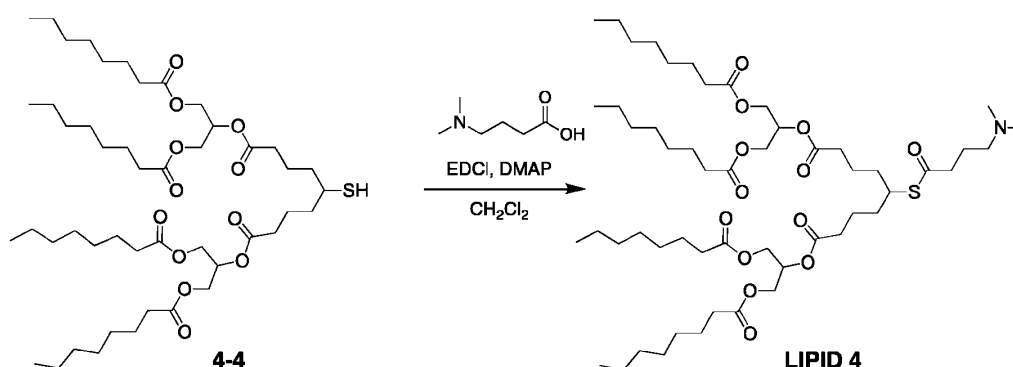
[0286] Synthesis of 4-4: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-mercaptanonanedioate



[0287] Into a 500-mL 3-necked round-bottom flask, was placed **4-3** (30.0 g, 32.077 mmol, 1.00 equiv) in DMF (300, 10 V), and the mixture was cooled in an ice-water bath under nitrogen.

This was followed by the addition of NaSH (9.0 g, 160.383 mmol, 5.00 equiv) in three portions over 1.5 hours at 0 °C. The resulting solution was stirred for 5 hours at room temperature. The reaction was then quenched by the addition of water/ice (100 mL). The mixture was extracted with EtOAc (3 x 100 mL). The combined organic phases were washed with brine (2 x 100 mL). The mixture was dried over anhydrous sodium sulfate and concentrated. This resulted in 10 g (crude) **4-4** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.17 min, m/z 872.57 (Calcd.), (found) 895.70 (M+Na).

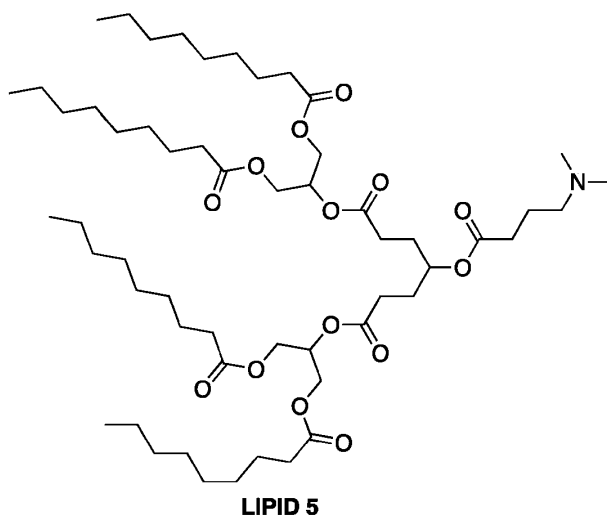
[0288] Synthesis of LIPID 4: bis(1,3-bis(Octanoyloxy)propan-2-yl) 5-((4-(dimethylamino)butanoyl)thio)nonanedioate



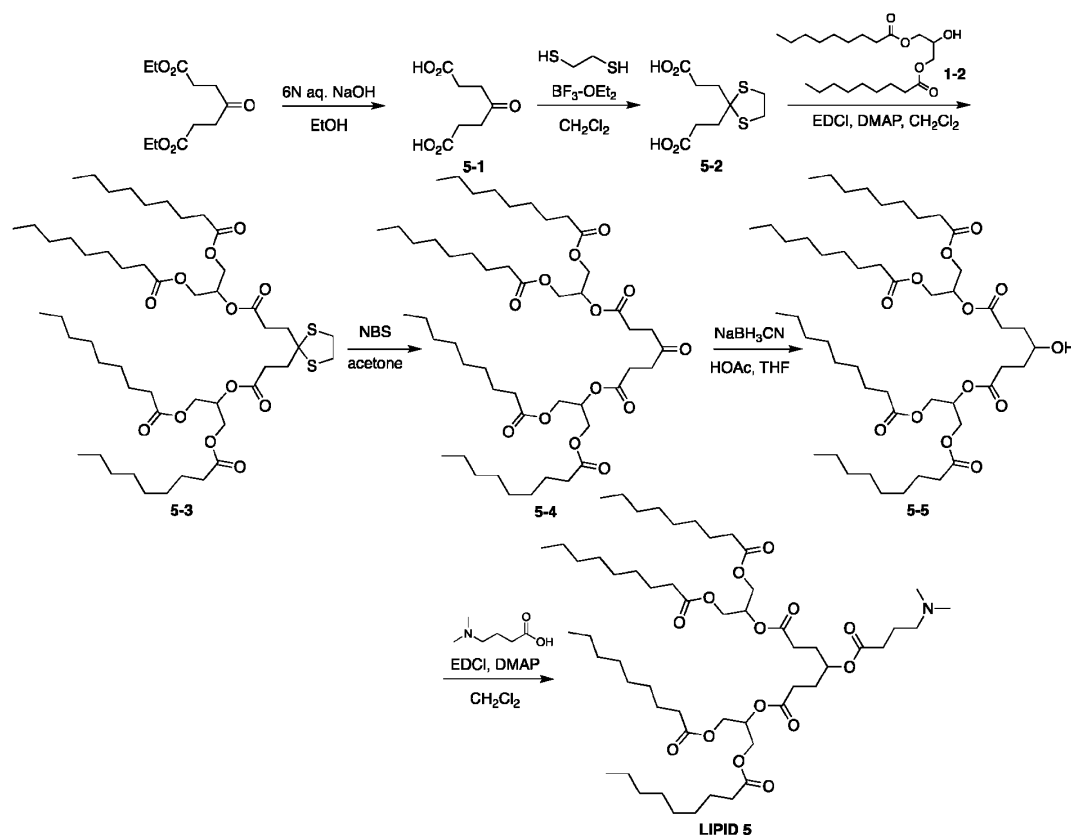
[0289] Into a 250-mL 3-necked round-bottom flask, was placed **4-4** (12.0 g, 13.742 mmol, 1.00 equiv), 4-(dimethylamino)butanoic acid (2.2 g, 16.490 mmol, 1.2 equiv) and DMAP (2.0 g, 16.490 mmol, 1.2 equiv) in DCM (120 mL), and the solution was cooled in an ice-water bath under nitrogen. This was followed by the addition of EDCI (3.16 g, 16.490 mmol, 1.2 equiv) in several batches at 0 °C. The resulting solution was stirred for 16 hours at room temperature. The reaction was then quenched by the addition of aqueous HCl (1 mol/L, 50 mL). The resulting solution was extracted with dichloromethane (2 x 100 mL). The combined organic phases were washed with brine (2 x 100 mL) and dried over anhydrous sodium sulfate and then concentrated. The crude material was adsorbed on the silica gel (25 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (200 g, type: ZCX-2, 100-200 mesh) with CH₂Cl₂/MeOH gradient from 100:0 to 25:1. The fractions containing pure product was pooled and concentrated under vacuum to get 1.5 g (three steps yield of 6.5%) **LIPID 4** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.15 min, m/z 985.66 (Calcd.), (found) 986.55 (M+Na); ¹H-NMR (400 MHz, CDCl₃): δ 5.26 (m, 2H),

4.31 (m, 4H), 4.15 (m, 4H), 3.53 (brs, 1H), 2.61 (t, $J = 7.5$ Hz, 2H), 2.39-2.28 (14H), 2.24 (s, 6H), 1.85 (m, 2H), 1.787-1.512 (16H), 1.34-1.29 (32H), 0.98-0.80 (12H).

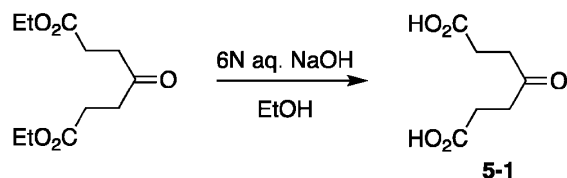
Example 5. Synthesis of LIPID 5: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-((4-(dimethylamino) butanoyl)oxy)heptanedioate



[0290] General Scheme:



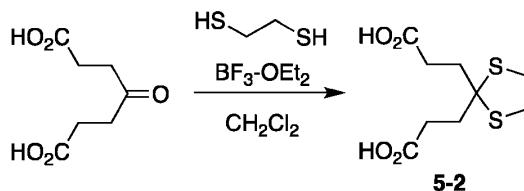
[0291] Synthesis of 5-1: 4-Oxoheptanedioic acid



[0292] To a three-neck round-bottom flask was added EtOH (25 mL, 5 V) and diethyl-4-oxoheptanedioate (5 g, 1 eq) at room temperature under nitrogen. The mixture was cooled in an ice-water bath, then aqueous sodium hydroxide (6N, 25 mL) was added slowly to the mixture at 0 °C. The resulting solution was then warmed and stirred for 2 h at 60 °C. After cooling to room temperature, brine (50 mL) and CH₂Cl₂ (50 mL) were added to the mixture and stirred for 10 minutes, then the aqueous phase was separated. The pH value of the aqueous phase was adjusted to 3~4 with 3 N HCl. The mixture was extracted with CH₂Cl₂ (100 mL). The organic phase was dried with anhydrous MgSO₄ and then filtered. Concentration under vacuum afforded **5-1** (3.2 g,

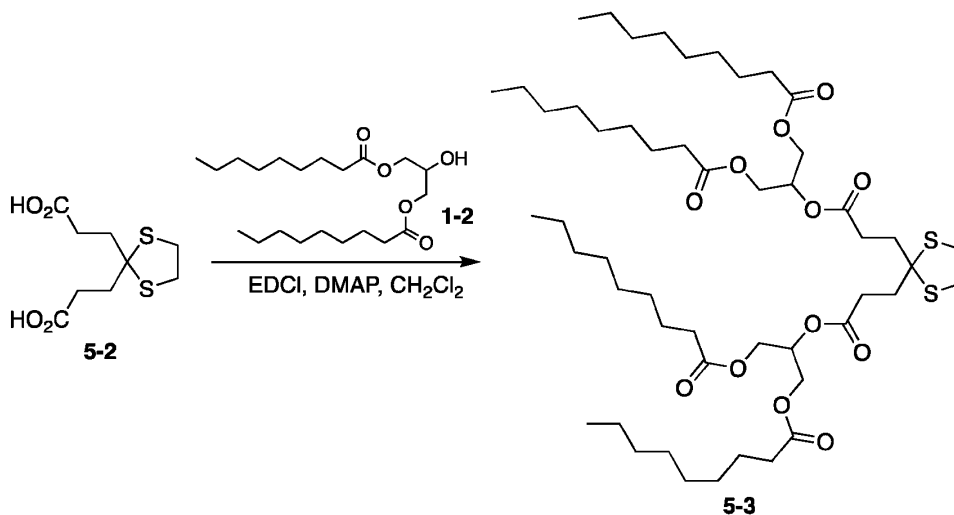
84.6% yield) as a light yellow solid. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 0.81 min, m/z 174.05 (Calcd.), (found) 197.06 (M+Na).

[0293] Synthesis of 5-2: 3,3'-(1,3-dithiolane-2,2-diyl)dipropionic acid



[0294] To a three-neck round-bottom flask was added CH₂Cl₂ (32 mL), **5-1** (3.2 g, 1 eq) and ethane-1,2-dithiol (2.1 g, 1.2 eq) one portion at room temperature. The mixture was cooled in an ice-water bath under nitrogen and BF₃·Et₂O (6.48 g, 2.5 eq) was added slowly to the mixture at 0 °C. The resulting solution was stirred for 16 h at 20 °C. The solid was collected by filtration. The solid was dried under vacuum to afford the **5-2** (4 g, 88% yield) as light yellow solid. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 0.20 min, m/z 250.03 (Calcd.), (found) 268.2 (M+Na).

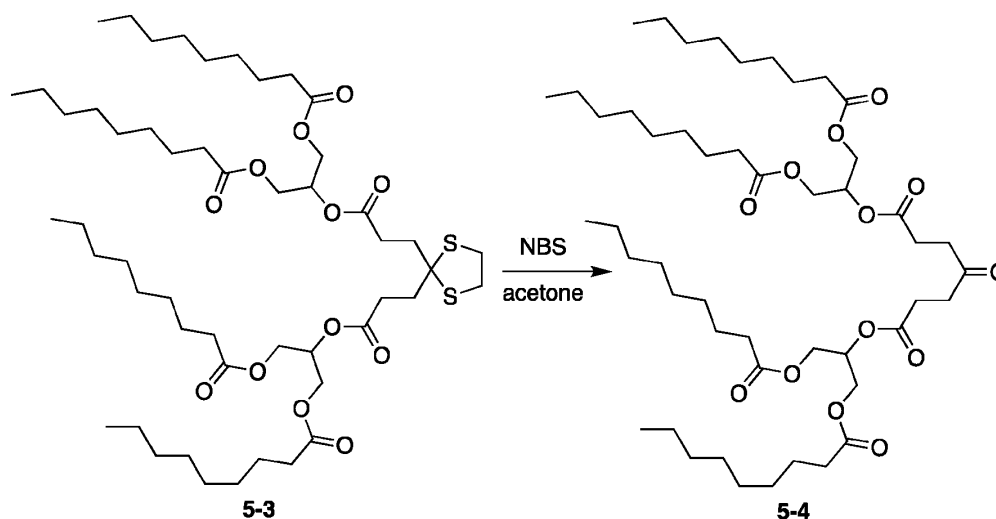
[0295] Synthesis of 5-3: ((3,3'-(1,3-Dithiolane-2,2-diyl)bis(propanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



[0296] To a three-neck round-bottom flask was added CH₂Cl₂ (100 mL), **5-2** (5 g, 1.0 eq), **1-2** (16.37 g, 2.2 eq) and DMAP (2.44 g, 1 eq) successively. The mixture was cooled in an ice-water bath under nitrogen, then EDCI (8.42 g, 2.2 eq) was added to the reaction mixture at 0 °C in

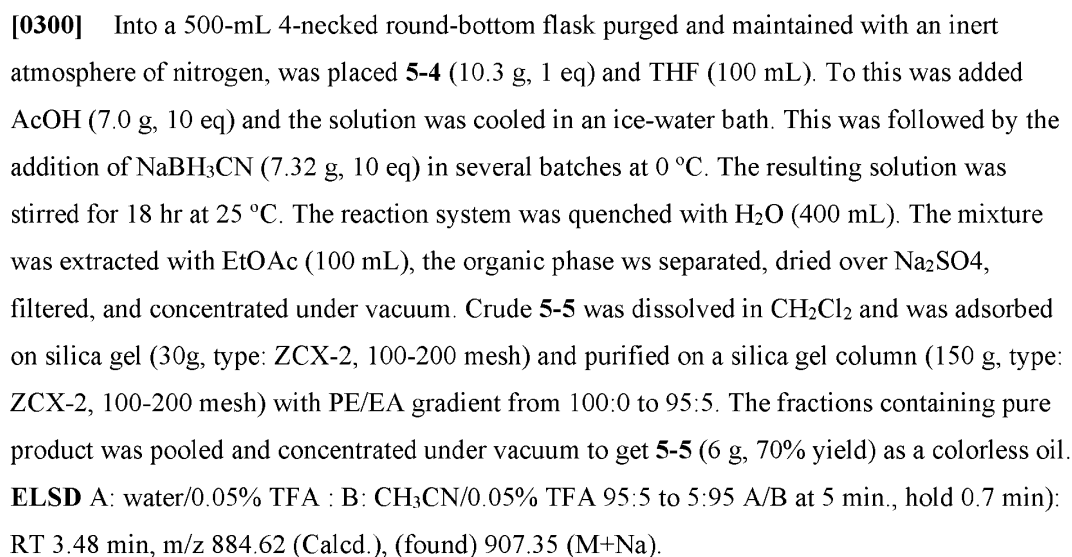
portions. The resulting solution was stirred for 16 h at 20 °C. The reaction system was quenched with 10% aq. citric acid (50 mL). The organic phase was separated, washed with 10% aq. citric acid (50 mL), brine (50 mL), and dried with anhydrous MgSO_4 and then filtered. Concentration under vacuum provided crude **5-3** which was dissolved in CH_2Cl_2 (50 mL) and the solution was adsorbed on silica gel (50g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (200 g, type: ZCX-2, 100-200 mesh) with PE/EA gradient from 100:0 to 98:2. The fractions containing pure product was pooled and concentrated under vacuum to give **5-3** (16.1 g, 84% yield) as a colorless oil. (Due to poor ionization no mass was observed. Hence, without further characterization the molecule was used in the next step).

[0297] Synthesis of 5-4: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-oxoheptanedioate



[0298] To a three-neck round-bottom flask was added acetone (400 mL) and **5-3** (16 g, 1.0 eq) and the solution was cooled to -20 °C under nitrogen. NBS (11.87 g, 4 eq) in acetone (80 mL) was added dropwise to the reaction mixture at -20 °C over a period of 15 mins. The resulting solution was stirred for 1 h at -20 °C,. The reaction was quenched with H_2O (320 mL) and warmed to room temperature. Acetone was removed by concentration under vacuum and the mixture was extracted with EtOAc (160 mL). The organic phase was dried over Na_2SO_4 , filtered and the solvent was removed under reduced pressure. The crude material was dissolved in CH_2Cl_2 (75mL), and was adsorbed on silica gel (30g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (200 g, type: ZCX-2, 100-200 mesh) with PE/EA gradient from 100:0 to 97:3. The fractions containing pure product was pooled and concentrated under vacuum to get **5-**

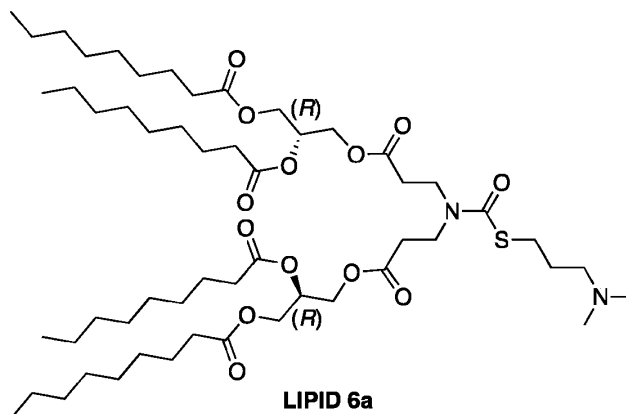
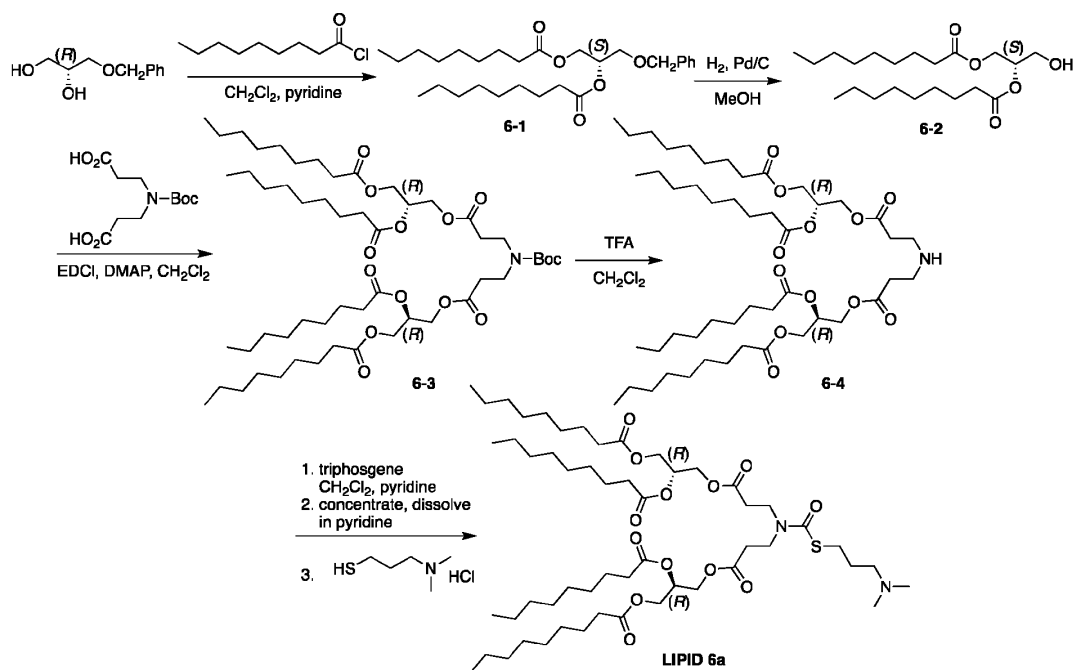
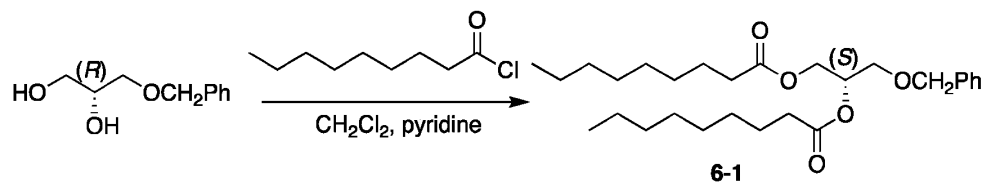
[0299] Synthesis of 5-5: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-hydroxyheptanedioate



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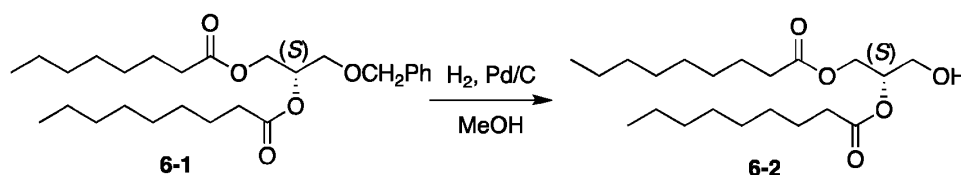


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Example 6. Synthesis of LIPID 6a**[0303] General Scheme:****[0304] Synthesis of 6-1: *(S)*-3-(benzyloxy)propane-1,2-diyl dinonanoate**

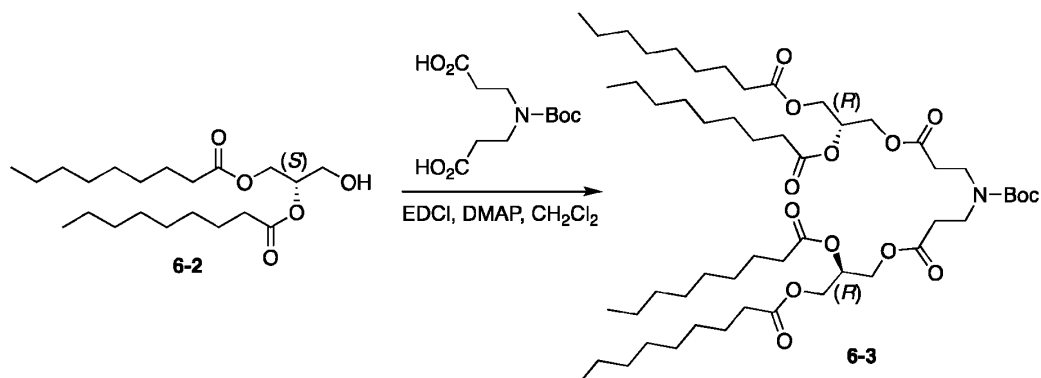
[0305] To a three-neck flask was added CH₂Cl₂ (60 mL), (*R*)-3-(benzyloxy)propane-1,2-diol (3 g, 1 eq), nonanoyl chloride (6.4 g, 2.2 eq) as one portion at room temperature, the mixture was cooled in an ice-water bath under nitrogen. Pyridine (3.90 g, 3 eq) was added to the reaction mixture at 0 °C over a period of 10 minutes. The resulting solution was stirred for 16 h at 20 °C. The reaction was then quenched by the addition of 30 mL of water and was stirred 10 min. The organic phase was separated. The aqueous layer was extracted with CH₂Cl₂ (75 mL). The combined organic layers were dried over anhydrous sodium sulfate then filtered. Concentration in vacuum provided crude **6-1** which was dissolved in CH₂Cl₂ (50 mL) which was adsorbed on 20 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (100 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using petroleum ether / EtOAc gradient from 100:0 to 50:1. Fractions containing pure products were analyzed, pooled, combined and concentrated under reduced pressure to afford the **6-1** (6.0 g, 80% yield) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.1 min, m/z (Calcd.) 462.33, (found) 485.2 (M+Na).

[0306] **Synthesis of 6-2: (*S*)-3-hydroxypropane-1,2-diyl dinonanoate**



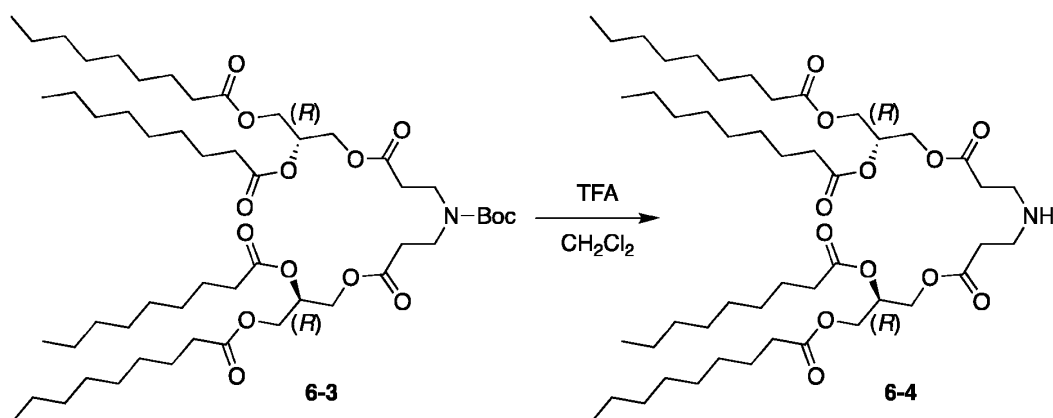
[0307] To a three-neck flask was added MeOH (60 mL), **6-1** (6 g, 1 eq), Pd/C (0.6 g, 10% wt) under nitrogen atmosphere at room temperature. The flask was evacuated and flushed three times with hydrogen. The mixture was stirred 16 h at room temperature under an atmosphere of hydrogen (balloon). Filtered and the filtrate was concentrated to dryness under vacuum to afford **6-2** (3.1 g, 64% yield) as colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 0.89 min, m/z (Calcd.) 3722.29, (found) 395.3 (M+Na).

[0308] **Synthesis of 6-3: (2*R*,2'*R*)-((3,3'-((*tert*-Butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(propane-3,1,2-triyl) tetranonanoate**



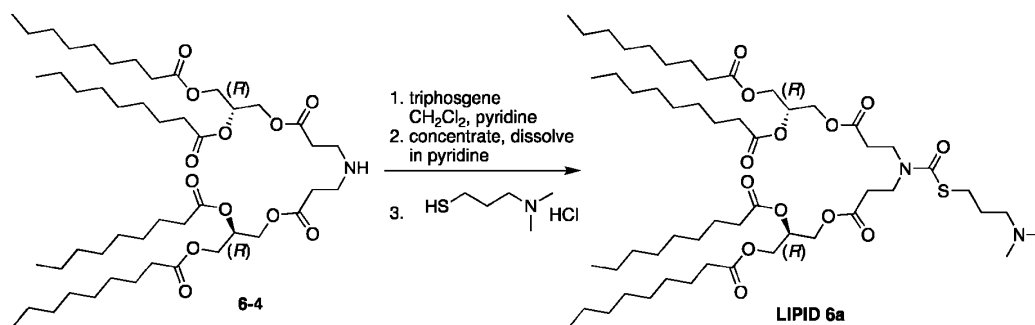
[0309] To a three-neck round-bottom flask was added CH₂Cl₂ (20 mL), 3,3'-((*tert*-butoxycarbonyl)azanediyl)dipropionic acid (commercially available, 1 g, 1.0 eq), **6-2** (3.10 g, 2.2 eq) and DMAP (0.47 g, 1 eq) successively, the mixture was cooled in an ice-water bath under nitrogen. EDCI (1.60 g, 2.2 eq) was added to the reaction mixture at 0 °C in portions over 10 minutes. The resulting solution was stirred for 16 h at 20 °C and the reaction was quenched with 10% aq. citric acid solution (10 mL). The organic phase was separated and washed with 10% aq. citric acid solution (10 mL), brine (10 mL), and dried with anhydrous MgSO₄ and then filtered. Concentration under vacuum gave crude **6-3** which was dissolved in CH₂Cl₂ (10 mL) and adsorbed on 5 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (25 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using petroleum ether / EtOAc gradient from 100:0 to 50:1. Fractions containing pure products were analyzed, pooled, combined and concentrated under reduced pressure to afford the **6-3** (3 g, 81% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.35 min, m/z (Calcd.) 969.68, (found) 992.5 (M+Na).

[0310] Synthesis of **6-4: (2*R*,2'*R*)-((3,3'-Azanediylbis(propanoyl))bis(oxy))bis(propane-3,1,2-triyl) tetranonanoate**



[0311] To a three-neck flask was added CH_2Cl_2 (60 mL), **6-3** (3 g, 1 eq) as one portion at room temperature, the mixture was cooled in an ice-water bath under nitrogen, then TFA (4.5 mL) was added slowly the reaction mixture at $0\sim 5^\circ\text{C}$. The reaction mixture was stirred for 2 h at 25°C . The reaction was then quenched by the addition of 5% aq. sodium carbonate solution (10 wt.%, 30 mL). The organic phase was separated. The organic phase was washed with brine (2x30 mL), dried with anhydrous MgSO_4 , and then filtered and concentrated to dryness under vacuum to afford the **6-4** (2.5 g, 94% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: $\text{CH}_3\text{CN}/0.05\%$ TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.87 min, m/z (Calcd.) 869.62, (found) 892.40 (M+Na).

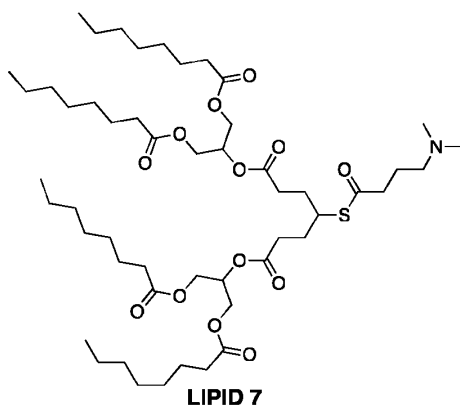
[0312] Synthesis of LIPID 6a



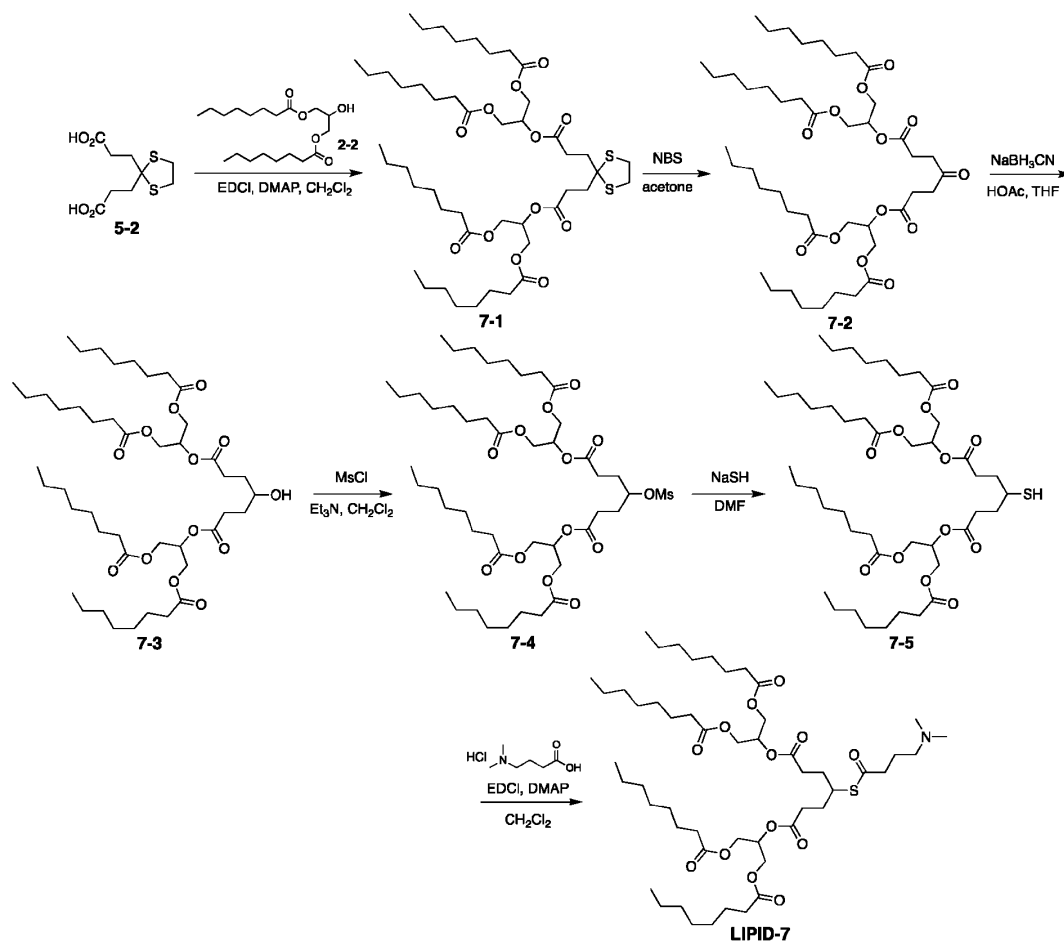
[0313] To a three-neck flask was added CH_2Cl_2 (50 mL, 20 V), **6-4** (2.50 g, 1 eq) as one portion at room temperature, the mixture was then cooled in an ice-water bath under nitrogen and then triphosgene (0.85 g, 1 eq) was added to the reaction mixture at $0\sim 5^\circ\text{C}$ in portions. Pyridine (1.13 g, 5 eq) was added slowly the reaction mixture over 2 ± 0.5 hours. After addition, the

reaction mixture was stirred for 2 h at room temperature. Solvent was evaporated under reduced pressure and the residue was dissolved in anhydrous pyridine (50 ml, 20 V) and cooled in an ice bath under nitrogen. To this was added 3-(dimethylamino)-1-propanethiol hydrochloride (0.41 g, 1.2 eq) at 0 °C. After addition, the above mixture was stirred for 18 h at room temperature. The solvent was removed by rotary evaporation under vacuum. The mixture was diluted with CH₂Cl₂ (50 mL). The organic phase was washed with 10% aq. citric acid solution (3 x 25 mL). The organic phase was dried with anhydrous MgSO₄ and then filtered. Concentration under vacuum gave crude LIPID 6a which was adsorbed on 10 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (50 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using CH₂Cl₂ / MeOH gradient from 100:0 to 98:2. Fractions containing pure products were analyzed, pooled, combined and concentrated under reduced pressure to afford 6 (1.2 g, 41% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.1 min, m/z (Calcd.) 1014.68, (found) 1015.68 (M+H); ¹H NMR (300 MHz, CDCl₃) δ 5.26 (m, 2H), 4.31 (m, 4H), 4.15 (m, 4H), 3.65 (t, *J* = 7.2 Hz, 4H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.65 (t, *J* = 7.1 Hz, 4H), 2.50-2.19 (16H), 1.52-1.70 (10H), 1.36-1.23 (40H), 0.93-0.83 (12H).

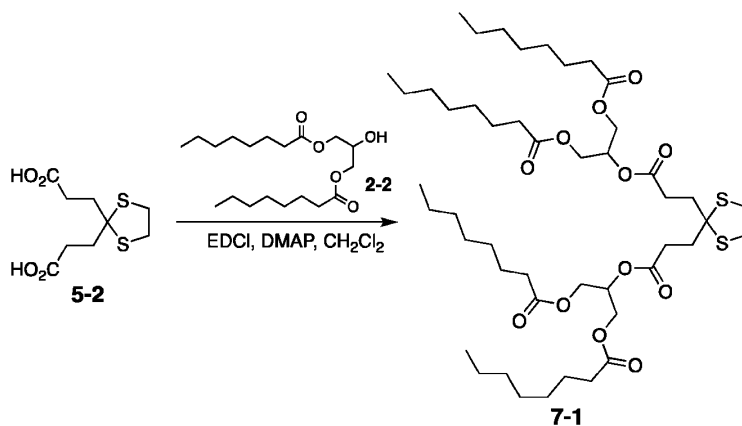
Example 7. Synthesis of LIPID 7: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-((4-(dimethylamino)butanoyl)thio)heptanedioate



[0314] General Scheme:

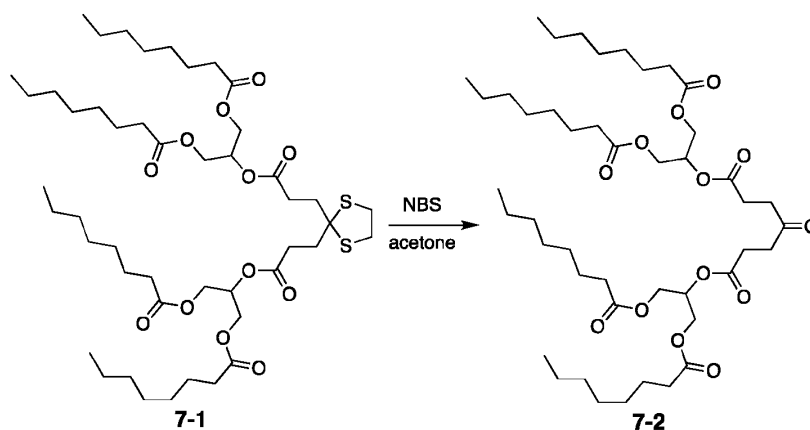


[0315] Synthesis of 7-1: ((3,3'-(1,3-Dithiolane-2,2-diyl)bis(propanoyl))bis(oxy)) bis(propene-2,1,3-triyl) tetraoctanoate



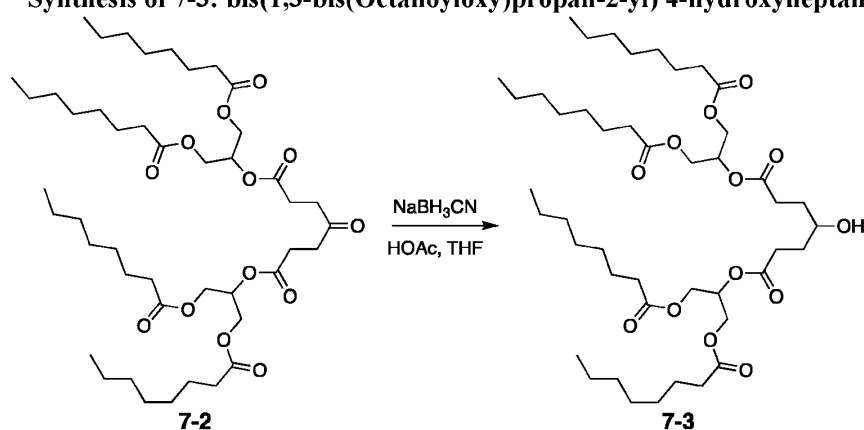
[0316] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **5-2** (16 g, 1.0 equiv) in CH_2Cl_2 (240 mL). This was followed by the addition of **2-2** (48 g, 2.0 equiv), DMAP (23 g, 1.0 equiv), and the mixture was cooled in an ice-water bath under nitrogen. To this cooled solution was added EDCI (36.8 g, 3.0 equiv) at 0 °C in portions over 45 minutes. The resulting solution was stirred for 16 h at room temperature. This reaction mixture was adsorbed on silica gel (110g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (800 g, type: ZCX-2, 100-200 mesh) with petroleum ether/EtOAc gradient from 100:0 to 90:10. The fractions containing pure product was pooled and concentrated under vacuum to afford the **7-1** (60 g, 95% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.60 min, m/z 902.52 (Calcd.), (found) 925.50 ($M+\text{Na}$).

[0317] Synthesis of 7-2: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-oxoheptanedioate



[0318] To a 3-L three-neck round-bottom flask was added acetone (1.5 L,) and 7-1 (60 g, 1.0 equiv), the mixture was cooled to -20°C under nitrogen, then a solution of NBS (47.3 g, 4.0 equiv) in acetone (300 mL) was added dropwise to the reaction mixture over 15 mins. The resulting solution was stirred for 1 h at -20 °C. The reaction was quenched with water (300 mL), warmed to room temperature and concentrated under vacuum to remove acetone. The mixture was extracted with EtOAc (600 mL), the organic phase was dried (Na₂SO₄), filtered, and concentrated under vacuum to give crude 7-2. The solvent was removed under reduced pressure. Crude 7-2 was dissolved in CH₂Cl₂ (200mL) and was adsorbed on silica gel (120g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (800 g, type: ZCX-2, 100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 90:10. The fractions containing pure product was pooled and concentrated under vacuum to afford the 7-2 (44 g, 80% yield) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.36 min, m/z 826.54 (Calcd.), (found) 849.50 (M+Na).

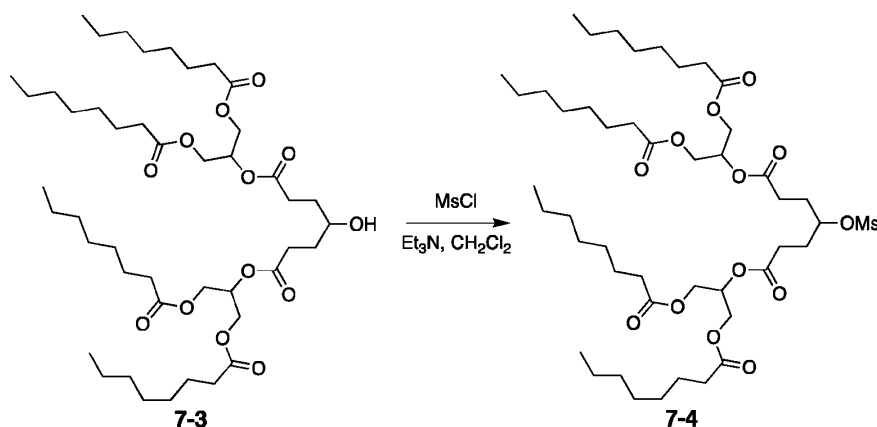
[0319] Synthesis of 7-3: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-hydroxyheptanedioate



[0320] Into a 1-L 4-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 7-2 (44 g, 1.0 equiv) in THF (400 mL). To this was added HOAc (37 g, 12.0 equiv) at 0 °C. This was followed by the addition of NaBH₃CN (39 g, 12.0 equiv) in several batches at 0 °C. The resulting solution was stirred for 18 hr at 25 °C. The reaction was quenched with water (800 mL,). The mixture was extracted with EA (800 mL). The organic phase was separated, dried over Na₂SO₄, filtered and concentrated under vacuum to give crude 7-3. Crude 7-3 was dissolved in CH₂Cl₂ (150mL) and was adsorbed on silica gel (80g, , type: ZCX-2, 100-200 mesh) and purified on a silica gel column (800 g, type: ZCX-2,

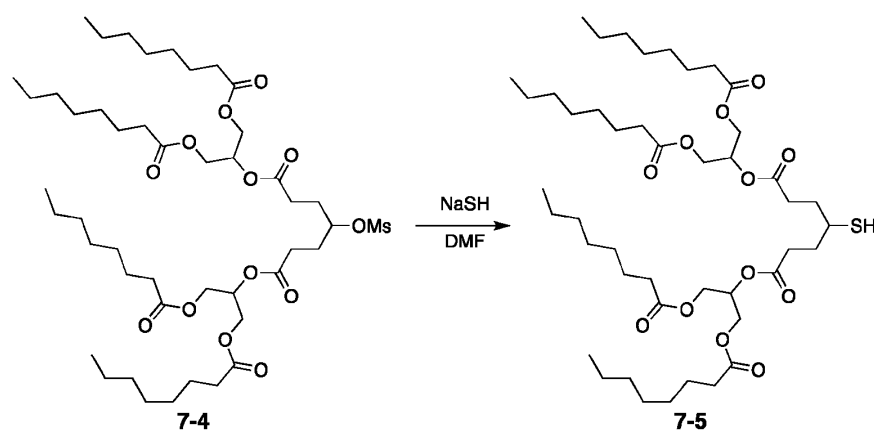
100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 80:20. The fractions containing pure product was pooled and concentrated under vacuum to afford **7-3** (16 g, 36% yield) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.32 min, m/z 828.56 (Calcd.), (found) 851.50 (M+Na).

[0321] Synthesis of 7-4: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-((methylsulfonyl)oxy) heptanedioate



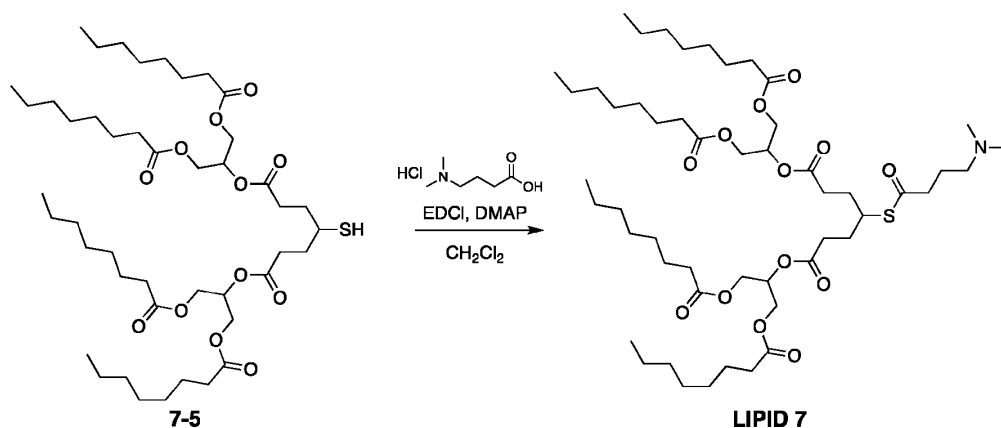
[0322] Into a 500-mL 3-necked round-bottom flask, was placed **7-3** (16 g, 1.0 equiv) and Et₃N (2.4 g, 1.2 equiv) in DCM (160 mL) and the solution was cooled in an ice-water bath under nitrogen. To this cooled solution was added MsCl (2.42 g, 1.1 equiv) dropwise with stirring at 0 °C over 20 min. The resulting solution was stirred for 3 hours at room temperature. The reaction was then quenched by the addition of water/ice (100 mL). The resulting solution was extracted with dichloromethane (2 x 100 mL). The combined organic phases were washed with brine (100 mL). The organic phase was separated, dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure to give crude **7-4**. Crude **7-4** was dissolved in CH₂Cl₂ (75 mL) and was adsorbed on silica gel (32g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (500 g, type: ZCX-2, 100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 80:20. The fractions containing pure product was pooled and concentrated under vacuum to afford **7-4** (10 g, 60% yield) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min, hold 0.7 min): RT 1.26 min, m/z 906.54 (Calcd.), (found) 929.50 (M+Na).

[0323] Synthesis of 7-5: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-((methylsulfonyl)oxy) heptanedioate



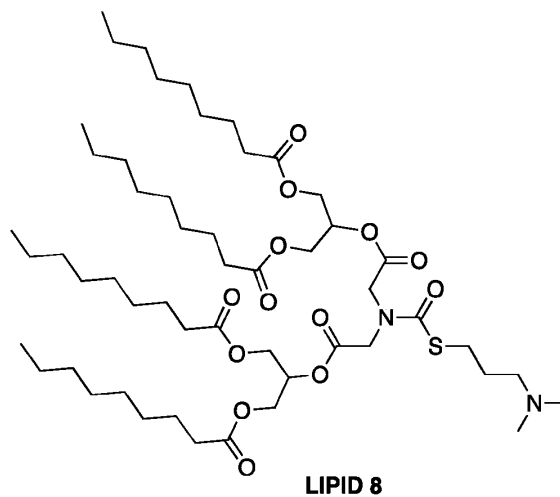
[0324] Into a 500-mL 3-necked round-bottom flask, was placed **7-4** (200 mg, 1.0 equiv) in DMF (4 ml) and the solution was cooled in an ice-water bath under nitrogen. This was followed by the addition of NaSH (37.5 mg, 3.0 equiv) at 0 °C. The resulting solution was stirred for 3 hours at 0 °C. The reaction was then quenched by the addition of water/ice (20 mL). The resulting solution was extracted with ethyl acetate (40 mL,). The organic phase was separated, washed with brine (2 x 30 mL). The same scale reaction process repeated 50 x. The combined mixture (from the repeated reactions) was dried over anhydrous sodium sulfate and concentrated under vacuum to give crude **7-5**. Crude **7-5** was dissolved in THF (75mL) and was adsorbed on silica gel (20 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (200 g, type: ZCX-2, 100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 80:20. The fractions containing pure product was pooled and concentrated under vacuum to afford the **7-5** (3 g, 32% yield) as a light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 1.26 min, m/z 844.54 (Calcd.), (found) 845.65 (M+H).

[0325] Synthesis of LIPID 7: bis(1,3-bis(Octanoyloxy)propan-2-yl) 4-((4-(dimethylamino)butanoyl) thio)heptanedioate

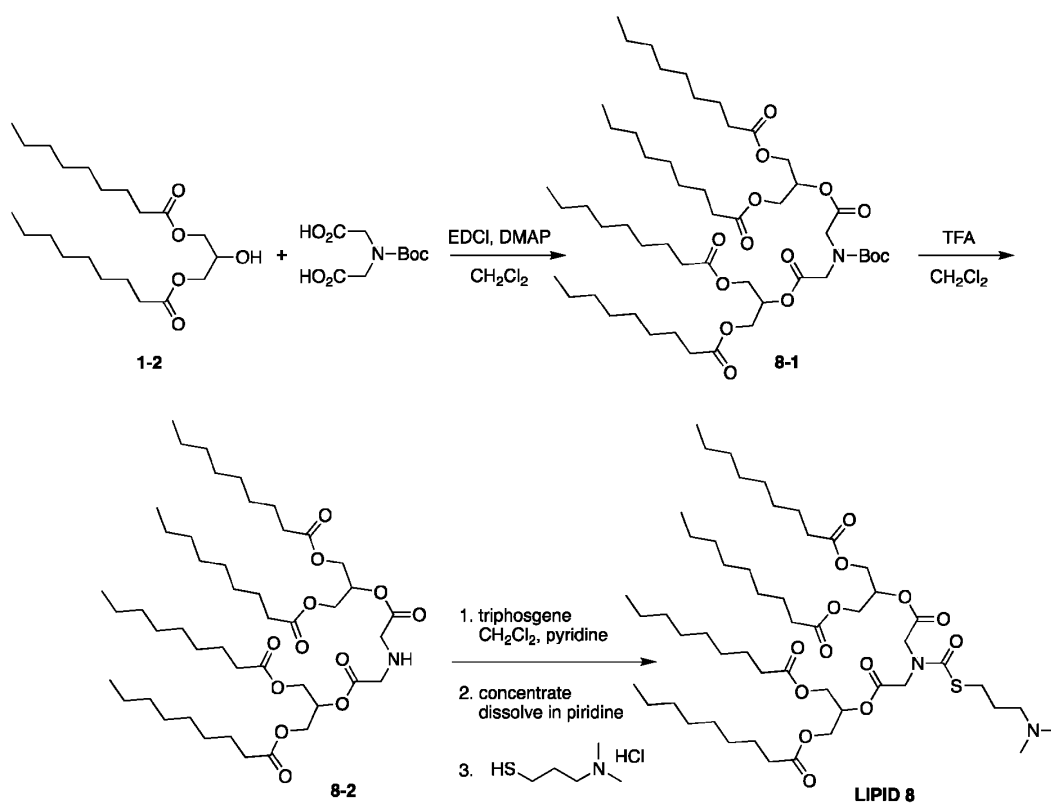


[0326] Into a 100-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **7-5** (3 g, 1.0 equiv) in CH₂Cl₂ (30 mL). 4-(dimethylamino)butanoic acid HCl salt (0.71 g, 1.2 equiv), DMAP (0.43 g, 1.0 equiv) were added and the mixture was cooled in an ice water bath. This was followed by the addition of EDCI (1.02 g, 1.5 equiv) in portions at 0 °C. The resulting solution was stirred for 16 h at room temperature. The reaction mixture was adsorbed on silica gel (250 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (250 g, type: ZCX-2, 100-200 mesh) with an n-heptane/acetone gradient from 100:0 to 50:50. The fractions containing pure product was pooled and concentrated under vacuum to afford LIPID **7** (1.1 g, 32% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 0.75 min, m/z 957.52 (Calcd.), (found) 958.50 (M+H). ¹H-NMR (300 MHz, CDCl₃): δ 5.25 (m, 2H), 4.29 (m, 4H), 4.15 (m, 4H), 3.54 (brm, 1H), 2.62 (t, *J* = 7.4 Hz, 2H), 2.52-2.21 (20H), 2.03 (m, 2H), 1.83 (m, 4H), 1.61 (m, 8H), 1.39-1.17 (28H), 0.97-0.80 (16H).

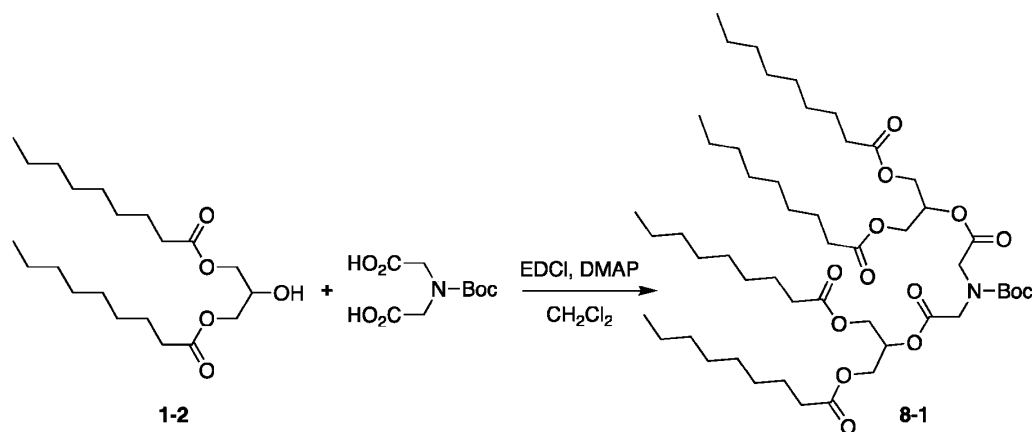
Example 8. Synthesis of LIPID 8: ((2,2'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(acetyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



[0327] General Scheme:

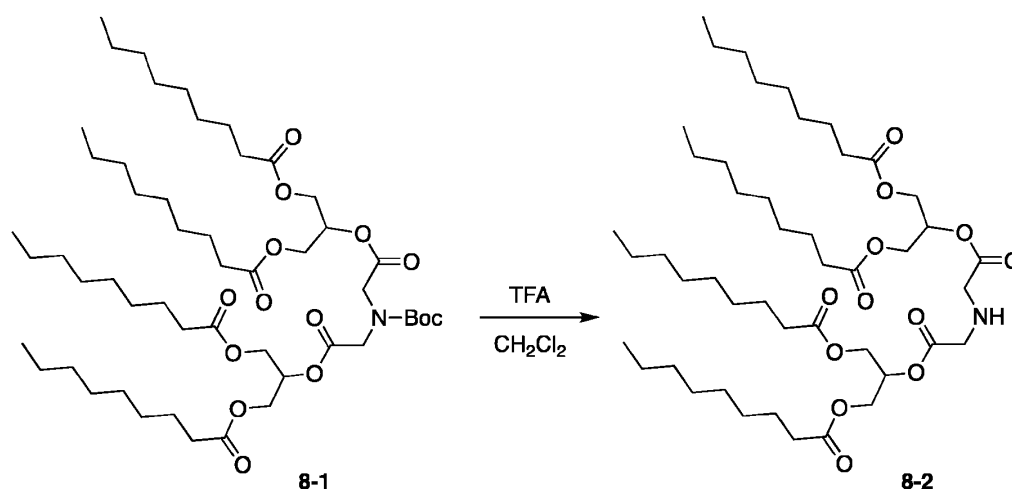


[0328] Synthesis of 8-1: ((2,2'-((*tert*-butoxycarbonyl)azanediyl)bis(acetyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



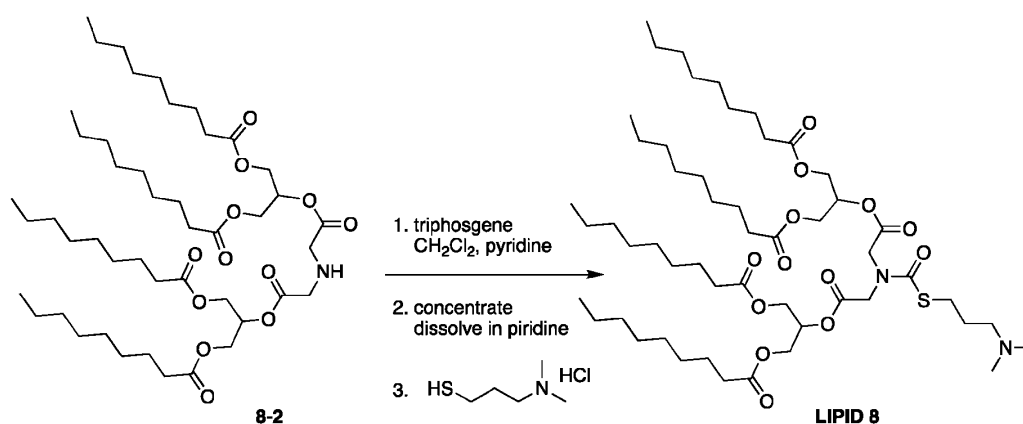
[0329] To a three-necked round-bottom flask was added 2,2'-((*tert*-butoxycarbonyl)azanediyl) diacetic acid (1 g, 1.0 eq), **1-2** (3.50 g, 2.2 eq) and DMAP (0.52 g, 1 eq) in DCM (20 ml), and the solution was cooled in an ice-water bath under nitrogen. To this cooled solution was added EDCI (1.80 g, 2.2 eq) at 0 °C in several portions. The resulting solution was stirred for 16 h at 20 °C. The reaction was quenched with 10% aqueous citric acid solution (10 mL). The organic phase was separated, washed with 10% aqueous citric acid solution (10 mL, 10 V), brine (10 mL, 10 V), and dried with anhydrous MgSO₄. Filtration and concentration under vacuum gave crude **8-1** which was dissolved in CH₂Cl₂ (15 mL), and adsorbed on 5 g of silica gel (type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (20 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using petroleum ether / EtOAc (volume ratio). (gradient from 100:0 to 50:1). Fractions containing pure products were analyzed, pooled, combined and concentrated under reduced pressure to afford **8-1** (3.27 g, 81% yield) as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.2 min, m/z (Calcd.) 941.64, (found) 964.60 (M+Na).

[0330] Synthesis of 8-2: ((2,2'-Azanediylbis(acetyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



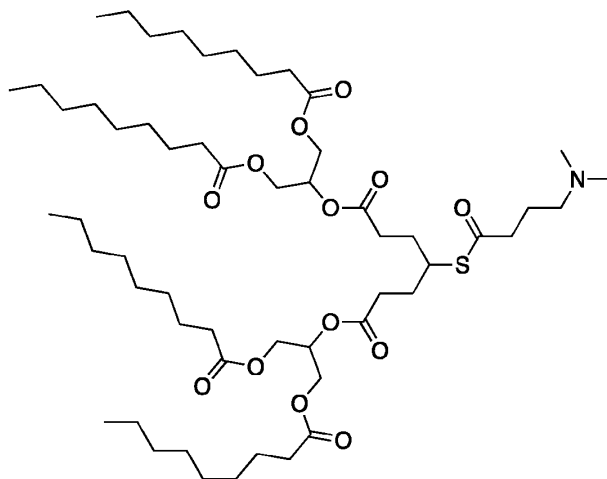
[0331] To a three-necked flask, was added **8-1** (3 g, 1 eq) in CH₂Cl₂ (60 mL) and the resulting solution was cooled in an ice-water bath under nitrogen, then, TFA (4.5 ml) was added slowly at 0~5 °C. The resulting solution was stirred for 2 h at 20 °C. The reaction was then quenched by the careful addition of 10% aqueous sodium carbonate solution (30 mL). The organic phase was separated and washed with brine (2 x 30 mL), dried with anhydrous MgSO₄ and filtered. The solvent was removed under vacuum to afford the **8-2** (2.5 g, 93% yield) as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.15 min, m/z (Calcd.) 841.59, (found) 842.51 (M+H).

[0332] Synthesis of LIPID 8: ((2,2'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(acetyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



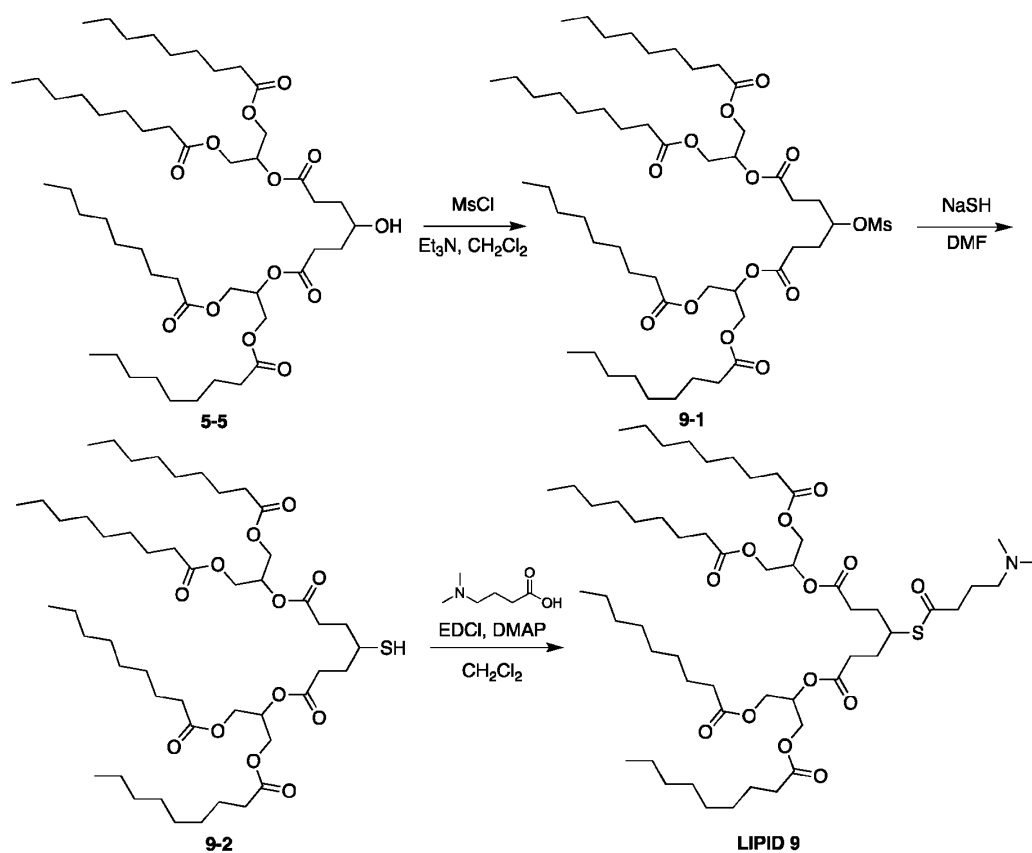
[0333] To a three-necked flash was added **8-2** (2.50 g, 1 eq) in CH₂Cl₂ (50 mL) as one portion at room temperature, the resulting solution was cooled in an ice-water bath under nitrogen, then triphosgene (0.88 g, 1 eq) was added to the reaction mixture at 0~5 °C over 5 minutes. Pyridine (1.17 g, 5 eq) was added slowly to the reaction mixture over 2 ± 0.5 hours. After addition, the reaction mixture was stirred for 2 h at room temperature. Solvent was evaporated under reduced pressure and the residue was dissolved in anhydrous pyridine (50 mL) and cooled in an ice-water bath. To this was added 3-(dimethylamino)-1-propanethiol hydrochloride (0.42 g, 1.2 eq) and the above mixture was stirred for 18 hours at room temperature. Solvent was removed by under vacuum. The residue was dissolved in CH₂Cl₂ (50 mL) and washed with 10% aqueous citric acid solution (3x25 ml). The organic phase was separated, dried with anhydrous MgSO₄ and then filtered. Concentration under vacuum afforded crude LIPID 8, the crude product dissolved in CH₂Cl₂ (15 mL), and adsorbed on silica gel (5g, type: ZCX-2, 100-200 mesh, 6.43 w./w.), and purified on a silica gel column (20 g of silica gel, type: ZCX-2, 100-200 mesh, 32.14 w./w.) using a gradient of CH₂Cl₂ / MeOH (volume ratio, gradient from 100/0 to 98:2). Fractions containing LIPID 8 were analyzed, pooled, combined and concentrated under reduced pressure to afford LIPID 8 (1.2 g, 41% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min): RT 0.88 min, m/z (Calcd.) 986.65, (found) 987.95 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.28 (m, 2H), 4.35 (m, 4H), 4.18 (m, 8H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.340-2.212 (16H), 1.78 (m, 2H), 1.71-1.57 (8H), 1.35-1.20 (40H), 0.94-0.80 (12H).

Example 9. Synthesis of LIPID 9: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-((4-(dimethylamino)butanoyl)thio)heptanedioate

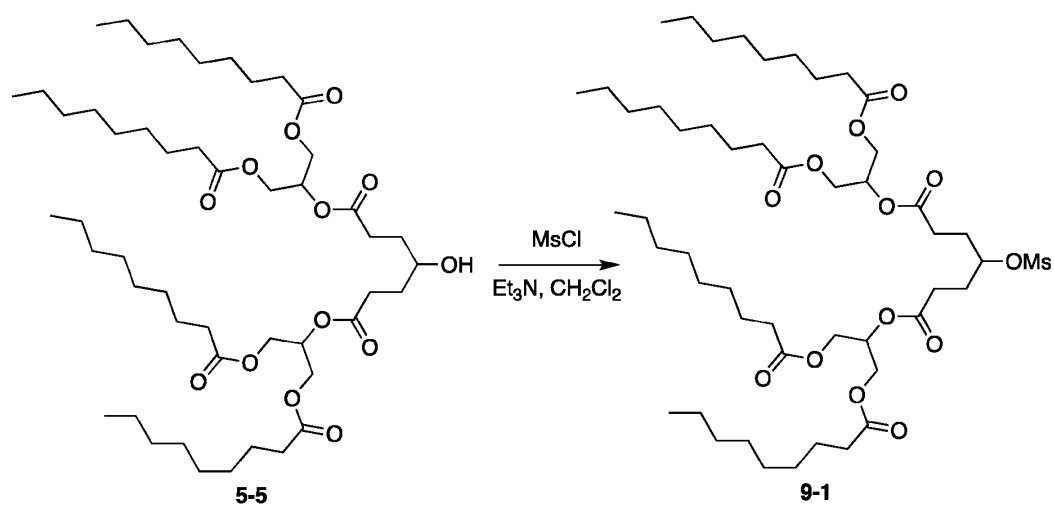


LIPID 9

[0334] General Scheme:

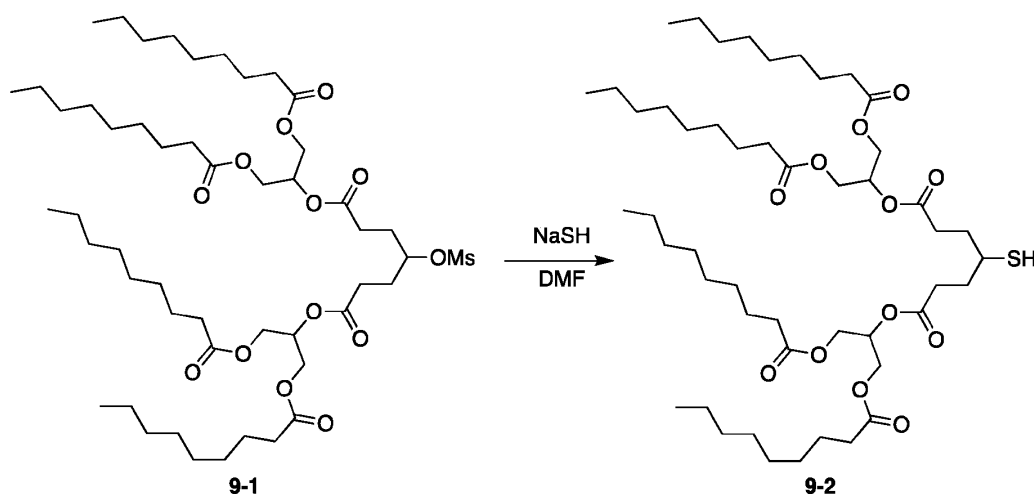


[0335] Synthesis of 9-1: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-((methanesulfonyl)oxy)heptanedioate



[0336] To a 250-mL four-necked round-bottle flask with mechanical stirring under N₂, was added **5-5** (6 g, 1.0 eq) in CH₂Cl₂ (90 mL). This was followed by the addition of Et₃N (2.06 g, 3.0 eq) and the resulting solution was cooled in an ice-water bath under nitrogen. To the cooled solution was added MsCl (1.16 g, 1.5 eq) dropwise with stirring at 0 °C. The resulting solution was stirred for 12 h at room temperature. The reaction was then quenched by the addition of H₂O (100 ml). The phases were separated, and the aqueous layer was extracted with CH₂Cl₂ (100 ml). Then the combined organic phases were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The crude **9-2** was purified by High-Flash-Prep-HPLC with the following conditions: column, XB-C18 silica gel; mobile phase, i-PrOH in 1 mmol NH₄HCO₃ in water, 65% to 95% gradient in 30 min; detector, UV ELSD. Concentrated to dryness under vacuum to afford the **9-1** (5 g, 44% overall yield in 2 steps) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 0.7 min); RT 1.66 min, m/z 962.60 (Calcd.), (found) 985.50 (M+Na).

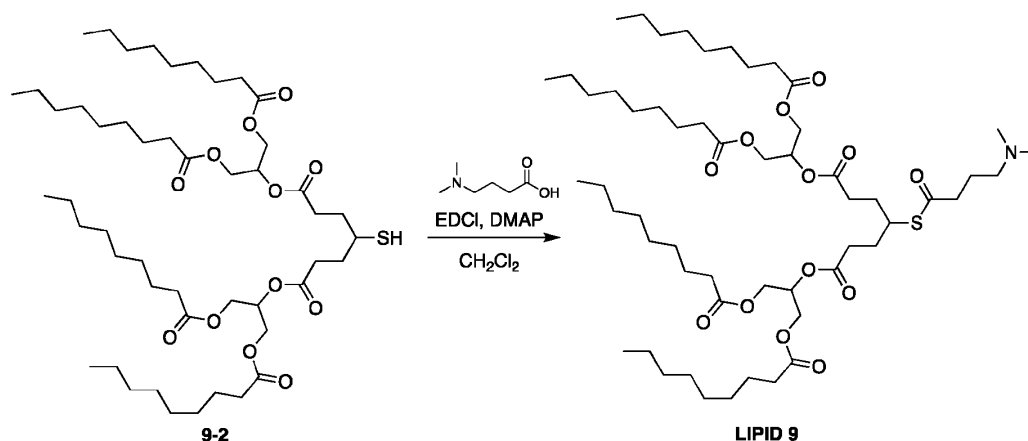
[0337] Synthesis of 9-2: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-mercaptoheptanedioate



[0338] Into a 100-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **9-1** (100 mg, 1.00 equiv) in DMF (0.5 mL), the resulting solution was cooled in an ice-water bath under nitrogen. This was followed by the addition of NaSH (29.1 mg, 5.00 eq) at 0 °C. The resulting solution was stirred for 24 h at 0 °C. This reaction was repeated for 49 x and overall mixture was combined to work up. The reaction was then quenched by the addition of water/ice (200 ml). The resulting solution was extracted with

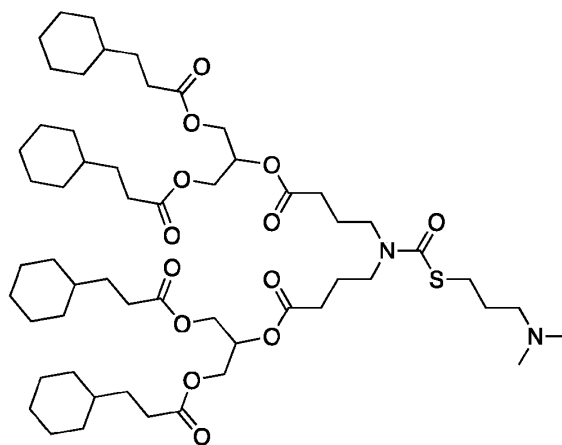
EtOAc (3x100 mL) and the organic phases were combined, dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to afford **9-2** (4.8 g, crude) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.08 min, m/z 900.60 (Calcd.), (found) 923.50 (M+Na).

[0339] Synthesis of LIPID 9: bis(1,3-bis(Nonanoyloxy)propan-2-yl) 4-((4-(dimethylamino)butanoyl)thio)heptanedioate



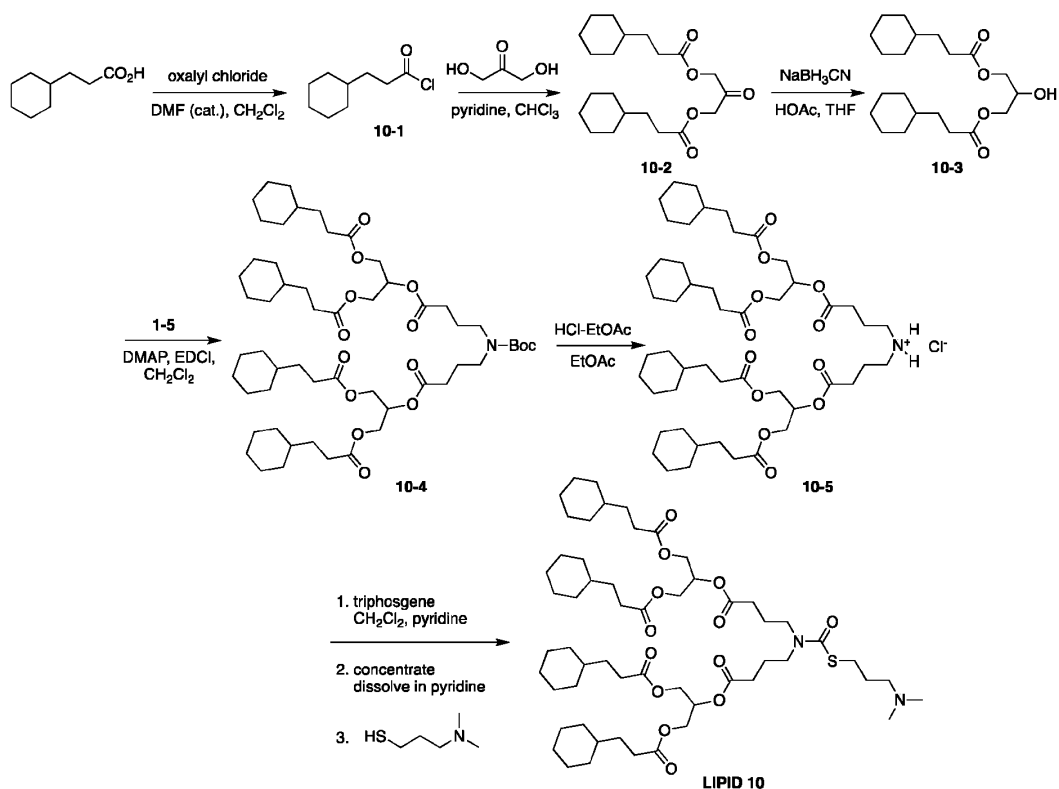
[0340] To a three-necked round-bottom flask was added **9-2** (4.8 g, 1.0 eq), 4-(dimethylamino)butanoic acid (1.16 g, 1.3 eq) and DMAP (0.46 g, 0.7 eq) in CH₂Cl₂ (72 mL), the resulting solution was cooled in an ice-water bath under nitrogen. EDCI (1.84g, 1.4 eq) was added to the reaction mixture at 0 °C in several portions. The resulting solution was stirred for 12 h at room temperature. The reaction system was quenched with 10% aqueous citric acid (48 mL). The organic phase was separated, washed with 10% aqueous citric acid (48 mL), brine (48 mL, 10 V), and dried with anhydrous MgSO₄. Filtration and concentration under vacuum gave crude **9** which was dissolved in CH₂Cl₂ (25 mL) and this was adsorbed on silica gel column (10 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (50 g, type: ZCX-2, 100-200 mesh) with an n-heptane/acetone gradient from 100:0 to 75:50. The fractions containing pure **9** was pooled and concentrated under vacuum to afford the **9** (0.9 g, 18% yield) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.24 min, m/z 1013.68 (Calcd.), (found) 1015.40 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.27 (q, *J* = 5.1 Hz, 2H), 4.30 (m, 4H), 4.16 (m, 4H), 3.54 (brm, 1H), 2.64 (t, *J* = 7.3 Hz, 2H), 2.46-2.23 (18H), 2.04 (m, 2H), 1.84 (m, 4H), 1.35-1.76 (13H), 1.00-1.32 (37H), 0.96-0.83 (12H).

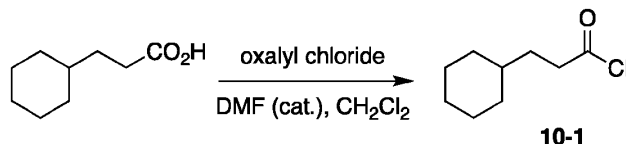
[0341] Example 10. Synthesis of LIPID 10: ((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



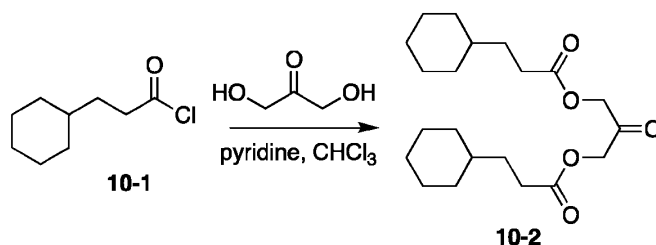
LIPID 10

[0342] General Scheme:



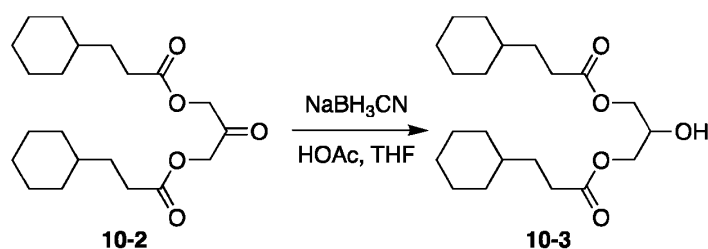
[0343] Synthesis of 10-1: 3-Cyclohexylpropanoyl chloride

[0344] Into a 2000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of 3-cyclohexylpropanoic acid (100 g, 1.0 eq) in CH_2Cl_2 (1 L) and added DMF (0.2 mL). Oxalyl chloride (161.00 g, 2.00 eq) was added in dropwise at r.t. The mixture was stirred at room temperature under nitrogen overnight. The mixture was concentrated under vacuum to give crude **10-1**. This was used as such in the next reaction.

[0345] Synthesis of 10-2: 2-Oxopropane-1,3-diyl bis(3-cyclohexylpropanoate)

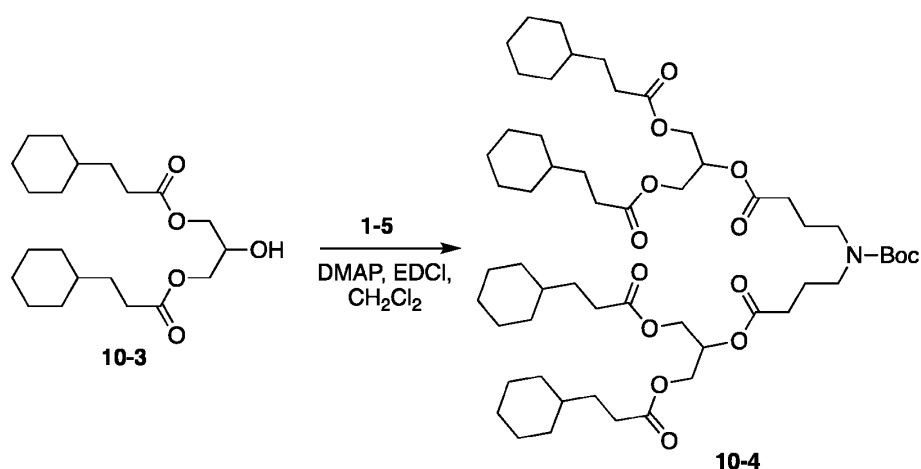
[0346] Into a 2000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **10-1** (83.00 g, 2.50 eq) and dihydroxyacetone (17.20 g, 1.00 eq) in CHCl_3 (1600 mL), the solution was cooled in an ice-water bath under nitrogen. To this cooled solution was added pyridine (61.00 g, 4.00 eq) while maintaining temperature at 0°C over 40 minutes. The mixture was stirred at room temperature under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with CH_2Cl_2 (200 mL). The combined filtrates were then washed with 5% aq. NaHCO_3 (2000 mL), 5% aq. HCl (2000 mL), brine (2000 mL), and dried over Na_2SO_4 . Concentration under vacuum gave 65 g (92.8% yield) of **10-2** as a yellow oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 2.56 min, m/z 366.24 (Calcd.), (found) 367.40 (M+H).

[0347] Synthesis of 10-3: 2-Hydroxypropane-1,3-diyl bis(3-cyclohexylpropanoate)



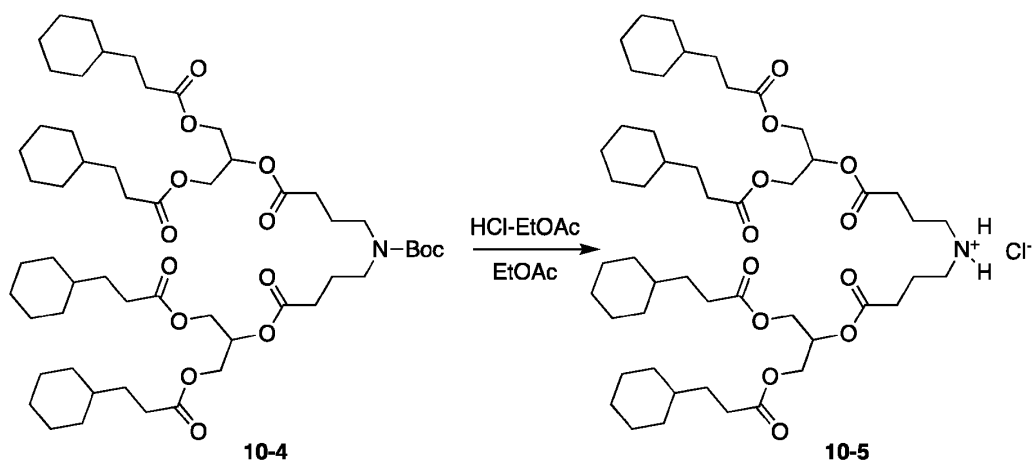
[0348] Into a 2000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **10-2** (85.00 g, 1.00 eq) in THF (1 L). To the solution was added HOAc (18.00 g, 1.30 eq) and the solution was cooled in an ice-water bath. To this cooled solution was added NaBH₃CN (18.00 g, 1.20 eq) at 0 °C. The mixture was stirred overnight at r.t. The reaction mixture was quenched with brine (1 L). The mixture was extracted with EtOAc (3 x 400 mL). The combined organic phases were then washed with 5% aq. NaHCO₃ (200 mL), 5% aq. HCl (200 mL), brine (200 mL), and dried over Na₂SO₄. Filtration and concentration under vacuum gave crude **10-3** which was dissolved in CH₂Cl₂ (500 mL) and adsorbed on silica gel (240 g, type: ZCX-2, 100-200 mesh). The crude material was purified on a silica gel column (800 g, type: ZCX-2, 100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 90:10. The fractions containing pure product were pooled and concentrated under vacuum to afford the **10-3** (61 g (70.1% yield)) as a yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.50 min, m/z 368.26 (Calcd.), (found) 351.2 (M-H₂O).

[0349] Synthesis of 10-4: ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



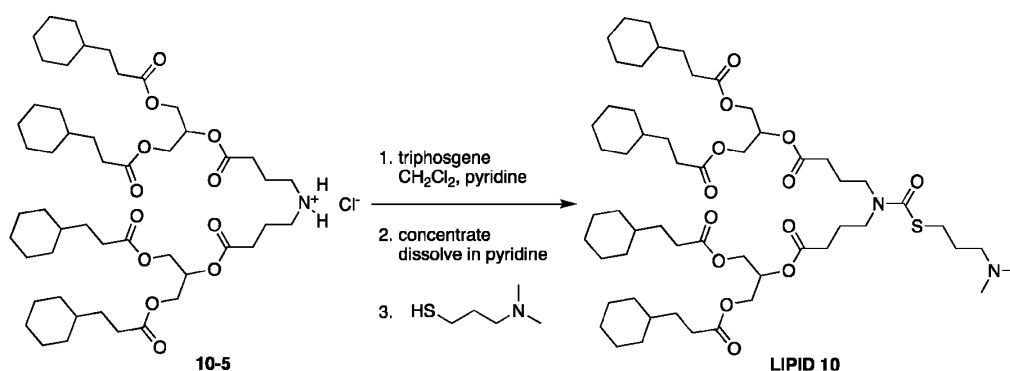
[0350] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **1-5** (4.00 g, 1 eq) and **10-3** (10.20 g, 2 equiv), and the mixture was dissolved in CH₂Cl₂ (80 mL). The solution was cooled in an ice-water bath and DMAP (1.69 g, 1.00 eq) and EDCI (10.60 g, 4.00 eq) were added in order at 0 °C. The reaction mixture was stirred overnight at r.t. The reaction was then quenched with 10% aq. citric acid (200 mL) and the organic phase was separated. The organic phase was washed with 10% aq. NaHCO₃ (200 mL), brine (200 mL), and was dried over anhydrous sodium sulfate. Filtration and concentration under vacuum gave crude **10-4** which was dissolved in CH₂Cl₂ (100 mL) and adsorbed on silica gel (50 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (400 g, type: ZCX-2, 100-200 mesh) with a petroleum ether/EtOAc gradient from 100:0 to 90:10. The fractions containing pure product was pooled and concentrated under vacuum to afford 11 g (77.7% yield) of **10-4** as light yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 1.79 min, m/z 989.64 (Calcd.), (found) 1012.50 (M+Na).

[0351] Synthesis of 10-5: bis(4-((1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl)oxy)-4-oxobutyl)ammonium chloride



[0352] Into a 250 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **10-4** (6.30 g, 1.00 eq) in EtOAc (20 mL) and the solution was cooled in an ice-water bath. To the cooled solution was added HCl in EtOAc (60 mL, 10 eq, 2M) dropwise at 0-10 °C. The resulting solution was stirred overnight at room temperature. The resulting mixture was concentrated under vacuum. This provided in 6 g (99% yield) of **10-5** as light yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 1.48 min, m/z 889.59 (Calcd.), (found) 890.50 (M+H).

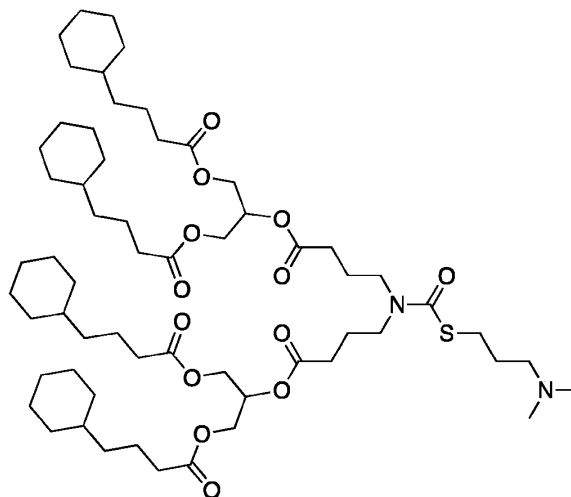
[0353] **Synthesis of LIPID 10: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)**



[0354] Into a 250-mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **10-5** (6.00 g, 1.00 eq) in CH₂Cl₂ (100 mL) and the solution was cooled in an ice-water bath under nitrogen. To the mixture was added

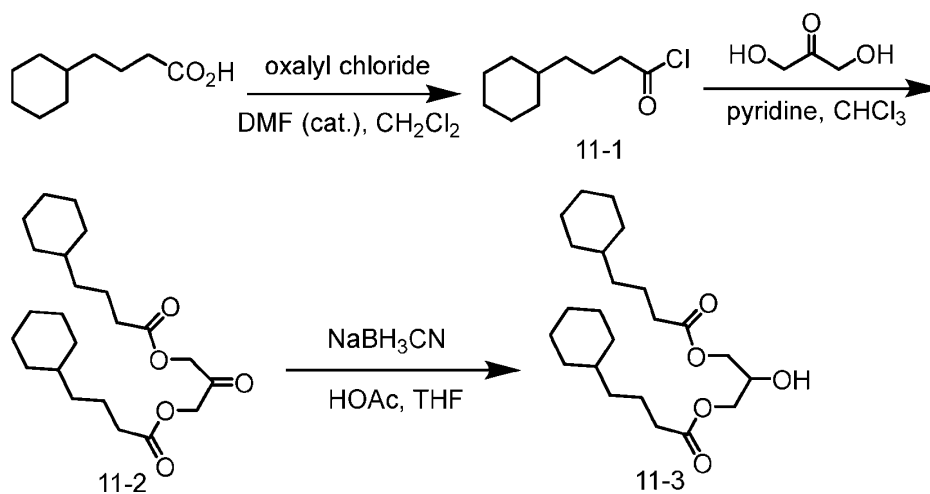
triphosgene (1.91 g, 1.62 eq) at 0 °C. This was followed by the addition of pyridine (2.56 g, 5.00 eq) dropwise with stirring at 0 °C. The mixture was stirred for 4 h at r.t and then concentrated under vacuum. The residue was dissolved with pyridine (100 mL) and was cooled in an ice-water bath under nitrogen. To this solution was added 3-(dimethylamino)propane-1-thiol (0.92 g, 1.93 eq) dropwise with stirring at 0 °C over 10 min. The resulting solution was stirred overnight at room temperature. The resulting mixture was concentrated under vacuum. The residue was diluted with CH₂Cl₂ (500 mL) and the solution was washed with 10% aq. citric acid (2 x 200 mL), saturated aq. NaHCO₃ (2 x 200 mL), brine (100 mL), dried over anhydrous sodium sulfate and concentrated under vacuum to give crude LIPID 10. The residue was dissolved in CH₂Cl₂ (100 mL) and was adsorbed on silica gel (50 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (250 g, type: ZCX-2, 100-200 mesh) with a CH₂Cl₂/MeOH gradient from 100:0 to 97:3. The fractions containing pure product were pooled and concentrated under vacuum. The product was dissolved in n-heptane (40 mL) and 0.22 g activated Charcoal powder was added. The mixture was stirred for 4 h at r.t and then filtered. The filtrate was concentrated under vacuum to yield 2 g (48% yield) of LIPID 10 as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.68 min, m/z 1034.65 (Calcd.), (found) 1035.65 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.25 (m, 2H), 4.31 (m, 4H), 4.14 (dd, *J* = 11.9, 5.7 Hz, 4H), 3.38 (brm, 4H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.44 – 2.22 (20H), 1.99-1.47 (26H), 1.39-1.17 (20H), 0.95 – 0.79 (12H).

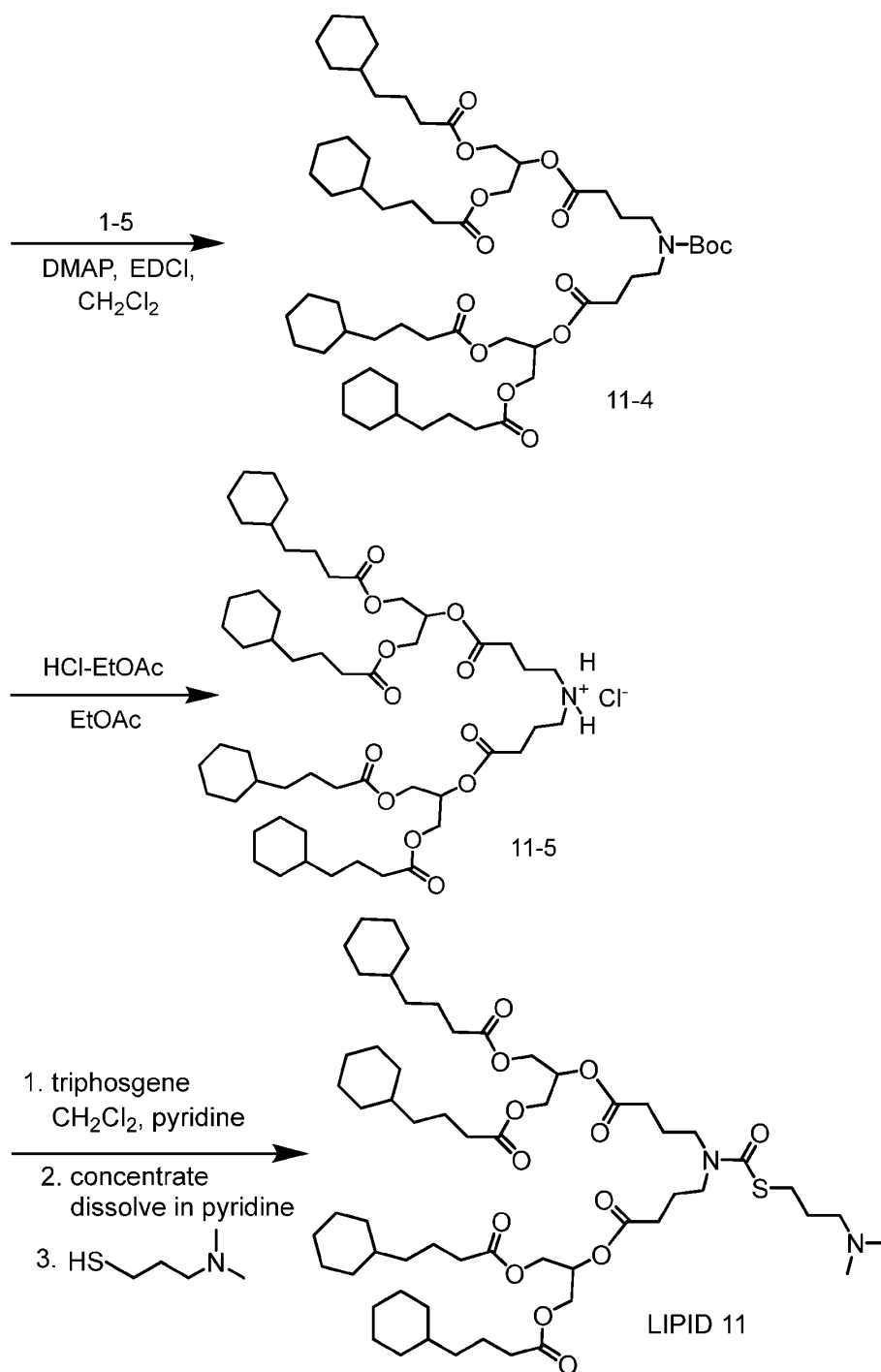
Example 11. Synthesis of LIPID 11: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-cyclohexylbutanoate)

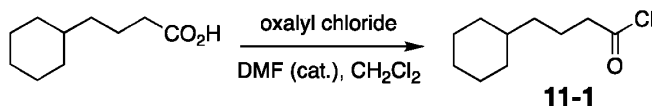


LIPID 11

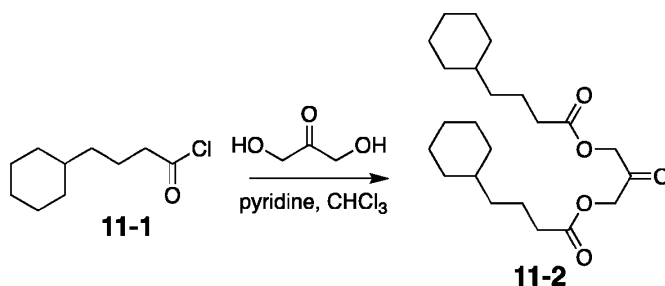
[0355] General Scheme:





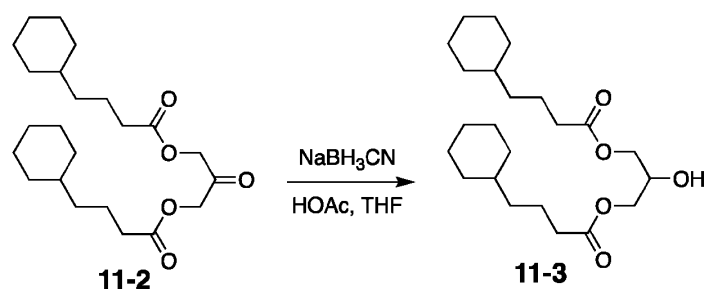
[0356] Synthesis of 11-1: 4-Cyclohexylbutanoyl chloride

[0357] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of 4-cyclohexylbutanoic acid (50 g, 1.0 eq) in CHCl_3 (500 ml), and then, DMF (0.25 ml) was added. The solution was cooled in an ice-water bath then oxalyl chloride (74.1 g, 2.0 eq) was added dropwise over a period of 30 minutes. The mixture was stirred at room temperature under nitrogen overnight. The mixture was concentrated under vacuum. This resulted in 55.7 g (99.9% yield) **11-1** as yellow oil that was used as such in the next step.

[0358] Synthesis of 11-2: 2-Oxopropane-1,3-diyl bis(4-cyclohexylbutanoate)

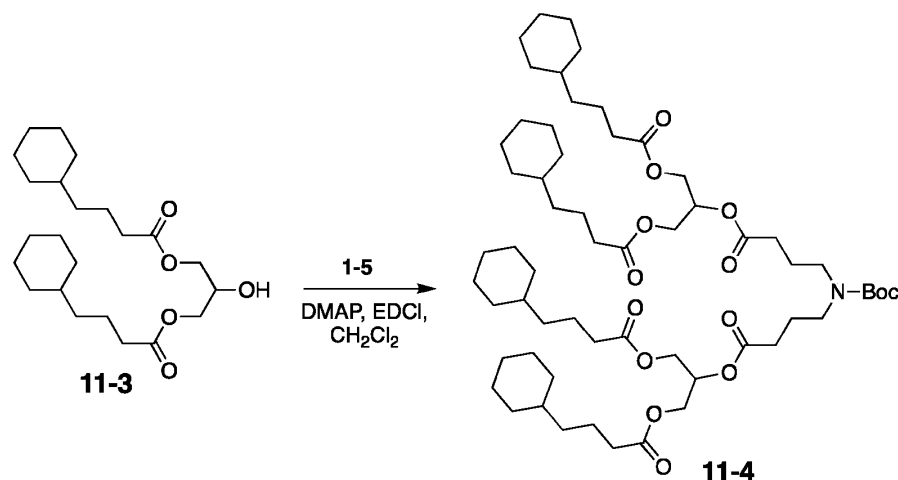
[0359] Into a 2 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **11-1** (55.7 g, 2.5 eq), the solution was cooled in an ice-water bath and 1,3-dihydroxyacetone (10.6 g, 1.0 eq) in CHCl_3 (1114 mL), was then added over a period of 1 hour. Pyridine (37.3 g, 4.0 eq) was added to the mixture while maintaining temperature at 0 °C over 40 minutes. The mixture was stirred at room temperature overnight under nitrogen. The reaction mixture was quenched with water (1 L). The organic phase were separated and the aqueous layer was extracted with CH_2Cl_2 (3 x 200 mL). The organic phases were combined and washed with 5% aqueous NaHCO_3 solution (300 mL), 5% aqueous HCl (300 mL), and brine (300 mL). The solution was then dried over anhydrous Na_2SO_4 and the product was obtained by evaporation. This resulted in 56.4 g of crude **11-2** as yellow oil that was used as such in the next reaction.

[0360] Synthesis of 11-3: 2-hydroxypropane-1,3-diyl bis(4-cyclohexylbutanoate)



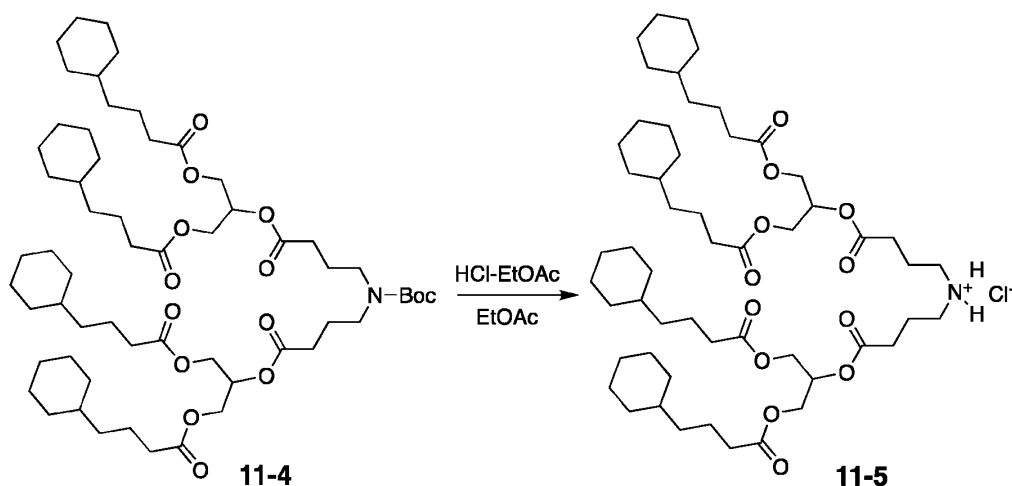
[0361] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **11-2** (56.4 g, 1.0 eq) in THF (550 ml), the mixture was cooled in an ice-water bath. To the cooled solution was added HOAc (11.13 g, 1.3 eq) at 0°C. And then to the mixture was added NaBH₃CN (10.79 g, 1.2 eq) at 0 °C. The mixture was stirred overnight at room temperature. The reaction mixture was quenched with water (500 mL). The mixture was extracted with CH₂Cl₂ (3 x 200 ml). The organic layers were combined and washed with 5% aqueous NaHCO₃ solution (200 ml), 5% aqueous HCl (200 ml), and brine (200 ml). The solution was then dried over anhydrous Na₂SO₄ and concentrated under vacuum. The residue was dissolved in CH₂Cl₂ (150 mL), silica gel was added (60g, type: ZCX-2, 100-200 mesh) and the crude product was adsorbed on the silica gel silica gel and purified on a silica gel column (240 g, type: ZCX-2, 100-200 mesh) with a petroleum ether / EtOAc gradient from 100:0 to 80:20. The fractions containing **11-3** were pooled and concentrated and dried under vacuum to get 30.3 g (65.0% overall yield in 2 steps) of **11-3** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 0.76 min, m/z 396.29 (Calcd.), (found) 419.29 (M+Na).

[0362] Synthesis of **11-4**: ((4,4'-((*tert*-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propene-2,1,3-triyl) tetrakis(4-cyclohexylbutanoate)



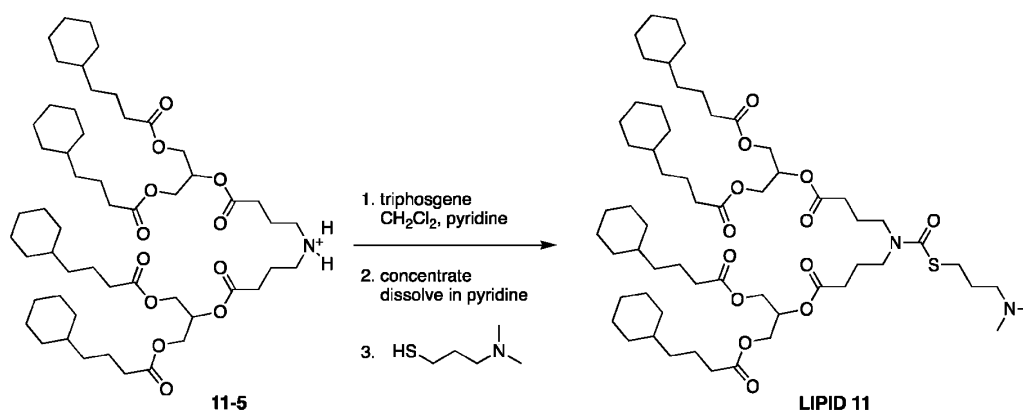
[0363] Into a 250 ml 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **11-3** (5.46 g, 2.0 eq) to the solution of **1-5** (2.05 g, 1.0 eq) in CH₂Cl₂ (100 mL), and the mixture was cooled in an ice-water bath. To the solution were added DMAP (2.3 g, 1.0 eq) followed by EDCI (14.51 g, 4.0 eq) at 0 °C. The reaction mixture was stirred overnight at room temperature. The reaction was then quenched with 10% aqueous citric acid (200 mL), the organic phase was separated and washed with 10% aqueous NaHCO₃ (200 mL), and brine (200 mL). The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. This material was combined with another 7.05 g scale reaction (based on **11-3**) and the combined material was adsorbed on silica gel (30 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (90 g, type: ZCX-2, 100-200 mesh) with a petroleum ether / EtOAc gradient from 100:0 to 65:35. The fractions containing **11-4** were pooled and concentrated and dried under vacuum to get 14.2 g (43.0%) **11-4** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 2.77 min, m/z 1045.71 (Calcd.), (found) 1068.55 (M+Na).

[0364] Synthesis of 11-5: bis(4-((1,3-bis((4-cyclohexylbutanoyl)oxy)propan-2-yl)oxy)-4-oxobutyl) ammonium chloride



[0365] Into a 250 ml round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed solution of **11-4** (14.17 g, 1.0 eq) in EtOAc (71 mL), and the solution was cooled in an ice-water bath. To the solution was added HCl in EtOAc (142 mL, 2 mol/L) dropwise at 0-10 °C. The resulting solution was stirred overnight at room temperature. The resulting mixture was concentrated under vacuum. This provided 13.6 g (99.9% yield) **11-5** as light yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min): RT 0.94 min, m/z 945.65 (Calcd.), (found) 946.60 (M+H).

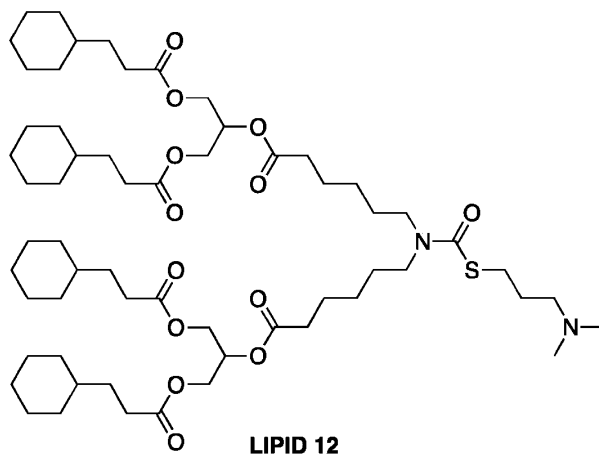
[0366] Synthesis of LIPID 11: ((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-cyclohexylbutanoate)



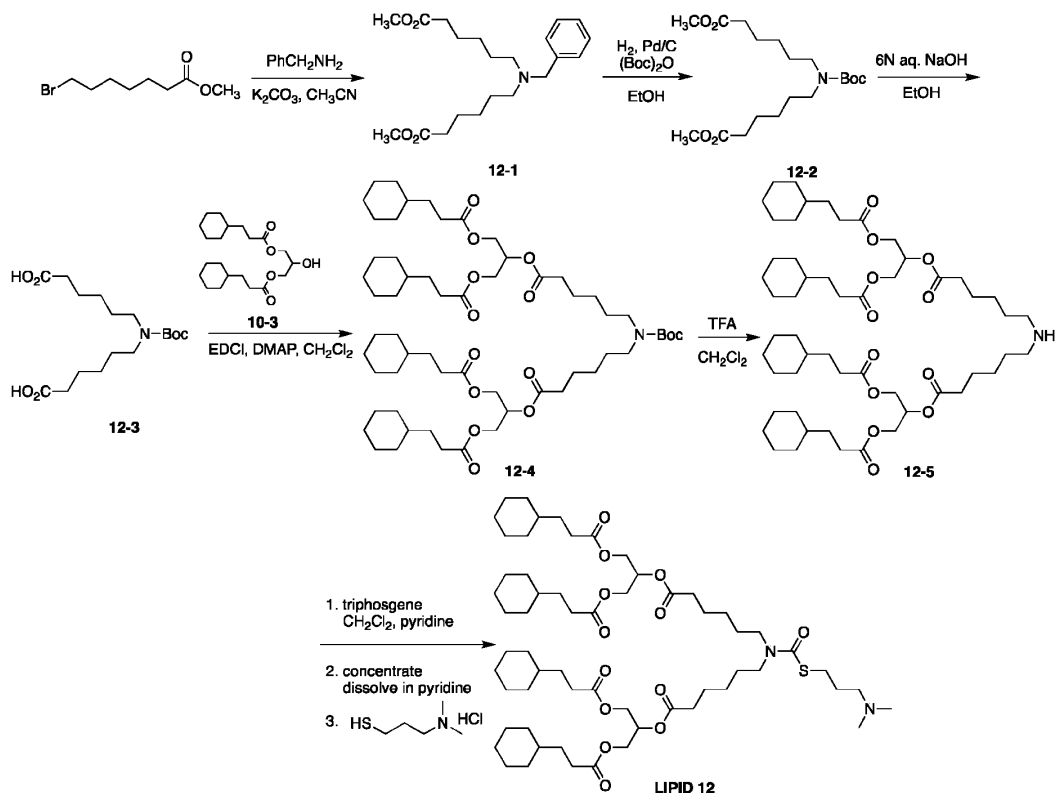
[0367] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **11-5** (12.0 g, 1.0 eq) in CH₂Cl₂ (420 mL) and the solution

was cooled in an ice-water bath. To the solution was added triphosgene (5.44 g, 1.5 eq) at 0°C. This was followed by the addition of pyridine (4.82 g, 5.0 eq) dropwise with stirring at 0°C. The mixture was stirred for 4 h at room temperature and then concentrated under vacuum. The residue was dissolved in pyridine (240 mL) and the resulting solution was cooled in an ice-water bath. To this solution was added 3-(dimethylamino)propane-1-thiol (2.91 g, 2.0 eq) dropwise with stirring at 0°C over 10 min. The resulting solution was stirred overnight at room temperature. The reaction mixture was concentrated under vacuum. The residue was diluted with DCM (200 mL). The mixture was washed with 10% aqueous NaHCO₃ (2 x 200 mL), brine (100 mL), dried over anhydrous sodium sulfate, filtered and concentrated under vacuum. The crude product was adsorbed on silica gel (20 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (80 g, type: ZCX-2, 100-200 mesh) with an n-heptane / acetone gradient from 100:0 to 65:35. The fractions containing pure product were pooled and concentrated and dried under vacuum to get 2 g **11** that was dissolved in n-heptane (40 mL, 20 V) and activated charcoal powder (0.22 g) was added. The mixture was stirred for 4 h at room temperature and then filtered. The filtrate was concentrated under vacuum. This resulted in 2 g (14.4% yield) of **11** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min, hold 0.7 min; RT 1.00 min, m/z 1090.71 (Calcd.), (found) 1091.60 (M+H); ¹H NMR (300 MHz, CDCl₃): δ 5.24 (m, 2H), 4.32 (dd, *J* = 11.9, 4.4 Hz, 4H), 4.15 (dd, *J* = 11.9, 5.7 Hz, 4H), 3.39 (brm, 4H), 2.92 (t, *J* = 7.3 Hz, 2H), 2.27-2.38 (14H), 2.05 (s, 6H), 1.85-2.00 (6H), 1.57-1.83 (29H), 1.11-1.28 (23H), 1.06-1.08 (8H).

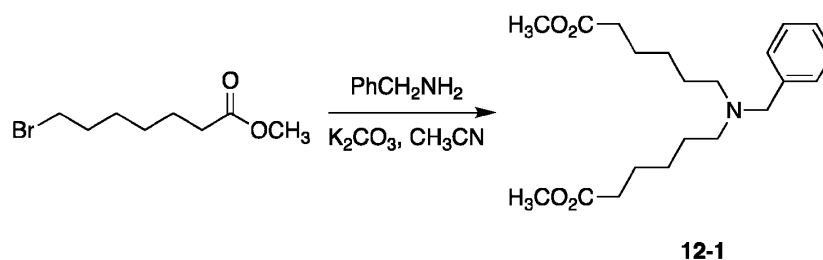
Example 12. Synthesis of LIPID 12: (((6,6'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(hexanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



[0368] General Scheme:

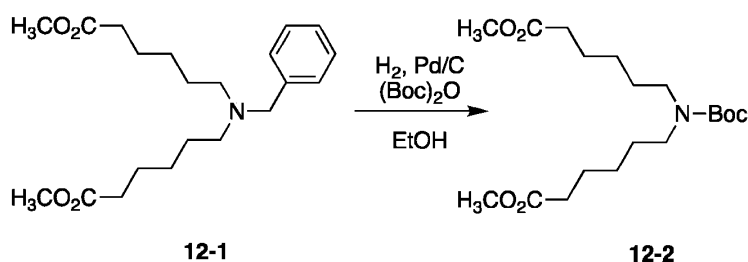


[0369] Synthesis of 12-1: Dimethyl 6,6'-((benzylazanediyl)di)hexanoate



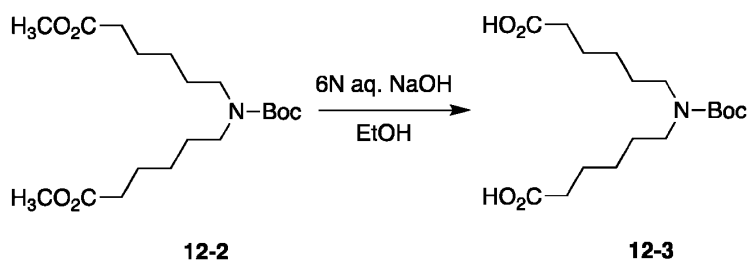
[0370] Charged K_2CO_3 (9.5 g, 2.3 eq), benzyl amine (3.2 g, 1.0 eq) and methyl 4-bromobutyrate (15.2 g, 2.3 eq) in CH_3CN (64 mL) to a 250 ml four neck round bottle flask at 25 °C with mechanical agitation under N_2 . The mixture was then heated (80°C) and stirred for 15 h, The mixture was then cooled to 25 °C and the mixture was cast into water (65 mL) and the mixture was extracted with EtOAc (2 x 65 ml). The combined organic phases were dried (Na_2SO_4), filtered, and concentrated under vacuum to give crude **12-1** (10 g, crude) as yellow oil that was used as such in the next reaction.

[0371] **Synthesis of 12-2: Dimethyl 6,6'-((*tert*-butoxycarbonyl)azanediyl)dihexanoate**



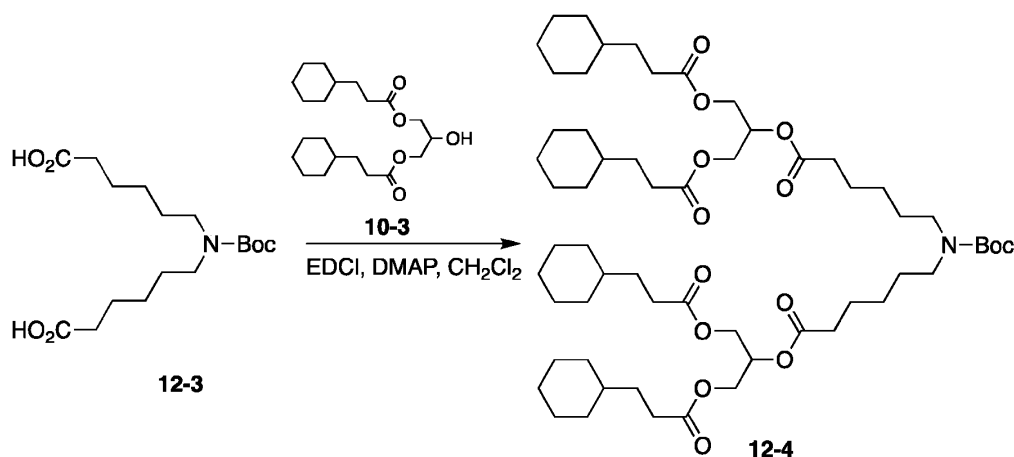
[0372] Charged **12-1** (10 g, 1.0 eq), $(Boc)_2O$ (6.6 g, 1.1 eq) and Pd/C (1 g, 10% w/w) in EtOH (100 mL) into the 250 ml hydrogenation autoclave at ambient temperature. The mixture was stirred for 16 hrs at room temperature under 5 atm in the hydrogen atmosphere. The reaction mixture was filtered and concentrated under vacuum at 40 °C. This resulted in **12-2** (11 g, crude) as light-brown oil. This was used as such in the next reaction.

[0373] **Synthesis of 12-3: 6,6'-((*tert*-Butoxycarbonyl)azanediyl)dihexanoic acid**



[0374] To a round bottom flask charged a solution of **12-2** (11 g, 1.0 eq) in ethanol (55 mL), at room temperature under nitrogen. 6 M aqueous NaOH (55 mL) was added at room temperature. After the addition was complete the mixture was heated to 60 °C for 2 hours. The mixture was cooled to room temperature and was cast into brine (110 mL). The solution was extracted twice with n-BuOH/n-heptane (2:1, 110 mL) to remove the organic impurities. The aqueous phase was acidified by the addition of 3 mol/L aqueous HCl to about pH=3 and then extracted with t-BuOH:n-heptane (2:1) (110 mL, 2 x). The combined organic phases were concentrated under reduced pressure to give a sticky solid. The residue was slurried with diethyl ether (22 mL) and filtered. Collected the filter cake to give **12-3** (5.6 g, 66% overall yield in three steps) as a white solid. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 1.64 min, m/z 345.22 (Calcd.), (found) 368.10 (M+Na).

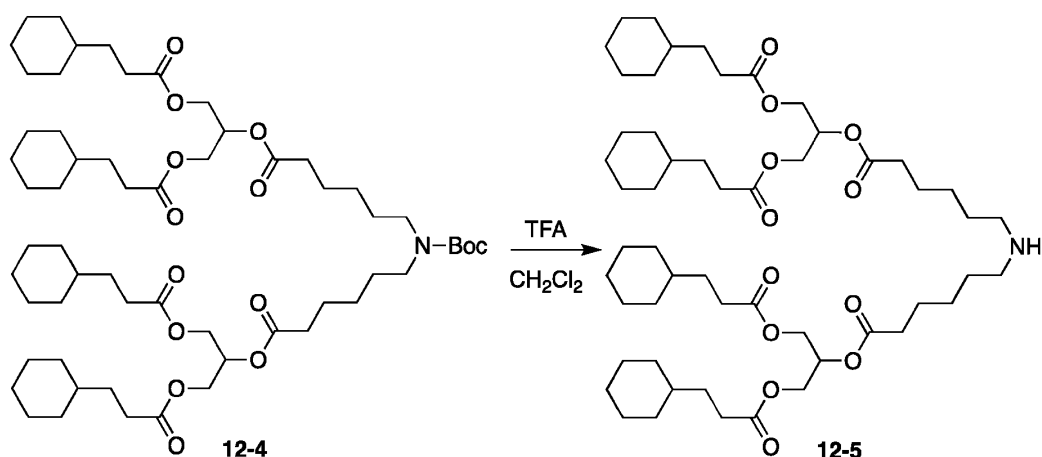
[0375] **Synthesis of 12-4: ((6,6'-((*tert*-Butoxycarbonyl)azanediyl)bis(hexanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)**



[0376] Into a 250 mL three-necked round-bottom flask, was placed **12-3** (5.6 g, 1.0 eq), **10-3** (10 g, 2.0 eq) and DMAP (1.65 g, 1.0 eq) in CH₂Cl₂ (85 mL), and the solution was cooled in an ice-water bath under nitrogen. EDCI (7.5 g, 2.2 eq) was added to the reaction mixture at 0 °C, in portions over 15 minutes. After the addition was complete the reaction was warmed to room temperature and was stirred for 16 h at 20 °C. The reaction mixture was cast into 10% aqueous citric acid (112 mL). The organic phase was separated, 10% aqueous citric acid (112 mL), brine (112 mL, dried with anhydrous MgSO₄ and then filtered. The solvent was removed under vacuum to give crude **12-4** which was dissolved with CH₂Cl₂ (65 mL) and the crude product was adsorbed

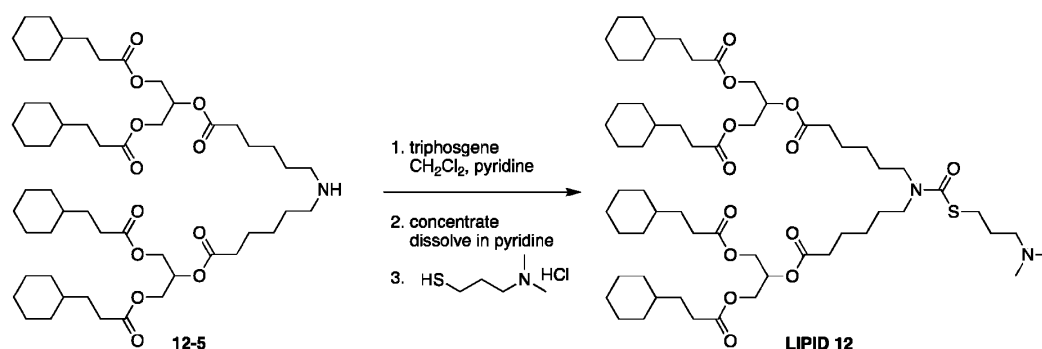
on silica gel (30 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (150 g, type: ZCX-2, 100-200 mesh) with a petroleum ether / EtOAc gradient from 100:0 to 88:12. The fractions containing pure product were pooled and concentrated and dried under vacuum to get 10.1 g (60%) of **12-4** as colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 2.2 min, m/z 1045.71 (Calcd.), (found) 1068.65 (M+Na).

[0377] Synthesis of 12-5: ((6,6'-Azanediylbis(hexanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



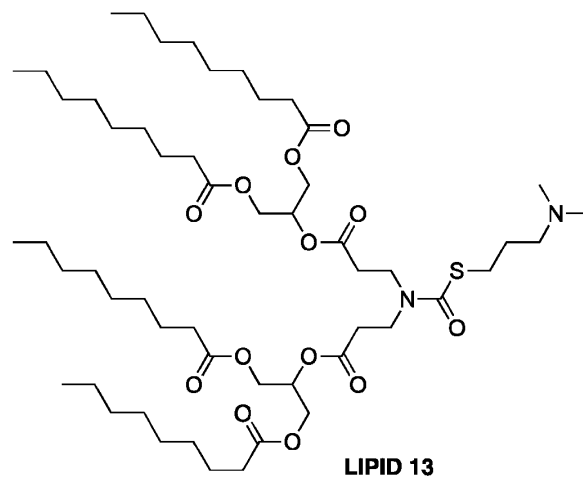
[0378] Into a 100 mL three-neck round-bottom flask, was placed **12-4** (5 g, 1.0 eq) in CH₂Cl₂ (50 mL) and the solution was cooled in an ice-water bath under nitrogen. Then TFA (7.5 mL) was added to reaction mixture at 0-15 °C. After the addition was complete the solution was allowed to warm to room temperature and was stirred for 2 h. The mixture was concentrated under vacuum at 30 °C, then n-heptane (100 mL) was added to the reaction mixture. The resulting cloudy mixture was washed with 17% aqueous sodium carbonate solution (500 mL), brine (250 mL, 3 x), and dried over anhydrous MgSO₄. Filtration and concentration under vacuum afforded **12-5** (4.5 g, 90% yield) as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.94 min, m/z 945.65 (Calcd.), (found) 946.60 (M+H).

[0379] Synthesis of LIPID 12: ((6,6'-(((3(dimethylamino)propyl)thio)carbonyl)azanediyl)bis(hexanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)

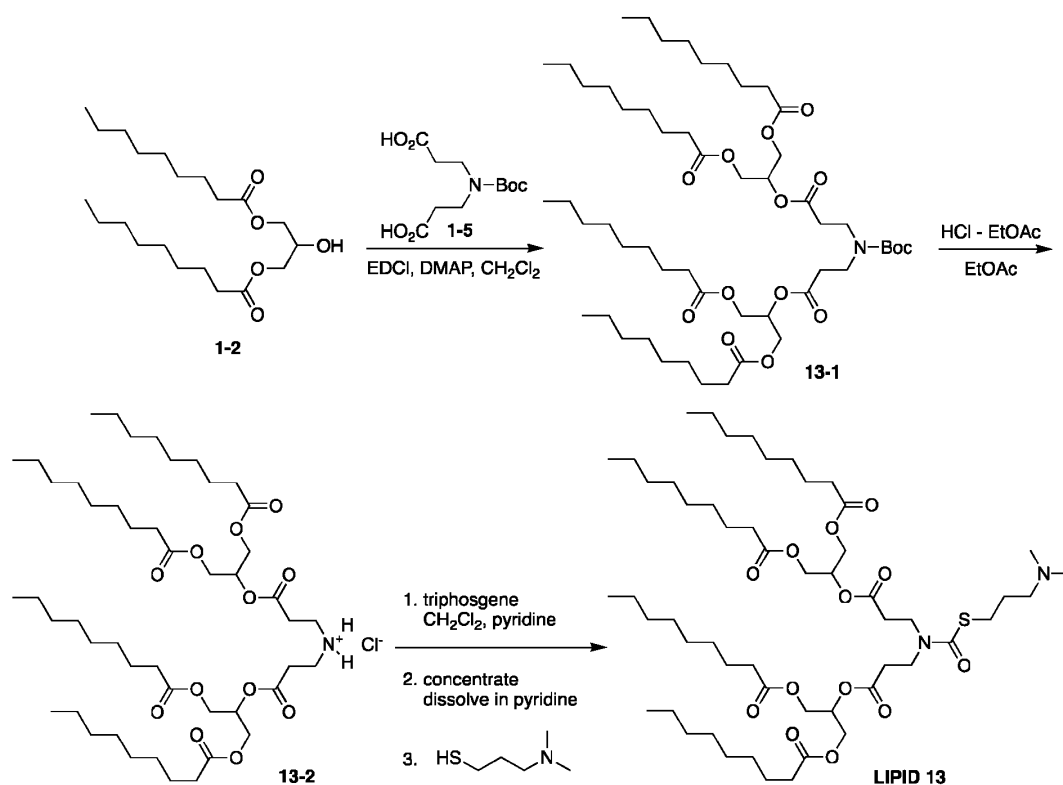


[0380] Into a 250 mL three-necked round-bottom flask was added **12-5** (4.5 g, 1.0 eq) and CH_2Cl_2 (68 mL), and the solution was cooled in an ice-water bath under nitrogen. Then triphosgene (1.4 g, 1.0 eq) was added to the cooled solution, followed by the addition of pyridine (1.88 g, 5.0 eq) over a period of 30 minutes. After addition was complete, the reaction mixture was warmed to room temperature and then stirred for 4 hours. The solvent was removed under vacuum and the residue was dissolved in pyridine (90 mL) and the mixture was cooled in an ice-water bath under nitrogen. To this cooled solution was added 3-(dimethylamino)-1-propanethiol hydrochloride (0.57 g, 1.1 eq). After the addition was complete the reaction mixture was warmed to room temperature and was stirred for 18 hours. The mixture was concentrated under vacuum, the temperature was kept less than or equal to 20 °C to provide crude LIPID 12 which was dissolved in CH_2Cl_2 (90 mL) and the resulting solution was washed with 10% aqueous citric acid solution (45 mL), brine (45 mL, 3 x), 10% aqueous sodium bicarbonate solution (45 mL), and brine (45 mL, 2 x). The organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced vacuum to give crude LIPID 12. Crude LIPID 12 was dissolved in CH_2Cl_2 (30 mL) and adsorbed on silica gel (15 g, type: ZCX-2, 100-200 mesh) and purified on a silica gel column (60 g, type: ZCX-2, 100-200 mesh) with a n-heptane / acetone gradient from 100:0 to 80:20. The fractions containing pure product were pooled and concentrated and dried under vacuum to get 1.5 g (29%) of LIPID 12 as a light-yellow oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 0.7 min): RT 0.87 min, m/z 1090.71 (Calcd.), (found) 1091.60 (M+H); ^1H NMR (300 MHz, CDCl_3): δ 5.27 (m, 2H), 4.30 (dd, J = 11.9, 4.4 Hz, 4H), 4.1 (dd, J = 11.9, 5.8 Hz, 4H), 3.27 (brm, 4H), 2.90 (t, J = 7.2 Hz, 2H), 2.45-2.20 (20H), 1.81 (m, 2H), 1.71-1.63 (22H), 1.55-1.47 (12H), 1.34-1.07 (22H), 0.94-0.77 (8H).

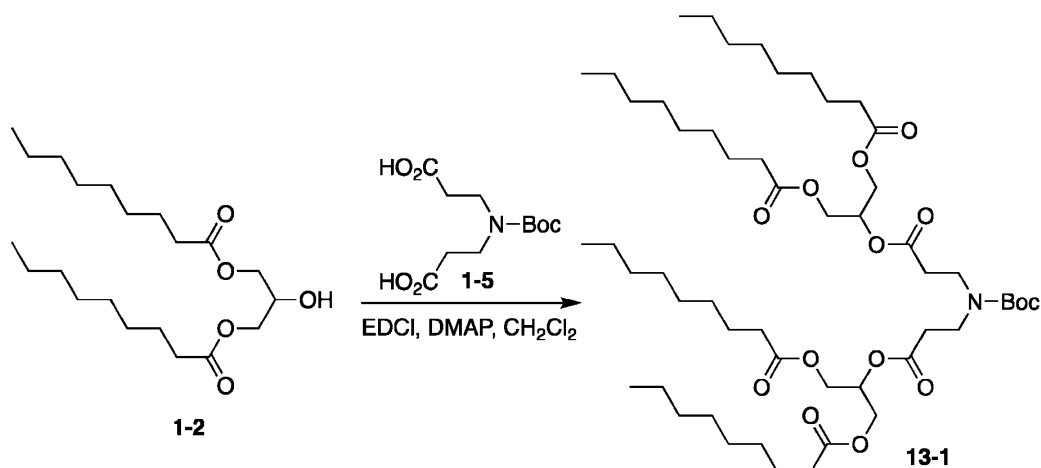
Example 13. Synthesis of LIPID 13: Nonanoic acid 2-(3-((3-dimethylamino-propylsulfanylcarbonyl)-[2-(2-nonanoyloxy-1-nonanoyloxymethyl-ethoxycarbonyl)-ethyl]-amino)-propionyloxy)-3-octanoyloxy-propyl ester



[0381] General Scheme:

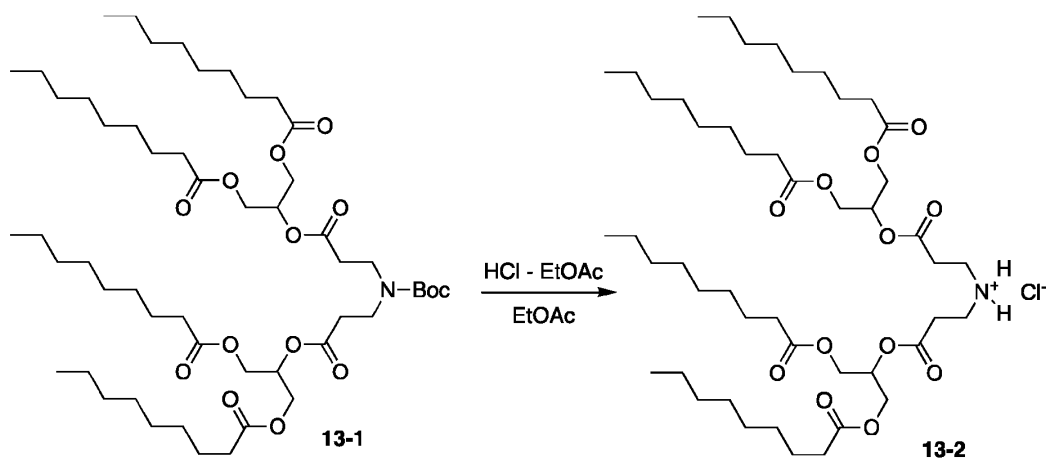


[0382] Synthesis of 13-1: ((3,3'-((*tert*-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



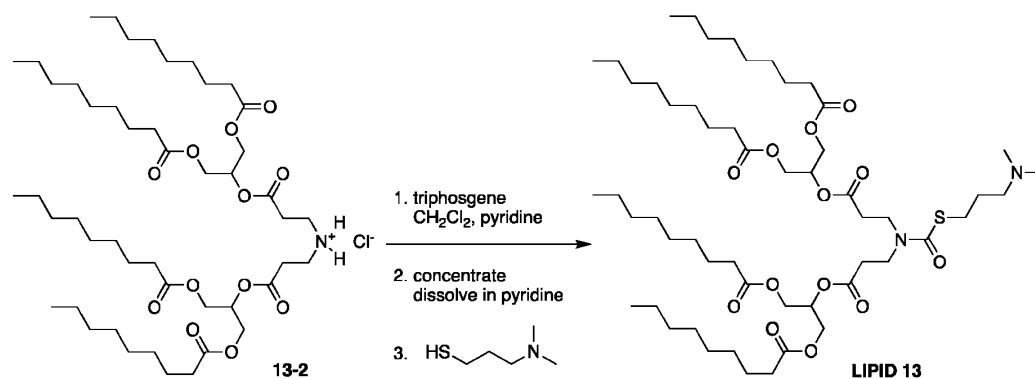
1-5 (7.3g, 28.0 mmol) and **1-2** (18.8g, 56.0 mmol) were dissolved in CH₂Cl₂ (110 mL) in a 500 mL 3-necked round bottom flask under nitrogen. The solution was cooled in an ice-water bath and DMAP (3.4g, 28.0 mmol) and EDCI (21.5g, 0.112 mol) were added in order. After the addition was complete, the mixture was warmed to room temperature and was allowed to stir for 16 hours. The mixture was cast into 10% aq. Citric acid (200 mL), the organic phase was separated, washed with brine (200 mL) and dried over anhydrous Na₂SO₄. The dessicant was removed by filtration through a sintered glass funnel and to the filtrate was added 50g of silica gel (type: ZCX-2, 100-200 mesh). The solvent was removed *in vacuo* with a rotary evaporator bath temperature of 35°C. The silica gel containing adsorbed **13-1** was placed atop a column of silica gel (50 mm OD, 200g silica gel, type: ZCX-2, 100-200 mesh). The column was eluted with a gradient of petroleum ether: EtOAc 100:0 to 97:3, 200 mL fractions. TLC analysis indicated fractions containing **13-1** which were combined and concentrated *in vacuo* to afford **13-1** (12.8g, 13.2 mmol, 47%) as a pale yellow, viscous oil. ¹H-NMR (300 MHz, DMSO-d₆): δ 5.18 (br m, 2H), 4.26 (m, 4H), 4.13 (m, 4), 2.29 (t, *J* = 7.2Hz, 8H), 1.40-1.60 (14H), 1.38 (s, 9H), 1.18-1.32 (42H), 0.86 (m, 12H).

[0383] Synthesis of 13-2: ((3,3'-Azanediyl)bis(propanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate hydrochloride salt



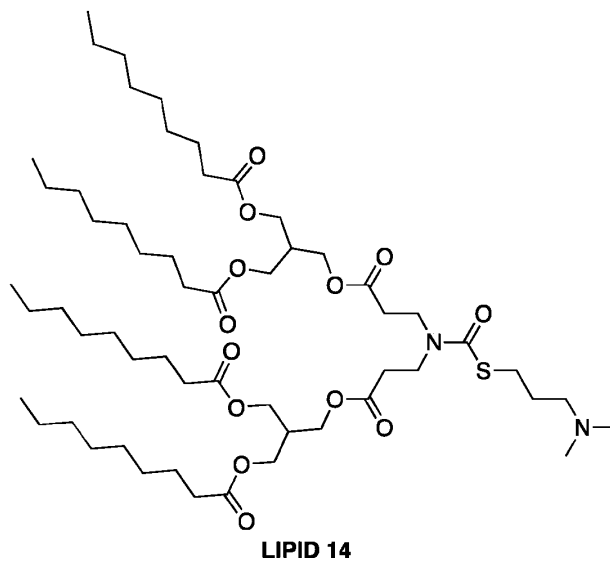
[0384] To a solution of **13-1** (12.8g, 13.2 mmol) in EtOAc (75 mL), cooled in an ice-water bath under nitrogen, was added a solution of HCl in EtOAc (2M, 80 mL, 0.160 mol) at such a rate that the internal temperature remained between 0-10 °C. After the addition was complete, the mixture was allowed to warm to room temperature and was stirred for 14 hours. Concentration *in vacuo* provided the HCl salt **13-2** (8.1g, 9.37 mmol, 71%) as a light yellow, viscous oil. ¹H-NMR (300 MHz, DMSO-d₆): δ 5.19 (br m, 2H), 4.25 (m, 4H), 4.14 (m, 4H), 2.73 (br t, *J* = 6.9Hz, 4H), 2.40 (br t, *J* = 6.9Hz, 4H), 2.29 (br t, *J* = 7.2Hz, 8H), 1.38-1.58 (8H), 1.16-1.32 (40H), 0.87 (m, 12H).

[0385] **Synthesis of LIPID 13: 2-((3-(((3-(Dimethylamino)propyl)thio)carbonyl)(3-((1-(nonanoyloxy)-3-(octanoyloxy)propan-2-yl)oxy)-3-oxopropyl)amino)propanoyl)oxy)propane-1,3-diyl dinonanoate**

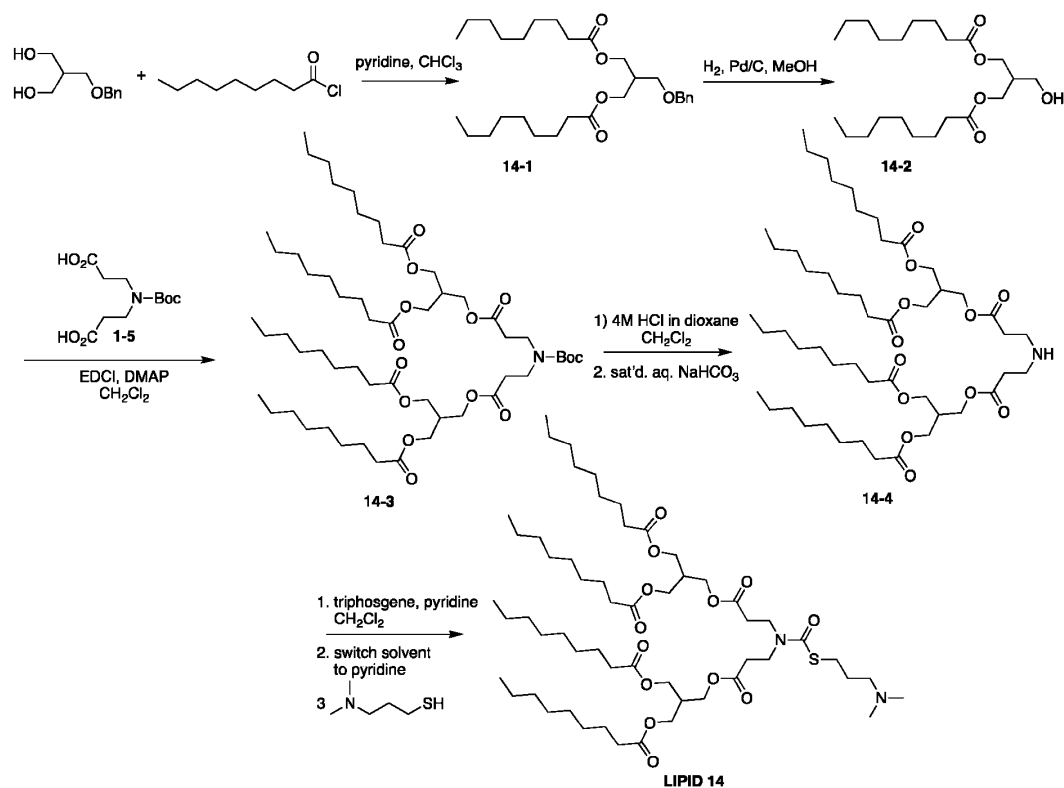


[0386] To a solution of **13-2** (8.1g, 9.37 mmol) in CH₂Cl₂ (280 mL), cooled in an ice-water bath under nitrogen, was added triphosgene (2.77g, 9.33 mmol) in one portion, followed by the

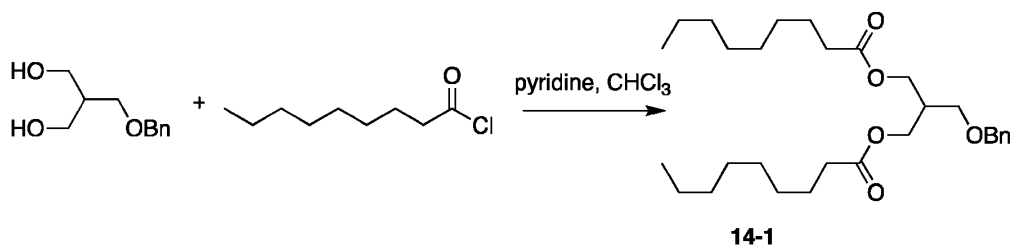
dropwise addition of pyridine (3.68g, 46.52 mmol). After the addition was complete the reaction mixture was allowed to warm to room temperature and stir for 4 hours. The solvent was removed *in vacuo* (bath temperature 25 °C) and the residue was dissolved in pyridine (160 mL). The solution was cooled in an ice-water bath under nitrogen and 3-dimethylamino-propane-1-thiol (1.32g, 11.1 mmol) was added dropwise over 10 minutes. After the addition was complete, the mixture was allowed to warm to room temperature and was stirred for 14 hours. The solvent was removed *in vacuo* and the residue was dissolved in CH₂Cl₂ (200 mL). The solution was washed with 10% aq. citric acid (100 mL), 5% aq. NaHCO₃ (100 mL), brine (100 mL), and was dried over anhydrous Na₂SO₄. The drying agent was removed by filtration through a sintered glass funnel and silica gel (16g, type ZCX02, 100-200 mesh) was added to the filtrate. The solvent was removed *in vacuo* (bath temperature 25 °C) and the silica gel containing adsorbed **13** was placed atop a column of silica gel (81 g silica gel type ZCX02, 100-200 mesh)– using a combi-flash. The column was eluted with a gradient of n-heptane/acetone from 100:0 to 90:10, 100 mL fractions. TLC was utilized to locate qualified fractions which were combined and concentrated *in vacuo* to give **13** (1.9g) which was judged to be 90% pure by HPLC. **13** was further purified by reverse phase prep-HPLC (C₁₈, A: water with 0.1% formic acid, B: acetonitrile, gradient 41% B to 58% B over 8 minutes. Qualified fractions were combined and concentrated *in vacuo* to yield **13** (1.01g, 1.00 mmol, 10.6%) as a clear, pale yellow, viscous oil. HPLC Purity: 99.65%; ES-MS (+ mode): Calcd. 1014.68, Found 1015.95 (M+H⁺); ¹H-NMR (300 MHz, CDCl₃): δ 5.26 (m, 2H), 4.33 (m, 4H), 4.16 (m, 4H), 3.68 (br t, *J* = 7.2Hz, 4H), 2.94 (t, *J* = 7.2Hz, 2H), 2.68 (br t, *J* = 7.2Hz, 4H), 2.20-2.40 (16H), 1.81 (m, 2H), 1.52-1.66 (8H), 1.16-1.38 (40H), 0.90 (m, 12H).

Example 14. Synthesis of LIPID 14

[0387] General Scheme:



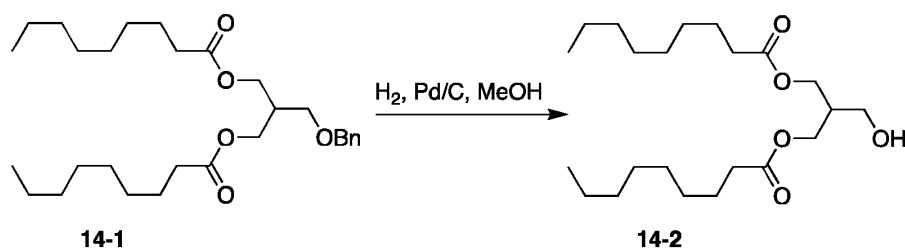
[0388] Synthesis of 14-1: 2-((Benzyloxy)methyl)propane-1,3-diyl dinonanoate



[0389] 2-[(Phenylmethoxy)methyl]-1,3-propanediol (*Bioorg. Med. Chem.* **2017**, 25, 4008-4030; 25.0g, 0.127 mol) was dissolved in CHCl_3 (500mL) and cooled in an ice water bath under nitrogen. To this solution was added nonyl chloride (56.5g, 0.318 mol) in one portion followed by the addition of pyridine (40.0g, 0.508 mol) dropwise over 40 minutes. The reaction mixture was allowed to warm to room temperature and then was stirred for 14 hours. The cloudy mixture was filtered through a pad of celite and the filtrate was washed with 5% aq. NaHCO_3 (250 mL), brine (250 mL) and dried over anhydrous Na_2SO_4 . The drying agent was removed by filtration

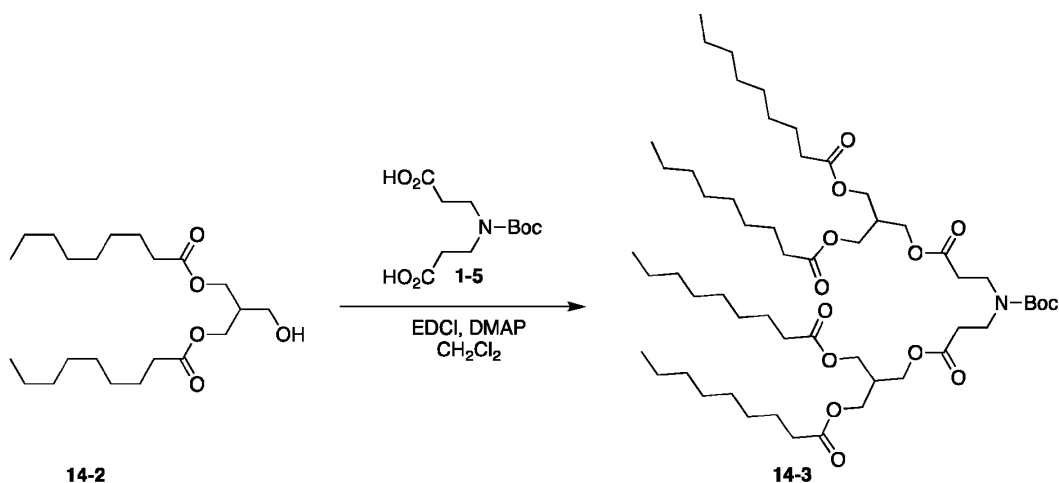
through a sintered glass funnel and silica gel (150g, type: ZCX-2, 100-200 mesh) was added to the filtrate. The solvent was removed *in vacuo* (bath temperature <35 °C) and the silica gel with adsorbed **14-1** was added onto the top of a combi flash column (600g, type: ZCX-2, 100-200 mesh, packed with petroleum ether:EtOAc 99:1 and eluted with petroleum ether:EtOAc 99:1 to 98:2, 1000 mL fractions). Qualified fractions were determined by TLC, combined and concentrated *in vacuo* to give **14-1** (60.0g, 0.124 mol, 98%) as a colorless oil. ¹H-NMR (300 MHz, CDCl₃): δ 7.28-7.40 (5H), 4.52 (s, 2H), 4.19 (m, 4H), 3.52 (d, *J* = 5.7Hz, 2H), 2.36 (m, 1H), 2.29 (t, *J* = 7.5Hz, 4H), 1.59 (m, 4H), 1.23-1.40 (18H), 0.92 (m, 6H).

[0390] Synthesis of 14-2: 2-(Hydroxymethyl)propane-1,3-diyl dinonanoate



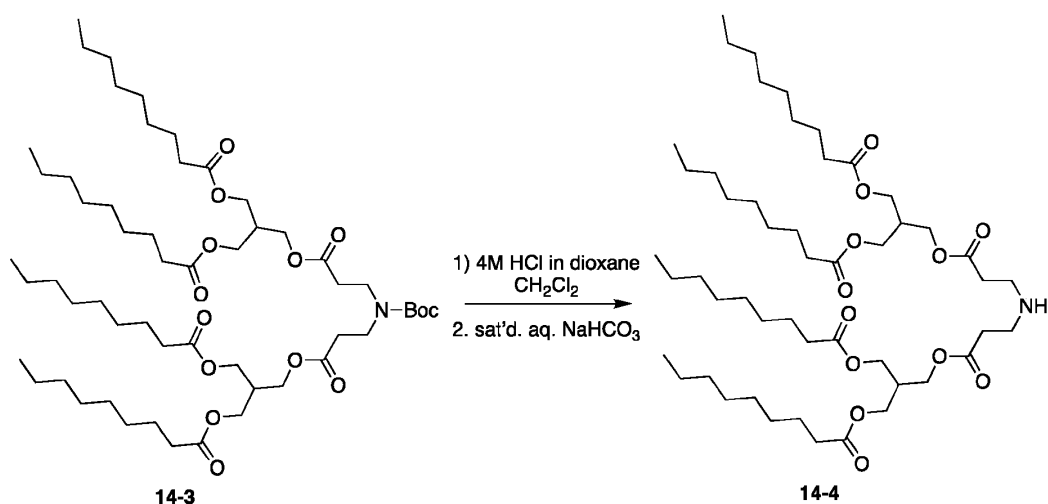
[0391] A solution of **14-1** (60.0g, 0.124 mol) in MeOH (600 mL), in 1 1.0L pressure vessel, was flushed with nitrogen 3X, then 10% Pd/C (18.0g) is added to the vessel and the mixture was placed under hydrogen pressure (3 atm). The mixture was stirred under hydrogen for 14 hours, then the vessel was vented and the solution was sparged with nitrogen. The Pd/C was removed by filtration through a pad of Celite, the filter cake was rinsed with MeOH (200 mL) and the combined filtrates were concentrated *in vacuo* to give **14-2** (32.0g, 82.8 mmol, 67%) as a clear, colorless oil. ¹H-NMR (300MHz, CDCl₃): δ 4.16 (m, 4H), 3.63 (d, *J* = 5.7Hz, 2H), 2.33 (t, *J* = 7.5Hz, 4H), 2.20 (m, 1H), 1.63 (m, 4H), 1.25-1.39 (20H), 0.90 (m, 6H).

[0392] Synthesis of 14-3: (((3,3'-((*tert*-butoxycarbonyl)azanediyl)bis(propanoyl))-bis(oxy))bis (methylene))bis(propane-2,1,3-triyl) tetranonanoate



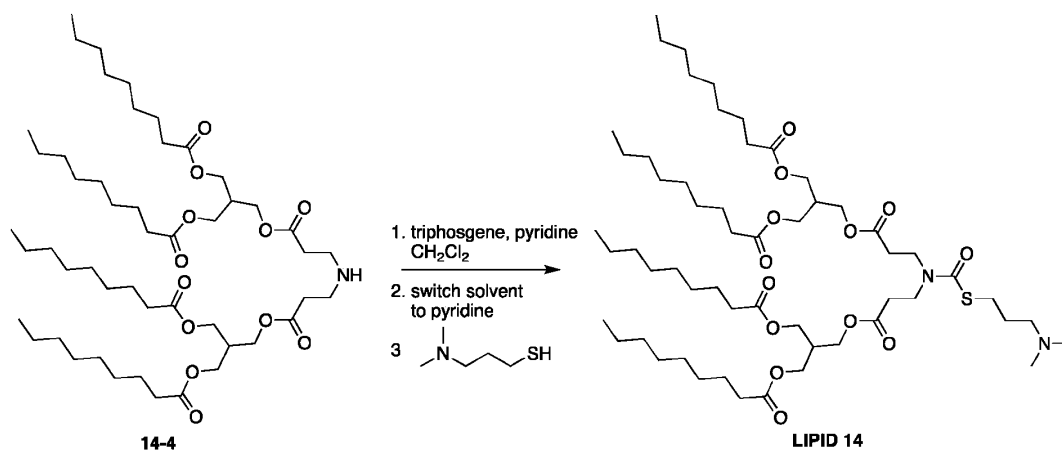
[0393] To a solution of **1-5** (5.00g, 19.1 mmol) in CH_2Cl_2 (75 mL), cooled in an ice water bath under nitrogen was added in order **14-2** (14.8g, 38.3 mmol), DMAP (2.34g, 19.1 mmol), and EDCI (14.7g, 76.7 mmol). The mixture was allowed to warm to room temperature and then was stirred for 14 hours. The reaction mixture was cast into 10% aq. citric acid (125 mL). The organic phase was separated, washed with brine (125 mL) and dried over anhydrous Na_2SO_4 . Filtration through a sintered glass funnel and concentration *in vacuo* gave crude **14-3** (14.0g, 14.0 mmol, 73%) which was carried forward without further purification.

[0394] **Synthesis of 14-4: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))-bis(propene-2,1,3-triyl) tetranonanoate**



[0395] To a solution of **14-3** (14.0g crude, assumed 14.0 mmol) in CH_2Cl_2 (80 mL), cooled in an ice water bath under nitrogen, was added 4.0M HCl in dioxane (35 mL, 0.140 mol) as such a rate that the internal temperature was maintained 0-10°C. The mixture was allowed to stir for 30 minutes after the addition was complete, then was allowed to warm to room temperature and stir for 16 hours. The reaction mixture was cast into saturated aq. NaHCO_3 (100 mL), the organic phase was separated, washed with saturated aq. NaHCO_3 (100 mL), brine (100 mL) and dried over anhydrous Na_2SO_4 . Filtration and concentration *in vacuo* afforded crude **14-4** as a viscous yellow oil which was dissolved in CH_2Cl_2 (200 mL) and silica gel (20g, type ZCX-2, 100-200 mesh) was added. Concentration *in vacuo* gave silica gel with adsorbed **14-4** which was placed atop a column of silica gel (100g, type ZCX-2, 100-200 mesh), eluted with a gradient from 67:33 to 50:50 using combi-flash. Qualified fractions were located by TLC, combined, and concentrated *in vacuo* to give **14-4** (5.20g, 5.79 mmol, 30% over 2 steps) as a clear, pale yellow, viscous oil. $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 4.12-4.18 (12H), 2.93 (t, $J = 6.6\text{Hz}$, 4H), 2.57 (t, $J = 6.6\text{Hz}$, 4H), 2.42 (m, 2H), 2.33 (t, $J = 7.5\text{Hz}$, 8H), 1.61 (m, 8H), 1.22-1.40 (40H), 0.90 (m, 12H).

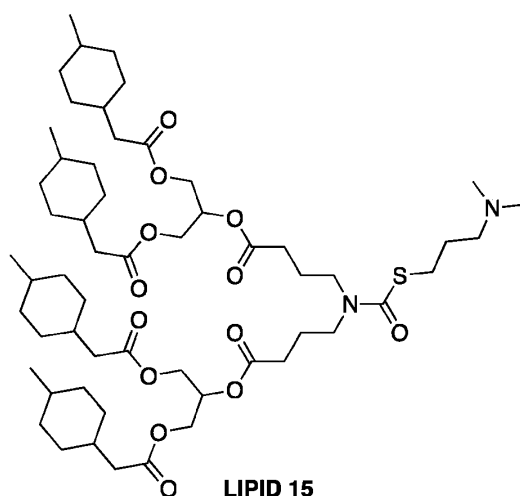
[0396] **Synthesis of LIPID 14**



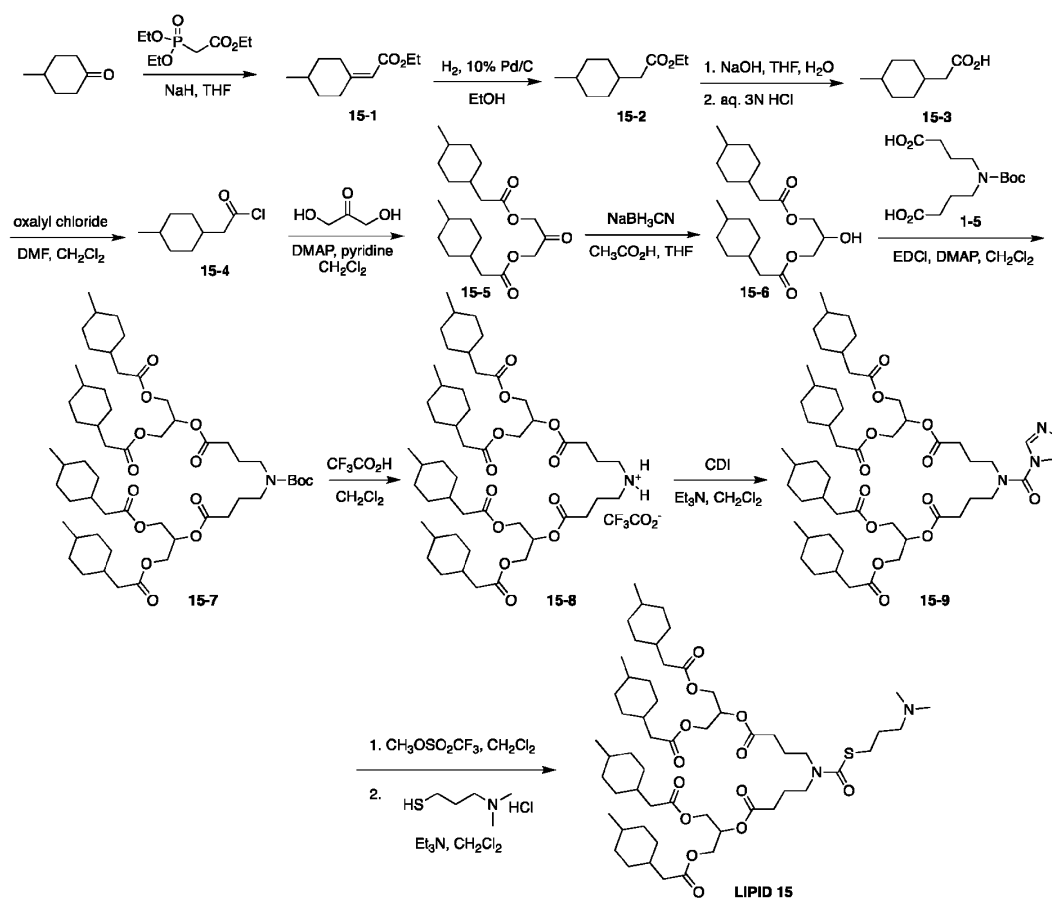
[0397] To a solution of **14-4** (5.20g, 5.79 mmol) in CH_2Cl_2 (175 mL), cooled in an ice-water bath under nitrogen, was added triphosgene (1.72g, 5.75 mmol) in one portion, followed by the addition of pyridine (2.29g, 28.9 mmol, 2.34 mL) at such a rate that the temperature remained at 0-5°C. The mixture was stirred for 30 minutes after the addition was complete, then was allowed to warm to room temperature and stir for 4 hours. The solvent was removed *in vacuo* and the residue was dissolved in pyridine (100 mL), and the solution was cooled in an ice water bath

under nitrogen. To this stirring solution was added 3-dimethylamino-propane-1-thiol (0.82g, 6.88 mmol) dropwise over a period of 10 minutes. The mixture was allowed to stir for 30 minutes after the addition was complete, then the mixture was warmed to room temperature and was stirred for 14 hours. The solvent was removed *in vacuo* and the residue was dissolved in CH₂Cl₂ (200 mL) and was washed with 10% aq. citric acid (2 x 100 mL), saturated aq. NaHCO₃ (2 x 100 mL), brine (2 x 100 mL), and dried over Na₂SO₄. Filtration and concentration *in vacuo* gave crude **14** as a viscous, yellow oil which was purified by reverse phase combi-flash chromatography (A: water + 0.1% CF₃CO₂H, B: acetonitrile; gradient 60% B to 80% B over 20 minutes then 100% for 20 minutes). Qualified fractions were combined and concentrated *in vacuo* to afford **14** (1.12g, 1.07 mmol, 18.5%) as a clear, pale yellow oil. ¹H-NMR (300MHz, CDCl₃): δ 4.12-4.20 (12H), 3.65 (brt, *J* = 7.2Hz, 4H), 3.11 (m, 2H), 2.93 (t, *J* = 6.9Hz, 2H), 2.82 (s, 6H), 2.65 (brm, 4H), 2.45 (m, 4H), 2.31 (t, *J* = 7.5Hz, 8H), 1.61 (m, 8H), 1.18-1.35 (40H, 0.88 (m, 12H).

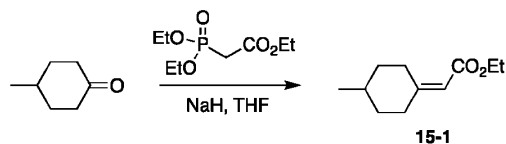
Example 15. Synthesis of LIPID 15: (((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl) acetate)



[0398] General Scheme:



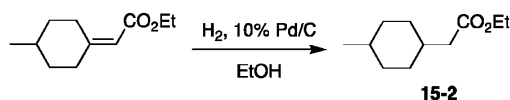
[0399] Synthesis of 15-1: Ethyl 2-(4-methylcyclohexylidene)acetate



[0400] To a suspension of NaH (12.5g, 60% in oil, washed with hexanes (2x25mL), 0.312 mol) in anhydrous THF (600 mL), under nitrogen and cooled in an ice-water bath, was added triethyl phosphonoacetate (70.0g, 0.312 mol) dropwise over 30 minutes. The mixture was stirred for 2 hours in the ice water bath, then 4-methyl-cyclohexanone (35.0g, 0.312 mol) was added over 30 minutes. The mixture was stirred for 30 minutes then was allowed to warm to room temperature and was stirred for 14 hours. The mixture was cast into water (1.2 L) and EtOAc (600 mL). The organic phase was separated and silica gel (200g, type: ZCX-2, m100-200 mesh) was added and the solvent was then remove *in vacuo* (bath temperature <35°C) to provide silica

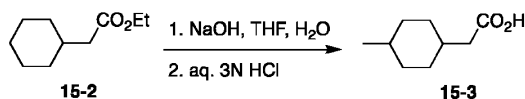
gel with adsorbed, crude **1**. The silica gel is placed atop a column of silica gel (1000g, type: ZCX-2, m100-200 mesh) eluted with a gradient to petroleum ether:EtOAc 100:0 to 95:5, collecting 1000 mL fractions using a combi-flash. Qualified fractions were located by TLC, combined and concentrated *in vacuo* to provide **15-1** (45.0g, 0.247 mol, 79%) as a clear, colorless oil. LC-MS (+mode): RT 1.804, 183.2 (M+H⁺); ¹H-NMR (300MHz, CDCl₃): δ 5.61 (s, 1H), 4.14 (q, *J* = 7.2Hz, 2H), 3.75 (m, 1H), 2.14-2.30 (2H), 1.90 (m, 1H), 1.80 (m, 2H), 1.62 (m, 1H), 1.28 (t, *J* = 7.2Hz, 3H), 1.00-1.15 (2H), 0.82 (d, *J* = 9.0Hz, 3H).

[0401] Synthesis of 15-2: Ethyl 2-(4-methylcyclohexyl)acetate



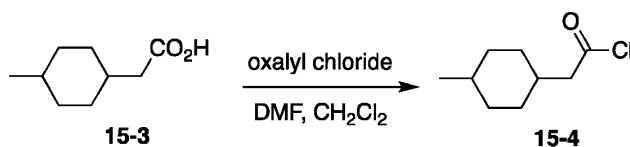
[0402] To ester **15-1** (45.0g, 0.247 mol), dissolved in EtOH (450 mL) under nitrogen at room temperature, was added 10% Pd/C (13.5 g). Hydrogen was then bubbled through the reaction mixture for 16 hours. The solvent was then sparged with nitrogen for 1 hours, the catalyst was removed by filtration through a pad of celite and the filter cake was rinsed with EtOH (450 mL). The combined filtrates were concentrated *in vacuo* to yield **15-2** (35.0g, 0.190 mol, 77%) as a pale yellow oil. ¹H-NMR (300MHz, CDCl₃): δ 4.14 (q, *J* = 7.2Hz, 2H), 2.25-2.40 (2H), 2.18 (m, 1H), 1.52-1.78 (3H), 1.28 (t, *J* = 7.2Hz, 3H), 0.78-1.03 (9H).

[0403] Synthesis of 15-3: 2-(4-Methylcyclohexyl)acetic acid



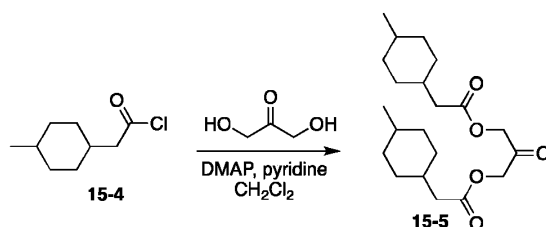
[0404] To a solution of **15-2** (35.0g, 0.190 mol) in THF:H₂O (350 mL, 50:50), at room temperature under nitrogen, was added solid NaOH (84.0g, 2.10 mol) over a period of 30 minutes. The mixture was stirred for 16 hours, then was concentrated *in vacuo* to remove the THF. The aqueous solution was then adjusted to pH 3.0 through the addition of 3N aq. HCl. The reaction mixture was extracted with EtOAc (350 mL) and the organic phase was dried over Na₂SO₄. Filtration and concentration *in vacuo* provided **15-3** (25.0g, 0.160 mol, 84%) as a white solid.

[0405] Synthesis of 15-4: 2-(4-Methylcyclohexyl)acetyl chloride



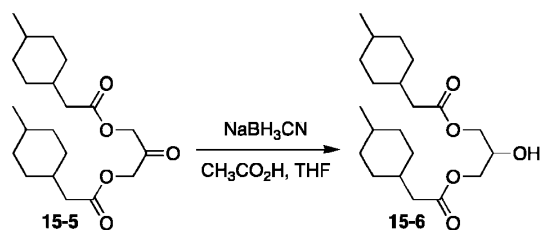
[0406] To **15-3** (25.0g, 0.160 mol) in CH_2Cl_2 (250 mL), containing DMF (1.0 mL), cooled in an ice-water bath under nitrogen, was added oxalyl chloride (40.7g, 0.321 mol), dropwise over a period of 20 minutes. The mixture was allowed to stir for 30 minutes after the addition was complete, then was warmed to room temperature and was stirred for 14 hours. Concentration *in vacuo*, bath temperature $<30^\circ\text{C}$, afforded **15-4** (25.2g, 0.144 mol, 90%) as a clear, colorless oil. $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 2.75 (m, 2H), 1.30-1.64 (5H), 1.18-1.28 (2H), 0.80-1.05 (6H).

[0407] Synthesis of **15-5**: 2-Oxopropane-1,3-diyl bis(2-(4-methylcyclohexyl)acetate)



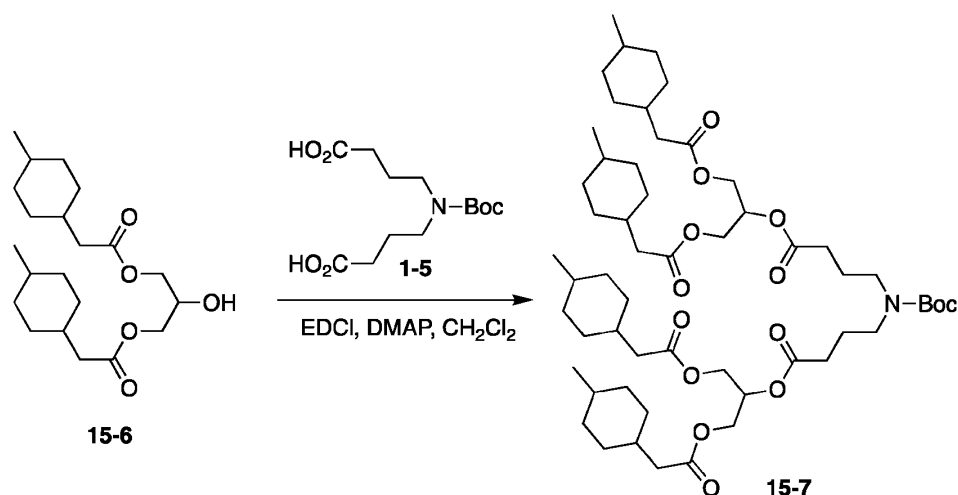
[0408] To 1,3-dihydroxyacetone (5.90g, 65.5 mmol), in CH_2Cl_2 (500 mL) under nitrogen at room temperature, was added DMAP (2.40g, 19.6 mmol) and pyridine (11.4g, 0.144 mol), followed by the addition of **15-4** (25.2g, 0.144 mol) dropwise over 30 minutes. The mixture was stirred for 16 hours at room temperature, then was cast into water (400 mL). The organic phase was separated, washed with water (400 mL), brine (400 mL), and dried over Na_2SO_4 . The drying agent was removed by filtration and silica gel (60g, type: ZCX-2, 100-200 mesh) was added to the filtrate. The solvent was removed *in vacuo* to give the silica gel with adsorbed, crude, **15-5**. The silica gel was plated atop a column of silica gel (300g, type: ZCX-2, 100-200 mesh), eluted with a gradient of petroleum ether:EtOAc from 100:0 to 90:10, 500 mL fractions, using a combi-flash. Qualified fractions were found using TLC, combined and concentrated *in vacuo* to furnish **15-5** (18.0g, 49.1 mmol, 75%) as a clear colorless oil. LC-MS (+-mode): RT 0.36min, 367.3 ($\text{M}+\text{H}^+$); $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 4.76 (s, 4H), 2.40 (d, $J = 6.0\text{Hz}$, 1H), 2.00-2.30 (4H), 0.75-1.10 (25H).

[0409] Synthesis of **15-6**: 2-Hydroxypropane-1,3-diyl bis(2-(4-methylcyclohexyl)acetate)



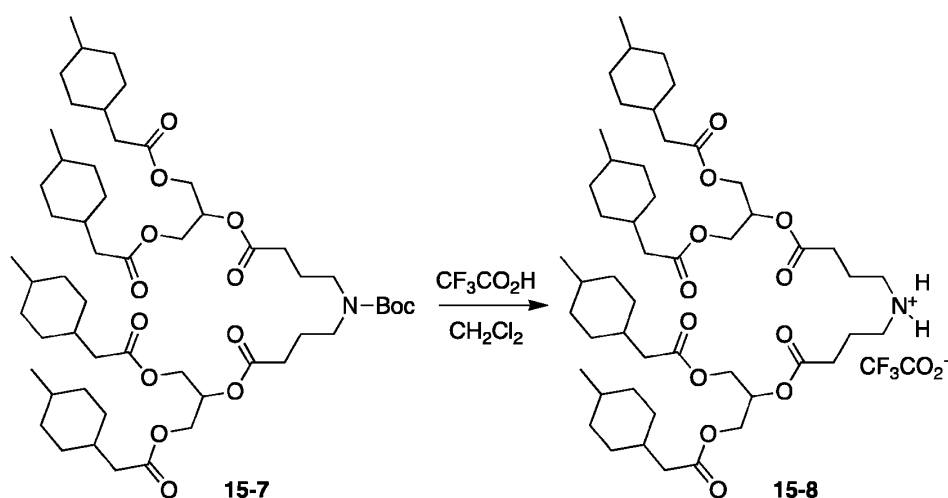
[0410] To a solution of **15-5** (18.0g, 49.1 mmol) in THF under nitrogen, cooled in an ice-water bath, was added $\text{CH}_3\text{CO}_2\text{H}$ (25.0g, 0.42 mol). To this stirring solution was added NaBH_3CN (12.9g, 0.205 mol) in portions over 20 minutes. The mixture was stirred for 30 minutes after the addition was complete then was warmed to room temperature and was stirred for 2 hours. The mixture was cast into water (100 mL), and was extracted with EtOAc (3 x 100 mL). The combined organic phases were dried over Na_2SO_4 , the drying agent was removed by filtration and silica gel (50g, type: ZCX-2, 100-200 mesh) was added to the filtrate. Concentration *in vacuo* provided silica gel containing adsorbed, crude **15-6** which was placed atop a column of silica gel (250g, type: ZCX-2, 100-200 mesh), eluted with a gradient of petroleum ether:EtOAc from 100:0 to 92:8, 500 mL fractions were collected using a combi-flash. Qualified fractions were found using TLC, then combined and concentrated *in vacuo* to provide **15-6** (17.0g, 46.1 mmol, 94%) as a clear, colorless oil. LC-MS (+-mode): RT 1.47min, 391.2 ($\text{M}+\text{Na}^+$); $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 5.30 (s, 1H), 4.00-4.20 (4H), 2.30 (m, 1H), 2.18 (m, 2H), 1.90-2.05 (2H), 1.18-1.75 (14H), 0.80-1.00 (12H).

[0411] **Synthesis of 15-7: ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propene-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)**



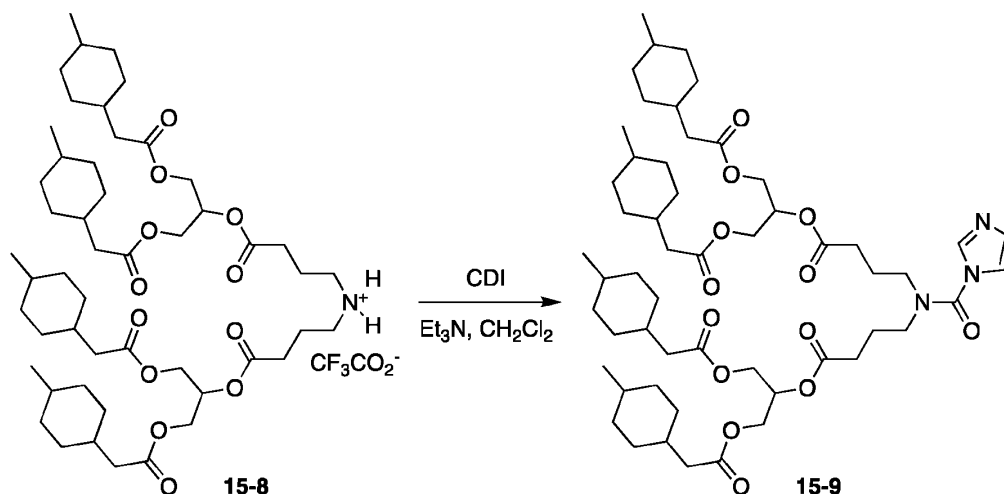
[0412] To **1-5** (6.10g, 21.1 mmol) in CH₂Cl₂ (120 mL), cooled in an ice-water bath under nitrogen was added DMAP (1.00g, 8.18 mmol) and **15-6** (17.0g, 46.1 mmol) in one portion followed by the addition of EDCI (9.70g, 50.6 mmol) in portions over a period of 30 minutes. The mixture was stirred for 30 minutes after the addition was complete, then was warmed to room temperature and stirred for 16 hours. The mixture was cast into brine (120 mL), the organic phase was separated and washed with brine (120 mL) and dried over Na₂SO₄. The drying agent was removed by filtration and silica gel (60g, type: ZCX-2, 100-200 mesh) was added to the filtrate. Concentration *in vacuo* provided silica gel containing adsorbed, crude **15-7** which was placed atop a column of silica gel (300g, type: ZCX-2, 100-200 mesh), eluted with a gradient of petroleum ether:EtOAc from 100:0 to 70:30, 500 mL fractions were collected using a combi-flash. Qualified fractions were found using TLC, then combined and concentrated *in vacuo* to provide **15-7B** (10.0g, 10.1 mmol, 48%) as a clear, colorless oil. LC-MS (+-mode): RT 1.654 min, 1012.9 (M+Na+); ¹H-NMR (300MHz, CDCl₃): δ 5.25 (m, 2H), 4.31 (m, 4H), 4.18 (m, 4H), 3.20 (brm, 4H), 2.31-2.40 (6H), 2.15-2.23 (5H), 2.00 (m, 1H), 1.78 (m, 4H), 1.50-1.75 (14H), 1.30-1.50 (3H), 1.35 (s, 9H), 1.18-1.30 (9H), 0.77-1.00 (26H).

[0413] Synthesis of 15-8: bis(4-((1,3-bis(2-(4-Methylcyclohexyl)acetoxy)propan-2-yl)oxy)-4-oxobutyl)ammonium trifluoroacetate



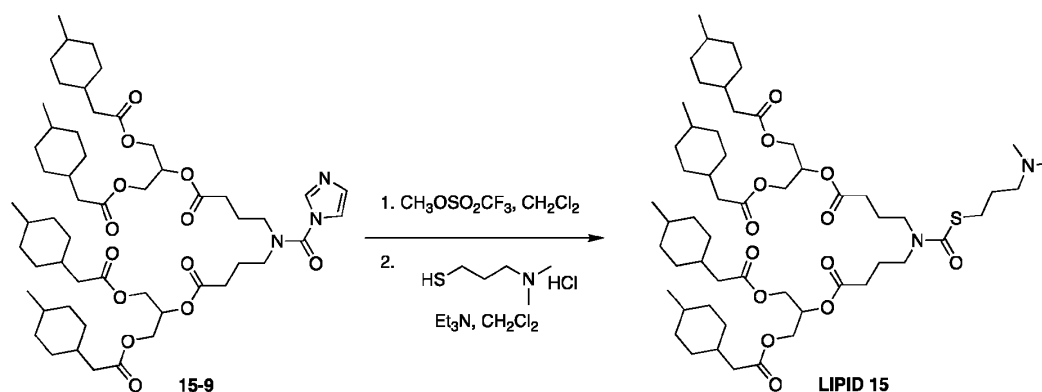
[0414] To a solution of **15-7** (10.0g, 10.1 mmol) in CH_2Cl_2 (40 mL), cooled in an ice-water bath under nitrogen, was added $\text{CF}_3\text{CO}_2\text{H}$ (5.00g, 43.9 mmol, 3.36 mL) in one portion. The mixture was stirred for 30 minutes after the addition then was warmed to room temperature and stirred for 4 hours. The mixture was concentrated *in vacuo* to give crude **15-8** (5.60g, 5.58 mmol, 55%) as a colorless, viscous oil. LC-MS (+-mode): RT 0.608 min, 890.6 ($\text{M}+\text{H}^+$); $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 11.10 (brs, 2H), 5.26 (m, 2H), 4.45 (m, 4H), 4.18 (m, 4H), 3.18 (m, 4H), 2.40 (t, $J = 6.5\text{Hz}$, 4H), 2.31 (t, $J = 7.2\text{Hz}$, 2H), 2.16-2.25 (6H), 1.82-2.08 (10H), 1.14-1.67 (22H), 0.75-1.00 (24H).

[0415] Synthesis of **15-9**: ((4,4'-((1*H*-Imidazole-1-carbonyl)azanediyl)bis(butanoyl))-bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)



[0416] To a solution of **15-8** (5.60g, 5.58 mmol) in CH_2Cl_2 (100 mL), under nitrogen, was added Et_3N (1.30g, 12.8 mmol, 1.21 mL) followed by carbonyldiimidazole (CDI, 2.00g, 12.33 mmol). The mixture was stirred at room temperature for 14 hours then was diluted with n-heptane (100 mL). The solution was washed with water (3 x 100 mL), and the organic phase was dried over Na_2SO_4 . Filtration and concentration *in vacuo* gave crude **15-9** (4.00g, 4.06 mmol, 73%) as a viscous, yellow oil. LC-MS (+ mode): RT 0.645 min 984.9 ($\text{M}+\text{H}^+$); $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 7.96 (m, 1H), 7.26 (m, 1H), 7.13 (m, 1H), 4.32 (m, 4H), 4.16 (m, 4H), 3.41 (m, 4H), 2.21-2.32 (6H), 2.08-2.16 (6H), 1.81-2.00 (6H), 1.50-1.72 (14H), 1.14-1.50 (12H), 0.75-1.00 (24H).

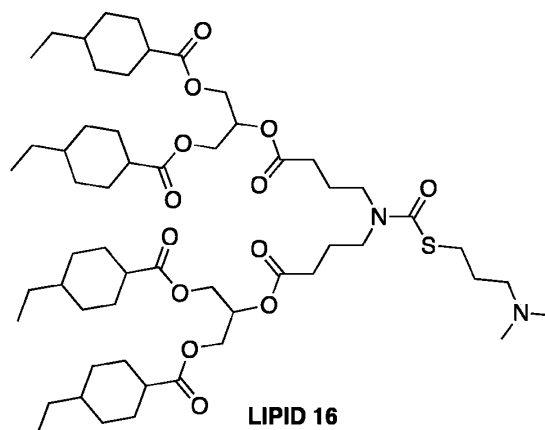
[0417] **Synthesis of LIPID 15: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)-azanediyl)bis(butanoyl) bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)**



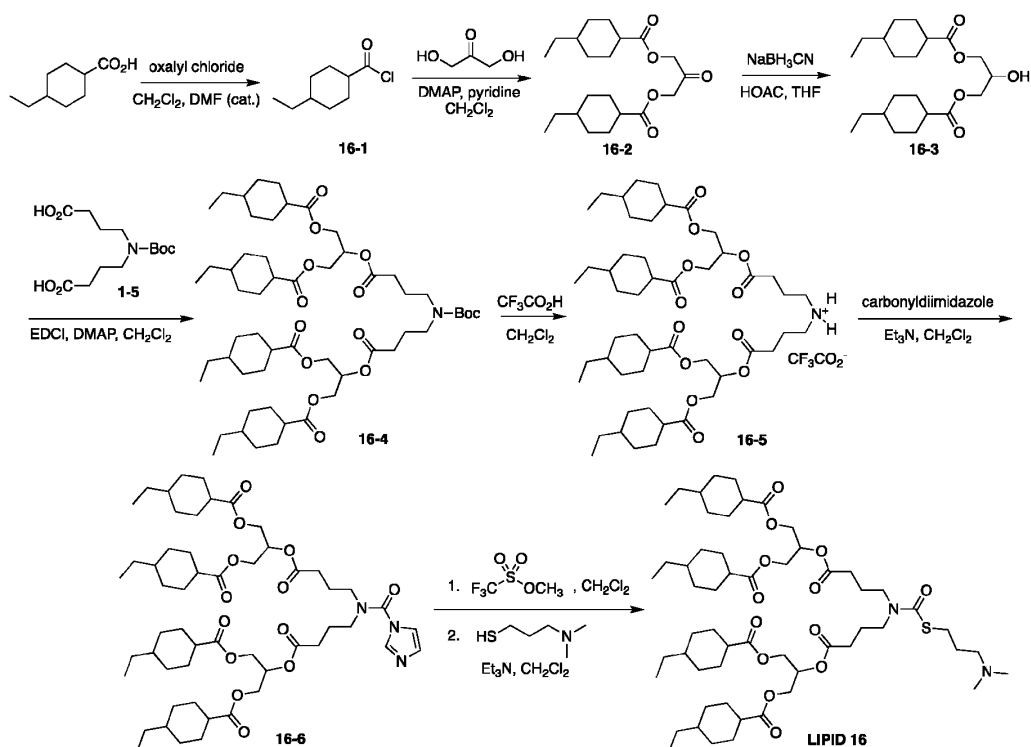
[0418] To a solution of **15-9** (4.00g, 4.06 mmol) in CH_2Cl_2 (80 mL), cooled in an ice-water bath under nitrogen, was added $\text{CF}_3\text{SO}_2\text{OCH}_3$ (0.70g, 4.27 mmol) over a period of 5 minutes. The mixture was stirred for 1 hour after the addition was complete, then Et_3N (0.80g, 7.90 mmol, 1.10 mL) and 3-dimethylamino-propane-1-thiol HCl salt (0.76g, 4.87 mmol) were added in order in one portion each. The mixture was allowed to stir for 30 minutes after the additions were complete, then the mixture was warmed to room temperature and stirred for 16 hours. Silica gel (15g, type: ZCX-2, 100-200 mesh) was added to the solution. Concentration *in vacuo* provided silica gel containing adsorbed, crude **15** which was placed atop a column of silica gel (75g, type: ZCX-2, 100-200 mesh), eluted with a gradient of CH_2Cl_2 :MeOH from 100:0 to 96:4, 300 mL

fractions were collected using a combi-flash. Qualified fractions were found using TLC, then combined and concentrated *in vacuo* to provide **LIPID 15** (1.70g) which was further purified by SFC (Column: Torus 2-PIC, 4.6 x 100 mm 5 μ m; mobile phase B: i-PrOH; flow rate 4 mL/min; gradient: isocratic 10% B; wave length 220 nM) to provide **LIPID 15** (1.00g, 0.965 mmol, 23.8%) as a clear, light yellow oil after concentration *in vacuo*. ES-MS: 1035.7 ($M+H^+$); HPLC Purity: 98.47%; 1H -NMR (300MHz, $CDCl_3$): δ 5.24 (m, 2H), 4.31 (m, 4H), 4.14 (m, 4H), 3.38 (brm, 4H), 2.92 (t, $J = 7.3$ Hz, 2H), 2.11-2.52 (18H), 1.56-2.11 (23H), 1.12-1.56 (14H), 0.75-1.11 (23H).

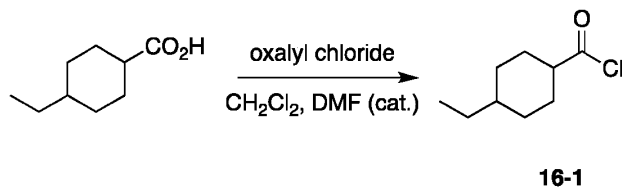
Example 16. Synthesis of LIPID 16: (((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanovyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate)



[0419] General Scheme:

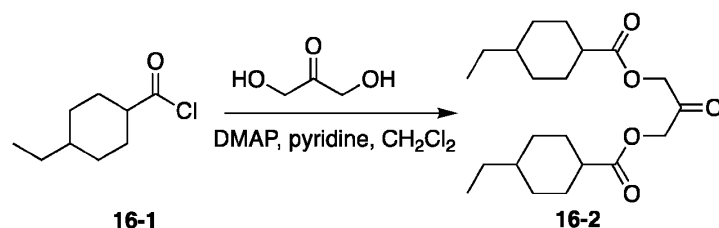


[0420] Synthesis of 16-1: 4-Ethylcyclohexane-1-carbonyl chloride



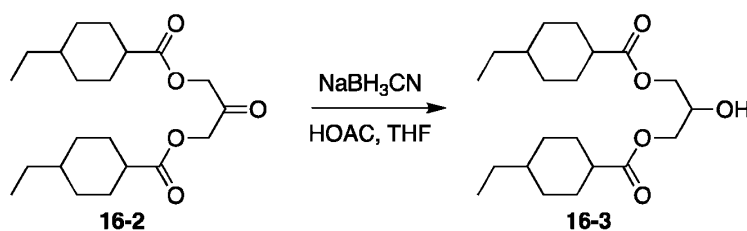
[0421] To a solution of 4-ethyl-cyclohexanecarboxylic acid (22.5g, 0.144mol) in CH_2Cl_2 (225mL), was cooled in an ice-water bath under nitrogen, was added DMF (0.5mL) followed by the addition of oxalyl chloride (36.6g, 0.288mol) over a period of 25 minutes. The mixture was allowed to stir for 30 minutes after the addition was complete, then was warmed to room temperature and was stirred for 16 hours. Concentration *in vacuo* afforded crude **16-1** (22.6g, 0.129mol, 90%) as a clear, colorless oil. $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 2.62 (m, 1H), 2.62 (m, 2H), 1.37-1.53 (2H), 1.10-1.27 (4H), 0.80-1.00 (6H).

[0422] Synthesis of 16-2: 2-Oxopropane-1,3-diyl bis(4-ethylcyclohexane-1-carboxylate)



[0423] To a solution of dihydroxy-acetone (5.30g, 58.8mmol) in CH_2Cl_2 (500mL), cooled in an ice-water bath under nitrogen was added DMAP (0.36g, 2.94mmol) and pyridine (10.24g, 0.129mol) in one portion, followed by the addition of **16-1** (22.6g, 0.129mol) over a period of 10 minutes. The mixture was allowed to stir for 30 minutes after the addition was complete, then was warmed to room temperature and stirred for 14 hours. The solvent was removed *in vacuo* and the residue was dissolved in CH_2Cl_2 (100mL). Silica gel (25g, type ZCX-2, 100-200 mesh) was added to the solution, then the solvent was removed *in vacuo* to provide silica gel impregnated with adsorbed **16-2**. The silica gel was placed atop a column of silica gel (250g, type ZCX-2, 100-200 mesh) and a combi-flash was used to purify the crude **16-2** by eluting with a gradient of petroleum ether:EtOAc from 100:0 to 90:10, collecting 300mL fractions. Qualified fractions were located by TLC, combined, and concentrated *in vacuo* to yield **16-2** (20.4g, 55.6mmol, 94%) as a clear, colorless oil. LC-MS (+ mode): RT 0.450 min, 367.3 ($\text{M}+\text{H}^+$); ^1H -NMR (300MHz, DMSO-d_6): δ 4.83 (s, 4H), 2.27 (m, 2H), 2.15 (m, 1H), 1.77-1.90 (5H), 1.48 (m, 4H), 1.00-1.33 (10H), 0.80-0.95 (8H).

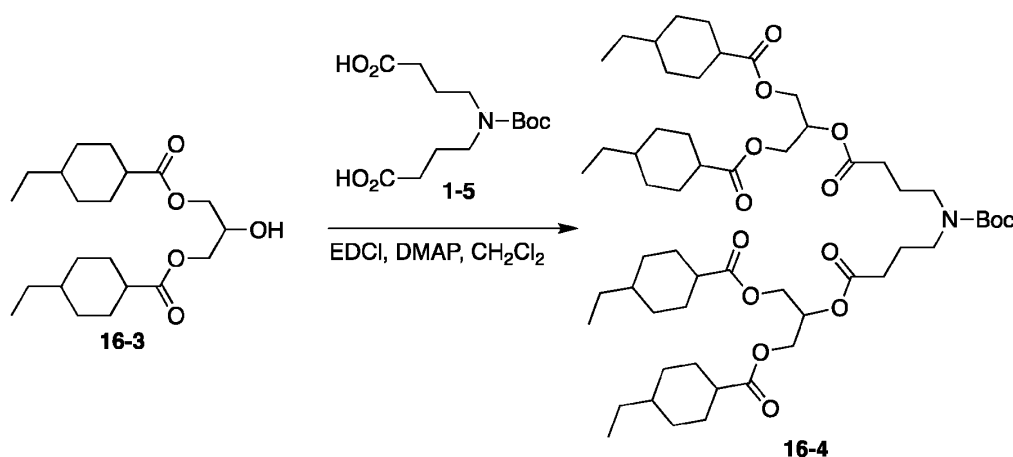
[0424] **Synthesis of 16-3: 2-Hydroxypropane-1,3-diyl bis(4-ethylcyclohexane-1-carboxylate)**



[0425] To a solution of **16-2** (20.4g, 55.66mmol) in THF (400mL), cooled in an ice-water bath under nitrogen as added HOAc (33.4g, 0.556mol) in one portion, followed by the addition of NaBH_3CN (17.5g, 0.278mol) in portions over a period of 30 minutes. The mixture was stirred for 30 minutes after the addition was complete, then was warmed to room temperature and was stirred for 2 hours. The mixture was cast into water (2.0L) and the resulting solution was

extracted with EtOAc (3 x 200mL). The combined organic phases were dried (Na₂SO₄), filtered, concentrated *in vacuo*, and the residue was dissolved in CH₂Cl₂ (100mL). To the solution of crude **16-3** was added silica gel (50g, type ZCX-2, 100-200 mesh) and the solvent was removed *in vacuo* to afford silica gel containing adsorbed, crude **16-3**. The silica gel was placed atop a column of silica gel (250g, type ZCX-2, 100-200 mesh) and a combi-flash was used to purify the crude **16-3** by eluting with a gradient of petroleum ether:EtOAc from 100:0 to 92:8, collecting 300mL fractions. Qualified fractions were located by TLC, combined, and concentrated *in vacuo* to yield **16-3** (16.0g, 43.42mmol, 78%) as a clear, colorless oil. LC-MS (+ mode): RT 1.463 min, 391.3 (M+Na⁺); ¹H-NMR (300MHz, CDCl₃): δ 4.00-4.25 (5H), 2.32 (brs, 1H), 2.25 (m, 2H), 1.91 (m, 4H), 1.77 (m, 4H), 1.37 (m, 4H), 1.00-1.25 (8H), 0.75-0.95 (8H).

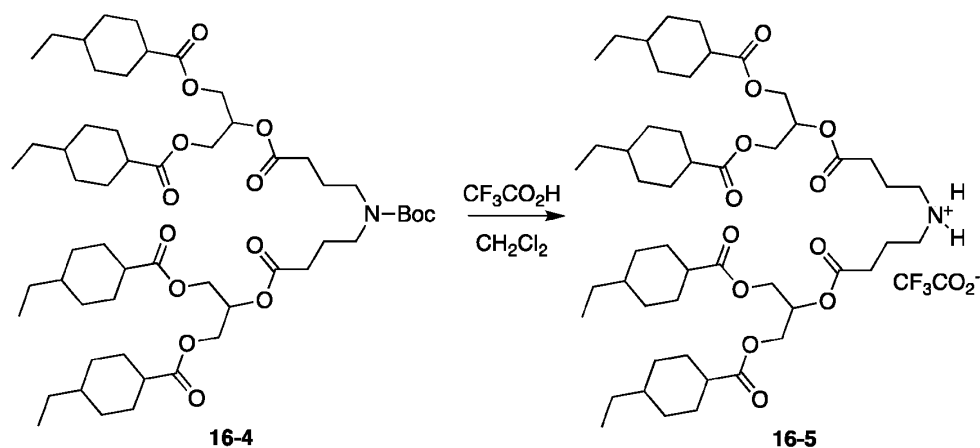
[0426] Synthesis of 16-4: ((4,4'-((tert-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate)



[0427] To a solution of **1-5** (5.42g, 18.73mmol) in CH₂Cl₂ (100mL), under nitrogen, was added in order: DMAP (0.91g, 7.49mmol), and **16-3** (15.2g, 41.21mmol). The resulting solution was cooled in an ice-water bath, then EDCI (8.60g, 44.96mmol) was added in 5 portions over a period of 30 minutes. The mixture was stirred for 30 minutes after the addition was complete, then the solution was warmed to room temperature and was allowed to stir for 14 hours. The mixture was cast into brine (100mL), the organic phase was separated, and dried over Na₂SO₄. Filtration gave a solution of crude **16-4** to which was added silica gel (15g, type ZCX-2, 100-200 mesh) and the solvent was removed *in vacuo* to afford silica gel containing adsorbed, crude **16-4**. The silica gel was placed atop a column of silica gel (75g, type ZCX-2, 100-200 mesh) and a combi-flash was used to purify the crude **16-4** by eluting with a gradient of petroleum

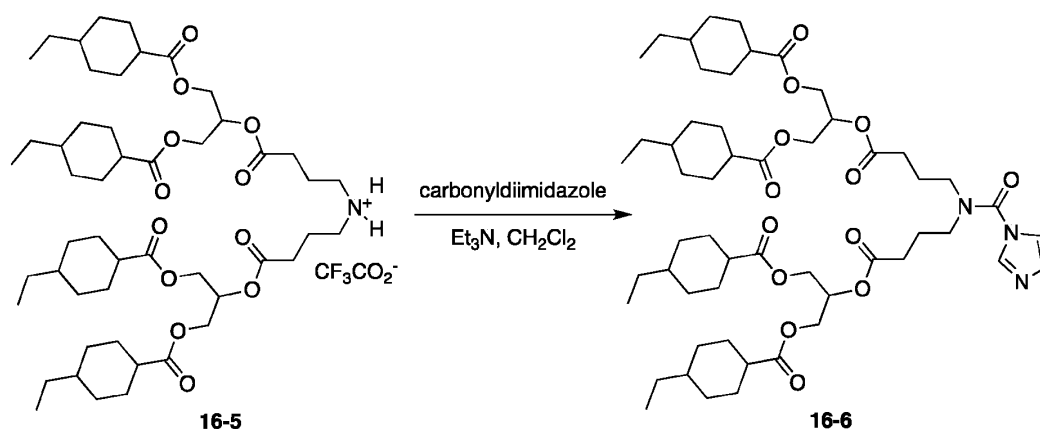
ether:EtOAc from 100:0 to 80:20, collecting 300mL fractions. Qualified fractions were located by TLC, combined, and concentrated *in vacuo* to yield **16-4** (12.98g, 13.10mmol, 70%) as a clear, colorless oil. LC-MS (+ mode): RT 1.703 min. 890.6 (M-Boc + 2H⁺); ¹H-NMR (300 MHz, CDCl₃): δ 5.50 (m, 2H), 4.32 (m, 4H), 4.21 (m, 4H), 3.20 (brm, 4H), 2.16-2.35 (7H), 1.82 (m, 8H), 1.75-1.80 (9H), 1.38 (s, 9H), 1.00-1.38 (24H), 0.75-0.90 (20H).

[0428] Synthesis of 16-5: bis(4-((1,3-bis((4-Ethylcyclohexane-1-carbonyl)oxy)propan-2-yl)oxy)-4-oxobutyl)ammonium trifluoroacetate



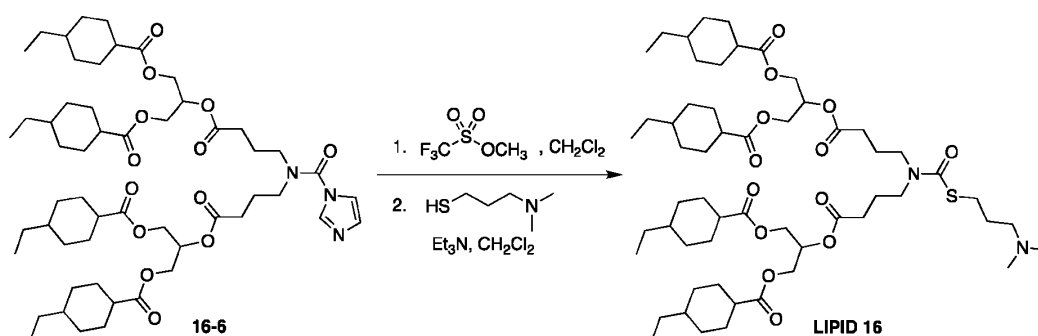
[0429] To a solution of **16-4** (12.98g, 13.11mmol) in CH₂Cl₂ (50mL), cooled in an ice-water bath under nitrogen, was added CF₃CO₂H (7.47g, 65.54mmol) over a period of 10 minutes. The mixture was allowed to stir for 15 minutes after the addition was complete, then it was warmed to room temperature and was stirred for 16 hours. Concentration *in vacuo* gave crude **16-5** (14.82g) as a colorless oil. LC-MS (+ mode): RT 0.677 min. 890.6 (M+H⁺); ¹H-NMR (300 MHz, CDCl₃): δ 5.16 (m, 2H), 4.41 (m, 4H), 4.11 (m, 4H), 3.19 (brm, 4H), 2.42 (m, 4H), 2.25 (m, 4H), 1.75-2.20 (18H), 1.00-1.50 (22H), 0.75-0.95 (20H).

[0430] Synthesis of 16-6: ((4,4'-((1*H*-Imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate)



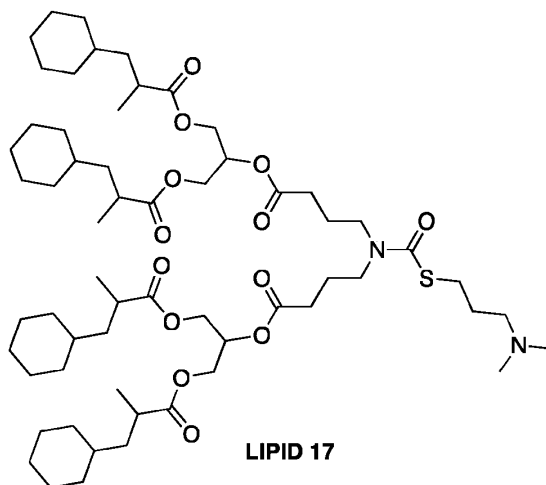
[0431] A solution of **16-5** (14.82g, crude) was dissolved in CH_2Cl_2 (300mL) and was cooled in an ice-water bath under nitrogen. To this solution was added in order Et_3N (6.74g, 66.59mmol) and carbonyldiimidazole (5.39g, 33.30mmol). The mixture was stirred for 30 minutes after the additions were complete, then the solution was warmed to room temperature and was stirred for 3 hours. The solvent was removed *in vacuo* and the residue was dissolved in n-heptane (300mL) and water was added to the flask. With vigorous stirring, the pH of the aqueous phase was adjusted to pH ca. 6.0 by the addition of 3% aq. citric acid solution. After the pH target was achieved, the organic phase was separated and was dried over Na_2SO_4 . Filtration and concentration *in vacuo* provided crude **16-6** (13.77) which was utilized in the next step without additional purification. LC-MS (+ mode): RT 0.773 min. 984.5 ($\text{M}+\text{H}^+$); ^1H -NMR (300 MHz, CDCl_3): δ 7.99 (m, 1H), 7.27 (m, 1H), 7.12 (m, 1H), 5.20 (m, 2H), 4.31 (m, 4H), 4.18 (m, 4H), 3.41 (m, 4H), 2.31 (m, 4H), 2.23 (m, 4H), 1.82-2.00 (12H), 1.79 (m, 8H), 1.00-1.40 (20H), 0.75-0.92 (20H).

[0432] **Synthesis of LIPID 16: ((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate)**

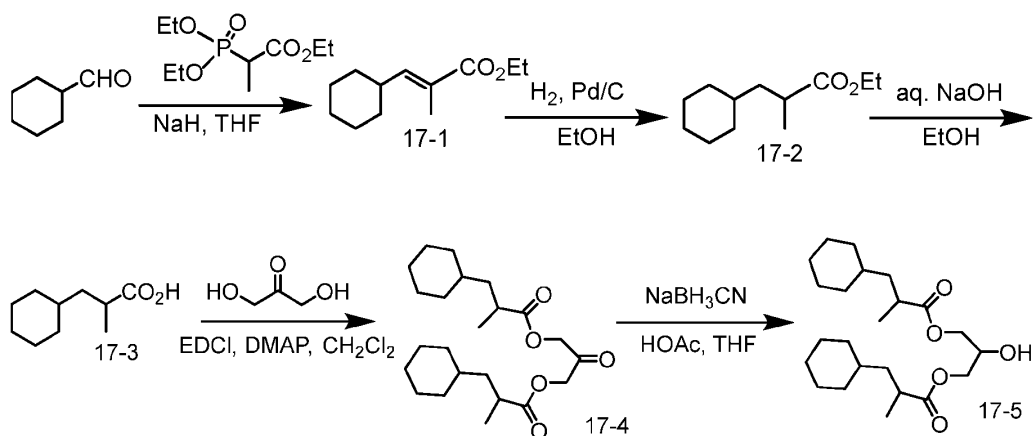


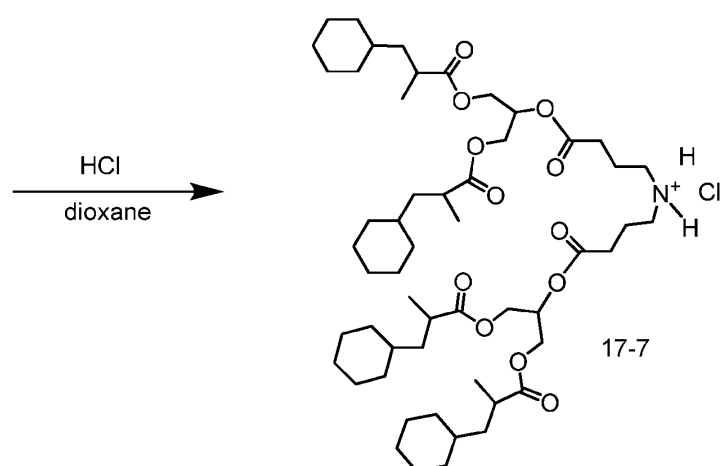
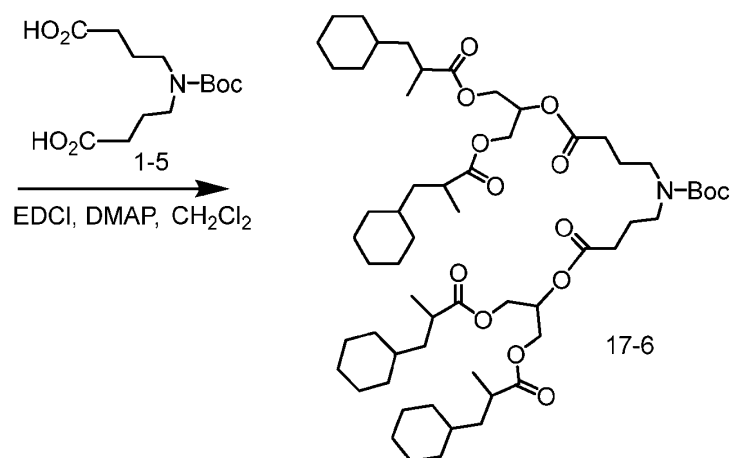
[0433] A solution of **16-6** (13.77g, crude) in CH_2Cl_2 (150mL) was cooled in an ice-water bath under nitrogen. To this cooled solution was added methyl trifluoromethanesulfonate (2.52 g, 20.99 mmol) over a period of 10 minutes. The mixture was stirred for 1 hour in the ice-water bath, then Et_3N (4.24g, 41.97mmol) was added over a period of 5 minutes followed by the addition of 3-dimethylamino-propane-1-thiol (2.49g, 20.99mmol) over a period of 5 minutes. The mixture was allowed to stir for 30 minutes, then it was warmed to room temperature and was stirred for 8 hours. The mixture was concentrated *in vacuo* and the residue was dissolved in CH_2Cl_2 (100mL) to which was added silica gel (30g, type ZCX-2, 100-200 mesh) and the solvent was removed *in vacuo* to afford silica gel containing adsorbed, crude **16**. The silica gel was placed atop a column of silica gel (80g, type ZCX-2, 100-200 mesh) and a combi-flash was used to purify the crude **LIPID 16** by eluting with a gradient of CH_2Cl_2 :MeOH from 100:0 to 96:4, collecting 300mL fractions. Qualified fractions were located by TLC, combined, and concentrated *in vacuo* to yield **LIPID 16** (10.14g, 9.797mmol, 75% yield over 3 steps) as a clear, light yellow oil. ES-MS: 1036.0 ($\text{M}+\text{H}^+$); HPLC Purity 94.24%; $^1\text{H-NMR}$ (300MHz, CDCl_3): δ 5.24 (m, 2H), 4.30 (dd, $J = 11.9, 4.6\text{Hz}$, 4H), 4.14 (dd, $J = 11.9, 5.7\text{Hz}$, 4H), 3.38 (brm, 4H), 2.94 (t, $J = 7.1\text{Hz}$, 2H), 2.46 (s, 6H), 2.34 (brm, 4H), 2.20 (m, 4H), 1.72-1.91 (20H), 1.37 (m, 8H), 1.08-1.22 (16H), 0.80-0.91 (20H).

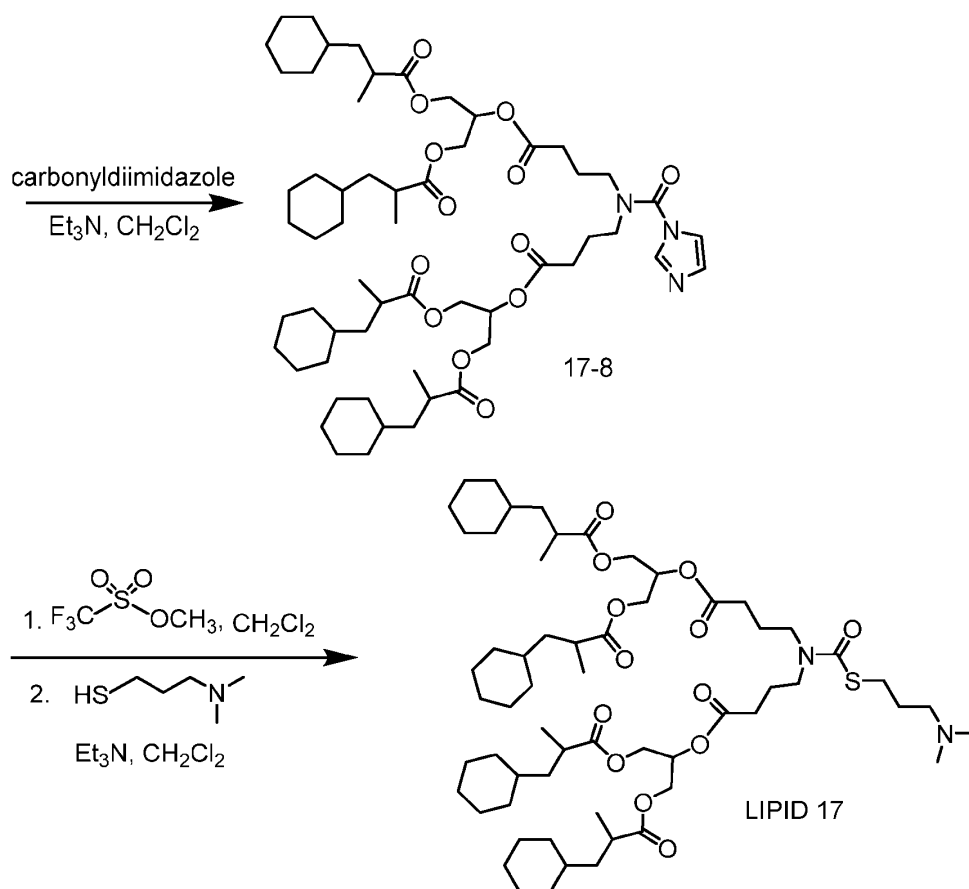
Example 17. Synthesis of LIPID 17: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexyl-2-methylpropanoate)



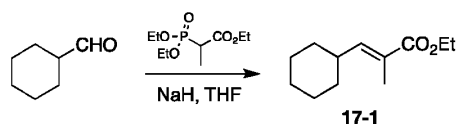
[0434] General Scheme:







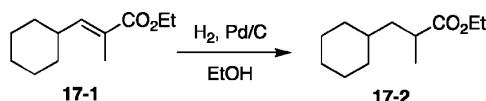
[0435] Synthesis of 17-1: Ethyl (*E*)-3-cyclohexyl-2-methylacrylate



[0436] Into a 2 L 4-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was charged NaH (60%, 21.42 g, 0.534 mol, 1.0 equiv) and THF (822 mL). Then, ethyl 2-(diethoxyphosphoryl)propanoate (127.2 g, 0.536 mol, 1.0 equiv) was added dropwise over 30 min at room temperature and the mixture was stirred for 1.5 h after the addition was complete. Cyclohexane carboxaldehyde (60.0 g, 0.536 mol, 1.0 equiv) in THF (318 mL) was added dropwise over 30 min and the mixture was stirred for 2 h at room temperature. The reaction was quenched with saturated aqueous NH₄Cl (1.5 L) and extracted with MTBE (2 x 0.75 L). The combined organic layers were washed with H₂O (0.75 L), brine (0.75 L, 12.5 V),

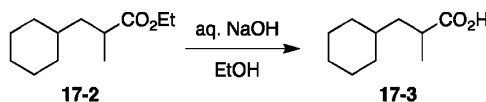
dried with anhydrous Na_2SO_4 , filtered and concentrated under vacuum. This resulted in 105 g **17-1** (crude) as a yellow oil that was used in the next step without further purification.

[0437] Synthesis of 17-2: Ethyl 3-cyclohexyl-2-methylpropanoate



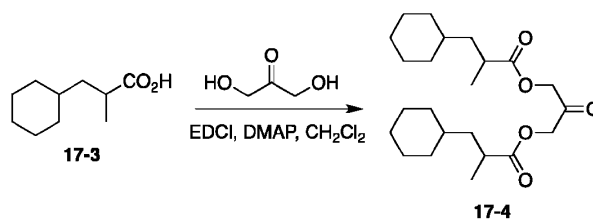
[0438] Into a 2 L round-bottom flask, flushed with nitrogen, was charged **17-1** (120.0 g, 1.0 equiv) in EtOH (1.2 L). Then, 10%wt Pd/C (36.0 g, 30%w./w.) was added in one portion. Then the mixture was stirred under H_2 atmosphere for 4 h at room temperature. Filtered and the filter cake was washed with CH_2Cl_2 (1.2 L). The filtrate was concentrated under vacuum to give crude **17-2**. Crude **17-2** was dissolved in CH_2Cl_2 (1 L) and added 200 g of silica gel (type: ZCX-2, 100-200 mesh, 1.67 w./w.), the solvent was then removed under vacuum while maintaining the temperature below 35°C . Charged 1 kg of silica gel (type: ZCX-2, 100-200 mesh, 8.33 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. A combi-flash was utilized to purify the product eluting with a petroleum ether / EtOAc gradient from 100:0 to 95:5, collecting 1000 mL fractions. Took samples for TLC analysis and combined qualified products. This resulted in 94 g (76% yield) of **17-2** as yellow oil.

[0439] Synthesis of 17-3: 3-Cyclohexyl-2-methylpropanoic acid



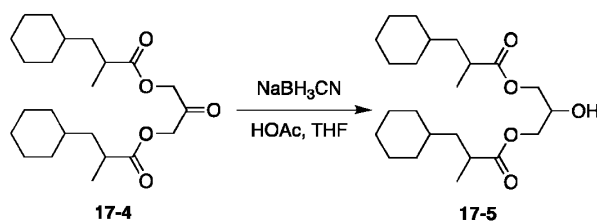
[0440] Into a 2 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **17-2** (57.0 g, 0.288 mol, 1.0 equiv) in EtOH (285 mL) at room temperature. Then, NaOH (17.3 g, 0.433 mol, 1.5 equiv) in H_2O (285 mL) was added in one portion. The resulting solution was then warmed to 70°C and was stirred for 3 h. The reaction was cooled to room temperature and extracted with n-heptane (2 x 200 mL). The H_2O layer was adjusted to pH=2 with aqueous HCl (12 mol/L), then extracted with MTBE (2 x 300 mL). The combined organic layers were washed with H_2O (2 x 150 mL), dried with anhydrous Na_2SO_4 , filtered and concentrated under vacuum. This resulted in 47 g (0.276 mol, 96 % yield) **17-3** as colorless oil. ELSD A: water/5mM $\text{NH}_4^+\text{HCO}_3^-$: B: CH_3CN 90:10 to 10:9 A/B at 2 min., hold 1 min); RT 0.56 min, m/z 170.1 (Calcd.), (found) 169.13 (M-H).

[0441] Synthesis of 17-4: 2-Oxopropane-1,3-diyl bis(3-cyclohexyl-2-methylpropanoate)



[0442] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added 1,3-dihydroxyacetone (17.5 g, 0.194 mol, 1.0 equiv) and **17-3** (66.0 g, 0.388 mol, 2.0 equiv) in CH_2Cl_2 (350 mL). The temperature was reduced to 0 °C in an ice/water bath. To the cooled solution was added DMAP (12.0 g, 0.098 mol, 0.5 equiv) and EDCI (112 g, 0.583 mol, 3.0 equiv) at 0 °C. The ice/water bath was removed, and the temperature was raised gradually. The reaction mixture was stirred overnight at room temperature. Directly, to the reaction mixture, was added 200 g of silica gel (type: ZCX-2, 100-200 mesh, 11.4 w./w.), and the solvent was removed under vacuum while maintaining the temperature below 35 °C. Charged 1 kg of silica gel (type: ZCX-2, 100-200 mesh, 57.1 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using combi-flash to purify the product, eluting with a petroleum ether / EtOAc gradient from 100:0 to 90:10, collecting 1000 fractions. Took sample for TLC analysis and combined qualified products. This resulted in 73 g (0.184 mol, 95% yield) of **17-4** as light-yellow oil. Product has no MS signal and used as such in the next step.

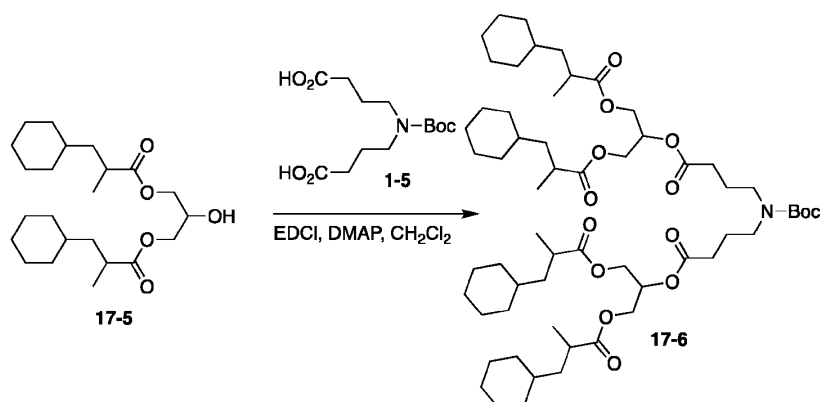
[0443] **Synthesis of 17-5: 2-Hydroxypropane-1,3-diyl bis(3-cyclohexyl-2-methylpropanoate)**



[0444] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **17-4** (56.0 g, 0.142 mol, 1.0 equiv) in THF (560 mL). The temperature was reduced to 0 °C in an ice/water bath. To the solution was added HOAc (12.8 g, 0.213 mol, 1.5 equiv) at 0 °C, and then to the mixture was added NaBH_3CN (12.5 g, 0.199 mol, 1.4 equiv) at 0 °C. The ice/water bath was removed, and the temperature was

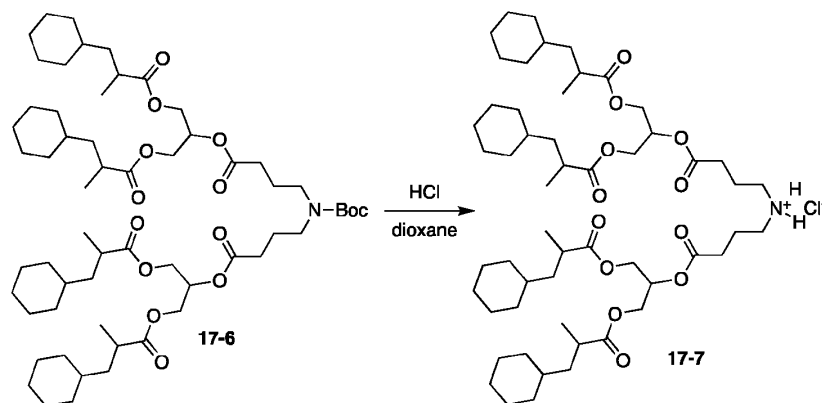
raised gradually. The reaction mixture was stirred for 8 h at room temperature. The reaction mixture was quenched with H₂O (1.1 L) and extracted with CH₂Cl₂ (1.6 L). The organic layer was washed with aqueous NaHCO₃ (560 mL), H₂O (2 x 280 mL), dried with anhydrous Na₂SO₄ and filtered. The filtrate was used for next step directly.

[0445] Synthesis of 17-6: ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexyl-2-methylpropanoate)



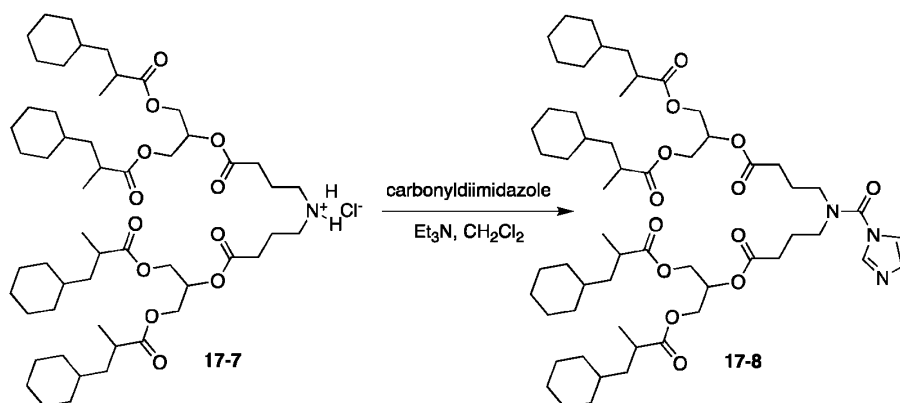
[0446] Into a 2 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **1-5** (20.0 g, 0.069 mol, 1.0 equiv) and **17-5** (solution from above, 0.138 mol, 2.0 equiv). The temperature was reduced to 0 °C in an ice/water bath. To the solution was added DMAP (8.4 g, 0.069 mol, 1.0 equiv) and EDCI (53 g, 0.277 mol, 4.0 equiv) at 0 °C. The ice/water bath was removed, and the temperature was raised gradually. The reaction mixture was stirred overnight at room temperature. Directly to the reaction mixture was added 200 g of silica gel (type: ZCX-2, 100-200 mesh, 10.0 w./w.), the mixture was then concentrated under vacuum while maintaining the temperature below 35 °C. Charged 1.5 kg of silica gel (type: ZCX-2, 100-200 mesh, 75.0 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using combi-flash to purify the product, eluting with a petroleum ether / EtOAc gradient from 100:0 to 90:10, collecting 500 mL fractions. Took sample for TLC analysis and combined qualified products. This resulted in 30.8 g (59.6 mmol, 42% for 2 steps) of **17-6** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 1.0 min): RT 2.08 min, m/z (Calcd.) 1045.7, (found) 946.6 (M-Boc+H).

[0447] Synthesis of 17-7: bis(4-((1,3-bis((3-Cyclohexyl-2-methylpropanoyl)oxy)propan-2-yl)oxy)-4-oxobutyl)ammonium chloride



[0448] Into a 1 L round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of 17-6 (48.0 g, 0.046 mol, 1.0 equiv) in 1,4-dioxane (240 mL) and the solution was cooled in an ice-water bath. To the solution was added 4M HCl in 1,4-dioxane (240 mL) dropwise at 0-10 °C over 10 min. The resulting solution was stirred overnight at room temperature. The mixture was concentrated under vacuum. This provided 48 g (crude) of 17-7 as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min., hold 1.0 min): RT 0.92 min, m/z (Calcd.) 945.6, (found) 946.6 (M+H).

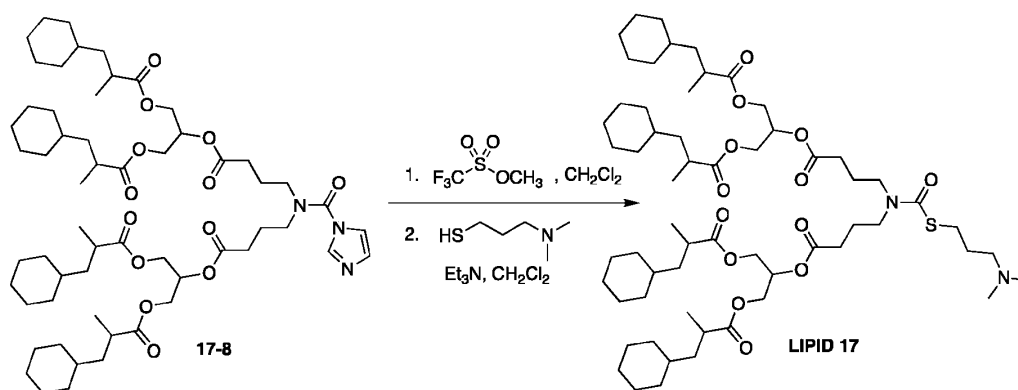
[0449] Synthesis of 17-8: ((4,4'-((1*H*-Imidazole-1-carbonyl)azanediyl)bis(butanoyl))-bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexyl-2-methylpropanoate)



[0450] Under nitrogen atmosphere, charged 17-7 (48.0 g, 0.046 mol, 1.0 equiv) in CH₂Cl₂ (1.06 L) into a 2 L 3-necked round-bottom bottle. Then, carbonyldiimidazole (15.9 g, 0.098 mol, 2.1 equiv) was added followed by pyridine (15.4 g, 0.196 mol, 4.26 equiv) and the mixture was

stirred overnight at room temperature. The resulting solution was washed with 3% aqueous citric acid (2 x 500 mL), H₂O (3 x 500 mL) and brine (500 mL), dried with anhydrous Na₂SO₄, and concentrated at 35 °C under vacuum. The crude mixture was dissolved in CH₂Cl₂ (800 mL) and 100 g of silica gel (type: ZCX-2, 100-200 mesh, 2.08 w./w.) was added and the solvent was removed under vacuum while maintaining the temperature below 35 °C. Charged 300 g of silica gel (type: ZCX-2, 100-200 mesh, 6.25 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the produce, eluting with a petroleum ether / EtOAc gradient from 100:0 to 70:30, collecting 400 mL fractions. Took samples for TLC analysis and combined qualified products. This resulted in 39 g (37.5 mmol, 81% yield) **17-8** as an oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min, hold 0.6 min): RT 1.0 min, m/z (Calcd.) 1039.6, (found) 1040.6 (M+H).

[0451] Synthesis of LIPID 17: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)-azanediyl)bis(butanoyl))bis(oxy))bis(propene-2,1,3-triyl) tetrakis(3-cyclohexyl-2-methylpropanoate)

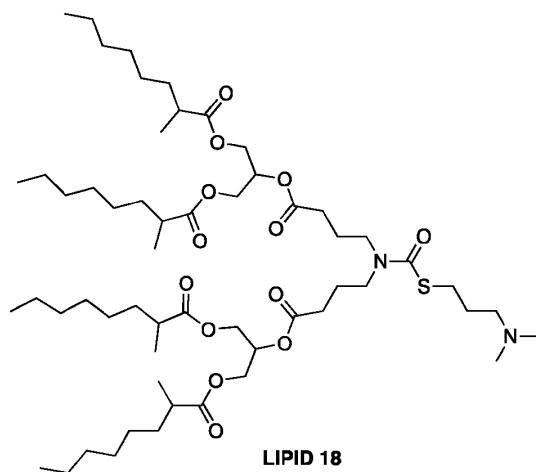


[0452] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **17-8** (20.0 g, 0.019 mol, 1.0 equiv) in CH₂Cl₂ (200 mL). The reaction temperature was reduced to 0 °C in an ice/water batch. To the mixture was added methyl trifluoromethanesulfonate (3.3 g, 0.020 mol, 1.05 equiv) at 0 °C. After addition, continue stirring the reaction for 3 hours at 0 °C. Charged the trimethylamine 2.0 M in THF (28.9 mL, 0.058 mol, 3.0 equiv) into the reactor at 0 °C. After addition, continue stirring the reaction for 0.5-1 hours at 0 °C. Charged 3-(dimethylamino)propane-1-thiol (3.0 g, 0.025 mol, 1.3 equiv) into the reactor at 0 °C. After addition, the reaction was allowed to come to room

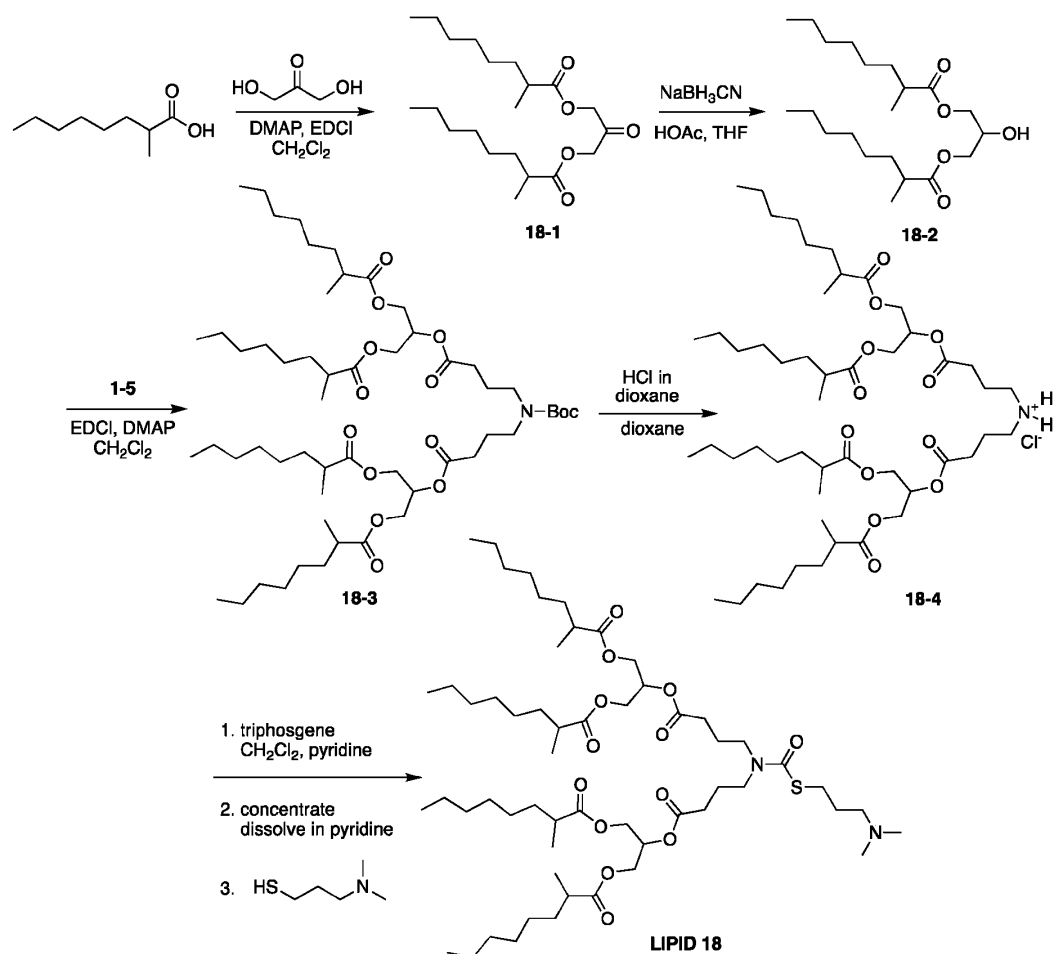
temperature and then continued to stir for 5.0 hours. Charged sodium chloride aqueous solution (10.0 wt.%, 200 mL) and 10% aqueous citric acid solution (10.0 wt%, 200 mL) into the reactor. Stirred for 15 minutes and then let reactor stand for 15 minutes to allow for phase separation at room temperature. Collected the organic layer. This operation was repeated one additional time. Charged sodium chloride aqueous solution (10.0 wt.%, 200 mL) and charge sodium bicarbonate aqueous solution (5.0 wt.%, 200 mL) into the reactor. Stirred for 15 minutes and then let reactor stand for 15 minutes to allow for phase separation at room temperature. Collect the organic layer. This operation was repeated one additional time. Charged sodium chloride aqueous solution (10.0 wt.%, 400 mL) into the reactor. Stirred for at least 15 minutes and then let reactor stand for at least 15 minutes to allow for phase separation at room temperature. Collected the organic layer. Charged the n-heptane (250 mL) into the reactor. Concentrated the solution to about 300 mL under vacuum while maintaining the temperature at 20-40 °C. Charged 10.0 wt.% citric acid methanol/water (10:1, 200 mL) solution into the reactor. After addition, stirred for 15 minutes and then let reactor stand for 15 minutes to allow phase separation at 36 ± 5 °C. Collected the MeOH/H₂O layer. Charged n-heptane (250 mL) into the reactor to wash the MeOH/H₂O phase. Repeat this n-heptane washing operation eight times. Charged n-heptane (500 mL), 15.0 wt.% sodium carbonate solution (250 mL) and 10.0 wt.% sodium chloride solution (250 mL) into the reactor. Stirred for 15 minutes and then let reactor stand for 15 minutes to allow for phase separation at room temperature. Collected the organic layer. Charged 5.0 wt.% sodium bicarbonate solution (400 mL) into the reactor. Stirred for 15 minutes and then let reactor stand for 15 minutes to allow for phase separation at room temperature. Collected the organic layer and dried over anhydrous sodium sulfate and concentrated under vacuum. To the residue in CH₂Cl₂ (300 ml) was added 30 g of silica gel (type: ZCX-2, 100-200 mesh, 1.5 w./w.), the mixture was then concentrated under vacuum while maintaining the temperature below 35 °C. Charged 200 g of silica gel (type: ZCX-2, 100-200 mesh, 10.0 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using combi-flash to purify the product, eluting with a CH₂Cl₂ / MeOH gradient from 100:0 to 90:10, collecting 400 mL fractions. Took samples for TLC analysis and combined qualified products. This resulted in 11.5 g (55% yield) of **LIPID 17** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 80:20 to 20:80 A/B at 3 min., hold 1 min): RT 0.97 min, m/z (Calcd.) 1090.7, (found) 1091.7 (M+H). ¹H-NMR (300 MHz, CDCl₃): δ 5.25 (m, 2H), 4.34 (dt, $J = 11.9, 4.0$ Hz, 4H), 4.15 (m,

4H), 3.38 (brn, 4H), 2.92 (t, $J = 7.3$ Hz, 2H), 2.67–2.49 (4H), 2.48 – 2.22 (12H), 1.89–1.84 (6H), 1.78 – 1.51 (24H), 1.35–1.07 (32H), 0.99 – 0.76 (8H).

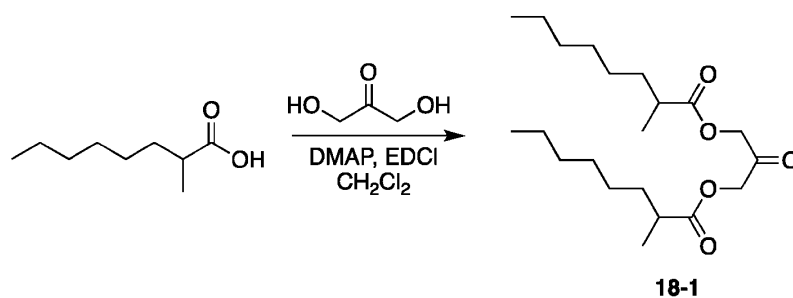
Example 18. Synthesis of LIPID 18: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanovl))bis(oxy))bis(propane-2,1,3-trivl) tetrakis(2-methyloctanoate)



[0453] General Scheme:



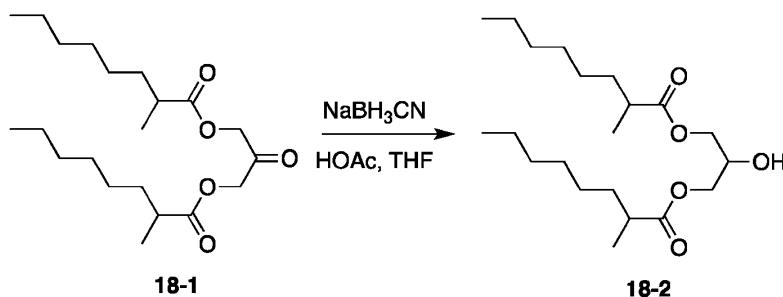
[0454] Synthesis of 18-1: 2-Oxopropane-1,3-diyl bis(2-methyloctanoate)



[0455] Into a 50 ml 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added 1,3-dihydroxyacetone (1.42 g, 1.0 equiv) and 2-methyloctanoic acid (5.0 g, 2.0 equiv, *Org. Biomol. Chem.* **2014**, *12*, 3649-3663) in CH_2Cl_2 (30

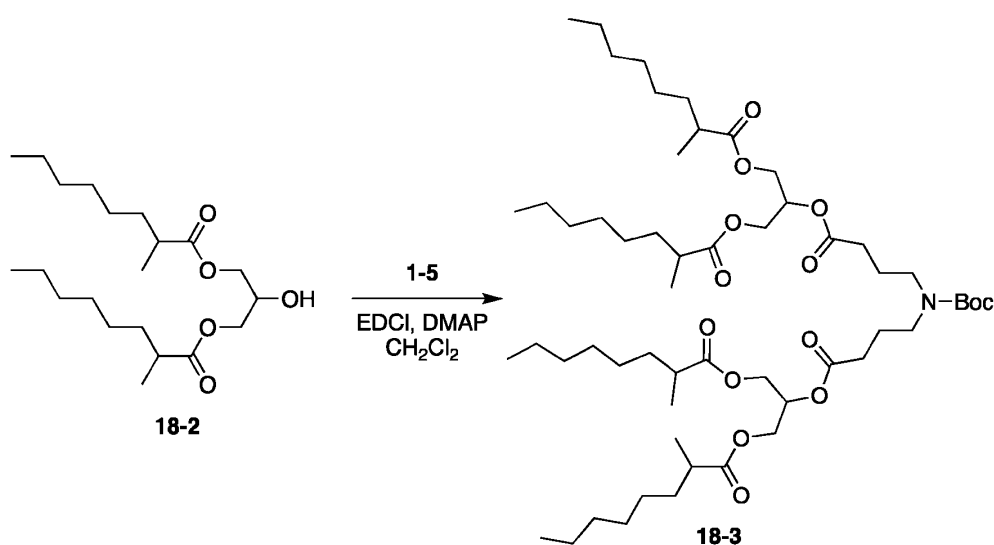
mL). The solution was cooled in an ice-water bath, then was added DMAP (0.96 g, 0.5 equiv) and EDCI (12.1 g, 4.0 equiv) at 0 °C. The reaction mixture was brought to room temperature and stirred overnight. To the mixture was added 20 g of silica gel (type: ZCX-2, 100-200 mesh, 15.5 w./w.), the solvent was removed under vacuum while maintaining the temperature below 35 °C. Charged 100 g of silica gel (type: ZCX-2, 100-200 mesh, 77.5 w/ w) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. The crude product was purified using a Combi Flash purification system, eluting with a petroleum ether / EtOAc gradient from 100:0 to 90:10 collected every 200±50 mL). Took sample for TLC analysis and qualified fractions were combined and concentrated under vacuum. This resulted in 4.9 g (85 % yield) of **18-1** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 1.3 min): RT 1.6 min, m/z (Calcd.) 370.3, (found) 371.3 (M+H).

[0456] Synthesis of 18-2: 2-Hydroxypropane-1,3-diyl bis(2-methyloctanoate)



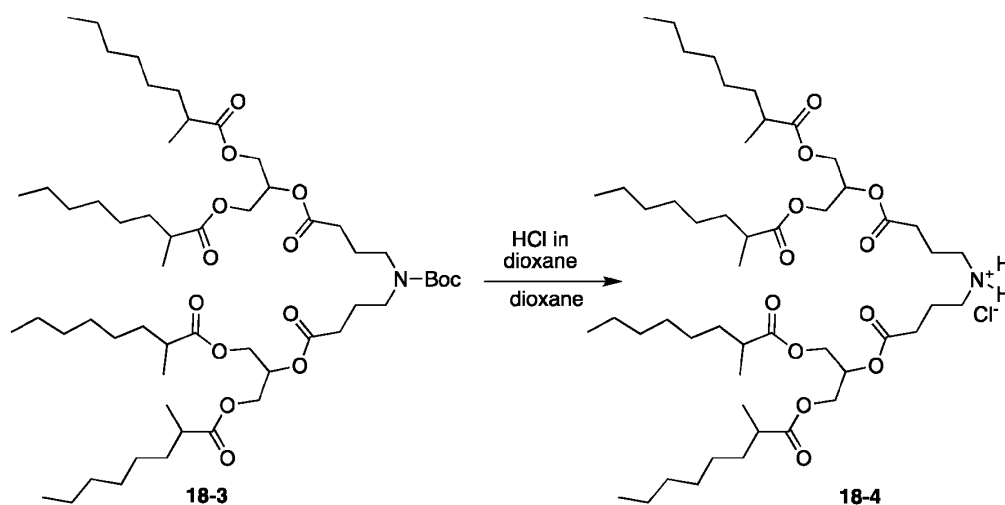
[0457] Into a 100 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **18-1** (4.9 g, 1.0 equiv) in THF (50 mL) and the solution was cooled in an ice-water bath. To the solution was added HOAc (1.03 g, 1.3 equiv) at 0 °C, followed by the addition NaBH₃CN (1.0 g, 1.2 equiv) at 0 °C. The ice water bath was removed after adding all reagents. The reaction mixture was stirred overnight at room temperature. The reaction mixture was quenched with H₂O (100 mL) and extracted with CH₂Cl₂ (150 mL). The organic layer was washed with 5% aqueous NaHCO₃ (50 mL), H₂O (2 x 50 mL), dried with anhydrous Na₂SO₄ and filtered. The filtrate, containing **18-2**, was used for next step directly.

[0458] Synthesis of 18-3: ((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)



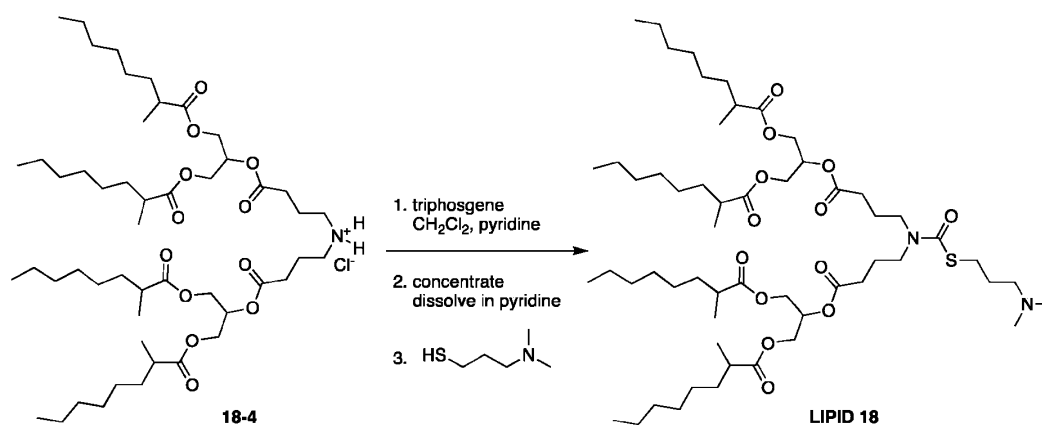
[0459] Into a 100 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **18-2** (8.8 g, 2.3 equiv) and **1-5** (3.0 g, 1.0 equiv) in CH₂Cl₂ (60 mL) and the mixture was cooled in an ice-water bath. To the solution was added DMAP (1.26 g, 1.0 equiv) and EDCI (7.96 g, 4.0 equiv) at 0 °C. The ice water bath was removed after adding all reagents. The reaction mixture was stirred overnight at room temperature. To the mixture was added 20 g of silica gel (type: ZCX-2, 100-200 mesh, 6.7 w./w.), and the solvent was removed under vacuum while maintaining the temperature below 35 °C. Charged 120 g of silica gel (type: ZCX-2, 100-200 mesh, 40.0 w/w) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. A combi-flash was utilized to purify the product, eluting with a petroleum ether / EtOAc gradient from 100:0 to 90:10, collecting 200 mL fractions. Took sample for TLC analysis and combined qualified products. This resulted in 4.3 g (42% yield) of **18-3** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 80:20 to 20:80 A/B at 3 min., hold 1 min): RT 1.97 min, m/z (Calcd.) 997.7, (found) 1020.6 (M+Na).

[0460] Synthesis of 18-4: bis(4-((1,3-bis((2-methyloctanoyl)oxy)propan-2-yl)oxy)-4-oxobutyl) ammonium chloride



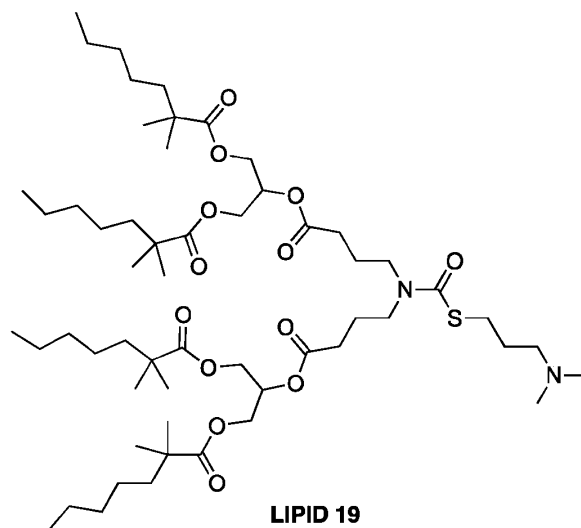
[0461] Into a 100 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **18-3** (4.3 g, 1.0 equiv) in 1,4-dioxane (21 mL) and the solution was cooled in an ice-water bath. To the cooled solution was added 4M HCl in 1,4-dioxane (21 mL) dropwise at 0-10 °C over 10 min. The resulting solution was stirred overnight at room temperature. The reaction mixture was concentrated under vacuum. This resulted in 4 g (crude) of **18-4** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 1.3 min): RT 1.6 min, m/z (Calcd.) 897.6, (found) 898.6 (M+H).

[0462] **Synthesis of LIPID 18: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)-azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

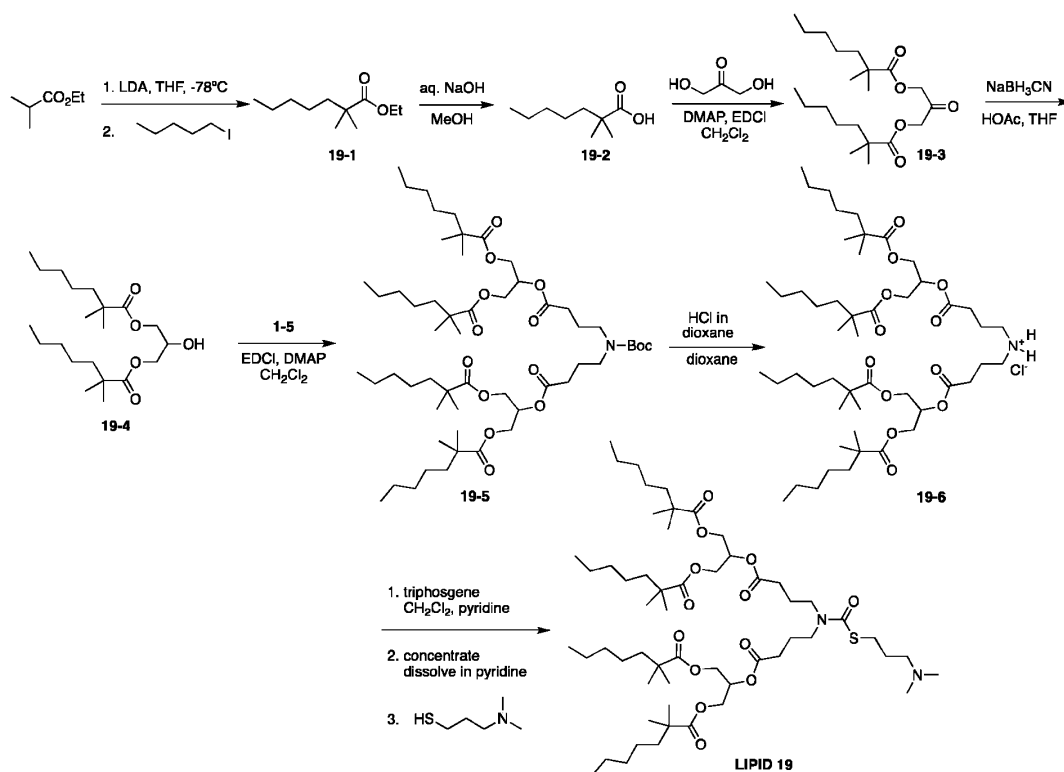


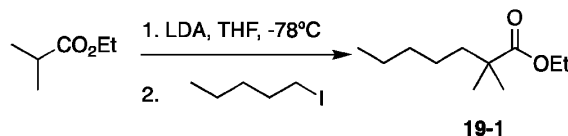
[0463] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **18-4** (3.8 g, 1.0 equiv) in CH₂Cl₂ (80 mL) and the solution was cooled in an ice-water bath. To the mixture was added triphosgene (1.26 g, 1.0 equiv) at 0 °C, followed by the addition of pyridine (1.67 g, 5.0 equiv) dropwise with stirring at 0 °C. The ice water bath was removed after adding all reagents. The mixture was stirred for 4 h at room temperature and then concentrated under vacuum (temperature <30°C). The residue was dissolved with pyridine (80 mL), cooled in an ice-water bath under nitrogen, then 3-(dimethylamino)propane-1-thiol (1.0 g, 2.0 equiv) was added dropwise with stirring at 0 °C in 10 min. The resulting solution was stirred for overnight at room temperature. The reaction mixture was concentrated under vacuum and the residue was diluted with CH₂Cl₂ (80 mL). The solution was washed with 10% aq. citric acid (40 mL), H₂O (40 mL), saturated NaHCO₃ (2 x 40 mL) and brine (40 mL, 10 V). The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. To the residue, dissolved in CH₂Cl₂ (60 mL), was added 8 g of silica gel (type: ZCX-2, 100-200 mesh, 2.11 w./w.), and the solvent was removed under vacuum while maintaining the temperature below 35 °C. Charged 100 g of silica gel (type: ZCX-2, 100-200 mesh, 26.3 w/ w) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the product, eluting with a CH₂Cl₂ / MeOH gradient from 100:0 to 90:10, collecting 100 mL fractions. Took samples for TLC analysis and combined qualified products. This resulted in 1.3 g (29% yield for 2 steps) of **LIPID 18** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 80:20 to 20:80 A/B at 3 min., hold 2.1 min): RT 1.1 min, m/z (Calcd.) 1042.7, (found) 1043.6 (M+H). ¹H-NMR (300 MHz, CDCl₃): δ 5.25 (m, 2H), 4.34 (m, 4H), 4.16 (m, 4H), 3.38 (brm, 4H), 2.92 (m, 4H), 2.66 (s, 6H), 2.46 (m, 4H), 2.35 (brs, 4H), 2.09 (m, 2H), 1.90 (brs, 4H), 1.64 (m, 4H), 1.47-1.20 (38H), 1.15-1.13 (12H), 0.95 – 0.81 (12H).

Example 19. Synthesis of LIPID 19: (((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2,2-dimethylheptanoate)

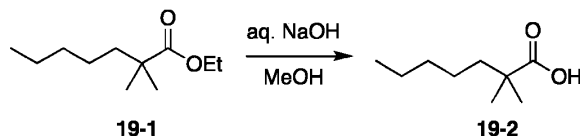


[0464] General Scheme:



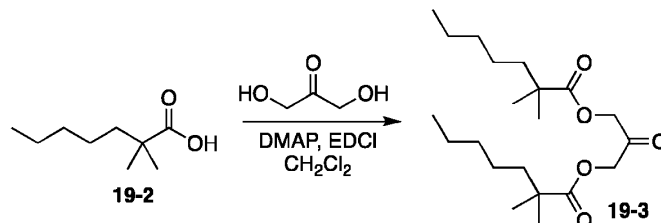
[0465] Synthesis of 19-1: Ethyl 2,2-dimethylheptanoate

[0466] Into a 500 ml 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed ethyl isobutyrate (40.0 g, 1.0 equiv) in THF (400 mL). The resulting solution was cooled to -78 °C, then LDA (205.6 ml, in hexane, 1.05 equiv) was added dropwise, and the resulting solution was stirred for 1 h at -78 °C. Then, 1-iodopentane (92.8 g, 1.2 equiv) was added dropwise, and the resulting solution was stirred for 5 h at -78 °C. The cooling bath was removed, and the solution was stirred overnight at room temperature. The pH value of the solution was adjusted to 6 with aqueous HCl solution (1 mol/L). The resulting solution was extracted with ethyl acetate (2 x 300 mL) and the organic layers were combined. The resulting mixture was washed with brine (500 mL), dried over anhydrous sodium sulfate and concentrated under vacuum. This resulted in 51 g (75.6 %) of **19-1** as yellow oil that was used in the next step without further purification.

[0467] Synthesis of 19-2: 2,2-Dimethyl heptanoic acid

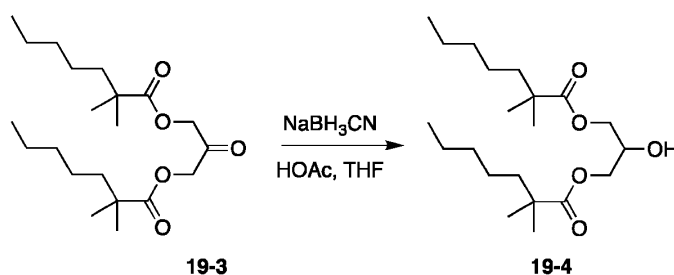
[0468] Into a 2-L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **19-1** (70.0 g, 1.0 equiv) in MeOH (700 mL). A solution of NaOH (49.0 g, 3.0 equiv) in H₂O (350 mL) was added dropwise to the solution at room temperature. The resulting solution was warmed and stirred for 4 h at 60 °C. The resulting mixture was concentrated under vacuum after cooling to room temperature. The residue was dissolved in H₂O (200 mL), extracted with MTBE (200 mL) and the aqueous layer was separated. The pH value of the aq. layer was adjusted to 5 with aqueous HCl solution (1 mol/L). The resulting solution was extracted with ethyl acetate (2 x 100 mL) and the organic layers combined. The resulting mixture was washed with brine (200 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. This resulted in 32 g (68%) of **19-2** as yellow oil which was used without purification.

[0469] Synthesis of 19-3: 2-Oxopropane-1,3-diyl bis(2,2-dimethylheptanoate)



[0470] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added 1,3-dihydroxyacetone (12.0 g, 1.0 equiv) in CH_2Cl_2 (240 mL). The solution was cooled to 0°C in an ice/water bath. To the solution were added **19-2** (44.0 g, 2.1 equiv), DMAP (16.3 g, 1.0 equiv) followed by EDCI (76.7 g, 3.0 equiv) at 0°C . The ice/water bath was removed and the reaction mixture was stirred overnight at room temperature. To the reaction solution was added 25 g of silica gel (type: ZCX-2, 100-200 mesh, 2.08 w./w.), the mixture was then concentrated under vacuum while maintaining the temperature below 35°C . Charged 500 g of silica gel (type: ZCX-2, 100-200 mesh, 41.7 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the product, eluting with a petroleum ether/EtOAc gradient from 95:5 to 90:10, collecting 1000 fractions. Took samples for TLC analysis and combined qualified products. Concentration under vacuum resulted in 40.6 g (75.8%) **19-3** as colorless oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 1.3 min): RT 1.8 min, m/z (Calcd.) 370.2, (found) 393.2 (M+Na).

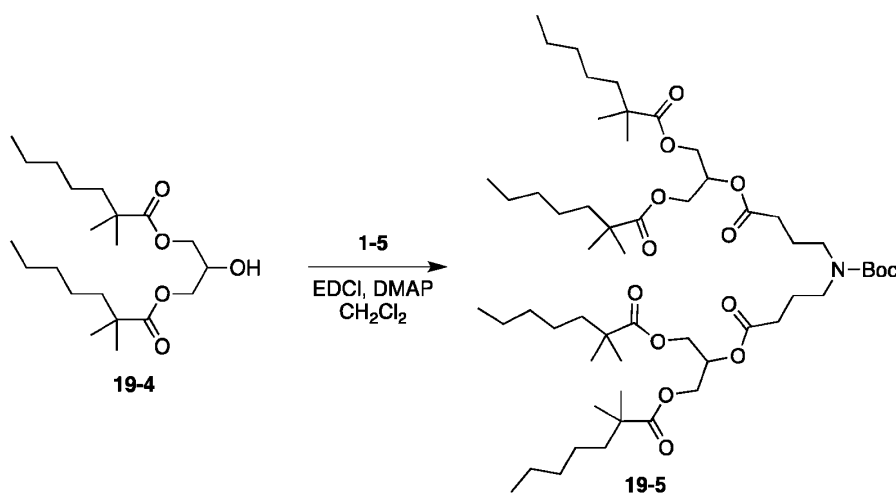
[0471] Synthesis of 19-4: 2-Hydroxypropane-1,3-diyl bis(2,2-dimethylheptanoate)



[0472] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **19-3** (15.5 g, 1.0 equiv) in THF (155 mL). The solution was cooled to 0°C in an ice/water bath. To the solution was added HOAc (3.26 g, 1.3 equiv) at 0°C ,

and then, to the mixture was added NaBH_3CN (3.16 g, 1.2 equiv) in one batch at 0 °C. The ice/water bath was removed, and the mixture was stirred for 16 h at room temperature. The reaction was quenched with water (200 mL). The mixture was extracted with CH_2Cl_2 (3 x 200 mL). The combined organic phases were washed with brine (500 mL), then dried with Na_2SO_4 . Filtration and concentration under vacuum gave crude **19-4** which was dissolved in CH_2Cl_2 (75 mL) and 30 g of silica gel (type: ZCX-2, 100-200 mesh, 1.94 w./w.) was added to the solution, the mixture was concentrated under vacuum while maintaining the temperature below 35 °C. Charged 200 g of silica gel (type: ZCX-2, 100-200 mesh, 12.9 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the product, eluting with a petroleum ether/EtOAc gradient from 90:10 to 85:15, collecting 400 mL fractions. Took samples for TLC analysis and combined qualified products. Concentration under vacuum gave in 12.3 g (79.3% yield) **19-4** as yellow oil. ELSD A: water/0.05% TFA : B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 2 min, hold 1.2 min): RT 1.5 min, m/z (Calcd.) 372.2, (found) 395.2 (M+Na).

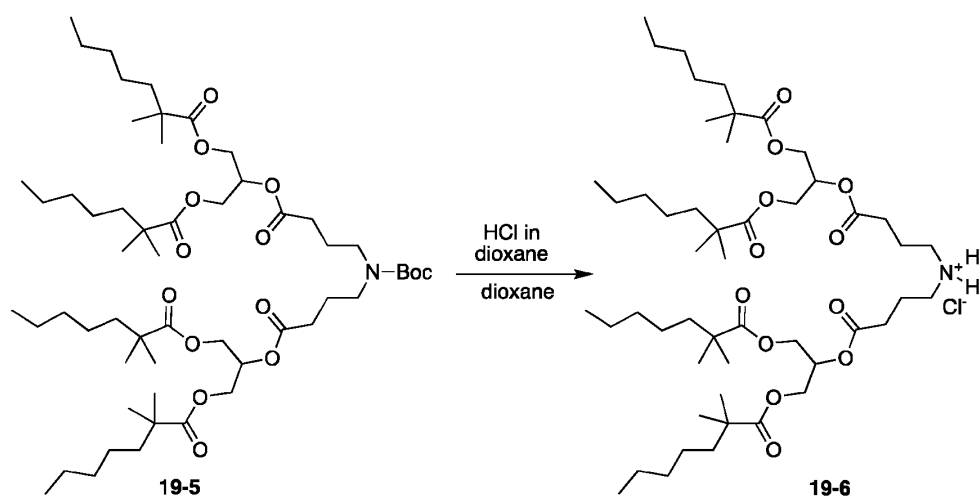
[0473] **Synthesis of 19-5: ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(proppane-2,1,3-triyl) tetrakis(2,2-dimethylheptanoate)**



[0474] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **1-5** (4.77 g, 1.0 equiv) in CH_2Cl_2 (80 mL). The solution was cooled to 0 °C in an ice/water bath, then **19-4** (12.3 g, 2.0 equiv), DMAP (2.0 g, 1.0 equiv) were added in order, followed by EDCI (9.5 g, 3.0 equiv) at 0 °C. The ice/water bath was removed and the resulting solution was stirred for 16 h at room temperature. To the reaction solution was

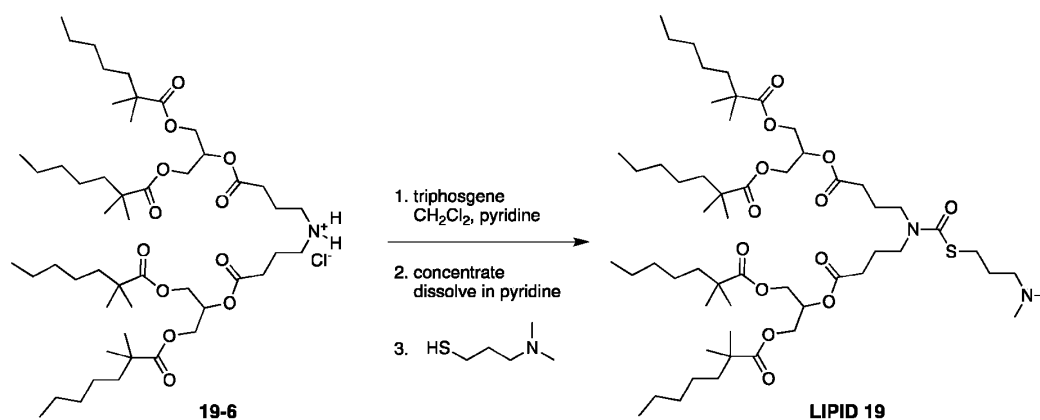
added 15 g of silica gel (type: ZCX-2, 100-200 mesh, 3.14 w./w.), the mixture was concentrated under vacuum while maintaining the temperature below 35 °C. Charged 200 g of silica gel (type: ZCX-2, 100-200 mesh, 41.9 w./w.) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the product, eluting with a petroleum ether/EtOAc gradient from 90:10 to 86:14, collecting 400 mL fractions. Took samples for TLC analysis and then combined qualified products. This resulted in 15.8 g (96.3 %) of **19-5** as yellow oil.

[0475] Synthesis of 19-6: bis(4-((1,3-bis((2,2-dimethylheptanoyl)oxy)propan-2-yl)oxy)-4-oxobutyl) ammonium chloride



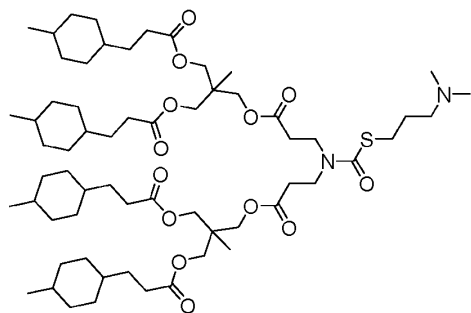
[0476] Into a 250 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **19-5** (6.0 g, 1.0 equiv) in CH₂Cl₂ (30 mL). The solution was cooled to 0 °C in an ice/water bath. To the solution was added HCl in dioxane (60 mL, 4 mol/L) dropwise at 0-10 °C. The ice/water bath was removed and the resulting solution was stirred overnight at room temperature. The reaction mixture was concentrated under vacuum resulting in 6.2 g (crude) of **19-6** as light-yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 1.2 min): RT 1.5 min, m/z (Calcd.) 897.6, (found) 898.5 (M+H).

[0477] Synthesis of LIPID 19: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)-azanediyl)bis(butanoyl)) bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2,2-dimethylheptanoate)



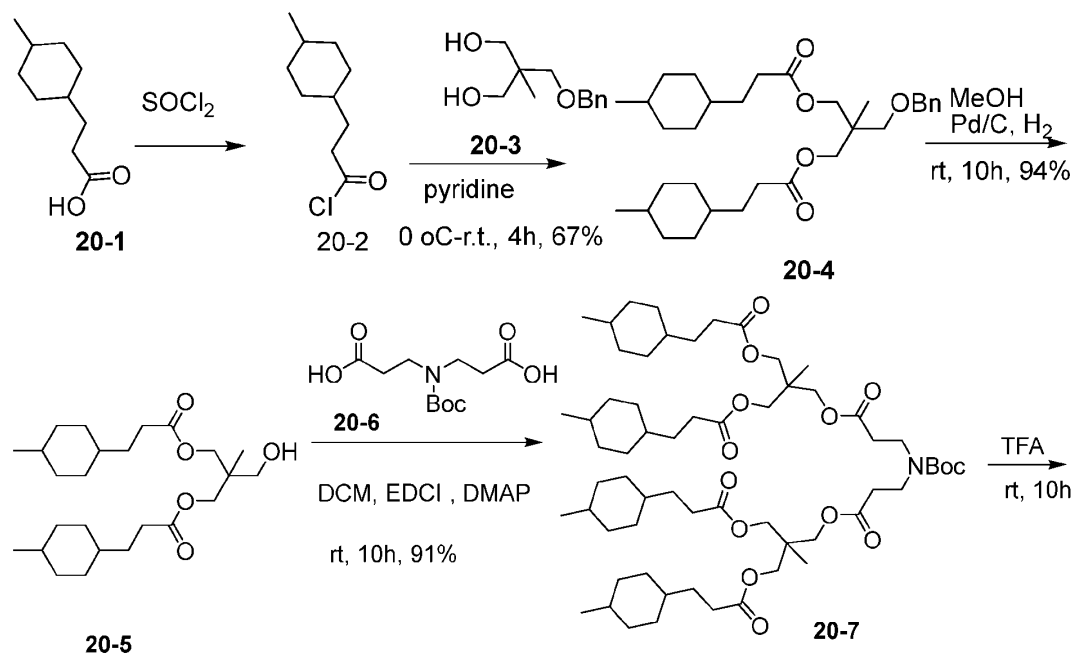
[0478] Into a 500 ml 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **19-6** (6.0 g, 1.0 equiv) in CH₂Cl₂ (210 mL). The solution was cooled to 0 °C in an ice/water bath. To the mixture was added triphosgene (2.69 g, 1.5 equiv) at 0 °C. This was followed by the addition of pyridine (2.53 g, 5.0 equiv) dropwise with stirring at 0 °C. The ice/water bath was removed and the mixture was stirred for 4 h at room temperature and then concentrated under vacuum (temperature <30 °C). The residue was dissolved with pyridine (120 mL, 20 V) and the solution was cooled to 0 °C in an ice/water bath. To this solution was added 3-(dimethylamino)propane-1-thiol (1.53 g, 2.0 equiv) dropwise with stirring at 0 °C in 10 min. The ice/water bath was removed and the resulting solution was stirred overnight at room temperature. The reaction mixture was concentrated under vacuum and crude **19** was dissolved in CH₂Cl₂ (100 mL) and 12 g of silica gel (type: ZCX-2, 100-200 mesh), was added and the mixture was concentrated vacuum while maintaining the temperature below 35 °C. Charged 100 g of silica gel (type: ZCX-2, 100-200 mesh) to the column, followed by the last step prepared dry silica gel which absorbed the reaction mixture. Using a combi-flash to purify the product, eluting with a CH₂Cl₂/acetone gradient from 75/25 to 70/30, collecting 200 mL fractions. Took samples for TLC analysis and combined qualified products. This resulted in 1.0 g (13.8% yield) **19** as yellow oil. ELSD A: water/0.05% TFA : B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 2 min., hold 1.2 min): RT 1.5 min, m/z (Calcd.) 1042.7, (found) 1043.9 (M+H). ¹H-NMR (300 MHz, CDCl₃): δ 5.28 (m, 2H), 4.32 (dd, *J* = 11.9, 4.4 Hz, 4H), 4.11 (dd, *J* = 11.9, 5.7 Hz, 4H), 3.38 (brm, 4H), 2.91 (m, 2H), 2.18-2.33 (6H), 2.23 (s, 6H), 1.78 (brm, 4H), 1.55 – 1.42 (8H), 1.35 – 1.10 (50H), 0.87 (t, *J* = 6.9 Hz, 12H).

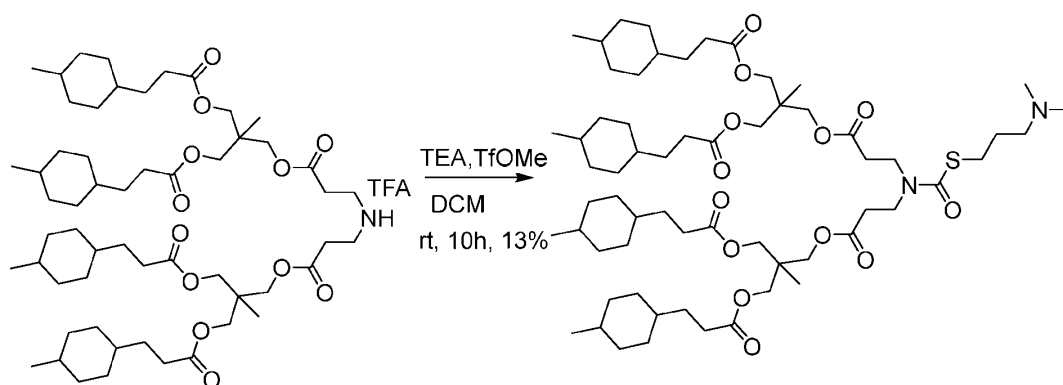
Example 20. Synthesis of LIPID 20: ((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)



[0479] LIPID 20

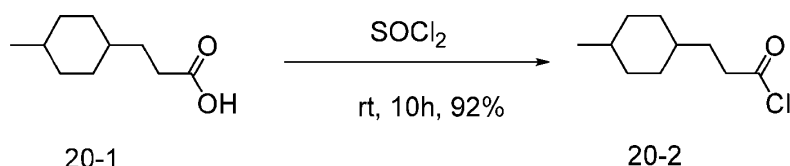
[0480] General scheme:





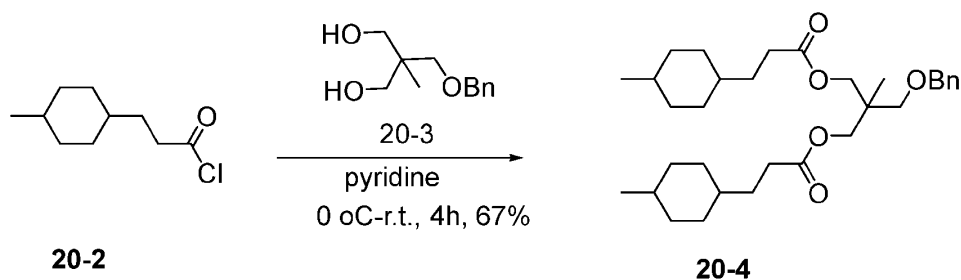
Lipid 20

[0481] Synthesis of 20-2: 3-(4-methylcyclohexyl)propanoyl chloride



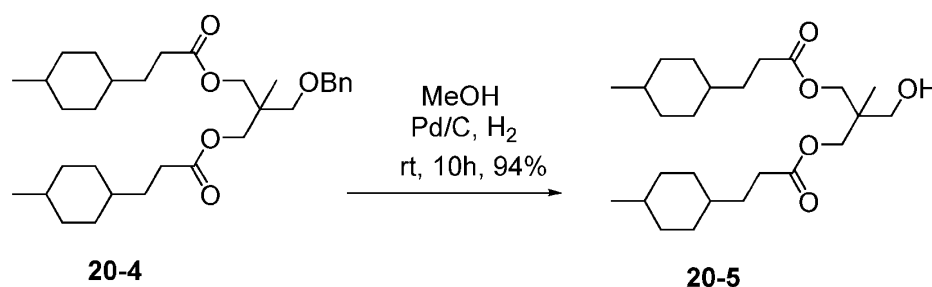
[0482] Into a 25mL 3-necked round-bottom flask were added SOCl₂ (100 mL, 1378.615 mmol, 4.69 equiv..) and commercially available 3-(4-methylcyclohexyl)propanoic acid (50 g, 293.682 mmol, 1 equiv..) at room temperature. The resulting mixture was stirred for 10h at room temperature and concentrated under reduced pressure to afford 3-(4-methylcyclohexyl)propanoyl chloride (51 g, 92.03%) as a yellow oil which was used as such without further purification or characterization.

[0483] Synthesis of 20-4: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(3-(4-methylcyclohexyl) -propanoate)



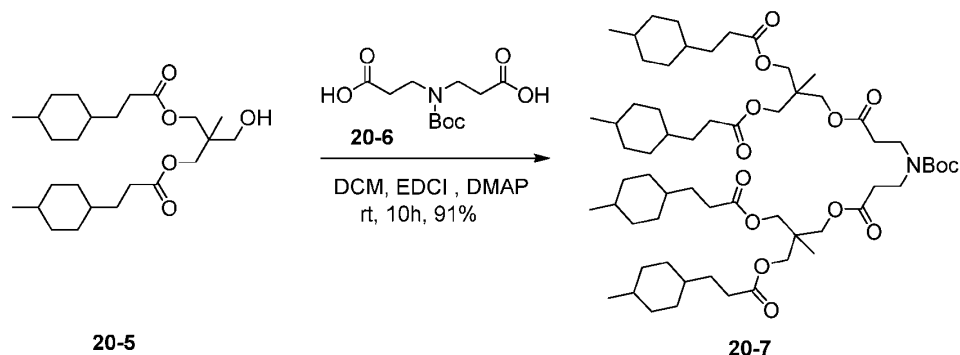
[0484] Into a 1L 3-necked round-bottom flask were added commercially available 2-[(benzyloxy)methyl]-2-methylpropane-1,3-diol (**20-3**, 27.1 g, 128.7 mmol, 1.00 equiv.), pyridine (25.45 g, 321.7 mmol, 2.5 equiv.) and CH₂Cl₂ (500 mL, 20V) at room temperature. The mixture was allowed to cool down to 0°C. To the above mixture was added **20-2** (51.00 g, 270.2 mmol, 2.1 equiv.) dropwise at 0°C. The resulting mixture was stirred for additional 4h at rt. The reaction was quenched by the addition of Water (1L) at 0°C. The resulting mixture was extracted with CH₂Cl₂ (3 x 500 mL). Combined organic layer was dried over anhydrous Na₂SO₄, filtered, and the filtrate was concentrated under reduced pressure. The residue was adsorbed on 540 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a 2700 g of silica gel column, using combi-flash purification system. Product was eluted with PE/EA (gradient from 100:0 to 70:30, collected every 500 ± 10 mL). After TLC analysis (EA:PE = 1:10) qualified fractions were and combined, concentrated and dried under vacuum to afford (45 g, 67.9%) **20-4** as colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN 95:5 to 5:95 A/B at 2 min., hold 1.2 min): RT 1.48 min, m/z (Calcd.) 514.4, (found) 537.5 (M+Na).

[0485] **Synthesis of 20-5: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(3-(4-methylcyclohexyl)-propanoate)**



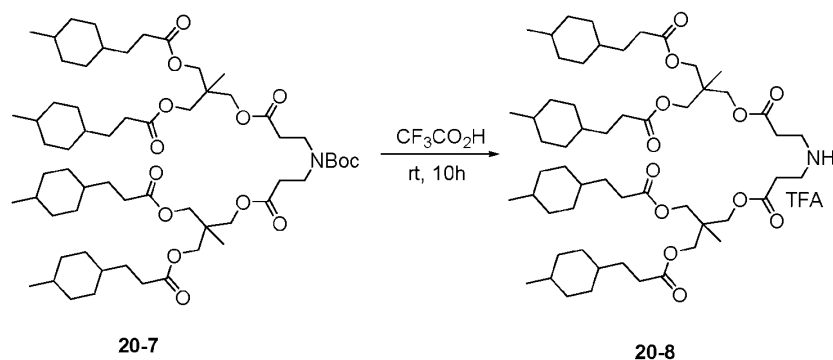
[0486] To a solution of **20-4** (45 g, 87.4 mmol, 1 equiv.) in MeOH (500 mL, 11V) was added Pd/C (10%, 4.5g) under nitrogen atmosphere in a 1L 3-necked round-bottom flask. The mixture was hydrogenated at room temperature for 10 h under hydrogen atmosphere using a hydrogen balloon. Reaction was filtered through a Celite pad and concentrated under reduced pressure to afford **20-5** (35 g, 94.3%) as a colorless oil. ELSD A: water/0.05% TFA : B: CH₃CN 95:5 to 5:95 A/B at 2 min., hold 1.2 min): RT 1.29 min, m/z (Calcd.) 424.3, (found) 447.4 (M+Na).

[0487] **Synthesis of 20-7: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**



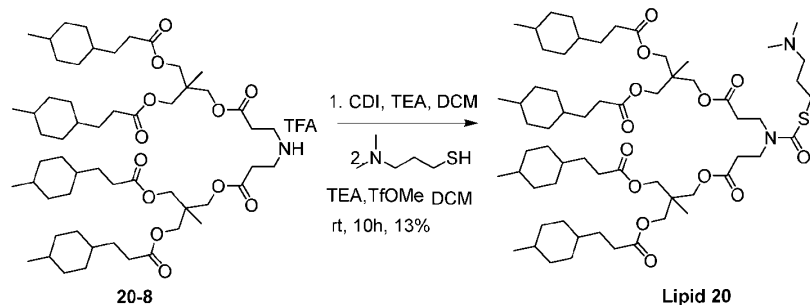
[0488] Into a 1L 3-necked round-bottom flask were added **20-5** (35.04 g, 82.5 mmol, 2.2 equiv.), 3-[(tert-butoxycarbonyl)(2-carboxyethyl)amino]propanoic acid (**20-6**, 9.8 g, 37.5 mmol, 1.00 equiv.), EDCI (14.38 g, 75.0 mmol, 2 equiv.), DCM (700 mL, 20V) and DMAP (4.58 g, 37.48 mmol, 1.00 equiv.) at room temperature. The resulting mixture was stirred for 10 h at room temperature and diluted with water (500 mL). The resulting mixture was extracted with CH₂Cl₂ (3 x 500 mL), and the combined organic layer was dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The residue was adsorbed on 540 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a 2.7 Kg of silica gel column, using combi-flash purification system. Product was eluted with PE/EA (gradient from 100:0 to 50:50, collected every 500 ± 10 mL). After TLC analysis (EA:PE = 1:10) qualified fractions were and combined, concentrated, and dried under vacuum to afford (37 g, 91.8%) **20-7** as colorless oil which was used in the next step, based on purity and structure by ¹H NMR.

[0489] Synthesis of **20-8**: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate) Trifluoroacetic acid salt



[0490] Into a 500 mL 3-necked round-bottom flask were added **20-7** (37 g, 34.4 mmol, 1 equiv.), DCM (370 mL) and trifluoroacetic acid (150 mL) at room temperature. The resulting mixture was stirred for 10h at room temperature. The resulting mixture was concentrated under reduced pressure to afford **20-8** as a colorless oil (40 g, crude). Both ¹H NMR and HPLC indicated ~94% pure product. It was used as such in the next reaction after drying under vacuum.

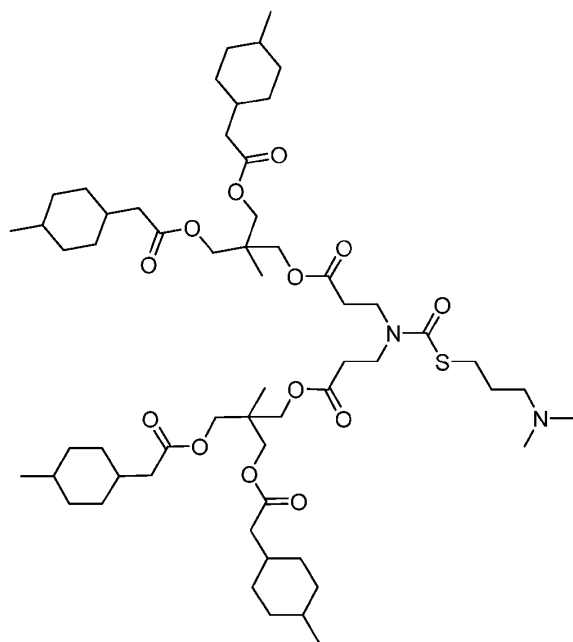
[0491] **Synthesis of LIPID 20: ((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**



[0492] Into a 1L 3-neck round-bottom flask were added **20-8** (37 g, 34 mmol, 1 equiv.), TEA (10.32 g, 102 mmol, 3 equiv.), CDI (6.06 g, 37.4 mmol, 1.1 equiv.) and DCM (1.48 L, 40V) at room temperature. The resulting mixture was stirred for 10 h at room temperature. The mixture was allowed to cool down to 0°C. To the above mixture was added methyl trifluoromethane sulfonate (6.14 g, 37.4 mmol, 1.1 equiv.) dropwise at 0°C. The resulting mixture was stirred for 1 h at 0°C and to the mixture was added 3-(dimethylamino)propane-1-thiol (4.46 g, 37.4 mmol, 1.1 equiv.) dropwise at 0°C. The resulting mixture was stirred for additional 10 h at room

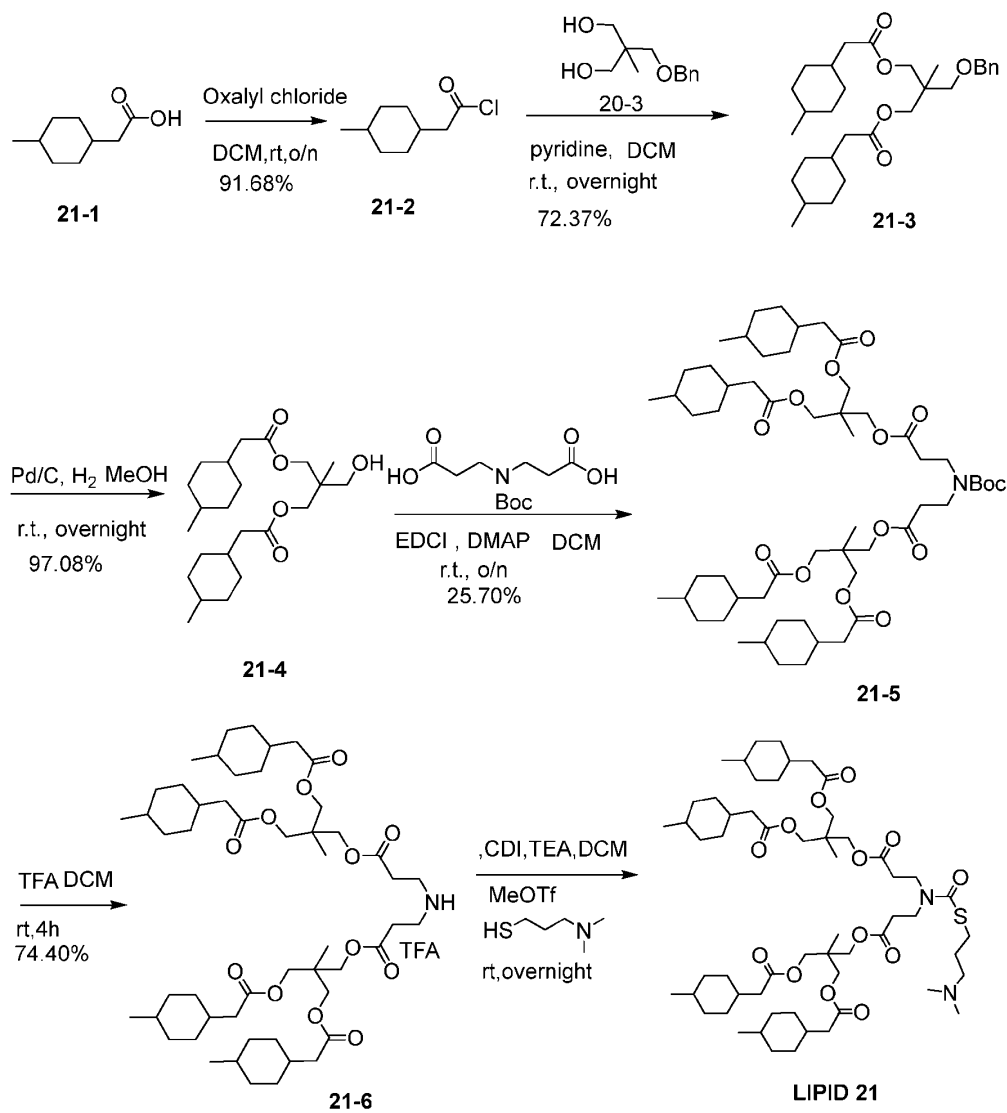
temperature, diluted with water (300 mL) and extracted with CH_2Cl_2 (3 x 300 mL). Combined organic layer was dried over anhydrous Na_2SO_4 , filtered, and evaporated. The residue was adsorbed on 74 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a 370 g of silica gel column, using combi-flash purification system. Product was eluted with PE/EA (gradient from 100:0 to 90:10, collected every 500 ± 10 mL). After TLC analysis (EA:PE = 1:10) qualified fractions were and combined, concentrated, and dried under vacuum to afford **LIPID 20** (5.1 g, 13.3%) as yellow oil. ELSD A: water/0.05% TFA: B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 25 min.): RT 10.3 min, m/z (Calcd.) 1118.8, (found) 1119.9 (M+H). ^1H NMR (300 MHz, Chloroform- d) δ 4.005 (d, $J = 6.6$ Hz, 12H), 3.654 (t, $J = 7.2$ Hz, 4H), 2.956 (t, $J = 7.0$ Hz, 2H), 2.755 – 2.598 (m, 6H), 2.519 (s, 6H), 2.373 – 2.248 (m, 8H), 2.023 – 1.899 (m, 2H), 1.769 – 1.092 (m, 46H), 1.044 – 0.995 (m, 6H), 0.941 – 0.830 (m, 16H).

[0493] Example 21. Synthesis of LIPID 21: ((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)

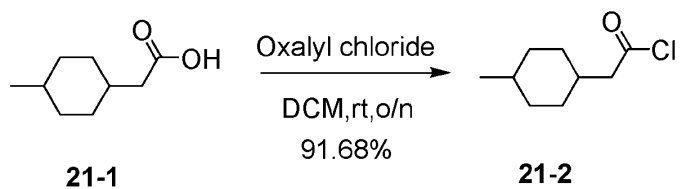


[0494] LIPID 21

[0495] General scheme

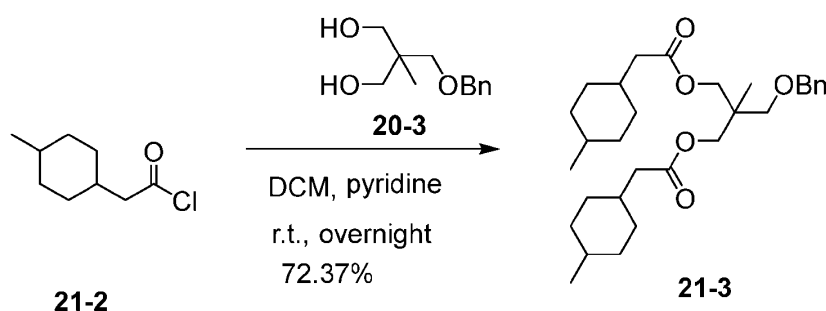


[0496] Synthesis of 21-2: 2-(4-methylcyclohexyl)acetyl chloride



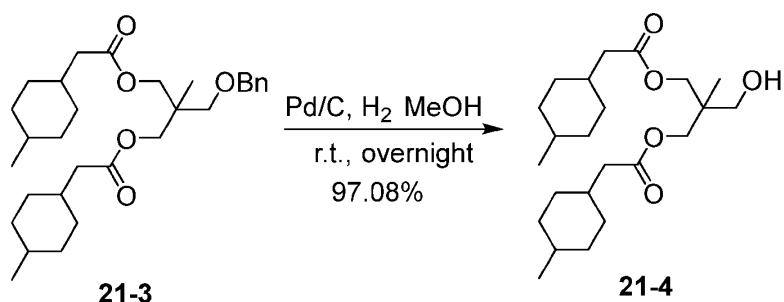
[0497] Into a 2 L three-necked round-bottle flask under nitrogen was added **21-1** (80 g, 512.0 mmol, 1.00 equiv.) in DCM (800 mL, 10 V). The solution was cooled to 0 °C in an ice/water bath and oxalyl chloride (130 g, 1024.2 mmol, 2.00 equiv.) was added dropwise at 0 °C. The ice/water bath was removed, and the reaction was stirred overnight at room temperature. The reaction mixture was concentrated under vacuum to get **21-2** (82 g, 91.7%) as colorless oil that was used in the next reaction as such.

[0498] **Synthesis of 21-3: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(2-(4-methylcyclohexyl) acetate)**



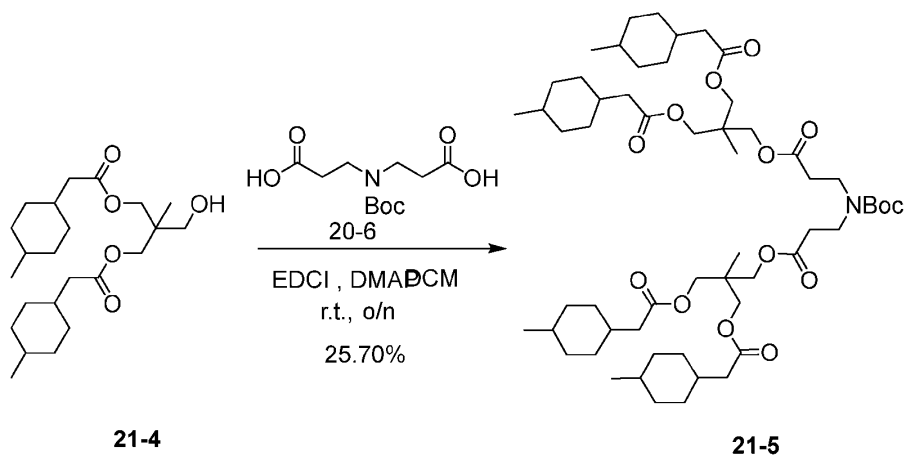
[0499] A solution of **20-3** (40 g, 190.2 mmol, 1.00 equiv.) in DCM (800 mL) was treated with pyridine (60.19 g, 760.9 mmol, 4.00 equiv.) and DMAP (6.97 g, 57.0 mmol, 0.30 equiv.) at 0 °C under nitrogen atmosphere followed by the addition of **21-2** (83.1 g, 475.6 mmol, 2.50 equiv.) dropwise at 0 °C. The mixture was stirred for 16 h at room temperature. The resulting mixture was diluted with water (500 mL) and acidified to pH 6 with HCl (aq.). The aqueous layer was extracted with DCM (2 x 200 mL). Combined organic layer was washed with of brine (1 x 300 mL), dried over anhydrous Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure to afford **21-3** (67 g, 72.4%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.); RT 2.49 min, m/z (Calcd.) 486.3, (found) 509.4 (M+Na).

[0500] **Synthesis of 21-4: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(2-(4-methylcyclohexyl) acetate)**



[0501] Into a 2 L 4-necked round-bottom flask was charged a solution of **21-3** (67 g, 137.66 mmol, 1.00 equiv.) in MeOH (670 mL 10V) was added Pd/C (20.1 g, 18.9 mmol, 0.14 equiv., 10% wt) in one portion and the resulting mixture was stirred for 16 h at room temperature under H₂. The reaction mixture was filtered, and the filter cake was washed with MeOH (1 x 300 mL). The filtrate was concentrated under vacuum to get **21-4** (53 g, 97.1 %) as colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.); RT 2.11 min, m/z (Calcd.) 396.3, (found) 397.2 (M+H).

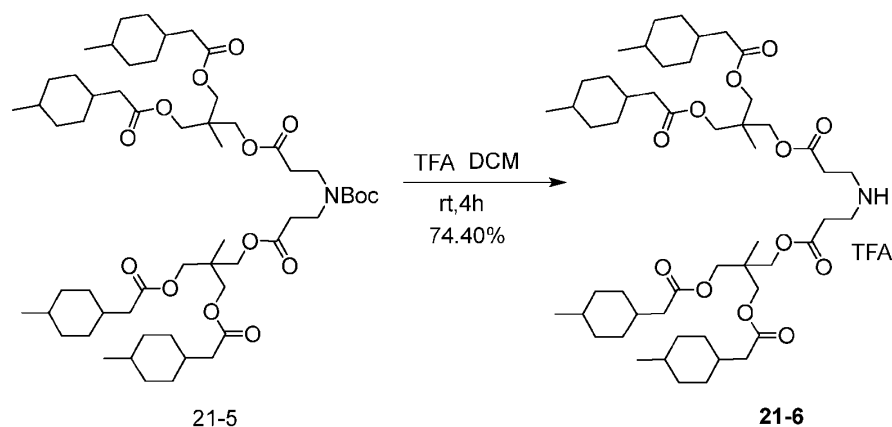
[0502] **Synthesis of 21-5: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)**



[0503] To a 2 L 4-necked round-bottom flask under nitrogen was added **21-4** (50 g, 126.1 mmol, 1.00 equiv.), followed by DMAP (15.40 g, 126.1 mmol, 1.00 equiv.) and **20-6** (72.47 g, 277.4 mmol, 2.20 equiv.) in DCM (1000 mL, 20V). The solution was cooled to 0 °C in an ice/water bath and to this was added EDCI (96.68 g, 504.3 mmol, 4.00 equiv.). The ice/water

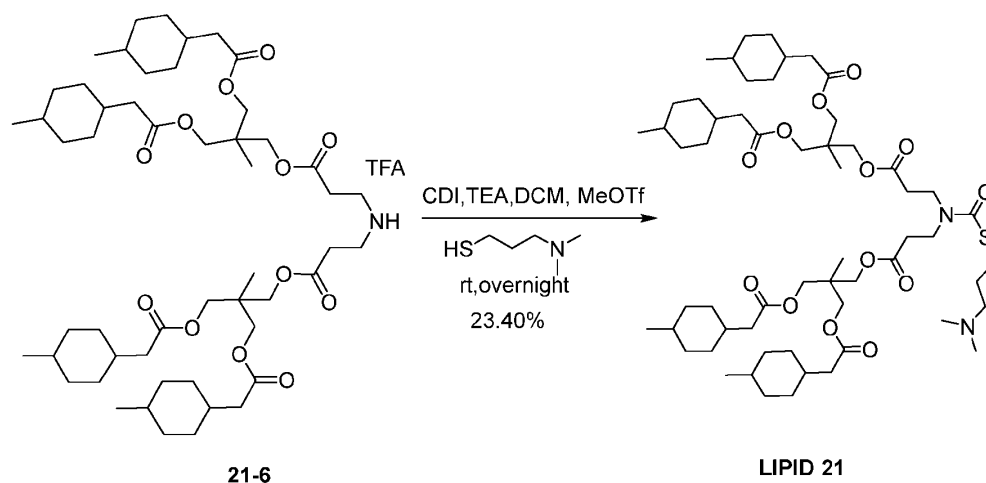
batch was removed, and the mixture was stirred for 16 h at room temperature. The mixture was washed with brine (1 x 1 L, 20 V) and the organic layer was dried over Na₂SO₄, filtered, and evaporated. The crude product was adsorbed on 300 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a 900 g of silica gel column, using combi-flash purification system. Product was eluted with PE/EA (gradient from 100:0 to 70:30, collected every 500 ± 10 mL). After TLC analysis (EA:PE 1:8) qualified fractions were and combined, concentrated, and dried under vacuum to afford **21-5** (33 g, 25.7%) as colorless oil. After verifying purity and identity (¹H NMR) the material was used in the next reaction.

[0504] Synthesis of 21-6: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate) Trifluoroacetic acid salt.



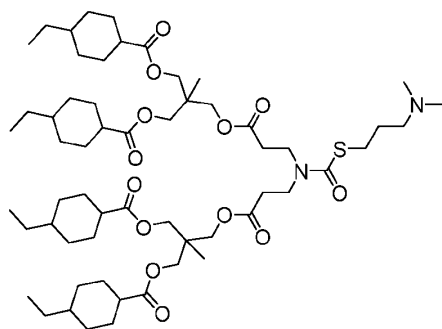
[0505] To a 250 mL three-necked round-bottom flask under nitrogen was added **21-5** (33 g, 32.4 mmol, 1.00 equiv.) in DCM (150 mL, 5V). The solution was cooled to 0 °C in an ice/water bath. To this was added trifluoroacetic acid (15.88 g, 162.0 mmol, 5.00 equiv.). The ice/water bath was removed, and the mixture was stirred for 4 h at room temperature. The reaction was concentrated under vacuum to get **21-6** (24.5 g, 74.4%) as colorless oil that was used as such in the next reaction.

[0506] Synthesis of LIPID 21: (((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-methylcyclohexyl)acetate)



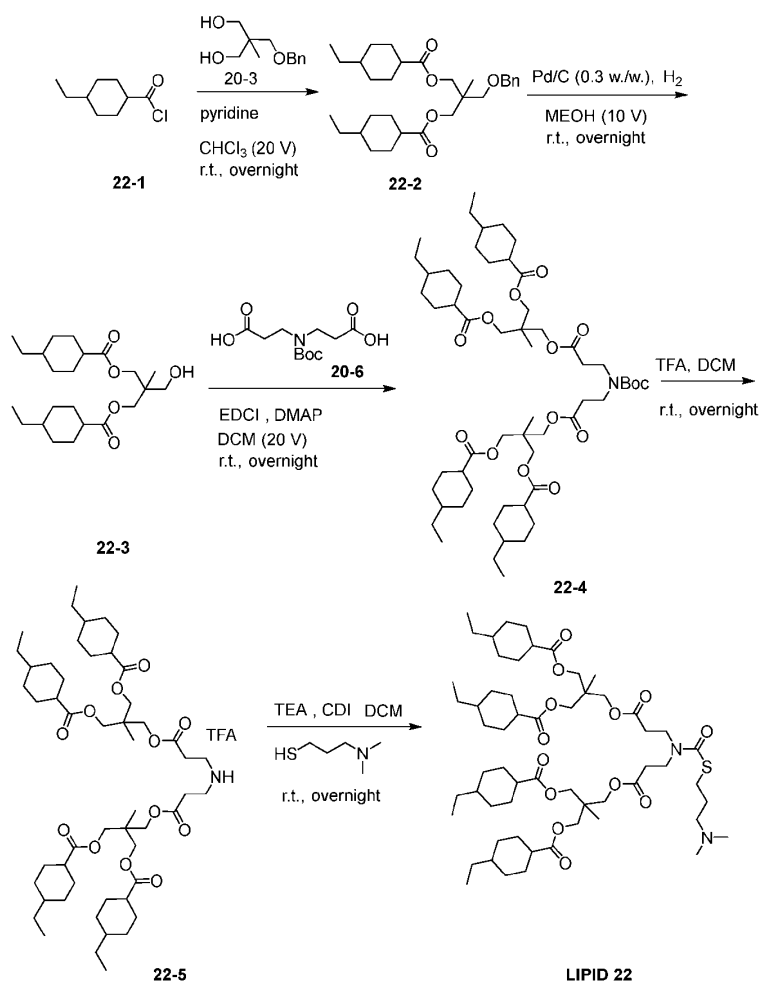
[0507] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen was placed a solution of **21-6** (24.5 g, 24.1 mmol, 1.00 equiv.) in DCM (500 mL, 20 V). To this was added the TEA (9.76 g, 96.4 mmol, 4.00 equiv.), followed by the addition of CDI (7.82 g, 48.21 mmol, 2.00 equiv.). The mixture was stirred overnight at room temperature. The solution was cooled to 0 °C in an ice/water bath. Then, methyltrifluoromethane sulfonate (4.35 g, 26.5 mmol, 1.10 equiv.) was added and the mixture was stirred at 0 °C for 1 hour. Then, 3-(dimethylamino)propane-1-thiol (3.45 g, 28.9 mmol, 1.20 equiv.) were added to the solution, ice/water bath was removed and the mixture was stirred overnight at room temperature. Crude compound was adsorbed on 50 g of silica gel (type: ZCX-2, 100-200 mesh, 3.75 w./w.) and purified on a 200 g of silica gel (type: ZCX-2, 300-400 mesh, 18.8 w./w.) using Combiflash system. Product was eluted with DCM/MeOH gradient from 100:0 to 96:4, collected every 300 ± 50 mL). Fractions were analyzed (TLC, DCM:MeOH = 10:1, R_f=0.5), and qualified fractions were combined and concentrated to get **LIPID 21** (6 g, 23.4%) as light yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05 TFA 75:25 to 25:75 A/B at 25 min.); RT 10.9 min, m/z (Calcd.) 1062.7, (found) 1063.8 (M+H). ¹H-NMR-**LIPID 21**: (400 MHz, CDCl₃, ppm): δ 4.02 (d, *J* = 8.2 Hz, 12H), 3.67 (t, *J* = 7.3 Hz, 4H), 2.95 (t, *J* = 7.2 Hz, 2H), 2.67 (t, *J* = 7.2 Hz, 4H), 2.53 (s, 2H), 2.45-2.28 (m, 8H), 2.24-2.16 (m, 6H), 2.01 (d, *J* = 3.4 Hz, 1H), 1.90 (t, *J* = 7.3 Hz, 2H), 1.71 (dd, *J* = 5.8, 3.1 Hz, 14H), 1.56-1.46 (m, 5H), 1.44-1.20 (m, 9H), 1.05-0.84 (m, 30H).

[0508] Example 22. Synthesis of LIPID 22: ((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl) azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-ethylcyclohexyl)acetate)

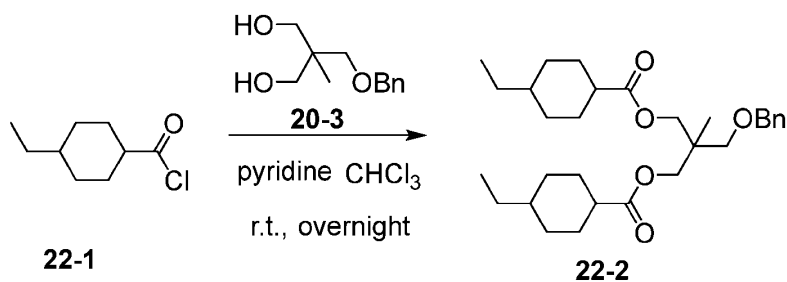


[0509] LIPID 22

[0510] General scheme



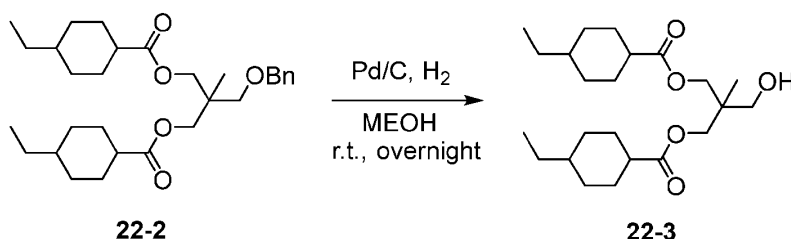
[0511] Synthesis of 22-2: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(4-ethylcyclohexane-1-carboxylate)



[0512] Into a 3 L three-necked round-bottom flask was added 20-3 (70.0 g, 0.33 mol, 1.00 equiv.), CHCl_3 (1400 mL, 20 V) and pyridine (105.0 g, 4.0 equiv.) at room temperature under

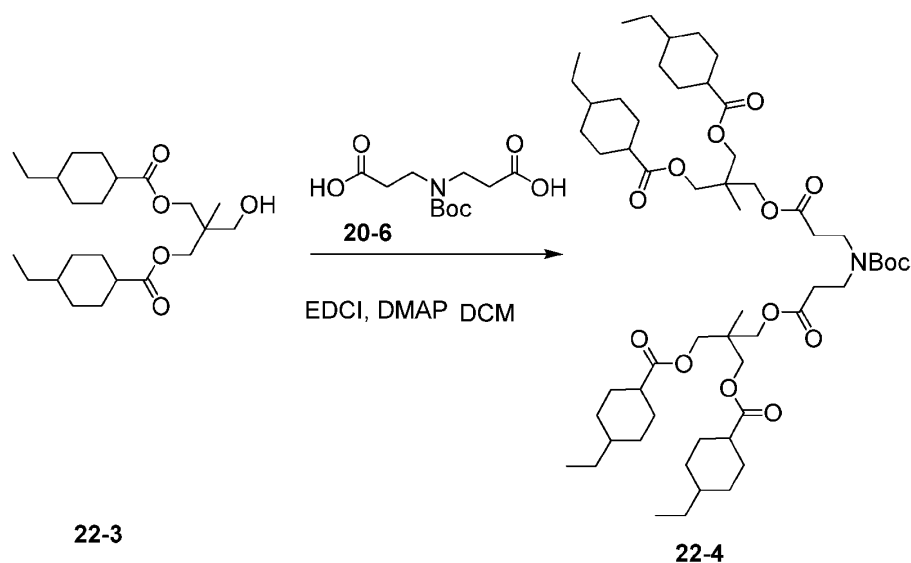
the N₂ atmosphere. Followed by the addition of **22-1** (127.0 g, 0.73 mol, 2.20 equiv.) dropwise at 0 °C. The resulting mixture was stirred for additional overnight at room temperature. The reaction was quenched with water (700 mL, 10 V) at room temperature and the organic layers was washed with saturated NaHCO₃ aqueous solution (1000 mL, 15 V), HCl (1000 mL, 15 V, 1 mol/L) and brine (1000 mL, 15 V). The organic phase was dried with anhydrous Na₂SO₄ and then filtered. After filtration, the filtrate was concentrated under vacuum. This resulted in (120 g, 0.25 mol, 74.0% yield) **22-2** as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 2 min.): RT 1.8 min, m/z (Calcd.) 486.3, (found) 509.5 (M+Na).

[0513] Synthesis of 22-3: 2-(hydroxymethyl)-2-methylpropane-1,3-diylbis(4-ethylcyclohexane-1-carboxylate)



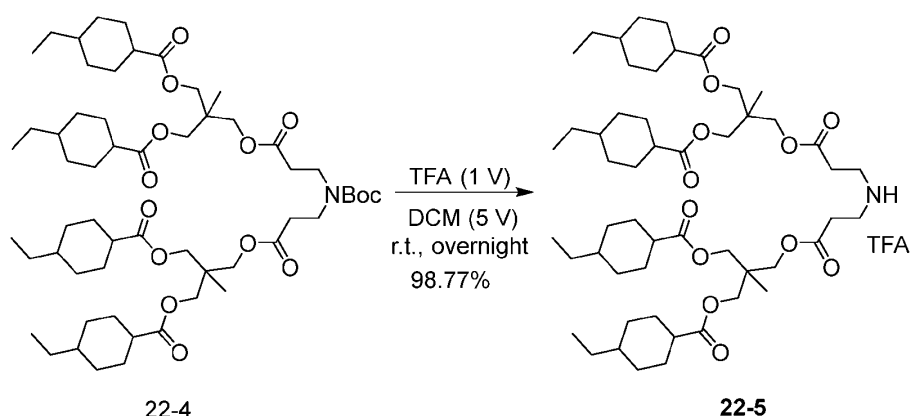
[0514] Into a 3 L three-necked round-bottom flask was added Pd/C (36.0 g, 0.3 w./w.) in MeOH (1.2 L, 10 V) at room temperature. Then, **22-2** (120.0 g, 0.25 mol, 1.00 equiv.) was added to the reaction mixture at room temperature. Replaced the reaction system with H₂ for three times. The resulting solution was stirred for overnight at room temperature under H₂ atmosphere. LCMS indicated completed consumption of **22-2**. The resulting mixture was filtered, the filter cake was washed with MeOH (2 x 1000 mL, 8 V). The filtrate was concentrated and dried under vacuum. This resulted in (90 g, 0.23 mol, 92.0% yield) **22-3** as yellow oil that was used without further purification. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 2 min.): RT 1.5 min, m/z (Calcd.) 396.3, (found) 397.3 (M+H).

[0515] Synthesis of 22-4: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate)



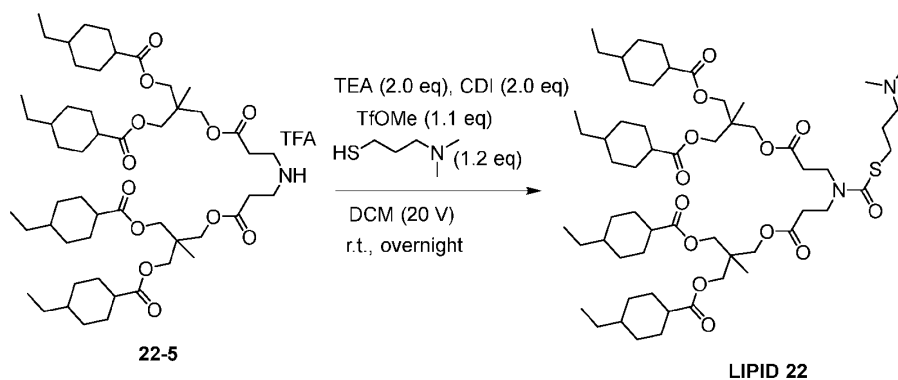
[0516] Into a 5 L four-neck round-bottom flask was added **22-3** (90 g, 0.22 mol, 2.20 equiv.), DCM (1.8 L, 20 V) and **20-6** (26.96 g, 0.10 mol, 1.00 equiv.) at room temperature under the N₂ atmosphere. Then, DMAP (12.61 g, 0.10 mol, 1.0 equiv.) and EDCI (79.12 g, 0.41 mol, 4.0 equiv.) was added to the reaction mixture at 0 °C. The resulting mixture was stirred for additional overnight at room temperature. The reaction system was quenched with water (1000 mL, 11 V). The organic phase was washed with brine (1000 mL, 11 V). The organic phase was dried with anhydrous Na₂SO₄ and then filtered. Crude product was adsorbed on 120 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (800 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using PE/THF (v/v) gradient from 100:0 to 95:5). Fractions were analyzed (TLC, THF:PE = 1:5), combined, concentrated and dried under vacuum to afford (70 g, 0.07 mol, 43.90% yield) **22-4** as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN 100:0 to 0:100 A/B at 3 min.; RT 1.0 min, m/z (Calcd.) 1017.7, (found) 1040.6 (M+Na).

[0517] Synthesis of **22-5**: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(4-ethylcyclohexane-1-carboxylate) Trifluoroacetic acid salt.



[0518] Into a 1 L three-necked flask was added **22-4** (70 g, 0.68 mol, 1.00 equiv.) in DCM (350 mL, 5 V) and TFA (70 mL, 1 V) at room temperature under the N₂ atmosphere. The resulting solution was stirred for overnight at room temperature. The resulting mixture was concentrated under vacuum. This resulted in (69 g, 0.06 mol, 98.8% yield) **22-5** (trifluoroacetic acid salt) as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.); RT 2.2 min, m/z (Calcd.) 917.6, (found) 918.5 (M+H).

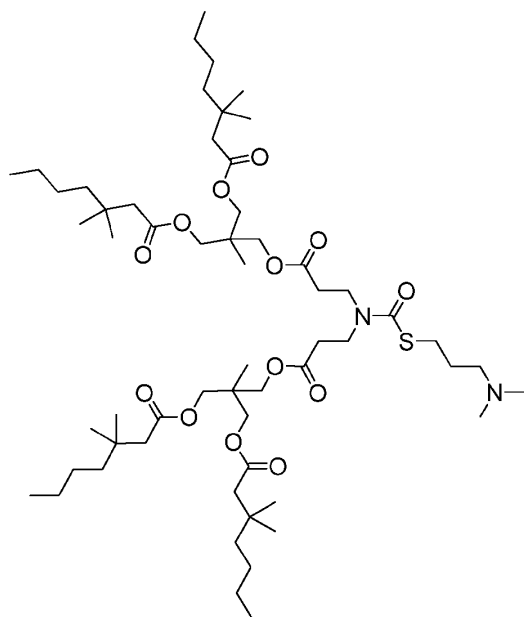
[0519] **Synthesis of LIPID 22: ((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2-(4-ethylcyclohexyl)acetate)**



[0520] Into a 3 L three-necked flask was added **22-5** (69.0 g, 0.06 mol, 1.00 equiv.) in DCM (1.38 L, 20 V) and TEA (13.74 g, 0.14 mol, 2.0 equiv.) followed by CDI (22.02 g, 0.14 mol, 2.0 equiv.) at room temperature under nitrogen atmosphere. The resulting mixture was stirred for additional 3 h at room temperature. Then into the reaction was added TfOMe (12.26 g, 0.07 mol,

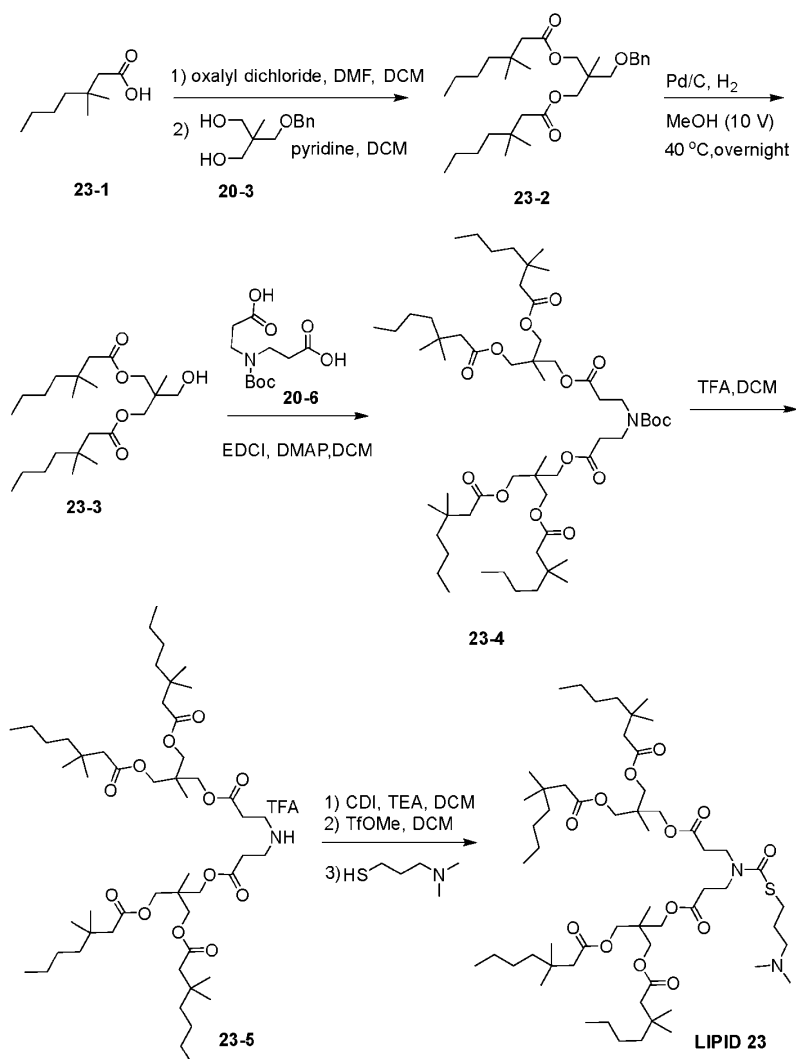
1.10 equiv.) at 0 °C and stirred for 1 h at 0 °C under nitrogen atmosphere. To the above mixture was added 3-(dimethylamino) propane-1-thiol (9.71 g, 81.5 mmol, 1.20 equiv.) at 0 °C and stirred overnight at room temperature. The reaction system was quenched with water (1L, 15 V) and organic phase was washed with brine (1 L, 15 V), dried with anhydrous Na₂SO₄, and filtered. Crude product was adsorbed on 90 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (800 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 70:30). Fractions were analyzed (TLC, THF:PE = 1:5), combined, concentrated, and dried under vacuum to afford 5.5 g of **LIPID 22** as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.), RT 2.2 min, m/z (Calcd.) 1062.6, (found) 1063.8 (M+H). ¹HNMR-**LIPID 22**: (400 MHz, CDCl₃, ppm) δ 4.01 (d, *J* = 12.8 Hz, 12H), 3.67 (t, *J* = 7.2 Hz, 4H), 2.95 (t, *J* = 7.2 Hz, 2H), 2.67 (t, *J* = 7.3 Hz, 4H), 2.36-2.18 (m, 10H), 2.01-1.91 (m, 8H), 1.894 -1.76 (m, 10H), 1.41 (qd, *J* = 13.0, 3.4 Hz, 8H), 1.30-1.08 (m, 13H), 1.03 (s, 6H), 0.98-0.83 (m, 20H).

[0521] **Example 23. Synthesis of LIPID 23: ((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3,3-dimethylheptanoate)**

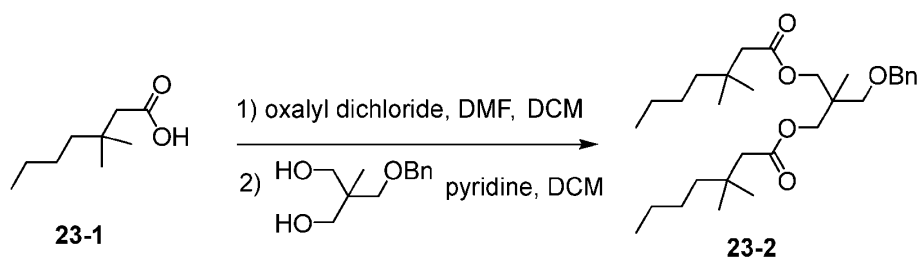


[0522] **LIPID 23**

[0523] **General scheme**

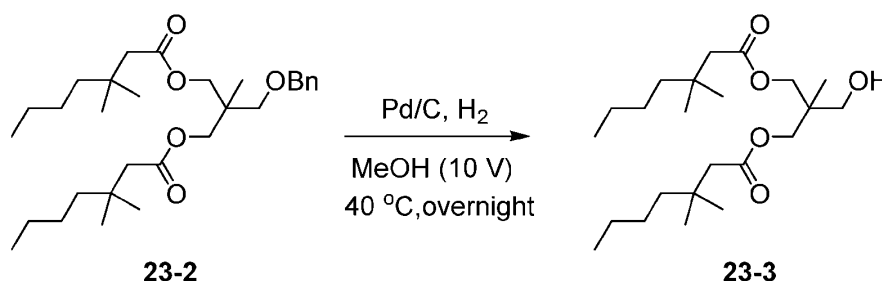


[0524] Synthesis of 23-2: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(3,3-dimethylheptanoate)



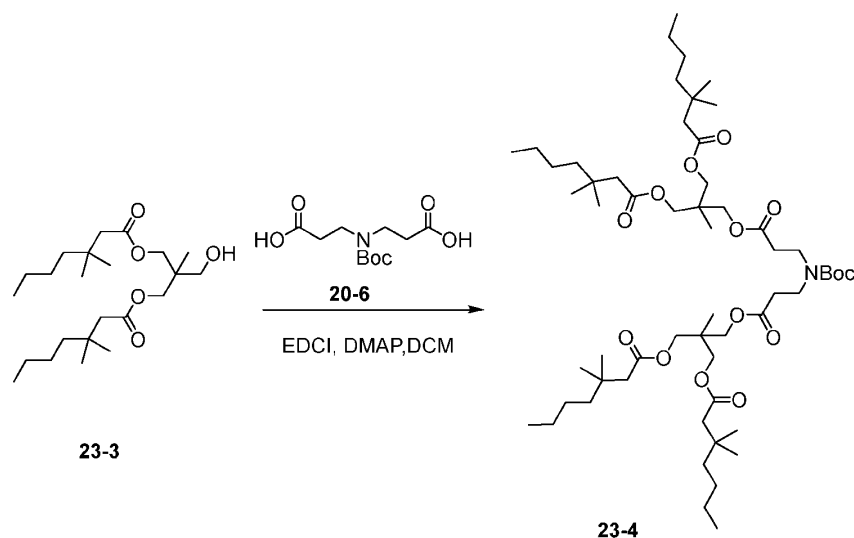
[0525] Into a stirred solution of **23-1** (84.66 g, 535.0 mmol, 2.50 equiv.) and DMF (1.56 g, 21.40 mmol, 0.10 equiv.) in DCM (1 L) was added oxalyl chloride (65.19 g, 513.61 mmol, 2.40 equiv.) dropwise at 0 °C under air atmosphere. The resulting mixture was stirred for 2 h at room temperature under air atmosphere. The resulting mixture was concentrated under vacuum and the residue was dissolved in DCM (200 mL). The above mixture was added dropwise to a stirred solution of **20-3** (45 g, 214.00 mmol, 1.00 equiv.) and pyridine (67.71 g, 856.0 mmol, 4.00 equiv.) in DCM (800 mL) at 0 °C under air atmosphere. The resulting mixture was stirred for 2 h at 0 °C under air atmosphere. The reaction was quenched by the addition of saturated aqueous NH₄Cl (1 L) at 0 °C. The resulting mixture was extracted with CH₂Cl₂ (2 x 500 mL). The combined organic layers were dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. Crude product was adsorbed on 200 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1.5 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 95:5). Fractions were analyzed (TLC, PE:EA = 20:1), combined, concentrated, and dried under vacuum to **23-2** (81 g, 77.13%) as a colorless oil. ELSD A: water/0.05% TFA; B: CH₃CN 95:5 to 5:95 A/B at 3 min.; RT 2.6 min, m/z (Calcd.) 490.4, (found) 491.5 (M+H).

[0526] **Synthesis of 23-3: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(3,3-dimethylheptanoate)**



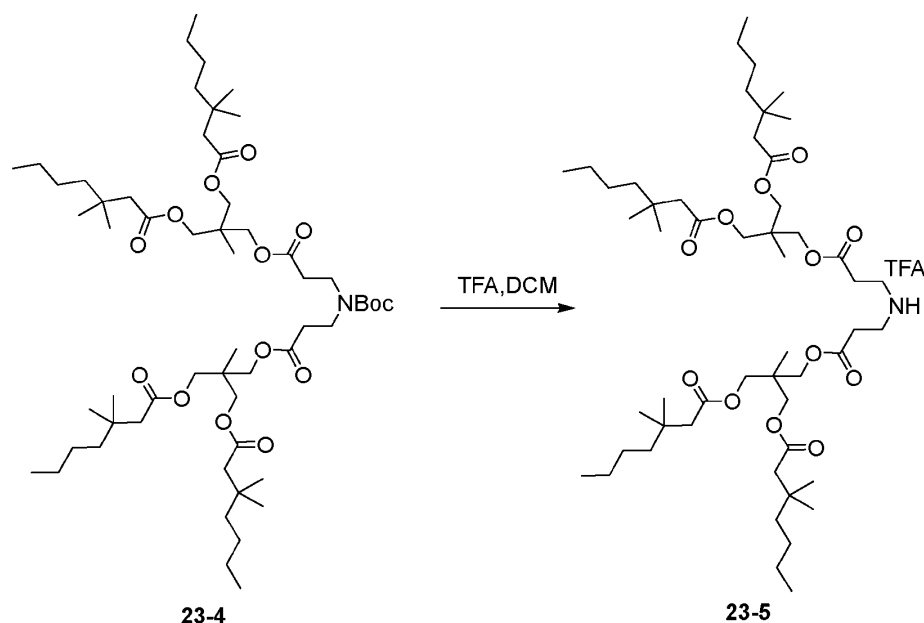
[0527] A solution of **23-2** (91 g, 185.4 mmol, 1.00 equiv.) and Pd/C (9.87 g, 92.7 mmol, 0.50 equiv.) in MeOH (2 L) was stirred for overnight at 40 °C under hydrogen (5 atm) atmosphere. The resulting mixture was filtered, the filter cake was washed with MeOH (2x100 mL). The filtrate was concentrated under reduced pressure. Crude product was adsorbed on 200 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 10:1), combined, concentrated,

[0528] Synthesis of 23-4: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3,3-dimethylheptanoate)



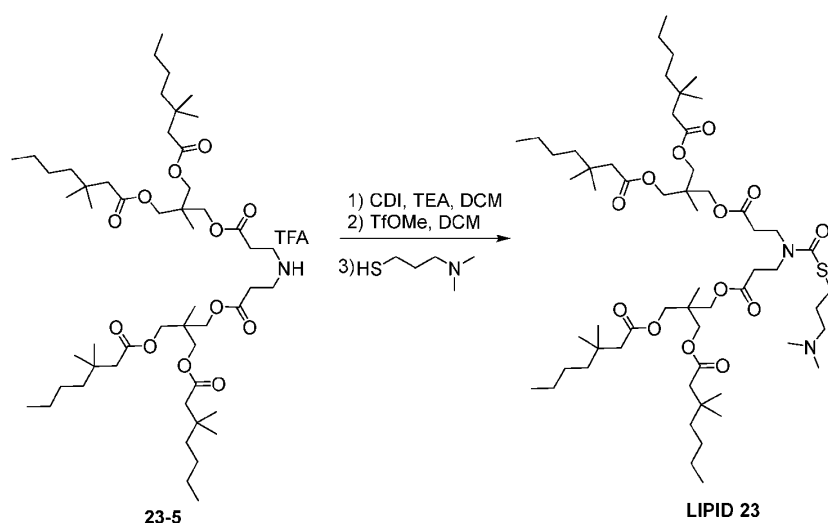
[0529] To a stirred solution of **20-6** (14.8 g, 56.64 mmol, 1.00 equiv.) and **23-3** (49.92 g, 124.62 mmol, 2.20 equiv.) in DCM (1 L) was added EDCI (27.15 g, 141.61 mmol, 2.50 equiv.) and DMAP (3.46 g, 28.32 mmol, 0.50 equiv.) at room temperature under air atmosphere. The resulting mixture was stirred for overnight at room temperature under air atmosphere. The reaction was quenched by the addition of ice/salt mixture (1 L) at room temperature. The resulting mixture was extracted with CH₂Cl₂ (2 x 500 mL). The combined organic layers were dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. Crude product was adsorbed on 150 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 75:25). Fractions were analyzed (TLC, PE:EA = 4:1), combined, concentrated, and dried under vacuum to **23-4** (41 g, 70.5%) as a colorless oil. Material was used in the next reaction after verifying identity and purity by NMR.

[0530] Synthesis of 23-5: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3,3-dimethylheptanoate) Trifluoroacetic acid salt



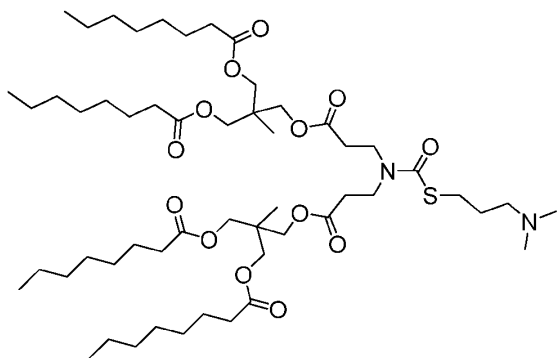
[0531] To a stirred solution of **23-4** (41 g, 39.94 mmol, 1.00 equiv.) in DCM (600 mL) was added TFA (60 mL) dropwise at room temperature under air atmosphere. The resulting mixture was stirred for overnight at room temperature. The resulting mixture was concentrated under reduced pressure and dried under vacuum. This resulted in **23-5** as its trifluoroacetic acid salt (42 g, 102.6%) as a light brown oil that was used as such. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 2 min.; RT 1.6 min, m/z (Calcd.) 925.7, (found) 949. (M+H+Na).

[0532] Synthesis of LIPID 23: (((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3,3-dimethylheptanoate)



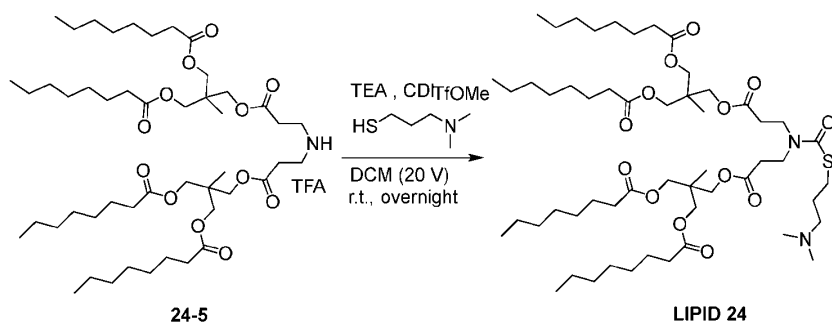
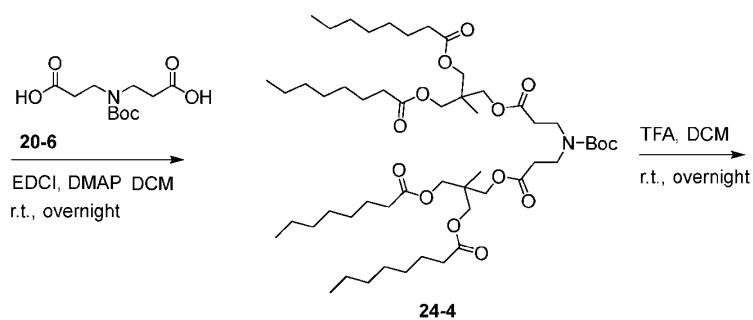
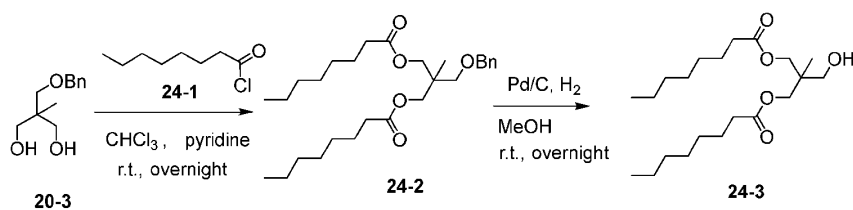
[0533] To a stirred solution of **23-5** (41 g, 40.0 mmol, 1.00 equiv.) and TEA (8.10 g, 80.0 mmol, 2.00 equiv.) in DCM (1 L) was added CDI (12.98 g, 80.0 mmol, 2 equiv.) at room temperature under air atmosphere. The resulting mixture was stirred for overnight at room temperature and quenched by the addition of water (1 L) at room temperature. The resulting mixture was extracted with CH₂Cl₂ (2 x 500 mL). The combined organic layer was dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure and the residue was dissolved in DCM (1 L). To the above mixture was added methyl triflate (7.22 g, 44.0 mmol, 1.10 equiv.) dropwise over 1 h at 0 °C. The resulting mixture was stirred for additional 2 h at 0°C. To the above mixture was added TEA (8.10 g, 80.0 mmol, 2.00 equiv.) and 3-(dimethylamino)propane-1-thiol (5.73 g, 48.0 mmol, 1.20 equiv.) at 0 °C and stirred overnight at room temperature. The resulting mixture was concentrated under reduced pressure. Crude product was adsorbed on 60 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w/w.) and purified on a silica gel column (600 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w/w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 50:50). Fractions were analyzed (TLC, PE:EA =1:1), combined, concentrated, and dried under vacuum to **LIPID 23** ((5.0327 g, 11.80%)) as a colorless oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05 TFA 95:5 to 5:95 A/B at 5 min.,): RT 3.9 min, m/z (Calcd.) 1070.7, (found) 1071.8 (M+H); ¹H-NMR-**LIPID 23**: (400 MHz, CDCl₃, *ppm*) δ 4.03 (s, 4H), 3.98 (s, 8H), 3.66 (t, *J* = 7.3 Hz, 4H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.67 (t, *J* = 7.2 Hz, 4H), 2.39 (d, *J* = 13.2 Hz, 2H), 2.28 (s, 6H), 2.22 (s, 8H), 1.84 (p, *J* = 7.4 Hz, 2H), 1.38-1.18 (m, 24H), 1.04 (s, 6H), 0.98 (s, 24H), 0.96-0.87 (m, 12H).

[0534] Example 24. Synthesis of LIPID 24: ((3,3'-((((3-(Dimethylamino)propyl)thio)carbonyl) azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(octanoate)

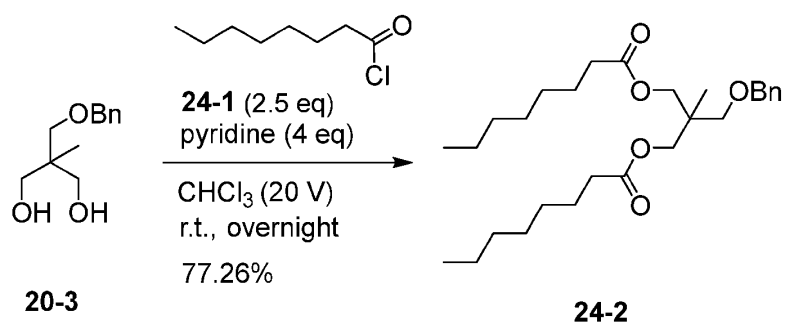


[0535] LIPID 24

[0536] General scheme

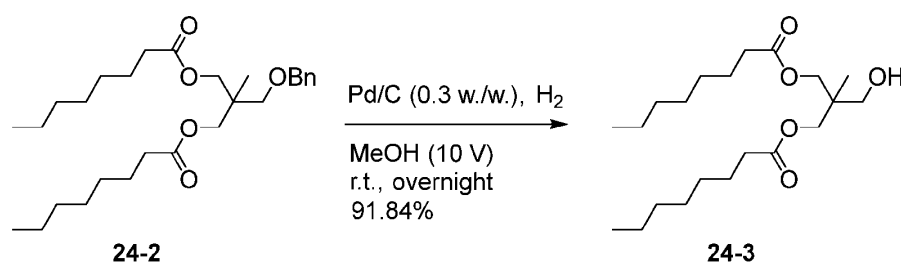


[0537] Synthesis of 24-2: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl dioctanoate



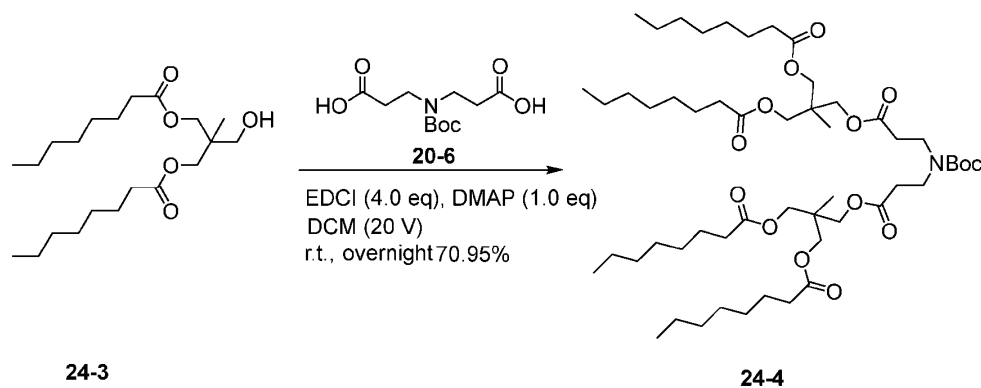
[0538] Into a 3 L three-necked round-bottom flask was added **20-3** (70.0 g, 0.33 mol, 1.00 equiv.), CHCl_3 (1.40 L, 20 V) and pyridine (105.3 g, 1.33 mol, 4.00 equiv.) at room temperature under the N_2 atmosphere, followed by addition a solution of **24-1** (119 g, 0.73 mol, 2.20 equiv.) in CHCl_3 (120 mL, 1V) dropwise at 0°C . The resulting mixture was stirred for additional overnight at room temperature. The reaction was quenched with water (700 mL, 10 V) at room temperature. The organic layer was washed with saturated NaHCO_3 aqueous solution (1000 mL, 15 V), HCl (1000 mL, 15 V, 1 mol/L) and brine (1000 mL, 15 V). The organic phase was dried with anhydrous Na_2SO_4 and then filtered. After filtration, the filtrate was concentrated and dried under vacuum. This was resulted in (119 g, 0.25 mol, 74.06%) **24-2** as yellow oil. ELSD A: water/0.05% TFA: B: CH_3CN 95:5 to 5:95 A/B at 2 min.); RT 1.7 min, m/z (Calcd.) 462.3, (found) 485.5 (M+Na).

[0539] **Synthesis of 24-3: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl dioctanoate**



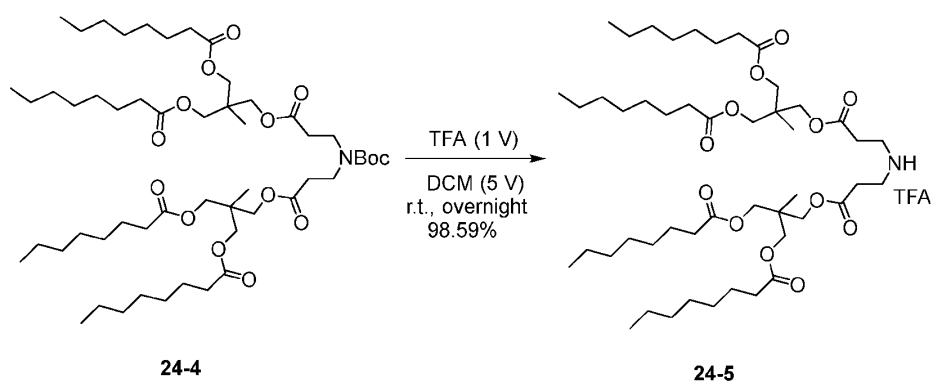
[0540] Into a 3 L three-necked round-bottom flask was added Pd/C (36.0 g, 0.3 w/w) and MeOH (1.2 L, 10 V) at room temperature. Then, **24-2** (119.0 g, 0.25 mol, 1.00 equiv.) was added to the reaction mixture at room temperature. Replaced the reaction system with H_2 for three times. The resulting solution was stirred for overnight at room temperature under H_2 atmosphere. LCMS indicated completed consumption of **24-2**. The resulting mixture was filtered, the filter cake was washed with MeOH (2 x 1000 mL, 8V) and the filtrate was concentrated under reduced pressure. This resulted in (88 g, 0.23 mol, 91.8%) **24-3** as yellow oil. ELSD A: water/0.05% TFA: B: CH_3CN 95:5 to 5:95 A/B at 2 min.); RT 1.5 min, m/z (Calcd.) 372.3, (found) 373.3 (M+H).

[0541] **Synthesis of 24-4: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetraoctanoate**



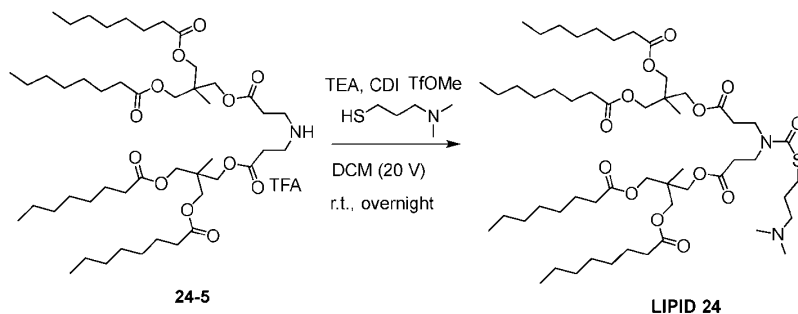
[0542] Into a 5 L four-necked flask was added **24-3** (75 g, 0.20 mol, 2.20 equiv.), DCM (1.5 L, 20 V) and **20-6** (23.91 g, 0.09 mol, 1.00 equiv.) at room temperature under the N₂ atmosphere. Then, DMAP (11.2 g, 0.09 mol, 1.00 equiv.) and EDCI (70.1 g, 0.36 mol, 4.00 equiv.) were added to the reaction mixture at 0 °C and stirred for additional overnight at room temperature. The reaction was quenched with water (900 mL, 12 V). The organic phase was washed with brine (900 mL, 12 V), dried with anhydrous Na₂SO₄, and filtered. Crude product was adsorbed on 130 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (900 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/THF (v/v) gradient from 100:0 to 95:5. Fractions were analyzed (TLC, PE:THF =1:6), combined, concentrated, and dried under vacuum to get **24-4** (63 g, 0.06 mol, 70.9% yield) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.); RT 0.78 min, m/z (Calcd.) 969.6, (found) 992.6 (M+Na).

[0543] Synthesis of **24-5**: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetraoctanoate trifluoroacetic acid salt



[0544] Into a 1 L three-necked flask was added **24-4** (63 g, 0.06 mol, 1.00 equiv.), DCM (315 mL, 5 V) and TFA (63 mL, 1V) at room temperature under the N₂ atmosphere. The resulting solution was stirred for overnight at room temperature. The mixture was concentrated and dried under vacuum. This resulted in (61.98 g, 0.06 mol, 98.6% yield) **24-5** as its trifluoroacetic acid salt as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.); RT 0.53 min, m/z (Calcd.) 869.6, (found) 870.5 (M+H).

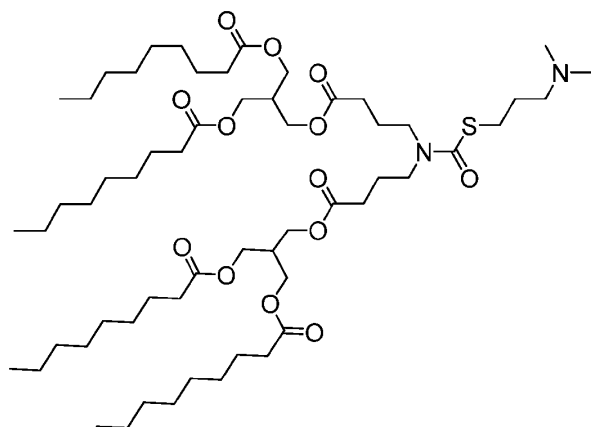
[0545] **Synthesis of LIPID 24: ((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(octanoate)**



[0546] Into a 3 L three-necked flask was added **24-5** (61.98 g, 0.06 mol, 1.00 equiv.), DCM (1.24 L, 20 V) and TEA (12.95 g, 0.12 mol, 2.00 equiv.) followed by the addition of CDI (20.76 g, 0.12 mol, 2.00 equiv.) at room temperature under nitrogen atmosphere. The resulting mixture was stirred for 3 h at room temperature. Then, into the reaction were added methyl triflate (17.69 g, 0.07 mol, 1.10 equiv.) at 0 °C and stirred for 1 h at 0 °C under nitrogen atmosphere. To the above mixture was added 3-(dimethylamino) propane-1-thiol (9.16 g, 0.07 mol, 1.20 equiv.) at 0 °C and stirred overnight at room temperature. The reaction system was quenched with water

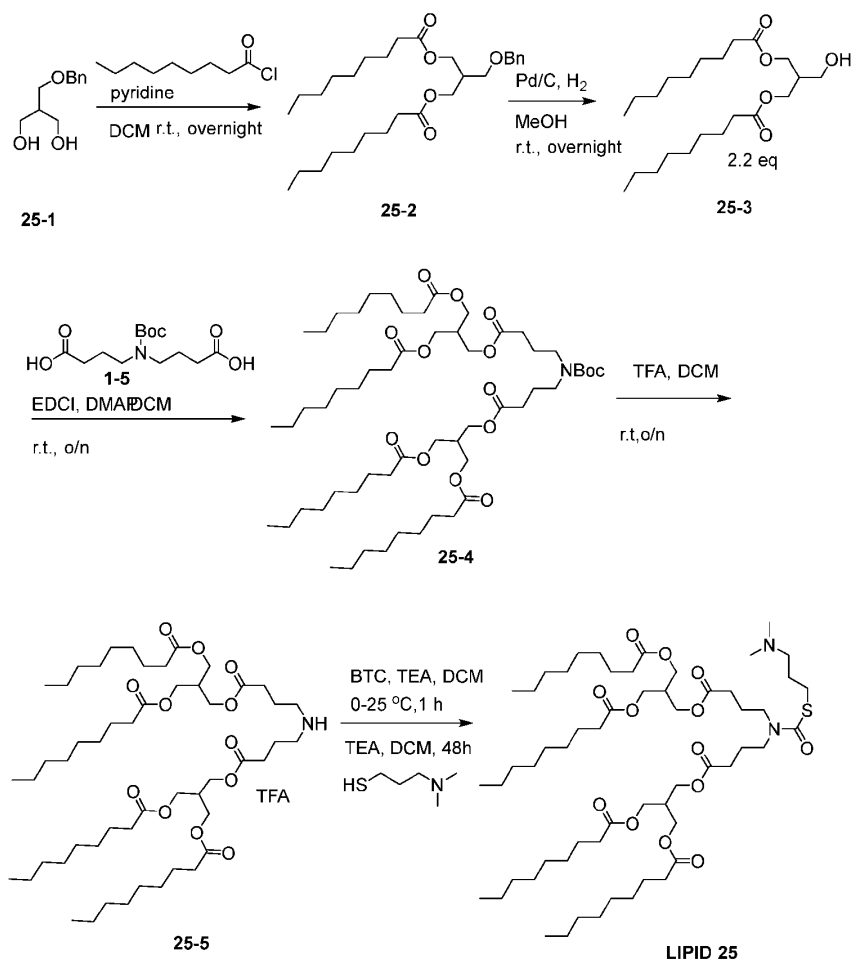
(1000 mL, 16 V). The organic phase was washed with brine (1000 mL, 16 V), dried with anhydrous Na_2SO_4 , and then filtered. Crude product was adsorbed on 90 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (650 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 30:70). Fractions were analyzed (TLC, heptane:EA = 1:10), combined, concentrated, and dried under vacuum to get **LIPID 24** (5.3 g, 8% yield) as a colorless oil. ELSD A: water/0.05% TFA: B: CH_3CN / 100:0 to 0:100 A/B at 5 min.); RT 3.46 min, m/z (Calcd.) 1014.8, (found) 1015.8 (M+H). $^1\text{H-NMR-LIPID 24}$: (400 MHz, $\text{DMSO-}d_6$, ppm) δ 4.00 (d, $J = 9.3$ Hz, 12H), 3.65 (t, $J = 7.2$ Hz, 4H), 2.93 (t, $J = 7.2$ Hz, 2H), 2.65 (t, $J = 7.3$ Hz, 4H), 2.46 (s, 2H), 2.31 (t, $J = 7.5$ Hz, 14H), 1.91-1.80 (m, 2H), 1.61 (dt, $J = 8.7, 4.8$ Hz, 8H), 1.34-1.22 (m, 32H), 1.01 (s, 6H), 0.93-0.82 (m, 12H).

[0547] **Example 25. Synthesis of LIPID 25: ((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl) azanediyl) bis(butanoyl))bis(oxy)) bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate**

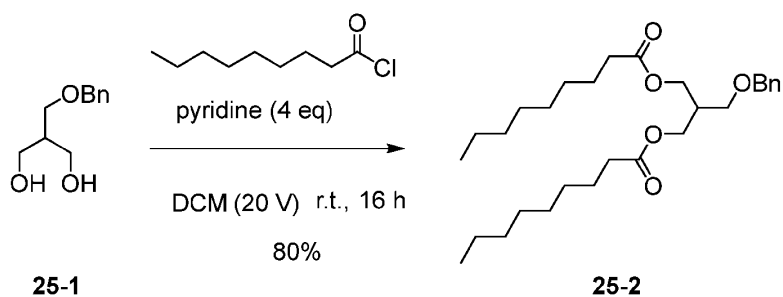


[0548] **LIPID 25**

[0549] **General scheme**



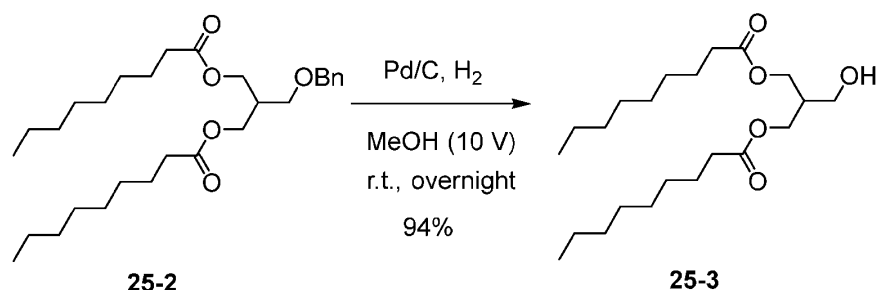
[0550] Synthesis of 25-2: ((benzyloxy)methyl)propane-1,3-diyl dinonanoate



[0551] Into a 5 L 4-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen were added **25-1** (100 g, 0.51 mol, 1.00 equiv.), pyridine (161.2 g, 2.04 mol, 4.00 equiv.) in DCM (2.0 L, 20 V). To the solution was added nonanoyl chloride (224.5 g, 1.27 mol,

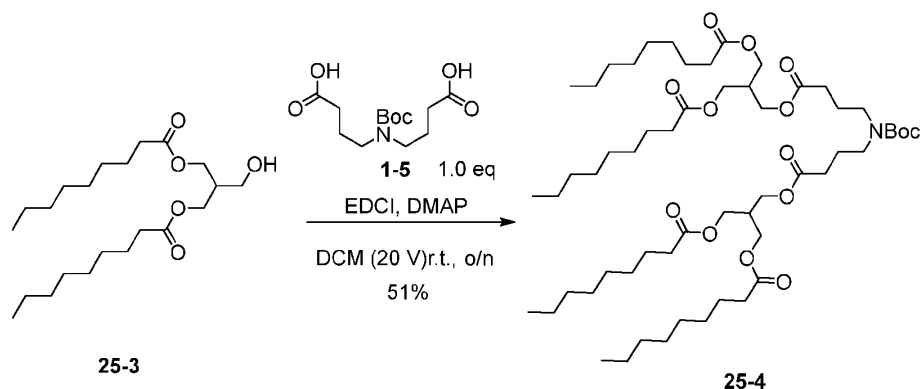
2.50 equiv.) dropwise at 20 °C for 30 min and stirred for 16 h at room temperature. The reaction was washed with water (1 x 1.0 L, 10 V) and brine (1 x 1.0 L mL, 10 V), organic layer was separated, dried, and evaporated. Crude product was adsorbed on 500 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (3 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using Pet. ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE :EA =6:1), combined, concentrated, and dried under vacuum to get **25-2** (194 g, 0.41 mol, 80.0% yield) as light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 95:5 to 5:95 A/B at 3 min.,): RT 2.5 min, m/z (Calcd.) 476.4, (found) 499.3 (M+Na).

[0552] Synthesis of 25-3: 2-(hydroxymethyl)propane-1,3-diyl dinonanoate



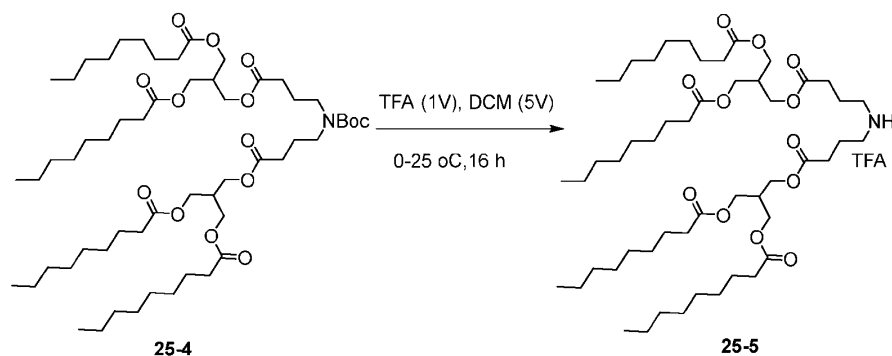
[0553] Into a 3 L 4-necked round-bottom flask was charged a solution of **25-2** (194.0 g, 0.41 mol, 1.00 equiv.) in MeOH (1.94 L, 10 V). Pd/C (38.8 g, 10%wt, 20%w/w) was added in one portion and hydrogenated for 16 h at room temperature. The reaction mixture was filtered, and the filter cake was washed with MeOH (0.97 L, 5 V). The filtrate was concentrated under vacuum to get **25-3** (148.0 g, 0.28 mol, 94.0% yield) as light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 95:5 to 5:95 A/B at 3 min.,): RT 2.2 min, m/z (Calcd.) 386.3, (found) 387.3 (M+H).

[0554] Synthesis of 25-4: (((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate



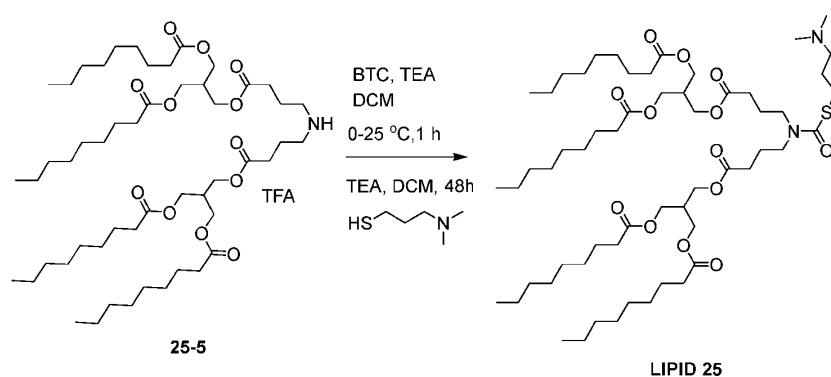
[0555] Into a 1000 mL 3-neck round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **25-3** (76.4 g, 0.19 mol, 2.00 equiv.) and **1-5** (26.0 g, 0.09 mol, 1.00 equiv.) in DCM (3 L, 20 V) followed by DMAP (11.0 g, 0.09 mol, 0.50 equiv.) and EDCI (41.4 g, 0.21 mol, 2.40 equiv.) at 0 °C. Ice water bath was removed and the reaction mixture was stirred for overnight at room temperature. The reaction was quenched with ice water (0.52 L, 20 V) and extracted with DCM (2 x 0.52 L, 20 V), washed with brine (1 x 0.52 L, 20 V), and the organic phase was dried with anhydrous Na₂SO₄ and filtered. Crude product was adsorbed on 150 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (900 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 70:30. Fractions were analyzed (TLC, PE :EA = 3:1), combined, concentrated, and dried under vacuum to get **25-4** (47 g, 46 mol, 80.0% yield) as light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.); RT 1.3 min, m/z (Calcd.) 1025.7, (found) 1048.0 (M+Na).

[0556] **Synthesis of (((4,4'-azanediylbis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate triufluoroacetic acid salt**



[0557] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **25-4** (47 g, 0.45 mol, 1.00 equiv.) in DCM (235 mL, 5V). To the solution was added TFA (47 mL, 1.0 V) dropwise at 0 - 5 °C for 20 min. The resulting solution was warmed to room temperature and stirred for 16 h at room temperature. The resulting mixture was concentrated under vacuum to get (49 g, 0.047 mol, crude) of **25-5** its trifluoroacetate salt as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 95:5 to 5:95 A/B at 3 min.; RT 2.3 min, m/z (Calcd.) 925.7, (found) 926.7 (M+H).

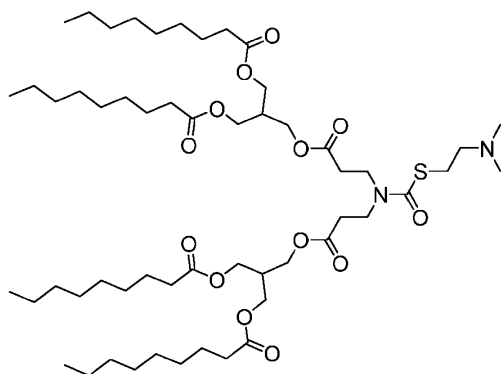
[0558] **Synthesis of LIPID 25: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate**



[0559] Into a 1 L 4-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **25-5** (30 g, 0.02 mol, 1.00 equiv.) in DCM (0.6 L, 20 V). To the mixture was added TEA (5.92 g, 0.05 mol, 2.00 equiv.) at 0 - 5 °C, followed by triphosgene (BTC, 0.5 equiv.) in batches at 0 - 5 °C. The ice water bath was removed, and the reaction mixture was stirred for 1 h at room temperature. After concentrating the reaction system it was dissolved in DCM (0.6 L, 20 V), TEA (8.86 g, 0.08 mol, 3.00 equiv.) and 3-(dimethylamino)propane-1-thiol (3.83 g, 0.03 mol, 1.10 equiv.) were added at 0 °C. The resulting solution was warmed to r.t and stirred for 48 h at room temperature. The reaction system was quenched with ice water (0.6 L, 20 V) and extracted with DCM (2 x 0.6 L, 20 V), washed with brine (2 x 0.6 L, 20 V). The organic phase was dried with anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. Crude product was adsorbed on 60 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (900 g of silica

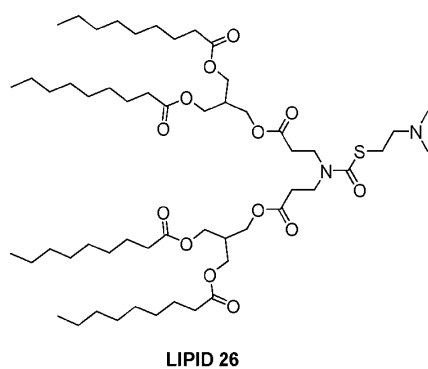
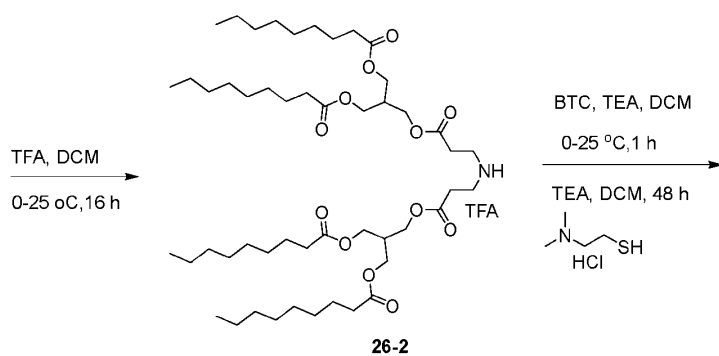
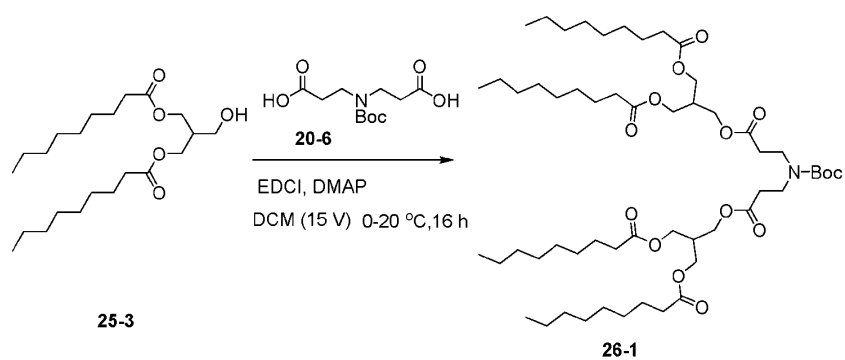
gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 70:30). Fractions were analyzed (TLC, Heptane :EA = 6:1), combined, concentrated, and dried under vacuum to get **LIPID 25** (5.5 g, 17.5%) as light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.), RT 2.2 min, m/z (Calcd.) 1070.7, (found) 1072.0 (M+H). ¹HNMR-**LIPID 25**: (300 MHz, CDCl₃, ppm): δ 4.15 (dd, *J* = 6.0, 3.1 Hz, 12H), 3.39 (s, 4H), 2.93 (t, *J* = 7.2 Hz, 2H), 2.48-2.21 (m, 22H), 2.01-1.76 (m, 6H), 1.71-1.53 (m, 8H), 1.29 (q, *J* = 4.3, 3.6 Hz, 40H), 0.98-0.81 (m, 12H).

[0560] **Example 26. LIPID 26: ((3,3'-(((2-(Dimethylamino)ethyl)thio)carbonyl)azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(propene-2,1,3-triyl) tetranonanoate**

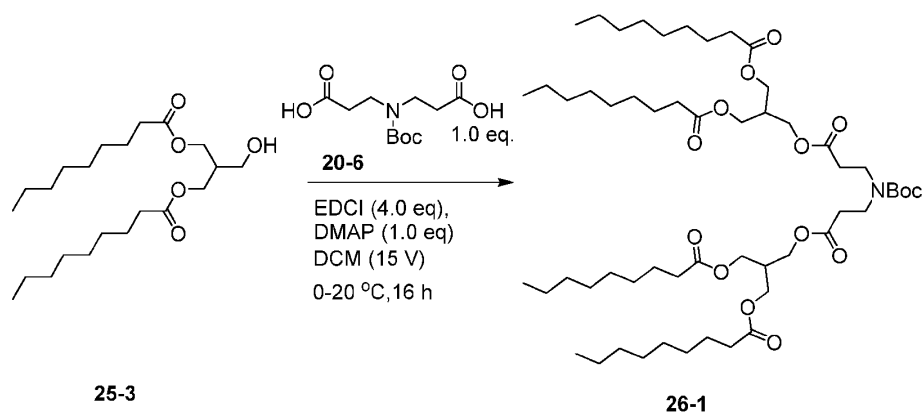


[0561] **LIPID 26**

[0562] **General scheme**

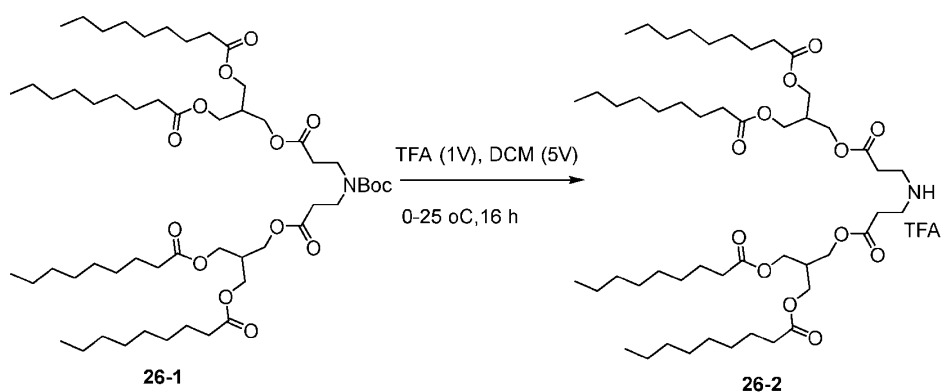


[0563] Synthesis of 26-1: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl)tetranonanoate



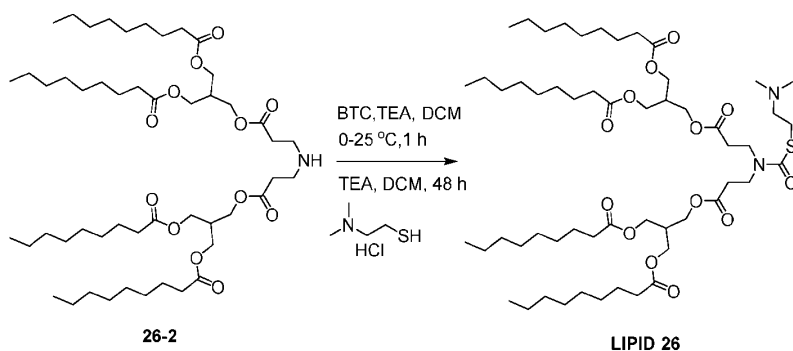
[0564] Into a 1000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **25-3** (84.6 g, 0.21 mol, 2.20 equiv.) and **20-6** (26.0 g, 0.099 mol, 1.00 equiv.) in DCM (520 mL, 20 V) followed by DMAP (12.1 g, 0.1 mol, 1.00 equiv.) and EDCI (45.9 g, 0.23 mol, 2.40 equiv.) at 0 °C. The reaction mixture was stirred for overnight at room temperature. The reaction was quenched with ice water (0.52 L, 20 V), extracted with DCM (2 x 0.52 L, 20 V), and washed with brine (1 x 0.52 L, 20 V). The organic phase was dried with anhydrous Na₂SO₄ and filtered. Crude product was adsorbed on 150 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (900 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 70:30). Fractions were analyzed (TLC, PE :EA = 3:1), combined, concentrated, and dried under vacuum to get **26-1** (49.6 g, 48.9%) as light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.,): RT 0.99 min, m/z (Calcd.) 997.7, (found) 1020.8 (M+Na).

[0565] Synthesis of **26-2**: (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate trifluoroacetic acid salt



[0566] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **26-1** (48.6 g, 0.45 mol, 1.00 equiv.) in DCM (243 mL, 5V). To the solution was added TFA (48.6 mL, 1.0 V) dropwise at 0 - 5 °C for 20 min. The resulting solution was warmed to room temperature and stirred for 16 h at room temperature. The resulting mixture was concentrated under vacuum to get 50.5 g, (0.050 mol, crude) of **26-2** as its trifluoroacetic acid salt as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 95:0 to 5:95 A/B at 3 min.); RT 2.3 min, m/z (Calcd.) 897.6, (found) 898.7 (M+H).

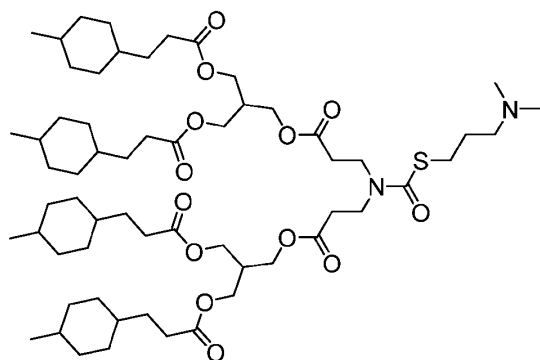
[0567] **Synthesis of LIPID 26: ((3,3'-(((2-(Dimethylamino)ethyl)thio)carbonyl) azanediyl) bis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetranonanoate**



[0568] Into a 1 L 4-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **26-2** (30 g, 0.03 mol, 1.00 equiv., crude) in DCM (0.6 L, 20 V). To the mixture was added TEA (6.08 g, 0.06 mol, 2.00 equiv.) into the reactor at 0 - 5 °C, then added BTC (4.47 g, 0.015 mol, 0.5 equiv.) in batches at 0 - 5 °C. The ice water bath was removed the reaction mixture was stirred for 1 h at room temperature. After

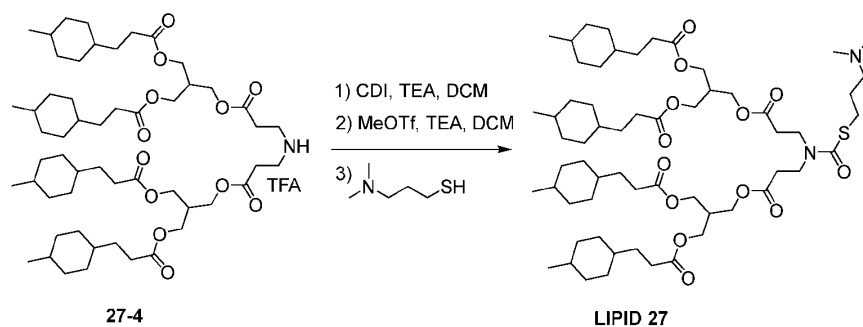
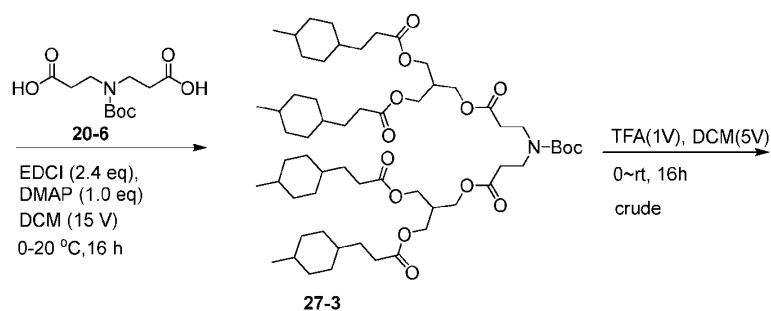
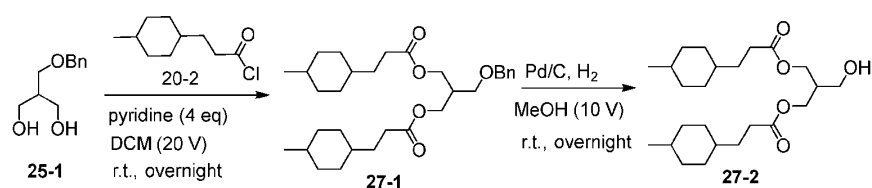
concentrating the reaction system it was dissolved in DCM (0.6 L, 20 V), TEA (12.16 g, 0.12 mol, 4.00 equiv.) and 2-(dimethylamino)ethane-1-thiol hydrochloride (4.67 g, 0.03 mol, 1.10 equiv.) were added at 0 °C. The resulting solution was warmed to room temperature and stirred for 48 h. The reaction was quenched with ice water (0.6 L, 20 V) and extracted with DCM (2 x 0.6 L, 20 V), washed with brine (2 x 0.6 L, 20 V), dried with anhydrous Na₂SO₄, and filtered. Crude product was adsorbed on 60 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (900 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 70:30. Fractions were analyzed (TLC, heptane :THF = 7:1), combined, concentrated, and dried under vacuum to get **LIPID 26** (5.4 g, 17.5%) as light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 100:0 to 0:100 A/B at 3 min.); RT 2.2 min, m/z (Calcd.) 1028.7, (found) 1030.0 (M+H). ¹HNMR-**LIPID 26**: (300 MHz, CDCl₃, ppm) : δ 4.15 (t, *J* = 5.5 Hz, 12H), 3.68 (t, *J* = 7.2 Hz, 4H), 3.07 (t, *J* = 7.0 Hz, 2H), 2.67 (t, *J* = 7.2 Hz, 4H), 2.59 (t, *J* = 7.0 Hz, 2H), 2.49-2.25 (m, 16H), 1.64 (q, *J* = 7.3 Hz, 8H), 1.30 (dd, *J* = 5.9, 3.0 Hz, 40H), 0.95-0.84 (m, 12H).

Example 27. LIPID 27: ((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)

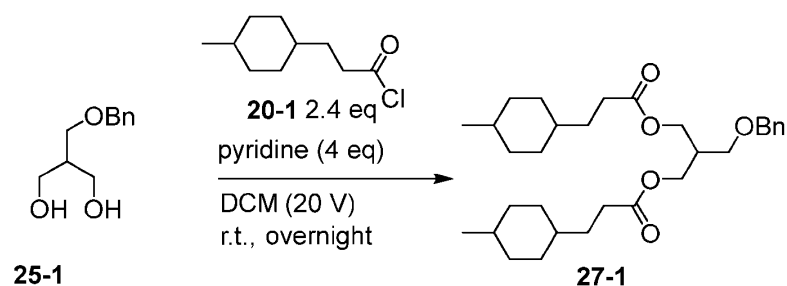


[0569] LIPID 27

[0570] General scheme

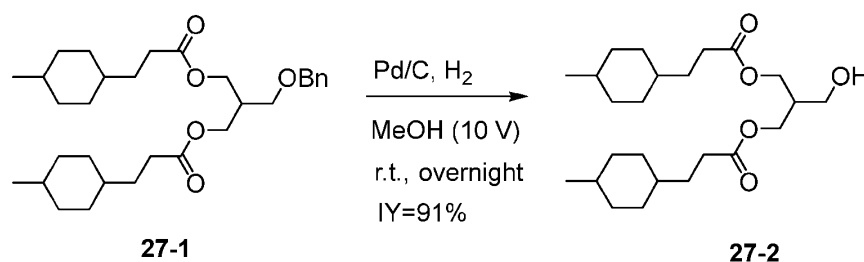


[0571] Synthesis of 27-1: 2-((benzyloxy)methyl)propane-1,3-diyl bis(3-(4-methylcyclohexyl)propanoate)



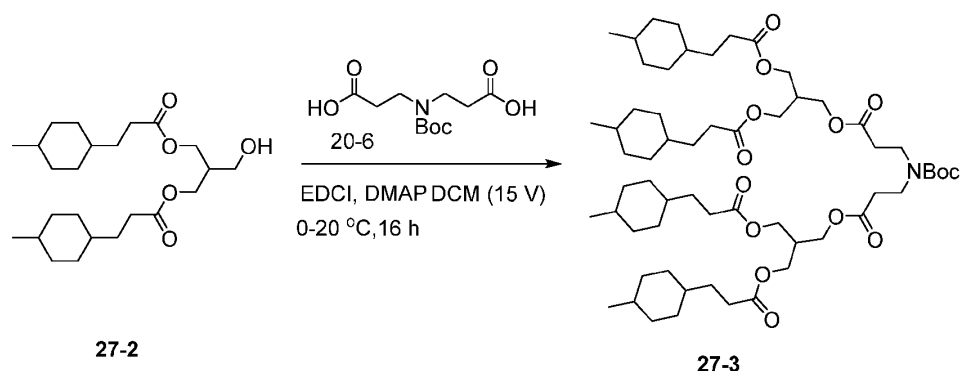
[0572] Into a 5 L 4-necked round-bottle flask purged and maintained with an inert atmosphere of nitrogen were added **25-1** (30 g, 0.15 mol, 1.00 equiv.), pyridine (48.3 g, 0.61 mol, 4.00 equiv.) in DCM (0.6 L, 20 V). To the solution was added **20-1** (69.0 g, 0.36 mol, 2.40 equiv.) dropwise at 0 °C for 30 min, then the resulting solution was stirred for overnight at room temperature. The reaction was washed with water (1 x 0.6 L, 10 V), brine (1 x 0.6 L, 10 V), dried (Na_2SO_4) and concentrated under reduced pressure. Crude product was adsorbed on 180 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 6:1), combined, concentrated, and dried under vacuum to get **27-1** (60 g, 75%) as light yellow oil. ELSD A: water/0.05% TFA: B: CH_3CN / 95:0 to 5:95 A/B at 3 min.): RT 2.5 min, m/z (Calcd.) 500.3, (found) 523.3 (M+Na).

[0573] **Synthesis of 27-2: 2-(hydroxymethyl)propane-1,3-diyl bis(3-(4-methylcyclohexyl)propanoate)**



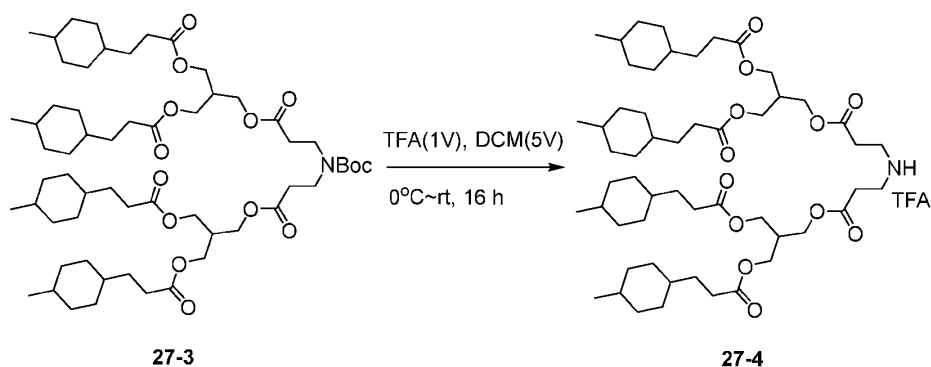
[0574] Into a 3 L 4-necked round-bottom flask was charged a solution of **27-1** (60.0 g, 0.12 mol, 1.00 equiv.) in MeOH (1.94 L, 10 V) and 10%wt Pd/C (12.0 g, 20%w./w.) was added in one portion. The reaction mixture was hydrogenated under hydrogen gas at room temperature. The reaction mixture was filtered, and the filter cake was washed with MeOH (300 mL, 5 V). The filtrate was concentrated under vacuum to get **27-2** (46.0 g, 0.12 mol, 91.0% yield) as light-yellow oil. ELSD A: water/0.05% TFA: B: CH_3CN / 95:0 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 410.3, (found) 411.3 (M+H).

[0575] **Synthesis of 27-3: (((3,3'-((tert-butoxycarbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**



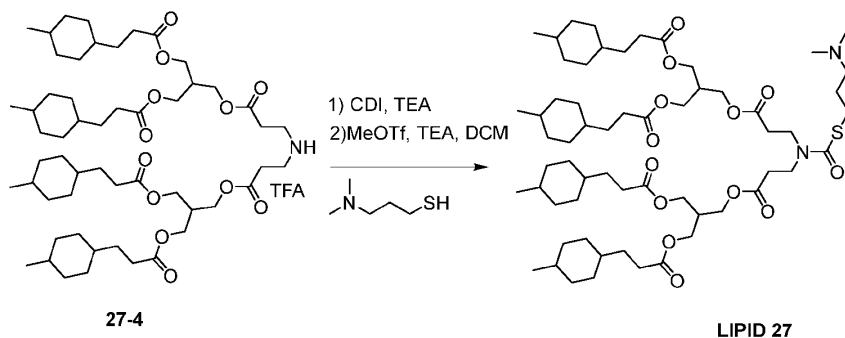
[0576] Into a 1000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was added **27-2** (103.6 g, 0.25 mol, 2.20 equiv.) and **20-6** (30.0 g, 0.11 mol, 1.00 equiv.) in DCM (600 mL, 20 V). To the solution was added DMAP (14.0 g, 0.11 mol, 1.00 equiv.) and EDCI (53.0 g, 0.27 mol, 2.40 equiv.) at 0 °C. The ice water bath was removed after adding all reagents. The reaction mixture was stirred for overnight at room temperature. The reaction system was quenched with ice water (0.60 L, 20 V). The system was extracted with DCM (2 x 0.60 L, 20 V) and washed with brine (1 x 0.60 L, 20 V). The organic phase was dried with anhydrous Na₂SO₄ and then filtered. Crude product was adsorbed on 260 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1.3 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 70:30. Fractions were analyzed (TLC, PE:EA = 3:1), combined, concentrated, and dried under vacuum to get **27-3** (38.2 g, 36.5 mmol, 31.8%) as light yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/ 100:0 to 0:100 A/B at 3 min): RT 1.2 min, m/z (Calcd.) 1045.7, (found) 1068.6 (M+Na).

[0577] **Synthesis of (((3,3'-azanediylbis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate) trifluoroacetic acid salt**



[0578] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **27-3** (38.2 g, 0.036 mol, 1.00 equiv.) in DCM (191 mL, 5V). To the solution was added TFA (38.2 mL, 1.0 V) dropwise at 0 - 5 °C for 20 min and stirred for 16 h at room temperature. The resulting mixture was concentrated and dried under vacuum to get (38.0 g, 0.036 mol, crude) of **27-4** as its trifluoroacetic acid salt as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/ 95:5 to 5:95 A/B at 3 min): RT 2.3 min, m/z (Calcd.) 945.6, (found) 946.6 (M+H).

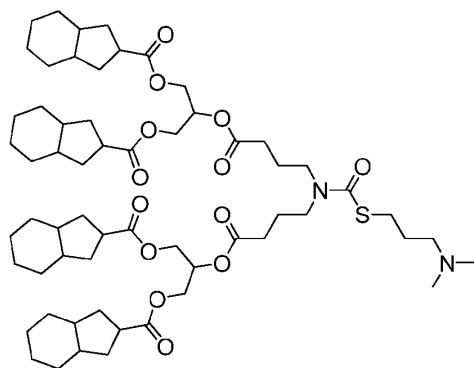
[0579] **Synthesis of LIPID 27: ((3,3'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(propanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**



[0580] Into a 1000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **27-4** (29.0 g, 0.02 mol, 1.00 equiv.) in DCM (580 mL, 20 V). To this was added TEA (4.2 g, 0.03 mol, 1.50 equiv.), followed by CDI (4.95 g, 0.02 mol, 1.10 equiv.). The mixture was stirred overnight at room temperature, LCMS showed

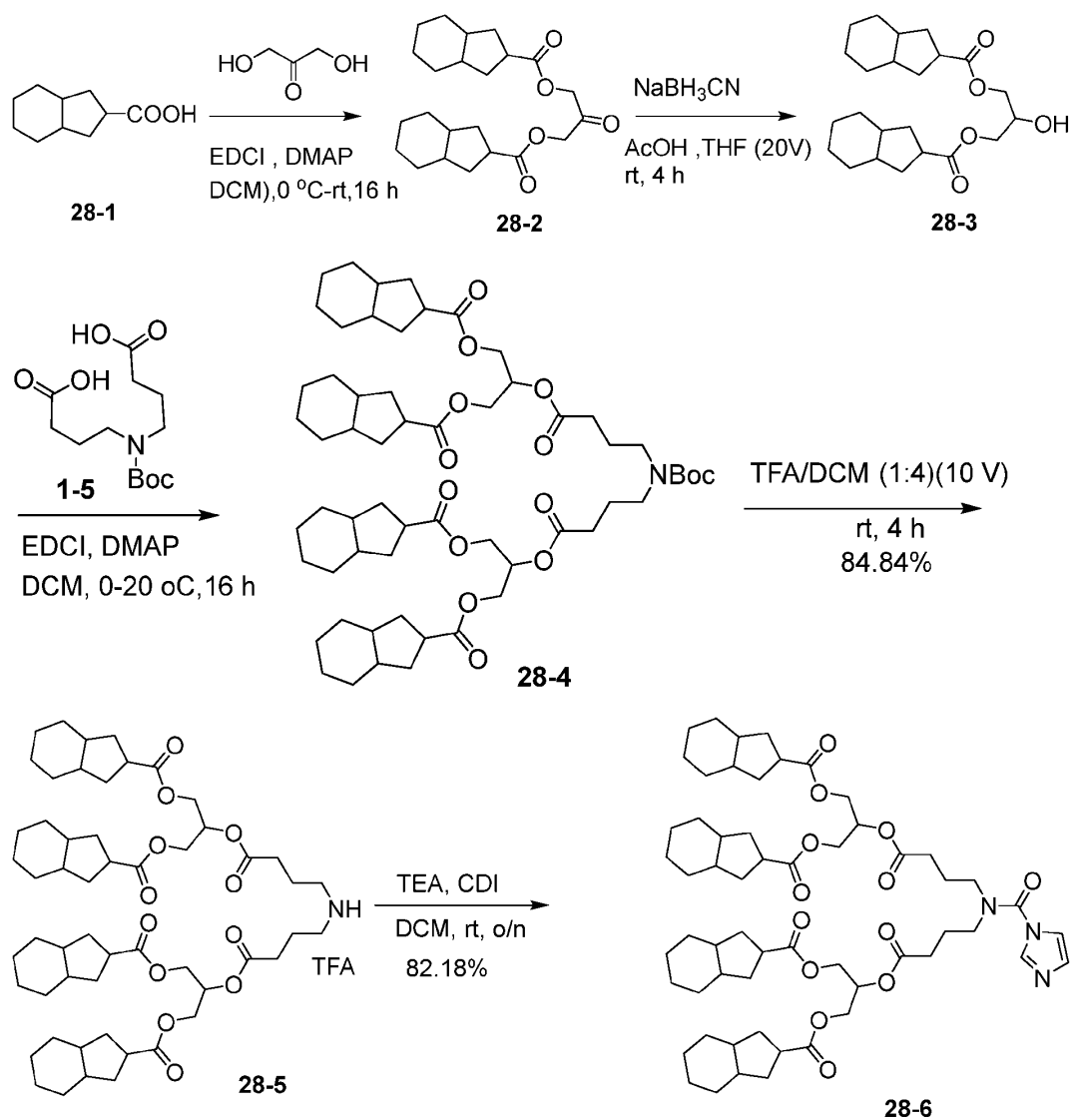
the reaction was completed. Then, TfOMe (5.01 g, 0.02 mol, 1.10 equiv.) was added and the mixture was stirred at 0 °C for 1 hour. Then, TEA (4.2 g, 0.03 mol, 1.50 equiv.) and 3-(dimethylamino) propane-1-thiol (3.64 g, 0.03 mol, 1.10 equiv.) were added to the solution. The ice/water bath was removed, and the mixture was stirred overnight at room temperature. The reaction system was quenched with ice water (580 mL, 20 V). The system was extracted with DCM (2 x 580 mL, 20 V) and washed with brine (2 x 580 mL, 20 V). The organic phase was dried with anhydrous Na₂SO₄ and then filtered. Crude product was adsorbed on 58 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (350 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 70:30). Fractions were analyzed (TLC, heptane:EA = 7:1), combined, concentrated, and dried under vacuum to get **LIPID 27** (5.4 g, 4.9 mmol, 17.8 %) as light yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/ 0.05% TFA 100:0 to 0:100 A/B at 25 min.): RT 11.7 min, m/z (Calcd.) 1090.7, (found) 1091.8 (M+H). ¹HNMR-**LIPID 27**: (300 MHz, CDCl₃, ppm): δ 4.24-4.08 (m, 12H), 3.67 (t, *J* = 7.2 Hz, 4H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.67 (t, *J* = 7.2 Hz, 4H), 2.51-2.29 (m, 12H), 1.82 (p, *J* = 7.3 Hz, 2H), 1.76-1.58 (m, 13H), 1.58-1.20 (m, 30H), 1.16 (tq, *J* = 10.9, 3.3 Hz, 1H), 0.90 (dd, *J* = 14.8, 6.7 Hz, 16H).

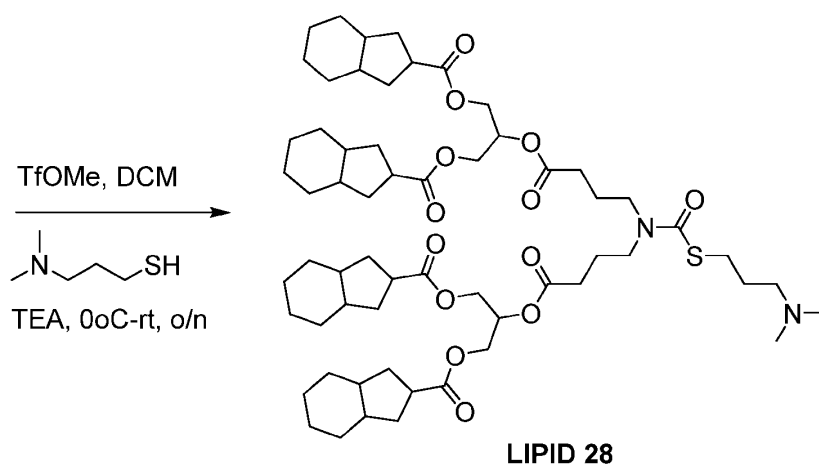
Example 28. LIPID 28: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl) azanediyl) bis(butanoyl))bis(oxy)bis(propene-2,1,3-triyl) tetrakis(octahydro-1H-indene)



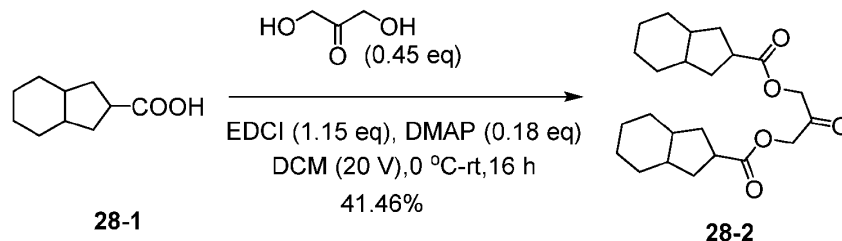
[0581] LIPID 28

[0582] General scheme



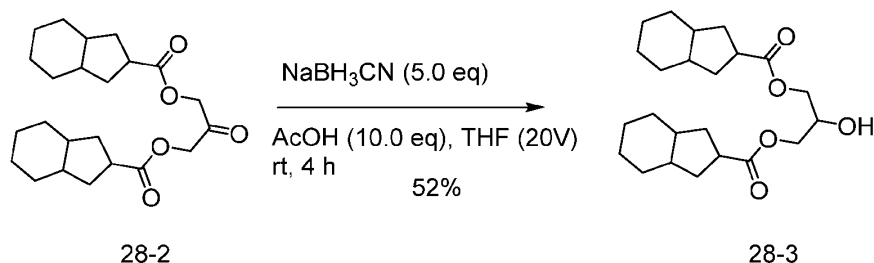


[0583] Synthesis of 28-2: 2-oxopropane-1,3-diyl bis(octahydro-1H-indene-2-carboxylate)



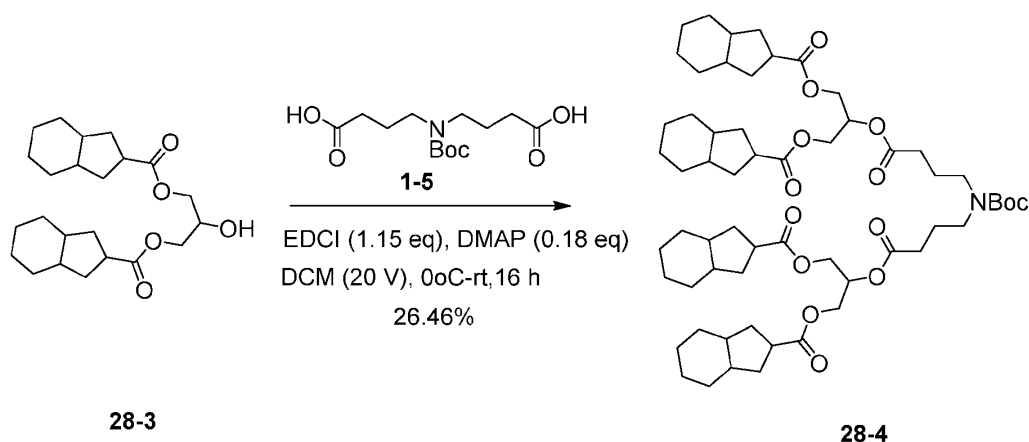
[0584] To 1.6 L (20V) of DCM in a 3 L 3-necked round-bottle flask was added **28-1** (80.00 g, 475.5 mmol, 1.00 equiv.), dihydroxyacetone (19.28 g, 214.0 mmol, 0.45 equiv.), DMAP (10.46 g, 85.6 mmol, 0.18 equiv.) and EDCI (84.90 g, 546.8 mmol, 1.15 equiv.) at 0°C. The reaction mixture was stirred for 16 h at room temperature. The resulting mixture was diluted with brine (1.6 L, 20 V). The organic solvent was removed under reduced pressure. The residual solution was extracted with ethyl acetate (2 x 1.6 L, 40 V). The combined organic layers were washed with brine (1.6 L, 20 V), dried over anhydrous Na₂SO₄, and then filtered. Crude product was adsorbed on 160 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1.6 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10. Fractions were analyzed (TLC, heptane:EA = 7:1), combined, concentrated, and dried under vacuum to get **28-2** (77 g, 0.197 mmol, 41.5%) as light yellow oil that was used in the next reaction.

[0585] Synthesis of 28-3: 2-hydroxypropane-1,3-diyl bis(octahydro-1H-indene-2-carboxylate)



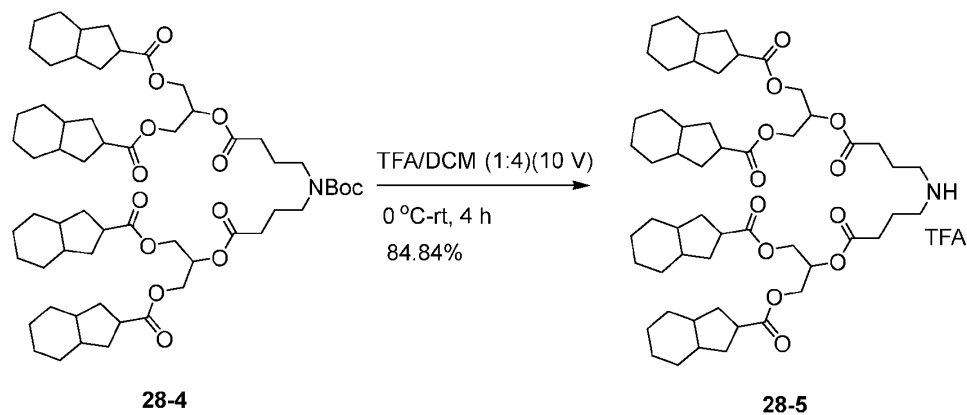
[0586] To a 3 L three-necked round-bottom flask were added **28-2** (75 g, 0.19 mol, 1.00 equiv.), THF (1.50 L, 20 V) and AcOH (115.3 g, 1.92 mmol, 10.0 equiv.) at room temperature. Then, NaBH₃CN (60.34 g, 0.96 mol, 5.0 equiv.) was added to the above mixture at 0°C in several portions. The reaction mixture was stirred for 4 h at room temperature. The reaction system was quenched with the addition of water (1.50 L, 20 V) at room temperature. The mixture was extracted with ethyl acetate (2 x 1.50 L, 40 V). The combined organic layers were washed with brine (1.5 L, 20 V), dried with anhydrous Na₂SO₄, and then filtered. Crude product was adsorbed on 150 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (1.2 Kg of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 87:13). Fractions were analyzed (TLC, PE:EA = 5:1), combined, concentrated, and dried under vacuum to get **28-3** (55 g, 0.14 mmol, 52%) as light yellow oil that was used in the next reaction. ELSD A: water/0.05% ammonia: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 392.3, (found) 375.2 (M-OH).

[0587] Synthesis of 28-4: ((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-2-carboxylate)



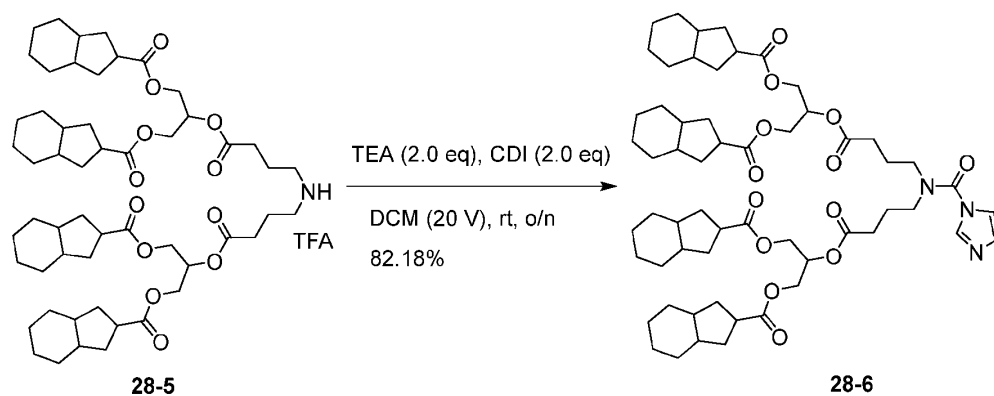
[0588] To a 2 L three-necked round-bottom flask was added **28-3** (40 g, 101.9 mmol, 1.00 equiv.), **28-5** (13.27 g, 45.85 mmol, 0.45 equiv.), DCM (0.8 L, 20 V), DMAP (2.24 g, 18.34 mmol, 0.18 equiv.). Then, EDCI (18.19 g, 117.2 mmol, 1.15 equiv.) was added to the above mixture at 0 °C in several portions. The reaction mixture was stirred for 16 h at room temperature. The reaction was monitored by LCMS. The resulting mixture was diluted with brine (0.8 L, 20 V). The mixture was extracted with ethyl acetate (2 x 0.8 L, 40V). The combined organic layers were washed with brine (0.8 L, 20V). The organic phase was dried with anhydrous Na₂SO₄ and then filtered. Crude product was adsorbed on 80 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (800 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 5:1), combined, concentrated, and dried under vacuum to get **28-4** (28 g, 0.027 mmol, 26.5%) as light yellow oil that was used in the next reaction.

[0589] **Synthesis of 28-5: ((4,4'-azanediylbis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis (octahydro-1H-indene-2-carboxylate) trifluoroacetic acid salt**



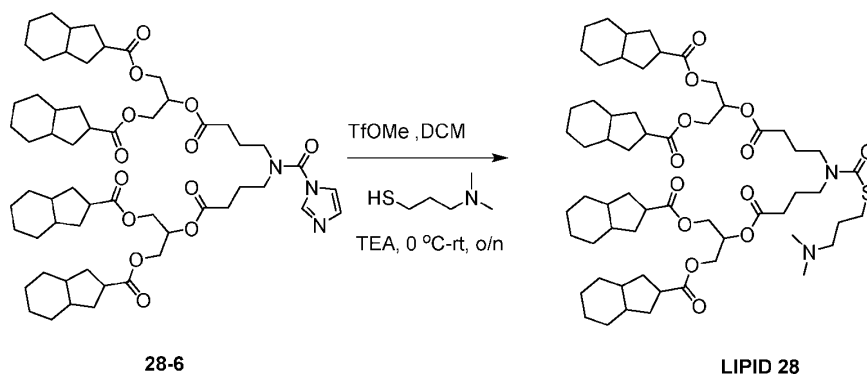
[0590] To a 500 mL round-bottle flask was added **28-4** (27 g, 26.00 mmol, 1.00 equiv.) in DCM (108 mL, 4 V) at room temperature. The solution was cooled to 0 °C in an ice/water bath. To this solution was added TFA (13.5 g, 0.5 V) at 0 °C. The ice/water bath was removed, and the mixture was stirred for 4 h at room temperature. The reaction was monitored by LCMS. The reaction was concentrated and dried under vacuum to get **28-5** as its trifluoroacetic acid salt (22 g, 0.021mmol, 84.8%) as a light brown oil that was used without further purification. ELSD A: water/0.05% TFA; B: CH₃CN 95:5 to 5:95 A/B at 3 min.); RT 2.0 min, m/z (Calcd.) 937.7, (found) 938.8 (M+H).

[0591] Synthesis of **28-6**: ((4,4'-((1H-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis (propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-2-carboxylate)



[0592] Into a 2 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **28-5** (22 g, 21.23 mmol, 1.00 equiv.) in DCM (440 mL). To this solution was added the TEA (4.30 g, 42.46 mmol, 2.00 equiv.) followed by CDI (6.88 g, 42.46 mmol, 2.00 equiv.) at room temperature. The mixture was stirred overnight at room temperature, LCMS showed the reaction was completed. The resulting mixture was diluted with water (400 mL). The mixture was extracted with CH₂Cl₂ (2 x 200 mL). The combined organic layers were washed with brine (400 mL), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. This resulted in **28-6** (18 g, 0.017mmol, 82.18%) as a brown oil which was used in the next step as such. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 1031.7, (found) 1032.8 (M+H).

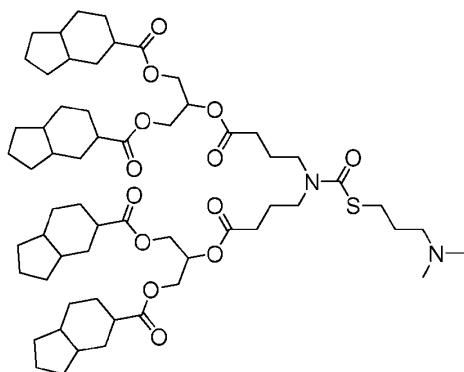
[0593] **Synthesis of LIPID 28: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(butanoyl))bis(oxy)bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene)**



[0594] Into a 1 L 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of **28-6** (15 g, 14.53 mmol, 1.00 equiv.) in DCM (300 mL, 20V). The solution was cooled to 0 °C in an ice/water bath. Then, TfOMe (2.62 g, 15.98 mmol, 1.10 equiv.) was added and the mixture was stirred at 0 °C for 1 hour. LCMS showed complete consumption of **28-6**. Then, TEA (2.94 g, 29.06 mmol, 2.00 equiv.) and 3-(dimethylamino)propane-1-thiol (2.08 g, 17.43 mmol, 1.20 equiv.) were added to the solution. The ice/water bath was removed, and the mixture was stirred overnight at room temperature.

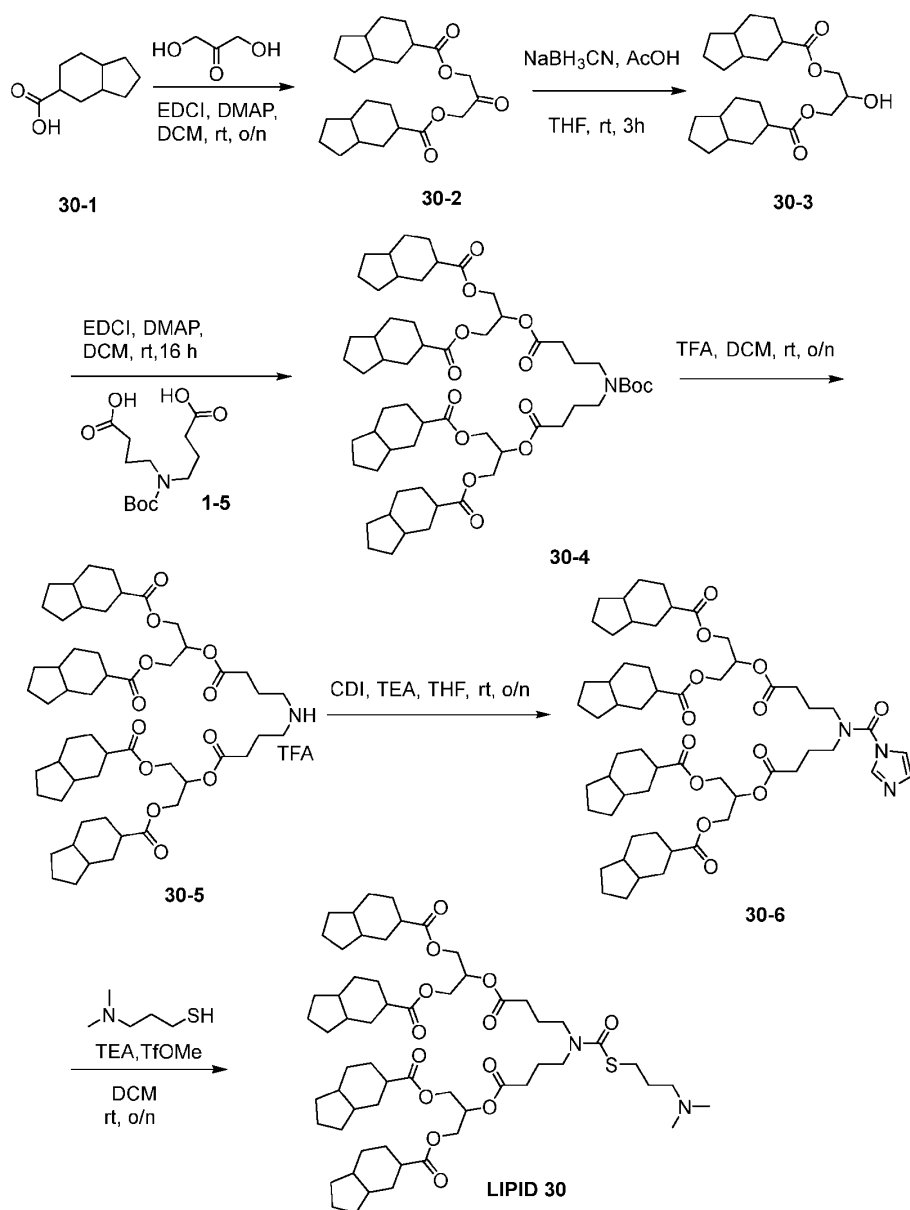
The reaction was monitored by LCMS. The resulting mixture was concentrated under reduced pressure. The residue was diluted with water (300 mL, 20 V) and extracted with ethyl acetate (2 x 300 mL, 40 V). The combined organic layers were washed with brine (300 mL), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by reverse flash chromatography with the following conditions: Welch Ultimate XB-Phenyl 50mm*250mm 10μm; mobile phase, (0.1% FA) water and CH₃CN; 50-90% in 15 min and hold 8min; flow rate: 90 ml/min; UV 220 nm This resulted in **LIPID 28** (3.2302 g, 20.52%) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN 95:5 to 5:95 A/B at 25 min.): RT 10.0 min, m/z (Calcd.) 1082.6, (found) 1083.7 (M+H). ¹H-NMR-**LIPID 28**: (400 MHz, CDCl₃, ppm): δ 5.29-5.26 (m, 2H), 4.34-4.29 (m, 4H), 4.18-4.13 (m, 5H), 3.69-3.67 (m, 1H), 3.38 (s, 4H), 2.97-2.82 (m, 7H), 2.38-2.34 (m, 6H), 2.24 (s, 6H), 2.11-1.99 (m, 4H), 1.97-1.84 (m, 18H), 1.83-1.75 (m, 12H), 1.56-1.15 (m, 34H), 1.09-0.91 (m, 1H).

Example 29. LIPID 30: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-5-carboxylate)

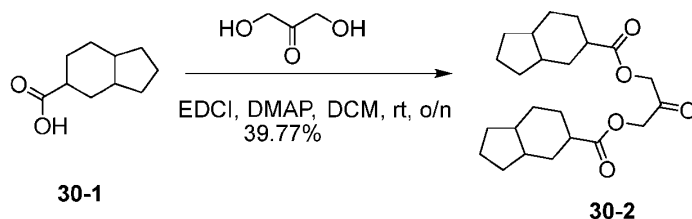


[0595] LIPID 30

[0596] General scheme

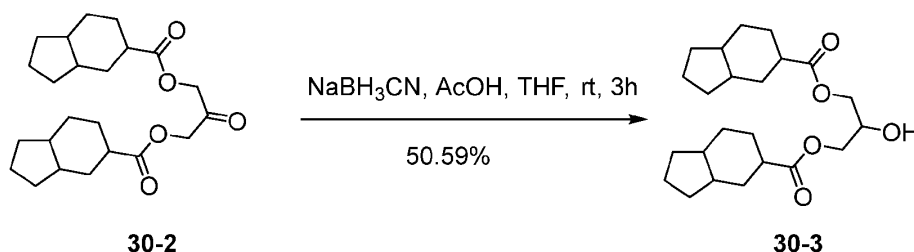


[0597] Synthesis of 30-2: 2-oxopropane-1,3-diyl bis(octahydro-1H-indene-5-carboxylate)



[0598] Into a 2 L 4-necked round-bottom flask was added commercially available octahydro-1H-indene-5-carboxylic acid (**30-1**, 65 g, 386.36 mmol, 1.00 equiv.) and dihydroxyacetone (15.66 g, 173.86 mmol, 0.45 equiv.) in DCM (800 mL). DMAP (9.44 g, 77.27 mmol, 0.20 equiv.) and EDC.HCl (88.88 g, 463.63 mmol, 1.20 equiv.) were added in portions at room temperature. The resulting mixture was stirred for overnight at room temperature and concentrated under vacuum. Crude product was adsorbed on 120 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (600 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10. Fractions were analyzed (TLC, PE:EA = 20:1), combined, concentrated, and dried under vacuum to get **30-2** (60 g, 0.15 mmol, 40%) as a colorless oil. ELSD A: water/0.05% TFA; B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 390.2, (found) 391.3 (M+H).

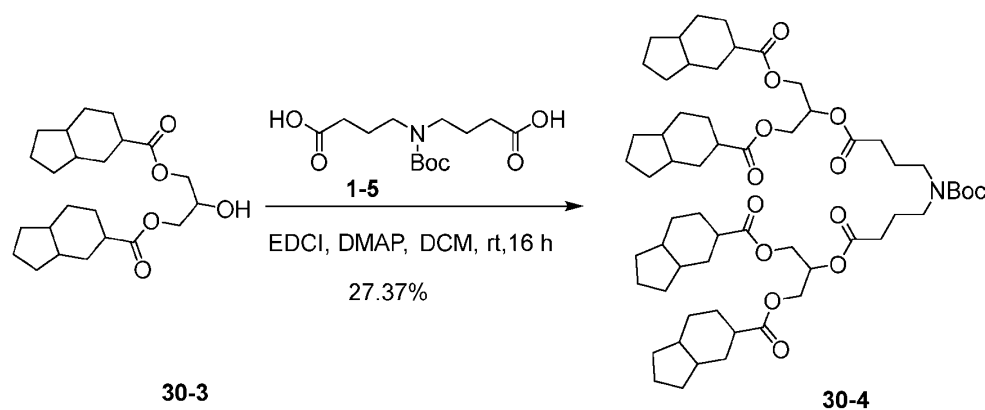
[0599] Synthesis of 30-3: 2-hydroxypropane-1,3-diyl bis(octahydro-1H-indene-5-carboxylate)



[0600] Into a 1000 mL 3-necked round-bottom flask was added **30-2** (59 g, 151.08 mmol, 1.00 equiv.) and AcOH (90.73 g, 1510.81 mmol, 10 equiv.) in THF (600 mL). NaBH₃CN (47.47 g, 755.40 mmol, 5.00 equiv.) was added in portions at room temperature and the mixture was stirred for 3 h. pH of the mixture was brought to 9 with saturated NaHCO₃ (aq.). The resulting mixture was extracted with EtOAc (2 x 500 mL). The combined organic layers were collected

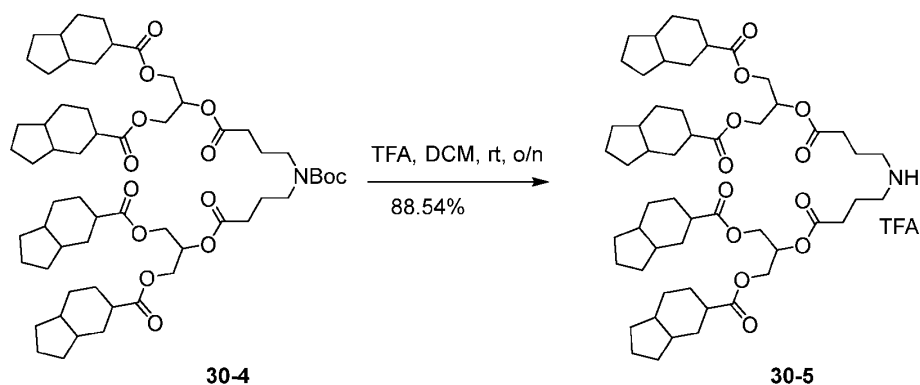
and dried over anhydrous Na_2SO_4 . After filtration, the filtrate was concentrated under reduced pressure. Crude product was adsorbed on 120 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (600 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 20:1), combined, concentrated, and dried under vacuum to get **30-3** (30 g, 0.076 mmol, 50.6%) as a colorless oil. ELSD A: water/0.02% TFA: B: CH_3CN 95:5 to 5:95 A/B at 3 min.): RT 2.1 min, m/z (Calcd.) 392.2, (found) 375.3 (M-OH).

[0601] Synthesis of 30-4: ((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propene-2,1,3-triyl) tetrakis(octahydro-1H-indene-5-carboxylate)



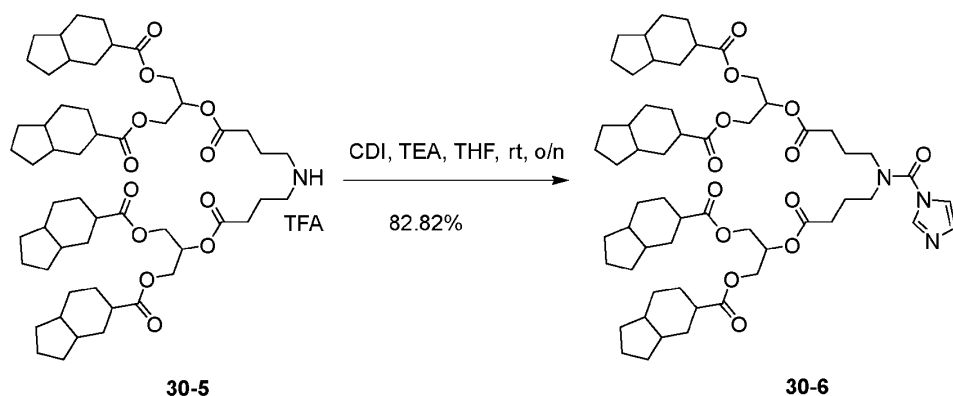
[0602] To a 1000 mL three-necked round-bottom flask was added **30-3** (29 g, 73.87 mmol, 1.00 equiv.) and **1-5** (9.62 g, 33.24 mmol, 0.45 equiv.) in DCM (600 mL). DMAP (1.81 g, 14.77 mmol, 0.20 equiv.) and EDC.HCl (13.76 g, 88.65 mmol, 1.20 equiv.) were added in portions at room temperature. The resulting mixture was stirred for 16 h at room temperature. The resulting mixture was concentrated under vacuum. Crude product was adsorbed on 60 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w/w) and purified on a silica gel column (400 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w/w) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 20:1), combined, concentrated, and dried under vacuum to get **30-4** (21 g, 0.02 mmol, 27.4%) as a colorless oil which was directly used in the next step.

[0603] Synthesis of ((4,4'-azanediylbis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-5-carboxylate) trifluoroacetic acid salt



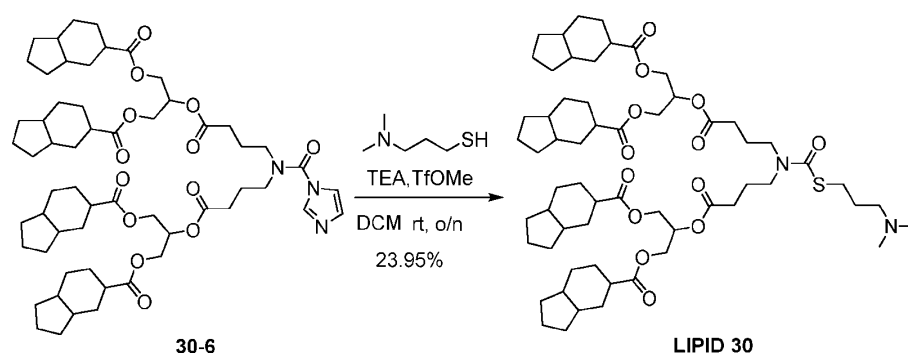
[0604] To a 1 L three-necked round-bottom flask was added **30-4** (20 g, 19.26 mmol, 1.00 equiv.) in DCM (80 mL) followed by TFA (10 mL, 134.63 mmol, 6.99 equiv.) drop-wise at room temperature. The resulting mixture was stirred overnight at room temperature and concentrated and dried under reduced pressure. This resulted in **30-5** as its trifluoroacetic acid salt (16 g, 0.015 mmol, 88.5%) as a light- yellow oil. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.0 min, m/z (Calcd.) 937.6, (found) 938.7 (M+H).

[0605] Synthesis of ((4,4'-((1H-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-5-carboxylate)



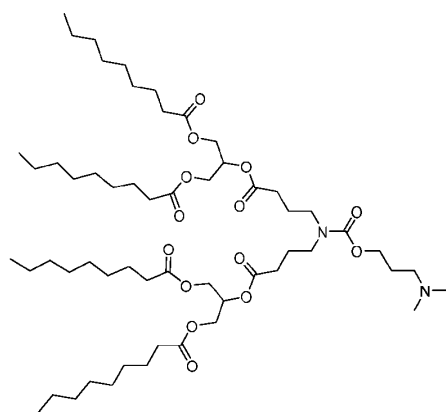
[0606] To a 500 mL three-necked round-bottom flask was added **30-5** (16 g, 17.05 mmol, 1.00 equiv.) and CDI (2.96 g, 18.24 mmol, 1.20 equiv.), TEA (3.08 g, 30.43 mmol, 2.00 equiv.) in THF (320 mL). The resulting mixture was stirred overnight at room temperature and concentrated under vacuum. To this was added water (500 mL), extracted with EtOAc (2 x 500 mL), combined organic layer was dried over anhydrous Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to afford **30-6** (13 g, 0.012 mmol, 82.82%) as a light-yellow oil which was immediately used in the next step. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.1 min, m/z (Calcd.) 1031.6, (found) 1032.7 (M+H).

[0607] **Synthesis of LIPID 30: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(octahydro-1H-indene-5-carboxylate)**



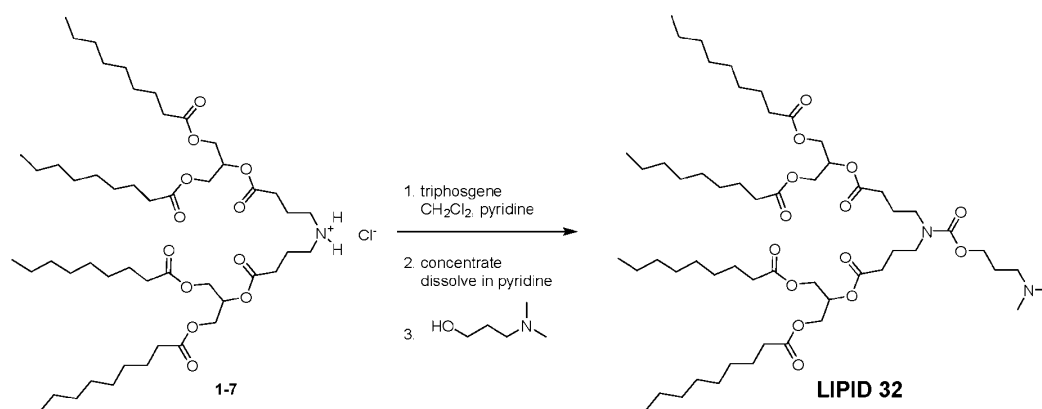
Hz, 2H), 2.61-2.18 (m, 17H), 2.16-1.80 (m, 20H), 1.80-1.4 (m, 39H), 1.42-1.40 (m, 6H), 1.31-1.0 (m, 6H).

Example 30. Synthesis of LIPID 32: ((4,4'-(((3-(dimethylamino)propoxy)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



[0609] The following method is contemplated.

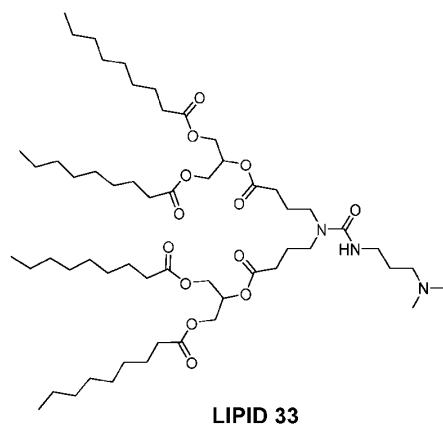
[0610] General Scheme:



[0611] Ammonium salt **1-7** (taken from the synthesis of **LIPID 1**) will be dissolved in CH_2Cl_2 , cooled in an ice-water bath under nitrogen, and triphosgene will be added. After the addition of the triphosgene, pyridine will be added and the mixture will be stirred for 4 hours, then the solvent will be removed *in vacuo* and the residue will be dissolved in pyridine and cooled in an ice-water bath under nitrogen. To this cooled solution 3-dimethylamino-1-propanol will be added

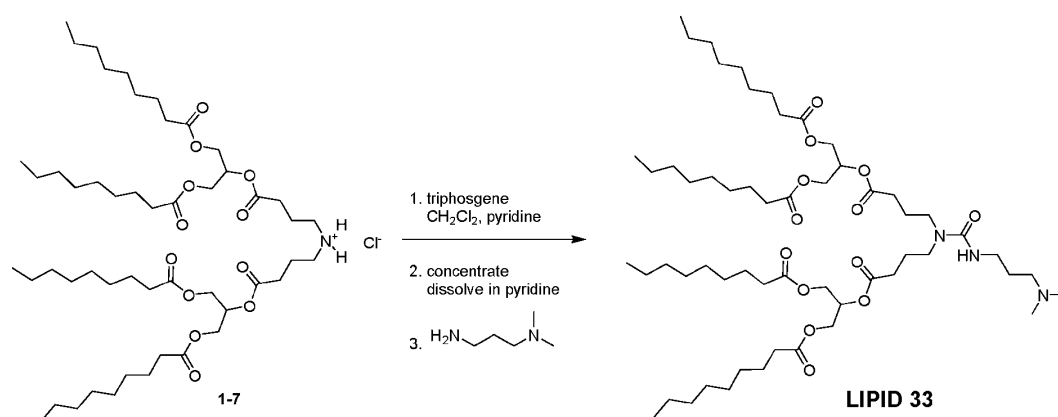
and the solution should be stirred for 30 minutes after the addition is made and then will be warmed to room temperature and will be allowed to stir for 14 hours. The mixture will then be concentrated *in vacuo* and the residue will be dissolved in CH_2Cl_2 , adsorbed on silica gel, and purified by chromatography using a Combi-flash apparatus. Fractions containing the desired carbamate product will be concentrated *in vacuo* dissolved in n-heptane, treated with activated charcoal, filtered, washed with a mixture of methanol and water, dried, and concentrated *in vacuo* to afford the target **LIPID 32**, ((4,4'-(((3-(dimethylamino)propoxy)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate.

Example 31. Synthesis of LIPID 33: ((4,4'-(((3-(dimethylamino)propyl)carbamoyl)azanediyl)bis(butanoyl)) bis(oxy))bis(propane-2,1,3-triyl) tetranonanoate



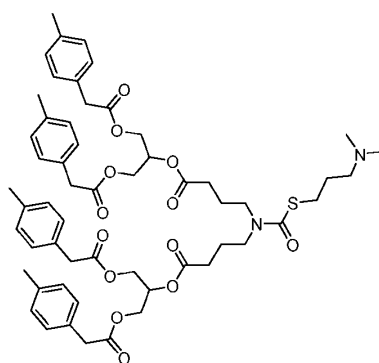
[0612] The following method is contemplated.

[0613] General Scheme:



[0614] Ammonium salt **1-7** (taken from the synthesis of **LIPID 1**) will be dissolved in CH_2Cl_2 , cooled in an ice-water bath under nitrogen, and triphosgene will be added. After the addition of the triphosgene, pyridine will be added and the mixture will be stirred for 4 hours, then the solvent will be removed under *in vacuo* and the residue will be dissolved in pyridine and cooled in an ice-water bath under nitrogen. To this cooled solution 3-dimethylamino-1-amino-propane will be added and the solution will be stirred for 30 minutes after the addition is made and then will be warmed to room temperature and will be allowed to stir for 14 hours. The mixture will then be concentrated *in vacuo* and the residue will be dissolved in CH_2Cl_2 , adsorbed on silica gel, and purified by chromatography using a Combi-flash apparatus. Fractions containing the desired urea product will be concentrated *in vacuo* dissolved in n-heptane, treated with activated charcoal, filtered, washed with a mixture of methanol and water, dried, and concentrated *in vacuo* to afford the target **LIPID 33**, ((4,4'-(((3-(dimethylamino)propyl)carbamoyl)azanediyl)bis(butanoyl))bis(oxy)) bis(propane-2,1,3-triyl) tetranonanoate.

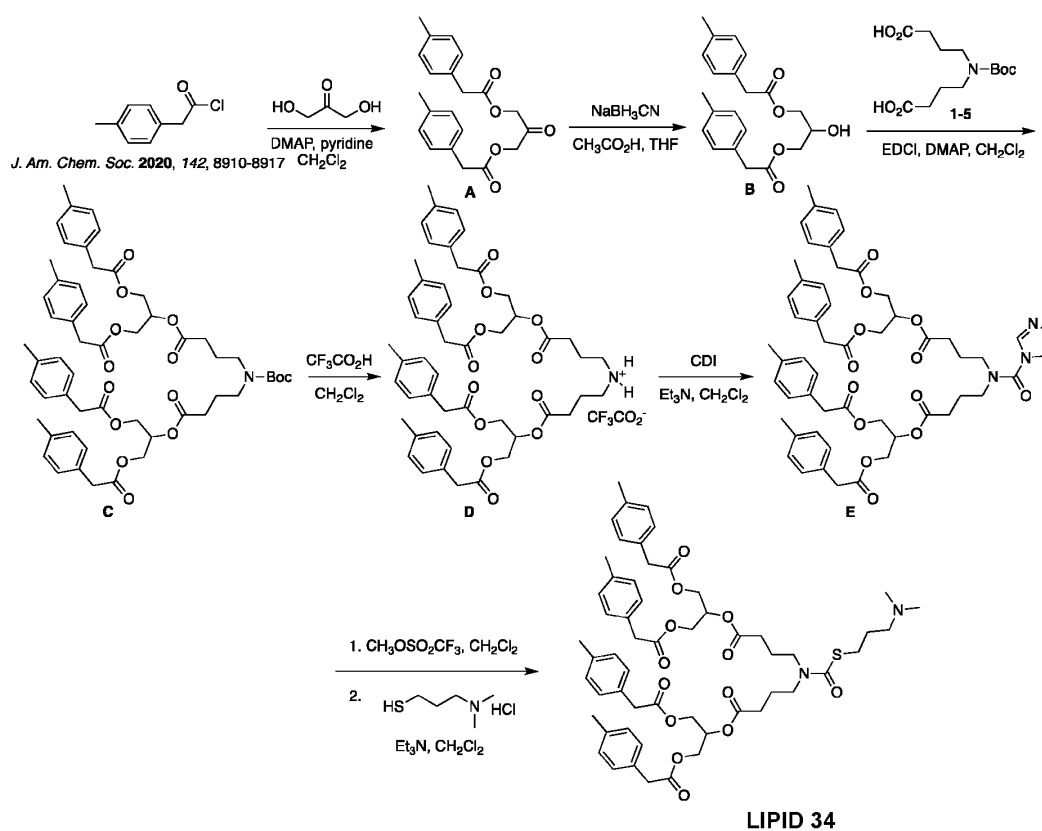
Example 32. Synthesis of LIPID 34: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl)) bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(p-tolyl)acetate)



LIPID 34

[0615] The following method is contemplated.

[0616] General Scheme:



[0617] 2-(*p*-Tolyl)acetyl chloride will be prepared from commercially available 2-(*p*-tolyl)acetic acid by the method of *J. Am. Chem. Soc.* **2020**, *142*, 8910-8917, and the acid chloride will be dissolved in CH₂Cl₂, 1,3-dihydroxyacetone will be added, followed by DMAP and pyridine. The mixture will be stirred at room temperature under nitrogen, then will be concentrated *in vacuo* to give crude 2-oxopropane-1,3-diyl bis(2-(*p*-tolyl)acetate) **A**.

[0618] Crude **A** will be dissolved in THF and will be cooled in an ice-water bath under nitrogen, then HOAc will be added followed by NaBH₃CN. The mixture will be warmed to room temperature and will stir for 14 hours. The mixture will be cast into water, extracted with EtOAc, and the combined organic phases will be dried, filtered, and concentrated *in vacuo* to give crude 2-hydroxypropane-1,3-diyl bis(2-(*p*-tolyl)acetate) **B**. Crude **B** will be adsorbed on silica gel and will be purified by Combi-flash chromatography to provide 2-hydroxypropane-1,3-diyl bis(2-(*p*-tolyl)acetate) **B**.

[0619] 2-Hydroxypropane-1,3-diyl bis(2-(*p*-tolyl)acetate) **B** will be dissolved in CH₂Cl₂, cooled in an ice-water bath under nitrogen and **1-5** (from the synthesis of LIPID 1) will be added. To the cooled mixture will be added DMAP and EDCI. The mixture will be warmed to room temperature, will be allowed to stir for 14 hours, then will be washed with brine and will be dried. The solution will be filtered, concentrated *in vacuo*, the resulting crude ((4,4'-((*tert*-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate) **C** will then be adsorbed onto silica gel and will be purified using a Combi-flash to give ((4,4'-((*tert*-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate) **C**.

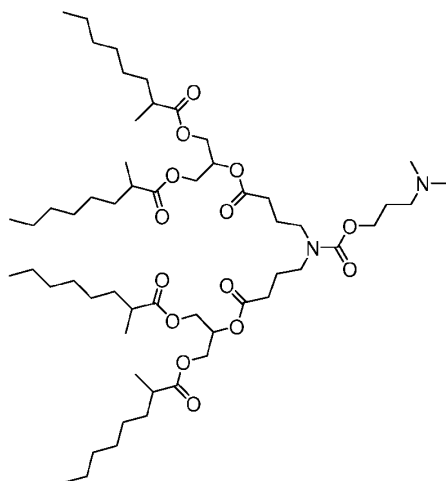
[0620] ((4,4'-((*tert*-Butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate) **C** will be dissolved in in CH₂Cl₂, cooled in an ice-water bath under nitrogen, and trifluoroacetic acid will be added. The mixture will be warmed to room temperature and will stir for 4 hours. The solvent will be removed *in vacuo* and this will gave crude bis(4-((1,3-bis(2-(*p*-tolyl)acetoxyl)propan-2-yl)oxy)-4-oxobutyl)ammonium trifluoroacetate **D**.

[0621] Crude bis(4-((1,3-bis(2-(*p*-tolyl)acetoxyl)propan-2-yl)oxy)-4-oxobutyl)ammonium trifluoroacetate **D** will be dissolved in CH₂Cl₂, under nitrogen, and Et₃N and carbonyldiimidazole will be added, then the mixture will be allowed to stir for 12 hours. The mixture will then be diluted with n-heptane, washed with water, and dried. Filtration and concentration *in vacuo* will give crude ((4,4'-((1*H*-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate) **E**.

[0622] Crude ((4,4'-((1*H*-imidazole-1-carbonyl)azanediyl) bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate) **E** will be dissolved in CH₂Cl₂, under nitrogen and cooled in an ice-water bath, then methyl triflate will be added, this will be followed by the addition of Et₃N and 3-dimethylamino-propane-1-thiol HCl salt. The mixture will be warmed to room temperature and will be allowed to stir, then will be concentrated *in vacuo* to give the crude lipid ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl) azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate). The crude lipid will be adsorbed on silica gel and purified using a Combi-flash. The lipid will then be dissolved in n-heptane and washed with a methanol / water mixture which will give purified **LIPID 34** ((4,4'-(((3-(dimethylamino)propyl)

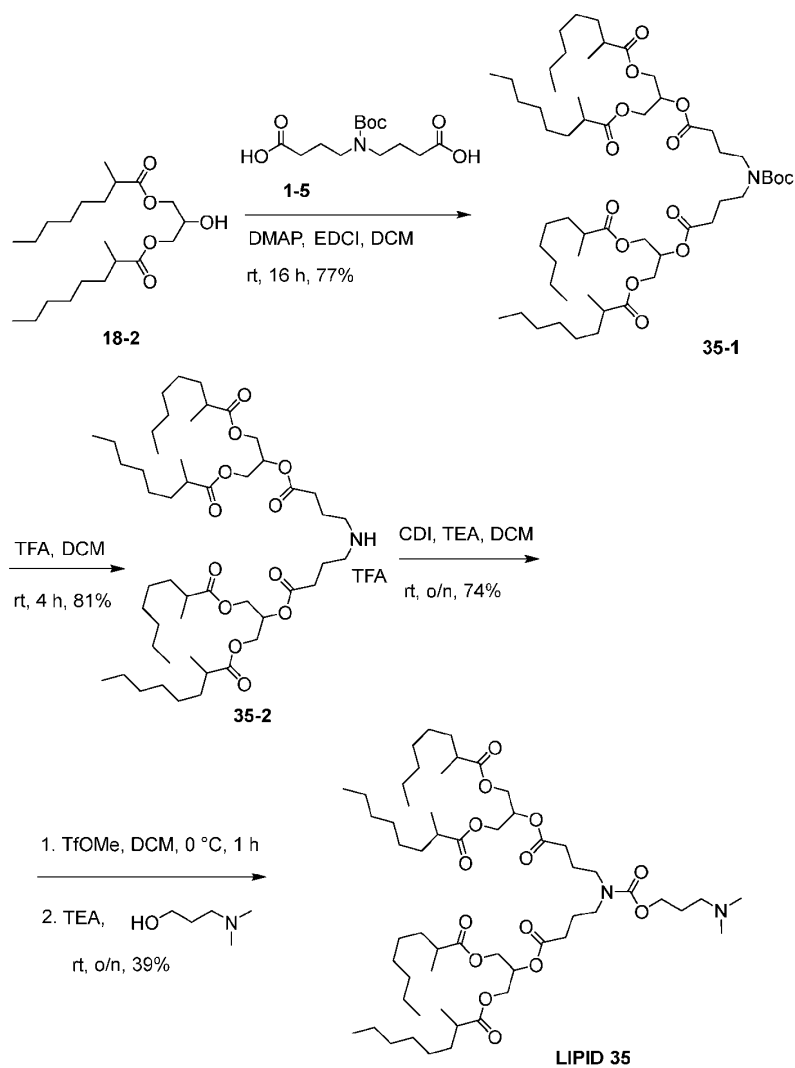
thio)carbonyl) azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-(*p*-tolyl)acetate).

Example 33. Synthesis of LIPID 35: [2-[4-[3-(dimethylamino)propoxycarbonyl]-4-[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanoyloxy)propyl] 2-methyloctanoate



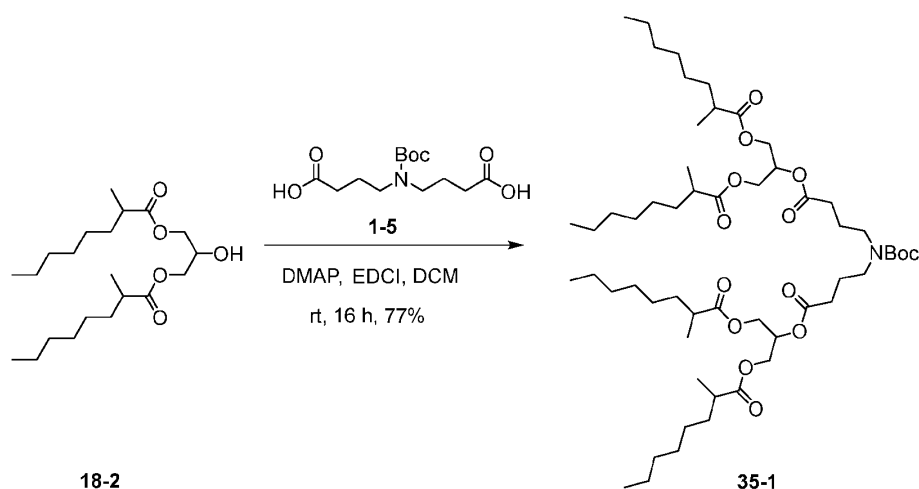
[0623] LIPID 35

[0624] General scheme



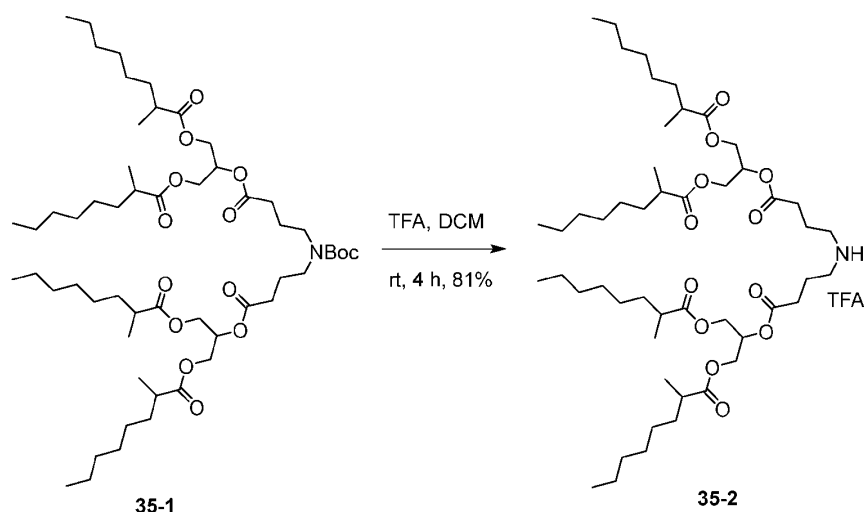
Lipid 35

[0625] Synthesis of 35-1: [2-[4-[tert-butoxycarbonyl-[4-[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanoyloxy)propyl] 2-methyloctanoate



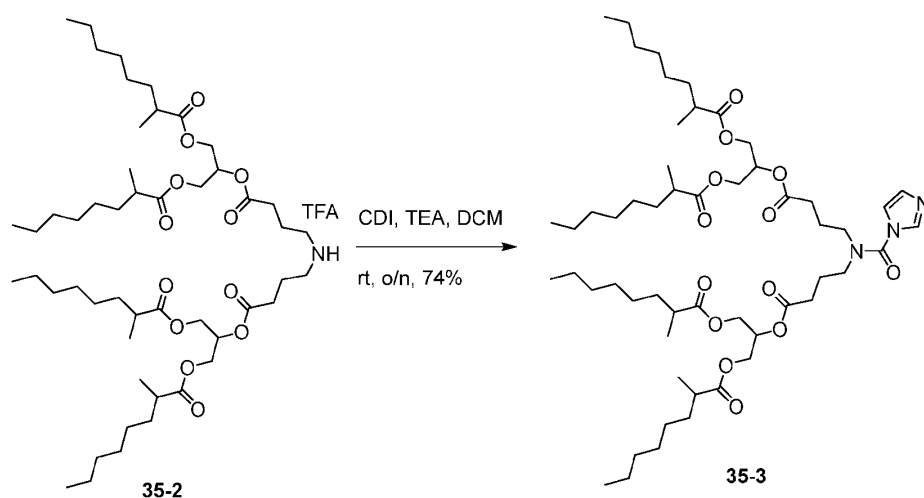
[0626] To a 2 L 3-necked round-bottom flask were added **18-2** (26 g, 69.84 mmol, 1.00 equiv.), **1-5** (9.09 g, 31.43 mmol, 0.45 equiv.), DCM (520 mL, 20 V) and DMAP (4.30 g, 34.92 mmol, 0.5 equiv.). The mixture was cooled to 0 °C and EDCI (12.99 g, 83.81 mmol, 1.2 equiv.) was added in several portions. The mixture was stirred for 15 min at 0 °C, and for additional 16 h at 25 °C. The resulting mixture was washed with water (2 × 260 mL) and brine (1 × 260 mL). The organic phase was collected and dried over anhydrous Na₂SO₄ and then filtered. Crude product was adsorbed on 50 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (400 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE: EA = 100:1), combined, concentrated, and dried under vacuum to get **35-1** (24 g, 77%) as a light-yellow oil. This material was used as such in the next reaction after confirming the identity by ¹H NMR. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.6 min, m/z (Calcd.) 997.7, (found) 1020.8 (M+Na).

[0627] **Synthesis of 35-2: [3-(2-methyloctanoyloxy)-2-[4-[[4-[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]propyl] 2-methyloctanoate trifluoroacetic acid salt**



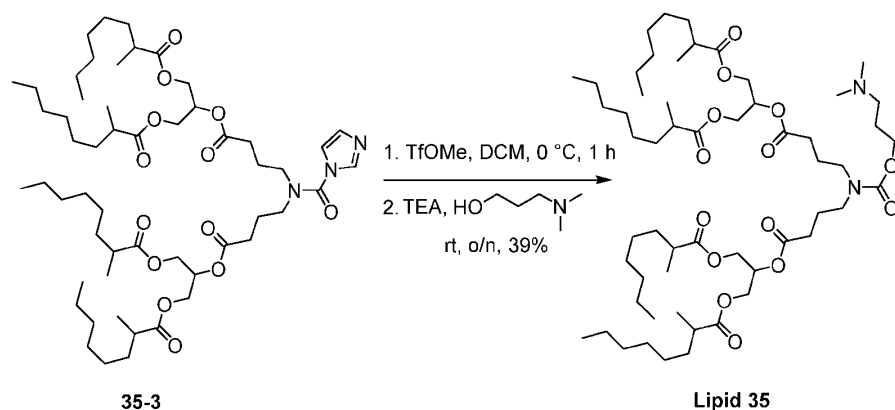
[0628] To a 250 mL round-bottle flask, a solution of **35-1** (24 g, 24.06 mmol, 1 equiv.) in DCM (96 mL, 4 V) was charged. The solution was cooled to 0 °C in an ice-water bath, and TFA (24 mL, 1 V) was added. The ice/water bath was removed, and the mixture was stirred for 4 h at room temperature. The reaction was concentrated under vacuum and diluted with n-heptane (168 mL) at 0±5 °C. While maintaining the temperature at 5±5 °C, the resulting solution was washed with a solution of K₂HPO₄ (24.14 g, 6.0 equiv.) in deionized water (192 mL). The organic phase was concentrated and dried under vacuum to afford **35-2** (19.5 g, 81%) as a light-yellow oil. This crude product was used as such in the next reaction.

[0629] Synthesis of 35-3: [2-[4-[imidazole-1-carbonyl-[4-[2-(2-methyloctanoyloxy)-1-(2-methyl octanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanoyl oxy)propyl] 2-methyloctanoate



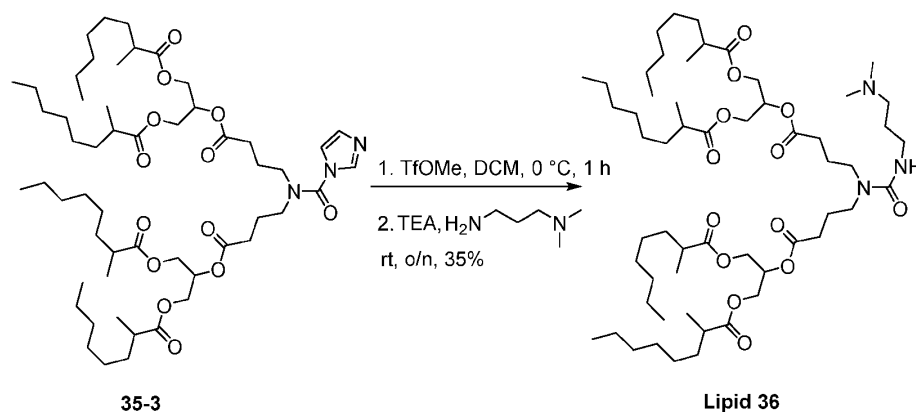
[0630] To a 1 L 3-necked round-bottom flask, purged and inserted with nitrogen, a solution of **35-2** (12 g, 12.05 mmol, 1 equiv.) in DCM (240 mL) was charged. To this solution, TEA (2.44 g, 24.1 mmol, 2.0 equiv.) and CDI (8.79 g, 54.24 mmol, 4.5 equiv.) were added at room temperature and the reaction mixture was stirred overnight. The resulting mixture was washed with 0.8 M HCl (1 × 120 mL). The organic layer was washed with 10% brine (120 mL), dried over anhydrous Na₂SO₄, and filtered. The filtrate was concentrated under reduced pressure. The residue was dissolved in n-heptane (240 mL), the resulting solution was washed with MeOH/H₂O (5:1) (2 × 240 mL). The heptane layer was dried over anhydrous Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to obtain **35-3** (8.9 g, 74%) as a light-brown oil, that was used as such in the next reaction.

[0631] Synthesis of LIPID 35: [2-[4-[3-(dimethylamino)propoxycarbonyl]-4-[2-(2-methyloctanoyl oxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanoyloxy) propyl] 2-methyloctanoate



[0632] To a 250 mL 3-necked round-bottom flask, purged and inerted with nitrogen, a solution of **35-3** (3.0 g, 3.02 mmol, 1 equiv.) in DCM (60 mL, 20V) was charged. The solution was cooled to 0 °C in an ice-water bath, and TfOMe (0.55 g, 3.33 mmol, 1.10 equiv.) was added. After stirring at 0 °C for 1 h, TEA (0.61 g, 6.05 mmol, 2.00 equiv.) and 3-(dimethylamino)propan-1-ol (0.37 g, 3.63 mmol, 1.20 equiv.) were added to the solution at cold. The reaction mixture was warmed to room temperature and stirred overnight. The mixture was diluted with DCM (100 mL) and washed subsequently with H₂O (2 × 100 mL) and brine (1 × 100 mL). The organic phase was dried over anhydrous Na₂SO₄ and filtered. Crude product was adsorbed on 20 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a 120 g of silica gel column using a combi-flash purification system. The column was eluted with heptane/EA (gradient from 100:0 to 60:40) and the eluent was collected in fractions. After TLC analysis (heptane: EA = 3:1), high-purity product fractions were combined and concentrated *in vacuo* to afford **LIPID 35** (1.6 g) as a yellow oil in 89.0% purity by HPLC-CAD. The **LIPID 35** (1.6 g) thus obtained, was purified by prep-achiral-SFC (Column: GreenSep Basic, 3*25 cm, 5 μm; A: CO₂, B: IPA: ACN=1: 1; 80 mL/min; isocratic 45% B; 35 °C; 220 nm). The enantio-rich product fractions were combined and concentrated to obtain **LIPID 35** (1.22 g, 39%) as a light-yellow oil. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 25 min.): RT 12.6 min, m/z (Calcd.) 1026.7, (found) 1027.7 [M+H]. ¹H NMR (300 MHz, Chloroform-d) δ 5.264-5.231 (m, 2H), 4.363-4.283 (m, 4H), 4.186-4.099 (m, 6H), 3.256 (d, *J*=8.1 Hz, 4H), 2.570-2.297 (m, 16H), 1.939-1.801 (m, 6H), 1.678-1.606 (m, 4H), 1.457-1.408 (m, 4H), 1.330-1.250 (m, 32H), 1.149 (d, *J*=6.9 Hz, 12H), 0.899-0.855 (m, 12H).

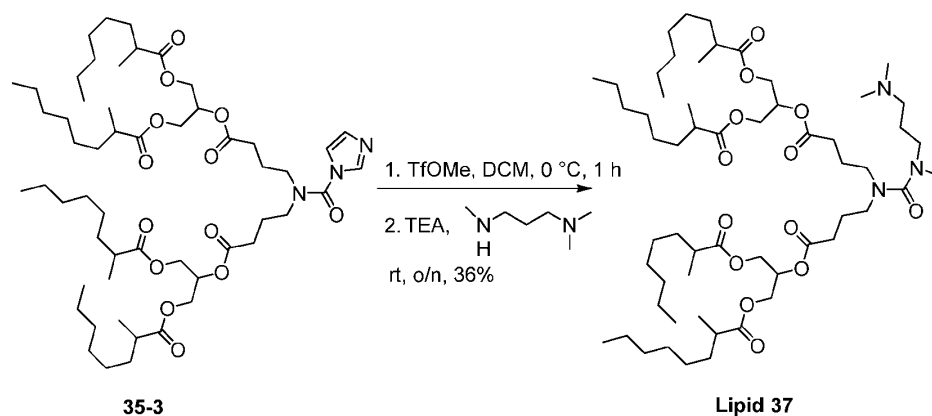
[0633] **Example 34. Synthesis of LIPID 36:** [2-[4-[3-(dimethylamino)propylcarbamoyl-[4-[2-(2-methyloctanoyl oxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyl octanoyloxy)propyl] 2-methyloctanoate



[0634] To a 250 mL 3-necked round-bottom flask, purged and inserted with nitrogen, **35-3** (3 g, 3.02 mmol, 1 equiv.) and DCM (60 mL, 20V) were added. To the mixture, methyl trifluoromethanesulfonate (0.55 g, 3.33 mmol, 1.10 equiv) was added dropwise at 0 °C. After stirring at 0 °C for 1 h, TEA (0.61 g, 6.05 mmol, 2.00 equiv.) and 3-(dimethylamino)propylamine (0.37 g, 3.63 mmol, 1.20 equiv.) were added. The reaction mixture was warmed to room temperature and stirred overnight. The mixture was diluted with DCM (200 mL) and washed with brine (1 × 100 mL). The organic layer was concentrated under vacuum, the residue was purified by reverse phase flash chromatography on a C18 silica gel column. The column was eluted with water/MeCN (gradient from 50:50 to 20:80 in 20 min) with monitoring by UV at 205 nm, the eluent was collected in fractions. High-purity product fractions were combined and concentrated *in vacuo* to afford **LIPID 36** (2.5 g) as a light-yellow oil in 93.03% purity by HPLC-CAD. The **LIPID 36** (2.5 g) thus obtained, was purified by prep-achiral-SFC (Column: GreenSep Basic, 3*25 cm, 5 µm; A: CO₂, B: IPA: ACN=1: 1; 80 mL/min; isocratic 45% B; 35 °C; 220 nm). The enantio-rich product fractions were combined and concentrated to obtain **LIPID 36** (1.09 g, 35%) as a light-yellow oil. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 1025.8, (found) 1026.7 [M+H]. ¹H NMR (300 MHz, CDCl₃, ppm): δ 6.227 (t, *J* = 6.0 Hz, 1H), 5.258-5.225 (m, 2H), 4.363-4.297 (m, 4H), 4.189-4.111 (m, 4H), 3.365-3.346 (m, 2H), 3.247 (t, *J* = 7.5 Hz, 4H), 3.001 (s, 2H), 2.710 (s,

6H), 2.483-2.328 (m, 8H), 2.008-1.978 (m, 2H), 1.868-1.818 (m, 4H), 1.677-1.606 (m, 4H), 1.431-1.345 (m, 4H), 1.266 (s, 32H), 1.149-1.126 (m, 12H), 0.877 (t, $J = 6.3$ Hz, 12H).

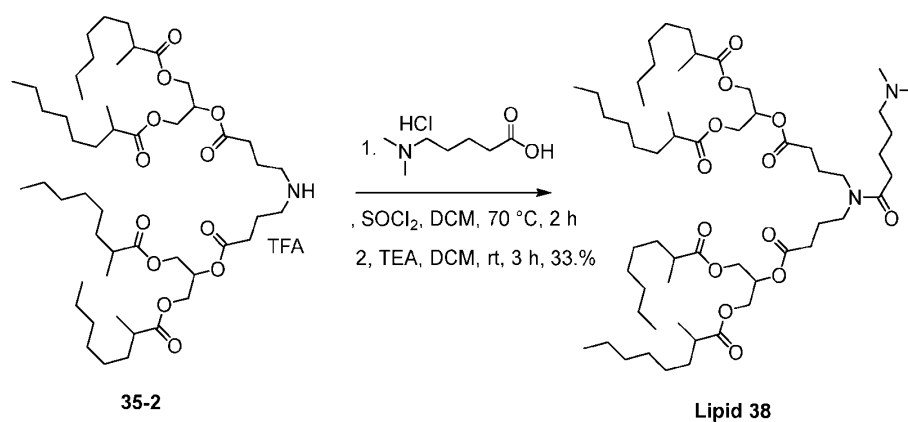
Example 35. Synthesis of LIPID 37: [2-[4-[3-(dimethylamino)propyl-methyl-carbamoyl]-4-[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanoyloxy)propyl] 2-methyloctanoate



[0635] To a 250 mL 3-necked round-bottom flask, purged and inserted with nitrogen, **35-3** (3 g, 3.02 mmol, 1 equiv.) and DCM (60 mL, 20V) were added. To the mixture, methyl trifluoromethanesulfonate (0.55 g, 3.33 mmol, 1.10 equiv.) was added dropwise at 0 °C. After stirring at 0 °C for 1 h, TEA (0.61 g, 6.05 mmol, 2.00 equiv.) and [3-(dimethylamino)propyl](methyl)amine (0.42 g, 3.63 mmol, 1.20 equiv.) were added. The reaction mixture was stirred for 1 h at 0 °C and then warmed to room temperature and stirred overnight. The mixture was diluted with DCM (200 mL) and washed with brine (1 × 100 mL). The organic layer was concentrated under vacuum, the residue was purified by reverse phase flash chromatography on a C18 silica gel column. The column was eluted with water/MeCN (gradient from 50:50 to 20:80 in 20 min) with monitoring by UV at 205 nm, the eluent was collected in fractions. High-purity product fractions were combined and concentrated *in vacuo* to afford **LIPID 37** (2.7 g) as a light-yellow oil in 91.42% purity by HPLC-CAD. The **LIPID 37** (2.7 g) thus obtained, was purified by prep-achiral-SFC (Column: GreenSep Basic, 3*25 cm, 5 μm; A: CO₂, B: IPA: ACN=1: 1; 80 mL/min; isocratic 45% B; 35 °C; 220 nm). The enantio-rich product fractions were combined and concentrated to obtain **LIPID 37** (1.13 g, 36%) as a light-yellow oil. ELSD A: water/0.02% TFA; B: CH₃CN 95:5 to 5:95 A/B at 25 min.): RT 13.2 min,

m/z (Calcd.) 1039.7, (found) 1041.2 $[M+H]^+$. 1H NMR (300 MHz, $CDCl_3$, ppm): δ 5.249-5.216 (m, 2H), 4.368-4.301 (m, 4H), 4.181-4.093 (m, 4H), 3.267-3.221 (m, 2H), 3.148 (t, $J = 7.5$ Hz, 4H), 2.858 (s, 3H), 2.821-2.702 (m, 2H), 2.595 (s, 6H), 2.480-2.281 (m, 8H), 1.970 (s, 2H), 1.877-1.805 (m, 4H), 1.676-1.604 (m, 4H), 1.430-1.362 (m, 4H), 1.266 (s, 32H), 1.148-1.125 (m, 12H), 0.878 (t, $J = 6.3$ Hz, 12H).

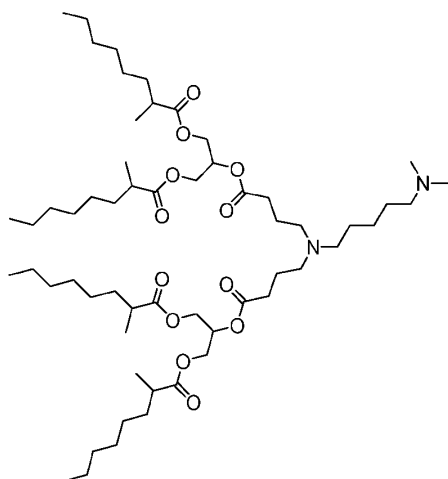
Example 36: Synthesis of LIPID 38: [2-[4-[5-(dimethylamino)pentanoyl]-4-[2-(2-methyl octanovloxy)-1-(2-methyloctanovloxymethyl)ethoxy]-4-oxo-butyl]amino]butanovloxy]-3-(2-methyloctanovloxy)propyl] 2-methyloctanoate



[0636] To a 100 mL 3-necked round-bottom flask, purged and inserted with nitrogen, a solution of the hydrochloride salt of 5-(dimethylamino)pentanoic acid (1.86 g, 1.57 mmol, 3 equiv.) in DCM (15 mL) was charged. After adding $SOCl_2$ (0.93 g, 7.83 mmol, 3 equiv.), the solution was heated to 70 °C and stirred for 2 h. The mixture was concentrated under reduced pressure, the residue was dissolved in DCM (52 mL). The solution was cooled to 0 °C, TEA (0.79 g, 7.83 mmol, 3.00 equiv.) and **35-2** (2.60 g, 2.61 mmol, 1.00 equiv.) were added at cold. The mixture was warmed to room temperature and stirred for 3 hours. The mixture was diluted with DCM (90 mL) and washed with water (2×50 mL) and brine (1×50 mL). The organic phase was dried over anhydrous Na_2SO_4 and filtered. Crude product was adsorbed on 20 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.), and was purified on a 120 g of silica gel column using a combi-flash purification system. The column was eluted with heptane/EA (gradient from 100:0 to 60:40) and the eluent was collected in fractions. After TLC analysis (heptane: EA = 5:1), high-purity product fractions were combined and concentrated *in vacuo* to

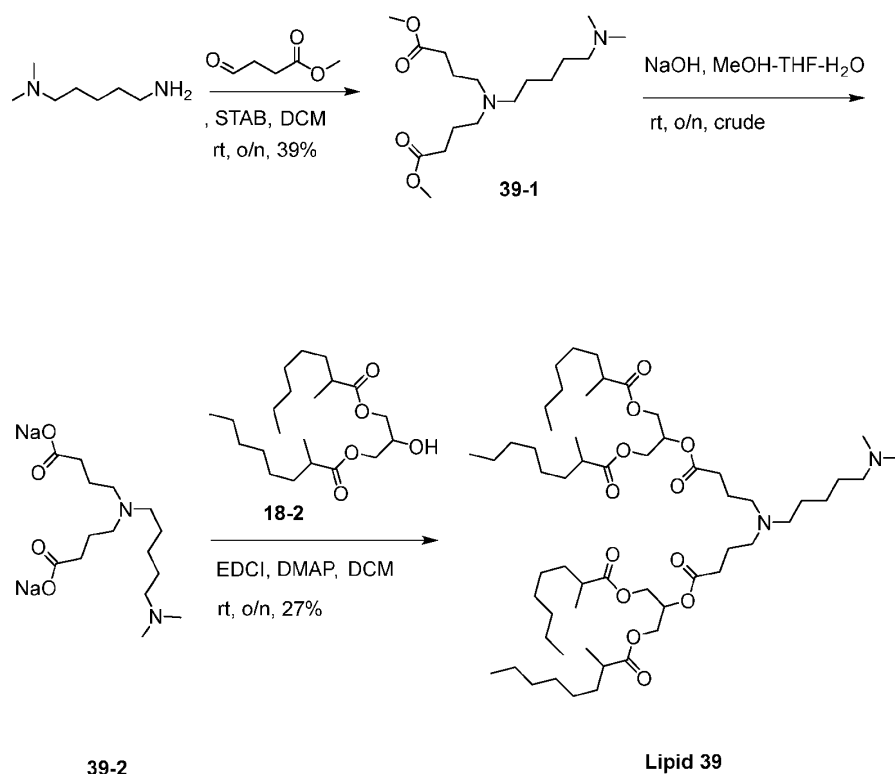
afford **LIPID 38** (1.6 g) as a yellow oil in 84.4% purity by HPLC-CAD. The **LIPID 38** (1.6 g) thus obtained, was purified by prep-achiral-SFC (Column: GreenSep Basic, 3*25 cm, 5 μ m; A: CO₂, B: IPA: ACN=1: 1; 80 mL/min; isocratic 45% B; 35 °C; 220 nm). The enantio-rich product fractions were combined and concentrated to obtain **LIPID 38** (0.89 g, 33%) as a yellow oil. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 25 min.): RT 14.0 min, m/z (Calcd.) 1024.7, (found) 1025.8 [M+H]. ¹H NMR (300 MHz, Chloroform-d) δ 5.246 (s, 2H), 4.384-4.279 (m, 4H), 4.180-4.094 (m, 4H), 3.379-3.273 (m, 4H), 2.481-2.299 (m, 18H), 1.860-1.812 (m, 4H), 1.693 (t, *J* = 12 Hz, 8H), 1.427-1.390 (m, 4H), 1.266 (s, 32H), 1.151-1.124 (m, 12H), 0.898-0.854 (m, 12H).

Example 37: Synthesis of LIPID 39: [2-[4-[5-(dimethylamino)pentyl]-4-[2-(2-methyloctanovloxy)-1-(2-methyloctanovloxymethyl)ethoxy]-4-oxo-butyl]amino]butanovloxy]-3-(2-methyloctanovloxy)propyl] 2-methyloctanoate

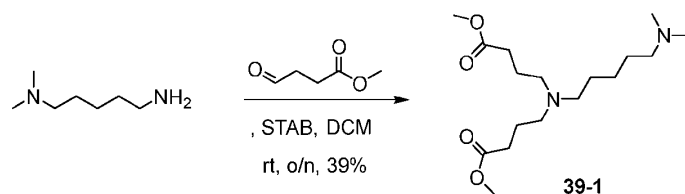


[0637] LIPID 39

[0638] General scheme



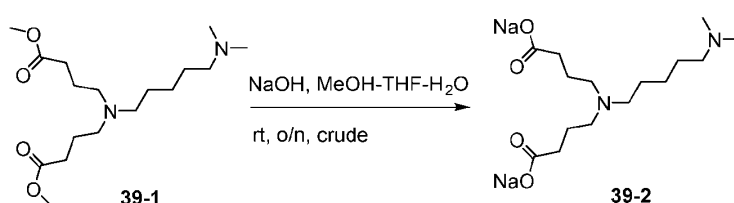
[0639] Synthesis of 39-1: methyl 4-[5-(dimethylamino)pentyl-(4-methoxy-4-oxo-butyl)amino]butanoate



[0640] A mixture of 5-(dimethylamino)amylamine (3.10 g, 23.80 mmol, 1 equiv.) and methyl 4-oxobutanoate (8.29 g, 71.41 mmol, 3.0 equiv.) in DCM (60 mL, 20V) was stirred for 30 min at room temperature. To the above mixture, sodium triacetoxycarboxylate (STAB, 25.22 g, 119.02 mmol, 5.0 equiv.) was added in portions at room temperature. The mixture was stirred overnight at room temperature. The reaction was quenched by the addition of sat. aqueous Na_2CO_3 solution (60 mL) at room temperature. The resulting mixture was extracted with DCM (2×60 mL), the combined organics were washed with brine (120 mL), dried over anhydrous Na_2SO_4 , and filtered. To the filtrate, 6 g silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) was added, after

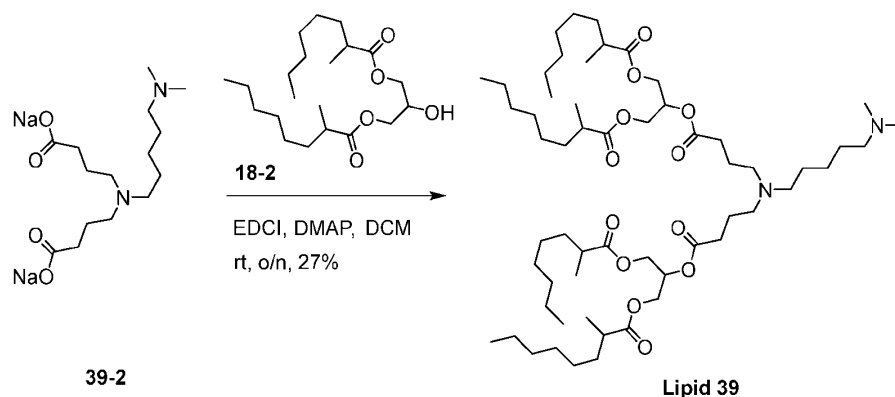
concentration to dryness, the residue was purified on a 30 g of silica gel column, using a combi-flash purification system. The column was eluted with DCM/MeOH (95:5) and the eluent was collected in fractions. After TLC analysis (DCM:MeOH = 8:1), pure product fractions were combined and concentrated *in vacuo* to afford **39-1** (3.1 g, 39%) as light-yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA, 95:5 to 5:95 A/B, RT 0.49 min), m/z (Calcd.) 330.3, (found) 331.5 [M+H].

[0641] Synthesis of 39-2: 4-[3-carboxypropyl-[5-(dimethylamino)pentyl]amino]butanoic acid disodium salt



[0642] To a solution of **39-1** (3.0 g, 9.08 mmol, 1 equiv.) in MeOH (6.0 mL, 2V) and THF (6.0 mL, 2V), an aqueous solution of NaOH (1.09 g, 27.23 mmol, 3.0 equiv.) in H₂O (3.0 mL, 1V) was added with stirring at room temperature. The resulting mixture was stirred overnight at room temperature and concentrated under reduced pressure. Crude **39-2** (4.1 g), obtained as a light-yellow solid was used in the next step, without further purification. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA, 95:5 to 5:95 A/B, RT 0.25 min), m/z (Calcd.) 302.2, (found) 303.4 [M+H].

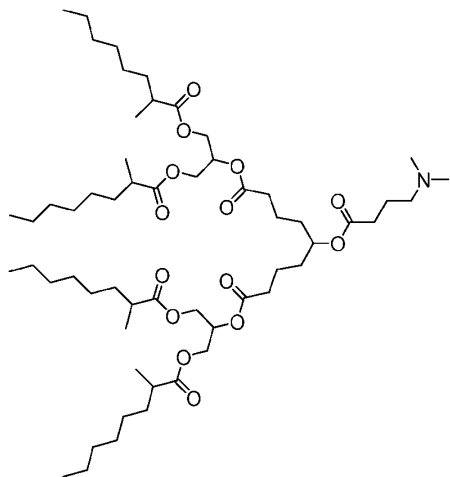
[0643] Synthesis of LIPID 39. [2-[4-[5-(dimethylamino)pentyl]-[4-[2-(2-methyloctanovloxy)-1-(2-methyloctanoyloxymethyl)ethoxy]-4-oxo-butyl]amino]butanoyloxy]-3-(2-methyloctanovl oxy)propyl] 2-methyloctanoate



[0644] To a solution of **39-2** (1.28 g, 3.70 mmol, 1 equiv.) and **18-2** (2.75 g, 7.39 mmol, 2.0 equiv.) in DCM (25 mL, 20V), DMAP (0.45 g, 3.70 mmol, 1.0 equiv.) and EDCI (4.25 g, 22.17 mmol, 6.0 equiv.) were added with stirring at room temperature, the reaction mixture was stirred overnight at room temperature. The resulting mixture was quenched with 10% acetic acid (50 mL) and extracted with DCM (2 × 50 mL). The combined organic layers were washed with aqueous 10% Na₂CO₃ (2 × 50 mL), dried over anhydrous Na₂SO₄ and filtered. Crude product was adsorbed on 6.4 g of silica gel (type: ZCX-2, 100-200 mesh, 5.00 w./w.) and was purified on 32 g of silica gel using combi-flash purification system. The column was eluted with DCM/MeOH (gradient from 100:0 to 90:10, and the eluent was collected in fractions. After TLC analysis, product fractions were combined and concentrated *in vacuo* to afford **LIPID 39** (1.70 g) in 90.1% purity by HPLC-CAD as a yellow oil. The **LIPID 39** (1.70 g) obtained was repurified by reverse-phase flash chromatography (Column: Ultimate XB-phenyl 50 × 250 mm, 10μm, A: water (0.1% TFA), B: 90% MeCN in water, 90 mL/min, 50% to 90% B in 15 min, ELSD detection) and the product fractions were combined and concentrated under reduced pressure. The residue was dissolved in n-heptane (50 mL), the solution was washed with saturated aqueous Na₂CO₃ (50 mL) and then with MeOH/H₂O (4:1) (2 × 50 mL). The heptane phase was dried over anhydrous Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to afford **LIPID 39** (1.01 g, 27%) as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA, 95:5 to 5:95 A/B, 25 min, RT 13.2 min. m/z (Calcd.) 1010.8, (found) 1011.8 [M+H]. ¹H NMR (400 MHz, Chloroform-*d*) δ 5.289-5.253 (m, 2H), 4.340-4.280 (m, 4H), 4.169-4.102 (m, 4H), 2.470-2.409 (m, 10H), 2.363-2.307 (m, 4H), 2.283-2.233 (m, 8H), 1.738-

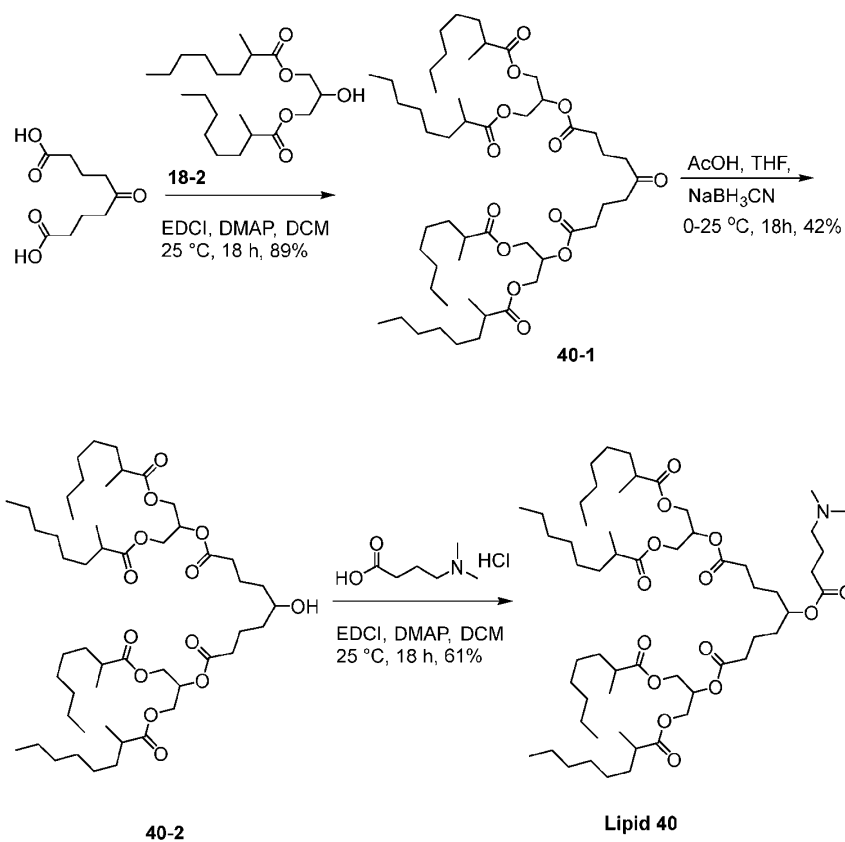
1.618 (m, 8H), 1.516-1.373 (m, 8H), 1.267 (s, 34H), 1.139 (d, $J=6.8$ Hz, 12H), 0.894-0.860 (m, 12H).

Example 38: Synthesis of LIPID 40: bis[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethyl] 5-[4-(dimethylamino)butanoyloxy]nonanedioate

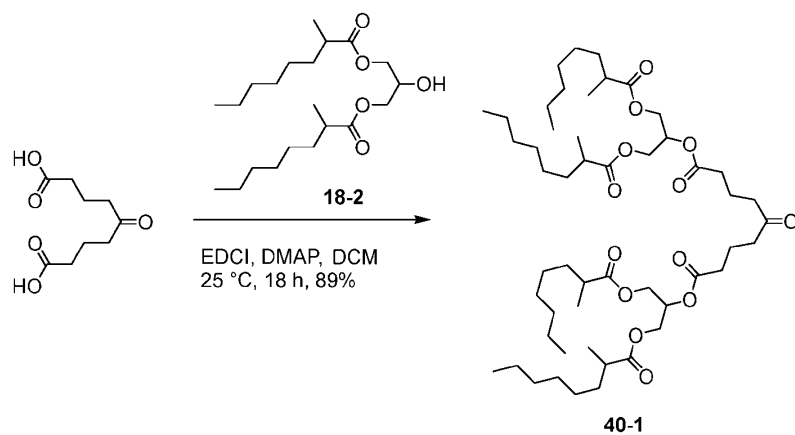


[0645] LIPID 40

[0646] General Scheme:

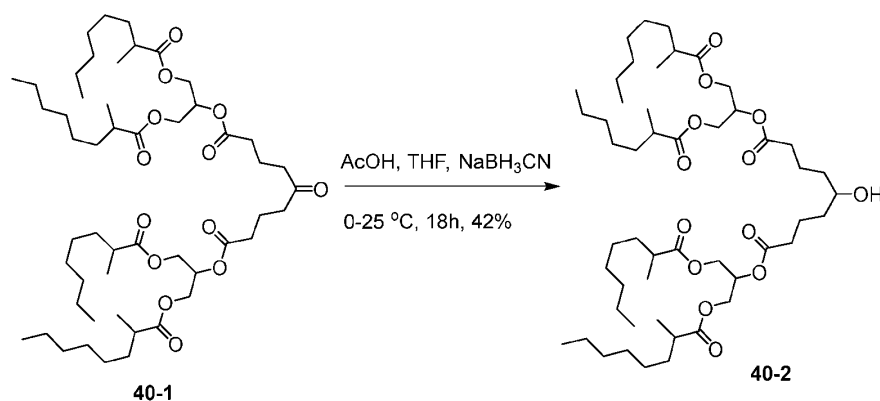


[0647] Synthesis of 40-1: bis[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethyl] 5-oxo nonanedioate



[0648] To a 250 mL round-bottom flask, 5-oxoazelaic acid (1.00 g, 4.95 mmol, 1.0 equiv.), **18-2** (3.87 g, 10.39 mmol, 2.1 equiv.) and DCM (80 mL) were added at 25 °C. DMAP (0.60 g, 4.95 mmol, 1.0 equiv.) and EDCI (2.09 g, 10.88 mmol, 2.2 equiv.) were added and the resulting mixture was stirred for 18 h at 25 °C. The reaction mixture was diluted with DCM (100 mL), washed with brine (2 × 40 mL), dried over anhydrous Na₂SO₄ and filtered. To the filtrate, 6 g of silica gel (type: ZCX-2, 100-200 mesh, 5.00 w./w.) was added, after concentration to dryness, the residue was purified on an 80 g silica gel column, using combi-flash to purification system. The column was eluted with heptane/EA. (gradient from 5:1 to 1:1) and the eluent was collected in fractions. After TLC analysis (heptane / EA = 2:1), the product fractions were combined and concentrated *in vacuo* to afford **40-1** (4.0 g, 89%) as a colorless oil. ELSD (A: water/0.05% TFA; B: CH₃CN/0.05% TFA, 95:5 to 5:95 A/B, RT 1.9 min), m/z (Calcd.) 910.6, (found) 933.9 [M+Na].

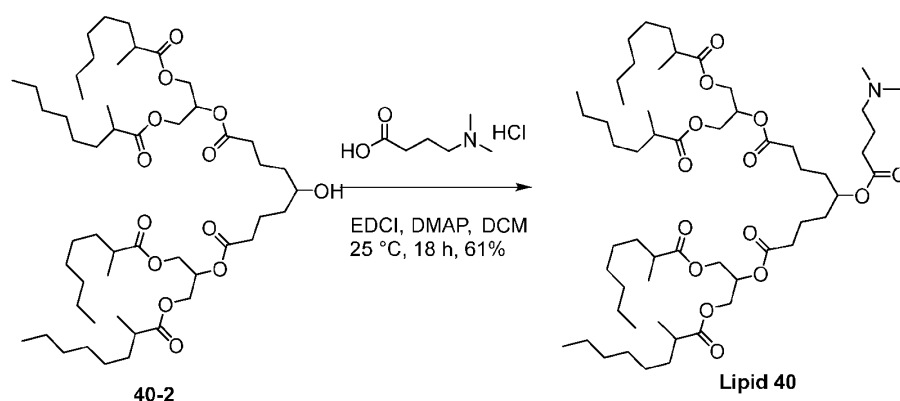
[0649] Synthesis of 40-2: bis[2-(2-methyloctanoyloxy)-1-(2-methyloctanoyloxymethyl)ethyl] 5-hydroxy nonanedioate



[0650] To a 250mL round-bottom flask **40-1** (3.6 g, 3.95 mmol, 1 equiv.) and tetrahydrofuran (80 mL) were added at 25 °C. The mixture was cooled to 0 °C, acetic acid (0.47 g, 7.90 mmol, 2 equiv.) was added, and stirring was continued for 10 min at cold. To the above mixture, NaBH₃CN (1.99 g, 31.61 mmol, 8 equiv.) was added in portions at 0 °C. The resulting mixture was stirred for an additional 1 h at 0 °C and then for 18 h at 25 °C. After quenching the reaction with water at 0 °C, the resulting mixture was extracted with DCM (2 x 50 mL). The combined organic layers were washed with 5% aqueous NaHCO₃ (2 x 50 mL) and H₂O (1 x 50 mL), and then dried over anhydrous Na₂SO₄. The organic phase was filtered, to the filtrate 6 g of silica gel

(type: ZCX-2, 100-200 mesh, 5.00 w./w.) was added, after concentration to dryness, the residue was purified on an 80 g of silica gel column using combi-flash purification system. The column was eluted with heptane/EA. (gradient from 5:1 to 1:1) and the eluent was collected in fractions. After TLC analysis (heptane / EA = 2:1), the pure product fractions were combined and concentrated *in vacuo* to afford **40-2** (1.5 g, 42%) as a colorless oil. The material was used after confirming identity by ^1H NMR.

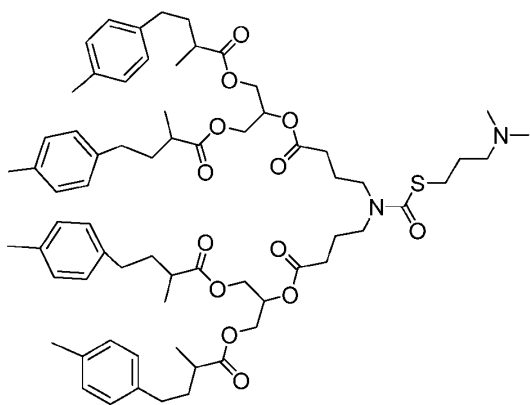
[0651] Synthesis of LIPID 40: bis[2-(2-methyloctanoyloxy) -1-(2-methyloctanoyl oxymethyl)ethyl] 5-[4-(dimethylamino)butanoyloxy]nonanedioate



[0652] To a 100 mL round-bottom flask, **40-2** (1.4 g, 1.53 mmol, 1 equiv.) and 4-(dimethylamino)butanoic acid hydrochloride (0.31 g, 1.84 mmol, 1.2 equiv.) in DCM (50 mL) were added at 25 °C. DMAP (37.46 mg, 0.31 mmol, 0.2 equiv.) and EDCI (0.38 g, 1.993 mmol, 1.3 equiv.) were added and the resulting mixture was stirred for 18 h at 25 °C. The reaction mixture was diluted with DCM (100 mL), washed with brine (2 × 50 mL), dried over anhydrous Na_2SO_4 , and filtered. To the filtrate, 6 g of silica gel (type: ZCX-2, 100-200 mesh, 5.00 w./w.) was added, after concentration to dryness, the residue was purified on an 80 g silica gel column, using combi-flash to purification system. The column was eluted with heptane/EA. (gradient from 15:1 to 3:1) and the eluent was collected in fractions. After TLC analysis (heptane / EA = 3:1), the pure product fractions were combined and concentrated *in vacuo* to afford **LIPID 40** (1.2 g) as a colorless oil in 91.2% purity by HPLC-CAD. The **LIPID 40** (1.2 g) thus obtained, was purified by prep-achiral-SFC (Column: GreenSep Basic, 3*25 cm, 5 μm ; A: CO_2 , B: IPA: ACN=1: 1; 80 mL/min; isocratic 45% B; 35 °C; 220 nm). The enantio-rich product containing fractions were combined and concentrated to obtain **LIPID 40** (0.95 g, 61%) as a colorless oil.

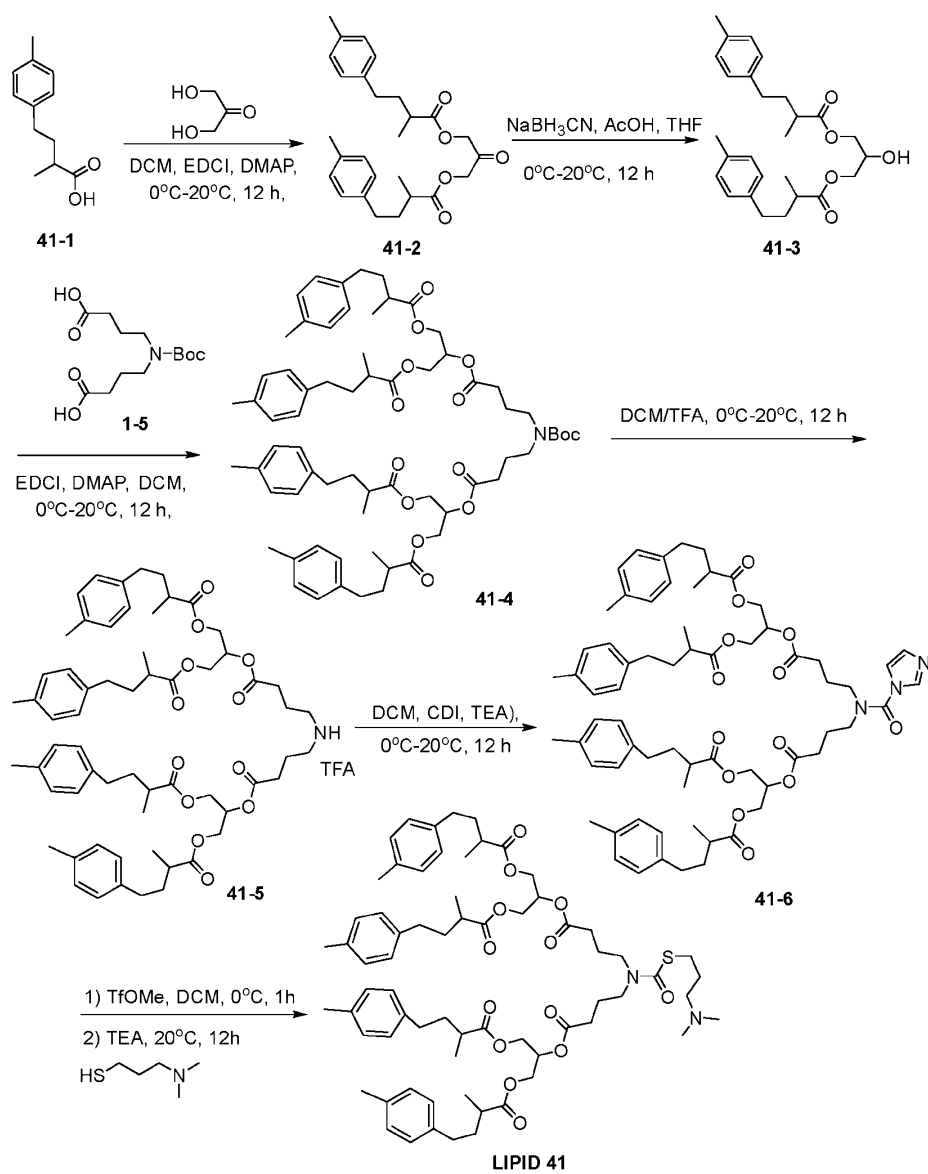
ELSD (A: water/0.05% TFA: B: CH₃CN/0.05% TFA, 95:5 to 5:95 A/B, 25 min. RT 12.8 min), m/z (Calcd.) 1025.7, (found) 1026.9 [M+H]. ¹H NMR (400 MHz, Chloroform-d, ppm) δ 5.303-5.215 (m, 2H), 4.945-4.838 (m, 1H), 4.391-4.263 (m, 4H), 4.201-4.072 (m, 4H), 2.504-2.402 (m, 4H), 2.376-2.268 (m, 8H), 2.225 (s, 6H), 1.855-1.746 (m, 2H), 1.720-1.531 (m, 12H), 1.469-1.347 (m, 4H), 1.340-1.205 (m, 34H), 1.185-1.098 (m, 12H), 0.912-0.843 (m, 12H).

Example 39. LIPID 41: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate)

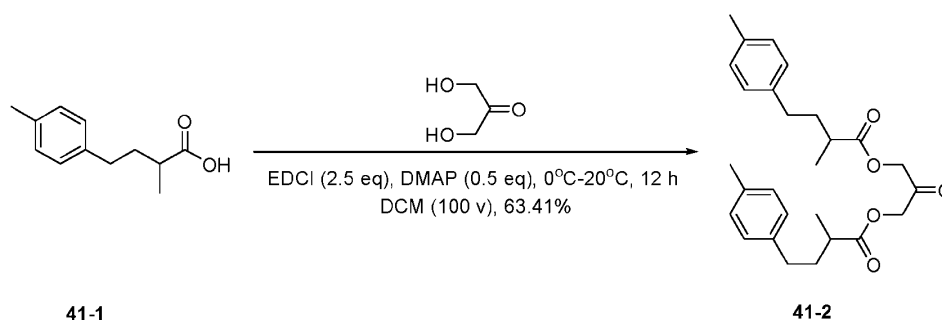


[0653] LIPID 41

[0654] General scheme

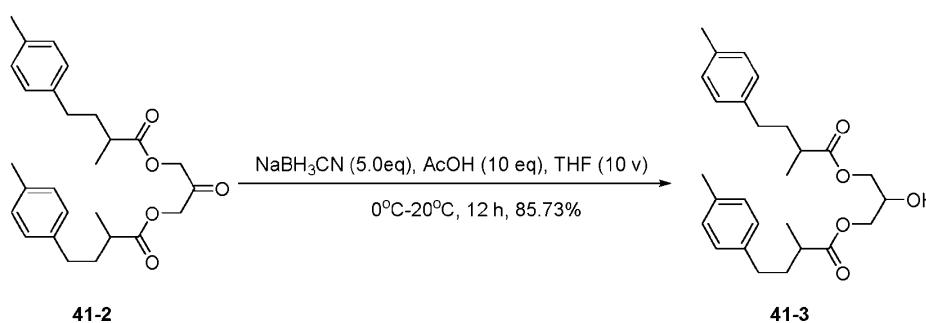


[0655] Synthesis of 41-2: 2-oxopropane-1,3-diyl bis(2-methyl-4-(p-tolyl)butanoate)



[0656] Into a 500 mL round-bottom flask, was placed dihydroxyacetone (2.5 g, 27.75 mmol, 1.00 equiv.), DCM (250 mL, 100 V), 2-methyl-4-(4-methylphenyl)butanoic acid (**41-1**, 12.27 g, 63.83 mmol, 2.3 equiv.), DMAP (1.70 g, 13.88 mmol, 0.5 equiv.). EDCI (13.30 g, 69.38 mmol, 2.5 equiv.) was added at 0 °C. Then the reaction was stirred at 20 °C for 12 h. The reaction mixture was diluted with DCM (500 mL, 200 V). The organic layer was washed with (500 mL, 200 V) of water, brine (250 mL, 100 V), dried with anhydrous Na₂SO₄, filtered, and concentrated under vacuum. Crude product was adsorbed on 25 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (200 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE:EA = 20:1), combined, concentrated, and dried under vacuum to get **41-2** (8.1 g, 18.49 mmol, 63.4%) as a colorless oil which was directly used in the next step. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.4 min, m/z (Calcd.) 438.2, (found) 456.2 (M+H₂O).

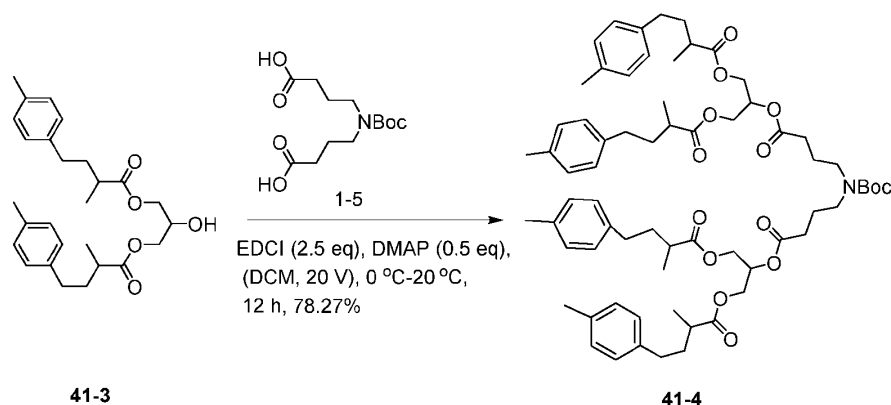
[0657] Synthesis of 41-3: 2-hydroxypropane-1,3-diyl bis(2-methyl-4-(p-tolyl)butanoate)



[0658] Into a 250 mL round-bottom flask, was placed **41-2** (8 g, 18.24 mmol, 1 equiv.), THF (80 mL), AcOH (10.95 g, 182.34 mmol, 10 equiv.), and NaBH₃CN (5.73 g, 91.18 mmol, 5.0

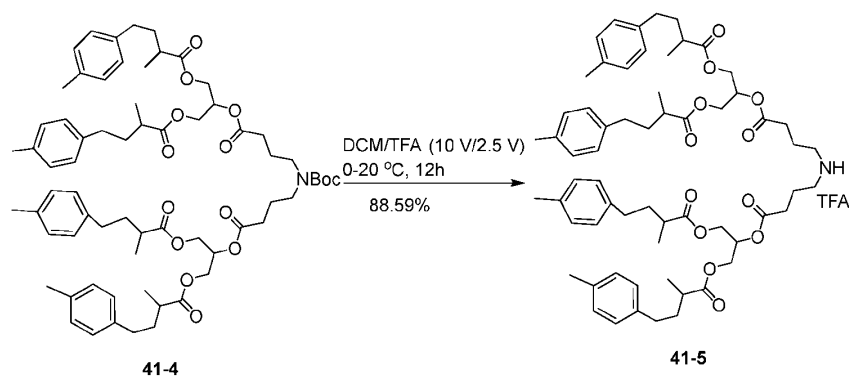
equiv.) was added at 0 °C. Then the reaction was stirred at 20 °C for 12 h. The reaction mixture was diluted with DCM (400 mL, 50 V). The organic layer was washed with (200 mL, 25 V) of water, brine (200 mL, 20 V), dried with anhydrous Na₂SO₄, filtered, and the filtrate was concentrated under vacuum. Crude product was adsorbed on 40 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (160 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE: EA = 20:1), combined, concentrated, and dried under vacuum to get **41-3** (6.6 g, 15.000 mmol, 85.7%) as a light-yellow oil. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 1.9 min, m/z (Calcd.) 440.2, (found) 463.2 (M+Na).

[0659] Synthesis of 41-4: ((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate)

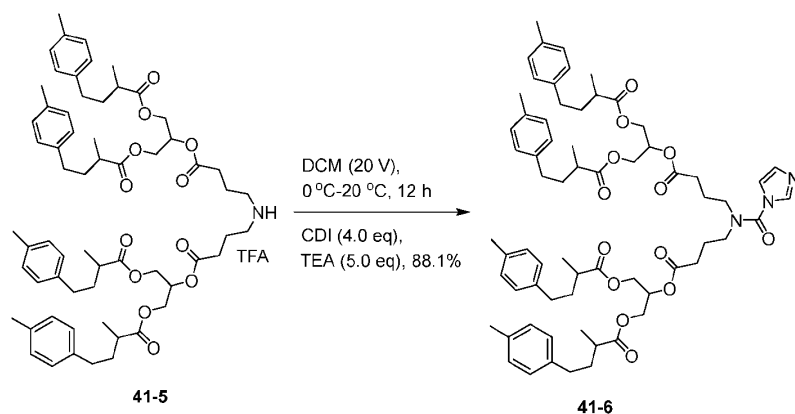


[0660] Into a 100 mL round-bottom flask, was placed **1-5** (2 g, 6.91 mmol, 1 equiv.) in DCM (40 mL, 20 V) followed by **41-3** (6.40 g, 14.52 mmol, 2.1 equiv.), DMAP (0.42 g, 3.46 mmol, 0.5 equiv.), and EDCI (3.31 g, 17.28 mmol, 2.5 equiv.) at 0 °C. Then the reaction was stirred at 20 °C for 12 h. The resulting mixture was diluted with DCM (200 mL, 100 V). The organic layer was washed with (100 mL, 50 V) of water, brine (100 mL, 50 V), dried with anhydrous Na₂SO₄, filtered, and the filtrate was concentrated under vacuum. Crude product was adsorbed on 20 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (100 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10). Fractions were analyzed (TLC, PE: EA = 100:1), combined,

[0661] Synthesis of 41-5: ((4,4'-azanediylobis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate) trifluoroacetic acid salt

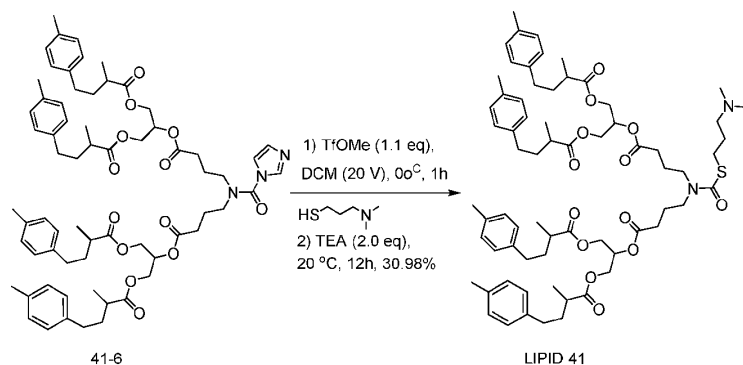


[0663] Synthesis of 41-6: ((4,4'-((1H-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis (propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate)



[0664] Into a 500 mL round-bottom flask **41-5** (5.0 g, 4.42mmol, 1 equiv.) in DCM (100 mL, 20 V) was added followed by CDI (2.86 g, 17.64 mmol, 4 equiv.) and TEA (2.23 g, 22.04 mmol, 5.0 equiv.) at 0°C. The reaction mixture was stirred at 20 °C for 12 h. The resulting mixture was diluted with DCM (200 mL, 40 V), washed with water (100 mL, 20 V), brine (100 mL, 20 V), dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to get **41-6** (4.5 g, 3.99 mmol, 88.1%) as a yellow oil that was used directly in the next reaction without purification. ELSD A: water/0.02% TFA: B: CH₃CN 95:5 to 5:95 A/B at 3 min.): RT 2.2 min, m/z (Calcd.) 1127.6, (found) 1128.6 (M+H).

[0665] Synthesis of LIPID 41: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate)



[0666] Into a 250 mL round-bottom flask was taken **41-6** (4.0 g, 3.55 mmol, 1 equiv.) in DCM (80 mL, 20 V). To this was added the methyl triflate (0.64 g, 3.90 mmol, 1.1 equiv.) at 0 °C. The reaction mixture was stirred at 0 °C for 1h. Then TEA (0.72 g, 7.12 mmol, 2.0 equiv.) and 3-(dimethylamino)propane-1-thiol (0.63 g, 5.28 mmol, 1.5 equiv.) were added at 0 °C. The reaction mixture was stirred at 20 °C for 12 h. The mixture was washed with H₂O (40 mL, 10 V) three times and brine (40 mL, 10 V), dried over Na₂SO₄, filtered and the crude product was adsorbed on 40 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (200 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using heptane/ethyl acetate (v/v) gradient from 100:0 to 40:60. Fractions were analyzed (TLC, Heptane: EA = 5:1), combined, concentrated, and dried under vacuum to get 3.1 g (89%) of **LIPID 41** which was further purified on Prep-Achiral-SFC (IntelFlash-1, Column: C18 silica gel; mobile phase A: IPA, phase B: Acetonitrile; 0% to 40% gradient in 25 min, 40% in 5 min; Detector, UV 220 nm/254 nm.). Qualified fractions were combined and concentrated to get pure **LIPID 41** (1.2 g, 1.0 mmol, 31%) as a colorless semi-solid. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.1 min, m/z (Calcd.) 1178.6, (found) 1179.8 (M+H). ¹H-NMR-**LIPID 41**: (300 MHz, Chloroform-*d*, ppm) δ 7.122-7.056 (m, 16H), 5.318-5.286 (m, 2H), 4.404-4.343 (m, 4H), 4.217-4.141 (m, 4H), 3.314 (brs, 4H), 2.913 (t, *J* = 7.2 Hz, 2H), 2.614-2.453 (m, 14H), 2.350-2.297 (m, 22H), 2.060-1.939 (m, 4H), 1.931-1.793 (m, 6H), 1.788-1.632 (m, 4H), 1.201 (d, *J* = 7.0 Hz, 12H).

Example 40. Biological Data of the Compounds of the Present Disclosure

[0667] A variety of assays were conducted to assess the efficacy of lipids of the present disclosure. A description of these assays follows.

Protocol for Factor VII Knock Down Evaluation

[0668] Lipid formulations comprising a FVII siRNA further described below were evaluated for their knockdown activity using the protocol of this example. In the FVII evaluation, seven to eight week-old, female Balb/C mice were purchased from Charles River Laboratories (Hollister, CA). The mice were held in a pathogen-free environment and all procedures involving the mice were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC). Lipid nanoparticles containing factor VII siRNA were administered intravenously at a dosing volume of 10 mL/kg and two dose levels (0.03 and 0.01 mg/kg). After 48 h, the mice were anesthetized with isoflurane and blood was collected retro-orbitally into

Microtainer® tubes coated with 0.109 M sodium citrate buffer (BD Biosciences, San Diego, CA) and processed to plasma. Plasma specimens were tested for factor VII levels immediately or stored at -80°C for later analysis. Measurement of FVII protein in plasma was determined using the colorimetric Biophen VII assay kit (Aniara Diagnostica, USA). Absorbance was measured at 405 nm and a calibration curve was generated using the serially diluted control plasma to determine levels of factor VII in plasma from treated animals, relative to the saline-treated control animals.

Protocol for hEPO mRNA Expression Evaluation

[0669] Lipid formulations comprising a hEPO mRNA below were evaluated for their ability to express hEPO in vivo according to the protocol of this example. All animal experiments were conducted using institutionally-approved protocols (IACUC). In this protocol, female Balb/c mice at least 6-8 weeks of age were purchased from Charles River Laboratory. The mice were intravenously injected with hEPO-LNPs via the tail vein with one of two dose levels of hEPO (0.1 and 0.03 mg/kg). After 6 hr, blood was collected with serum separation tubes, and the serum was isolated by centrifugation. Serum hEPO levels were then measured using an ELISA assay (Human Erythropoietin Quantikine IVD ELISA Kit, R&D Systems, Minneapolis, MD).

Mouse Plasma Stability

[0670] Lipid stock solution was prepared by dissolution of the lipid in isopropanol at the concentration of 5 mg/mL. A requisite volume of the lipid-isopropanol solution was then diluted to 100 μM concentration at a total volume of 1.0 mL with in 50:50 (v/v) ethanol / water. Ten microliters of this 100 μM solution was spiked into 1.0 mL of mouse plasma (BioIVT, Cat. No.: MSE00PLNHUNN, CD-1 mouse, anticoagulant: sodium heparin, not filtered) that was pre-warmed to 37°C and and was stirred at 50 rpm with a magnetic stir bar. The starting concentration of lipids in plasma was thus 1 μM . At time points 0, 15, 30, 45, 60 and 120 min, 0.1 mL of the plasma was withdrawn from the reaction mixture and the protein was precipitated by adding 0.9 mL of ice-cold 4:1 (v/v) acetonitrile/methanol with 1 $\mu\text{g/mL}$ of a selected internal standard lipid added. After filtration through a 0.45 micron 96-well filtering plate, the filtrates were analyzed by LC-MS (Thermo Fisher's Vanquish UHPLC – LTQ XL linear ion trap Mass Spectrometer); Waters XBridge BEH Shield RP18 2.5 micron (2.1 x 100mm) column with its matching guard column. Mobile phase A was 0.1 % formic acid in water, and mobile phase B was 0.1 % formic acid in 1:1 (v/v) acetonitrile/methanol. Flow rate was 0.5 ml/min. Elution gradient was: Time 0

– 1 min: 10% B; 1- 6 min: 10% - 95% B; 6 – 8.5 min: 95% B; 8.5 - 9 min: 95% - 10% B; 9 - 10 min: 10% B. Mass spectrometry was in positive scanning mode from 600 – 1100 m/z. The peak of the molecular ion of the lipids was integrated in the extracted ion chromatography (XIC) using Xcalibur software (Thermo Fisher). The relative peak area compared to T=0, after normalization by the peak area of the internal standard, was used as the percentage of the lipid remaining at each time point. $T_{1/2}$ values were calculated using the first-order decay model.

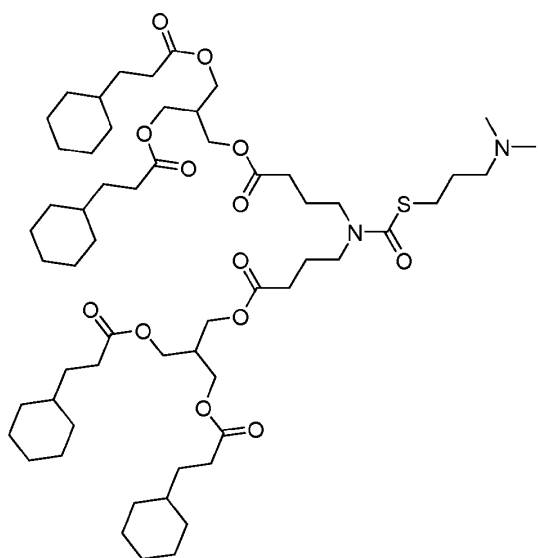
In vivo biodegradability assay

[0671] In vivo biodegradability assay was performed to assess the biodegradability of lipids in the LNP. Briefly, mice were injected with either 0.1 or 0.03 mg/Kg dose and after 24 or 48 hours mice livers were collected. To measure the concentration of lipids in the mouse liver, liver samples were homogenized in appropriate buffer in 1 - 10 dilution and mixed with the same amount of stabilized plasma. The samples were then mixed with organic solvents spiked with internal standard to precipitate proteins. After centrifugation, supernatant was diluted further with organic solvent before sample analysis by LC-MS. In LC-MS analysis, positive electrospray ionization was used, and multiple reaction monitoring (MRM) parameters were set up to specifically target the lipid analyte and internal standard. Calibration standards were prepared in stabilized plasma and mixed with same amount of homogenization buffer before protein precipitation. Quality control samples with known amounts of lipid was prepared in blank liver homogenate to monitor the precision and accuracy of the assay.

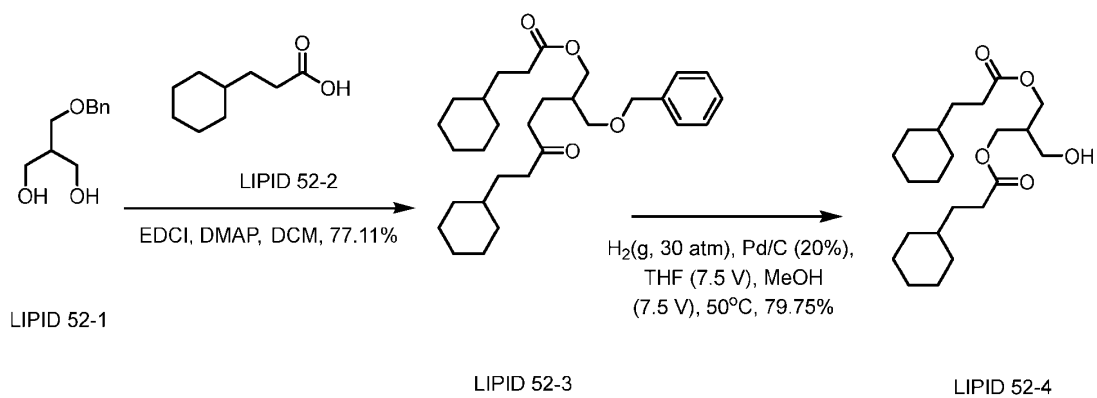
Example 41. LogD and pKa Data of the Compounds of the Present Disclosure

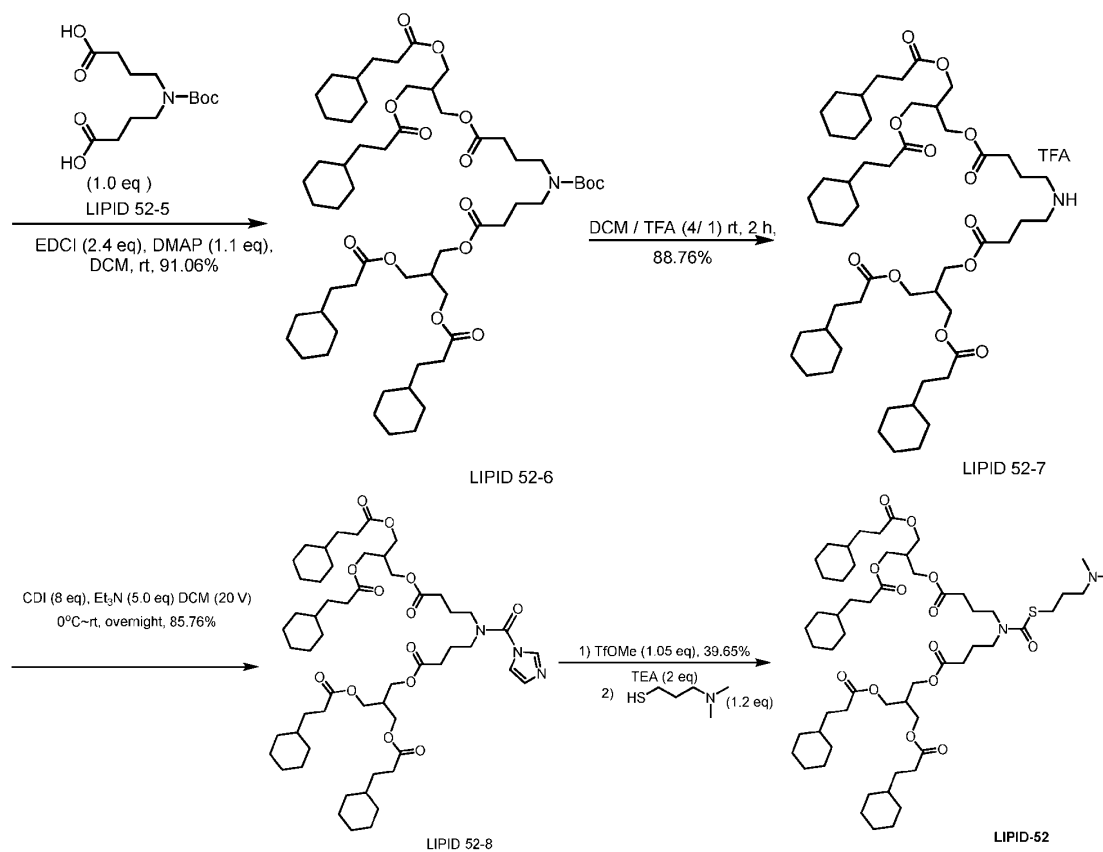
[0672] Calculated LogD (cLogD) and calculated pKa (cpKa) values for lipid compounds were determined using ACD Labs Version B and ACD Labs Structure Designer version 12.0, respectively. Measured pKa values were determined based on the pH measured in the indicated formulation (i.e., EPO or FVII formulation). The data are summarized in Table 1 and Table 2.

[0673] **Example 42. LIPID 52: ((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)**

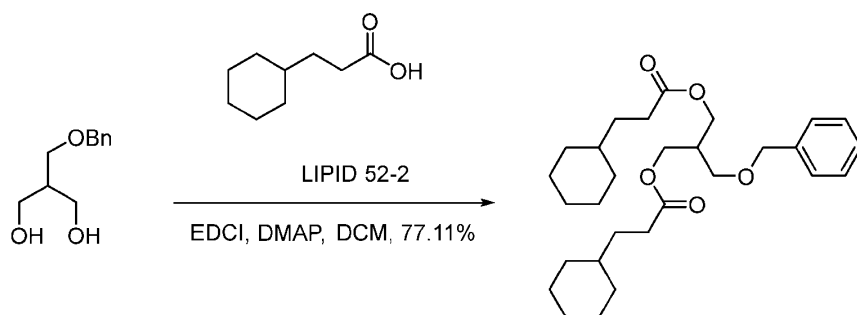


Lipid 52

[0674] General scheme



[0675] Synthesis of Lipid 52-3: 3-cyclohexylpropanoic acid-2-((benzyloxy)methyl)propane-1,3-diyl bis(3-cyclohexylpropanoate)--2-((benzyloxy)methyl)propane-1,3-diol (1/1/1)

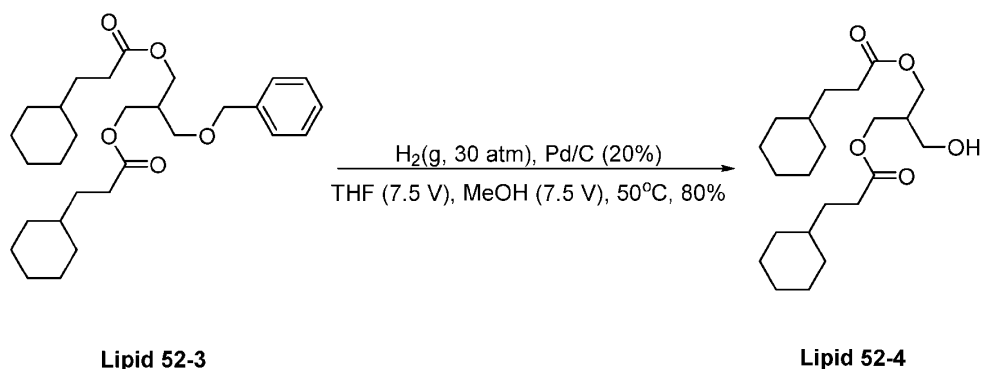


LIPID 52-1

LIPID 52-3

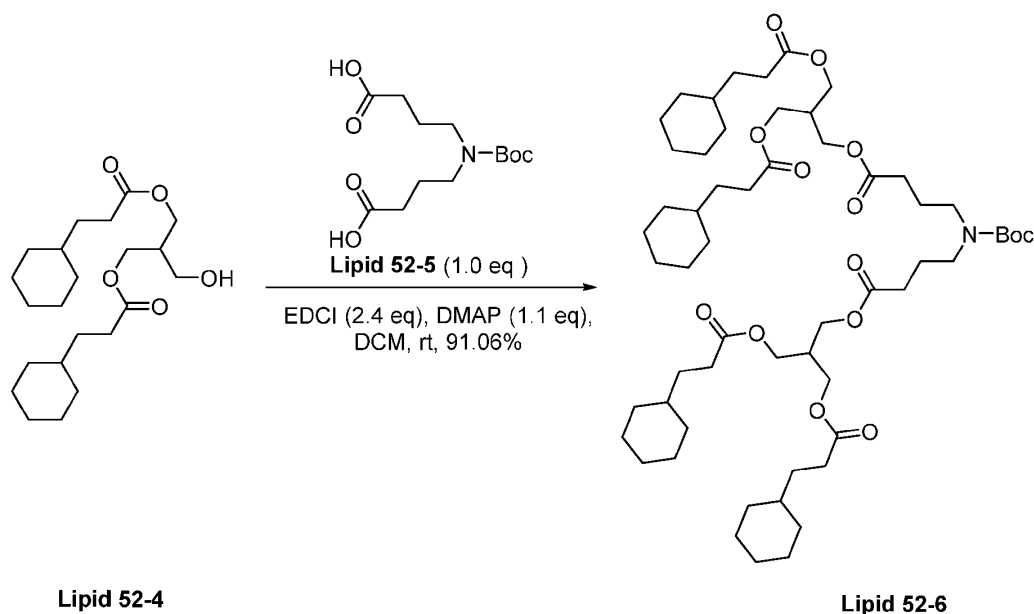
[0676] Into a 500 mL 3-necked round-bottom flask were added 2-[(benzyloxy)methyl]propane-1,3-diol (7 g, 35.670 mmol, 1 equiv.), cyclohexane propionic acid (12.3 g, 78.733 mmol, 2.21 equiv.), DCM (210 mL) and DMAP (2.6 g, 21.282 mmol, 0.60 equiv.) at room temperature. To the above mixture was added EDCI (17.1 g, 89.202 mmol, 2.50 equiv.) in portions at room temperature and the resulting mixture was stirred overnight. The reaction was diluted with DCM (200 mL) and washed with 2x200 mL of water, 100 mL of aqueous citric acid (5 %) and sat. aqueous NaHCO₃. The organic solution was washed with 1x200 mL of brine, dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. Crude product was adsorbed on 30 g of silica gel (type: ZCX-2, 100-200 mesh, 1.40 w./w.) and purified on a silica gel column (250 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) using petroleum ether/ethyl acetate (v/v) gradient from 100:0 to 90:10. Fractions were analyzed (TLC, PE:EA = 6:1), combined, concentrated, and dried under vacuum to get **Lipid 52-3** (13 g, 77%) as light colorless oil. HPLC purity 97.2%. This was used as such in the next reaction.

[0677] Synthesis of Lipid 52-4: 2-(hydroxymethyl)propane-1,3-diyl bis(3-cyclohexylpropanoate)



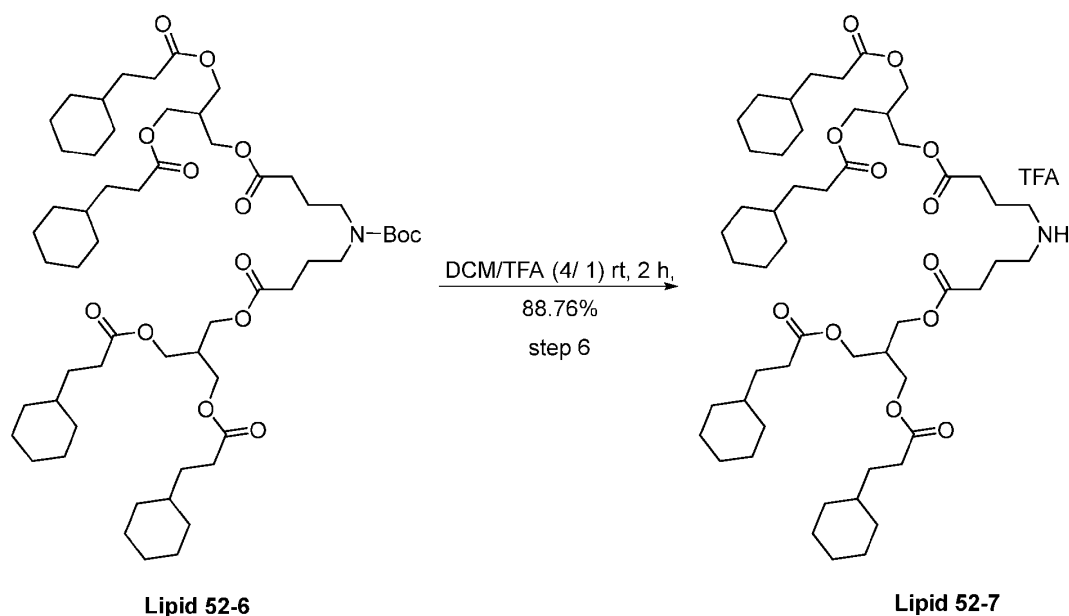
[0678] Into a 250 mL pressure tank reactor were added 3-(benzyloxy)-2-{[(3-cyclohexylpropyl)oxy]methyl}propyl 3-cyclohexylpropanoate (11 g, 23.272 mmol, 1 equiv.), MeOH (80 mL), THF (80 mL) and Pd/C (2.2 g, 20% W/W) at room temperature. The resulting mixture was stirred for additional 2 days at 50 °C under hydrogen atmosphere (30 atm). The mixture was allowed to cool down to room temperature. The resulting mixture was filtered, the filter cake was washed with THF (2x20 mL). To the filtrate was added 20 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and evaporated to adsorb the material on silica gel. The crude product thus adsorbed was purified on a silica gel column (250 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with PE/DCM (volume ratio). (gradient from 100:0 to 90:10 and collected product eluent PE/DCM=5/95). After TLC analysis. (PE: DCM = 1:10, 0.2) fractions were pooled and evaporated under reduced pressure to afford Lipid 52-4 (7.1 g, 80%) as a colorless oil. HPLC purity 97.25%.

[0679] Synthesis of Lipid 52-6 : (((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



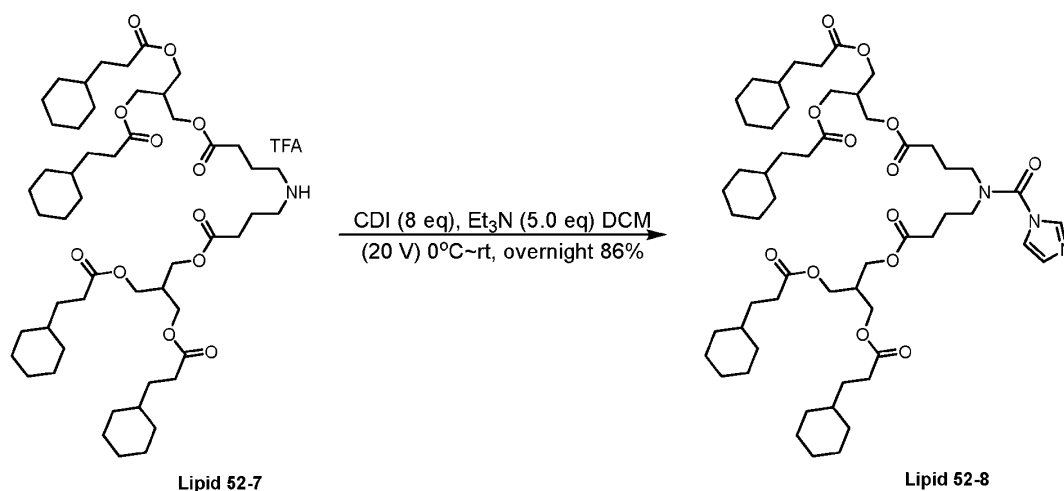
[0680] To a 250 ml three-necked round-bottle flask was added **Lipid 52-4** (7 g, 18.299 mmol, 2.2 equiv.), **Lipid 52-5** (commercially sourced, 2.38 g, 8.235 mmol, 1.0 equiv.), DCM (140 mL) and DMAP (1.12 g, 9.149 mmol, 1.1 equiv.). Then EDC.HCl (3.86 g, 20.129 mmol, 2.4 equiv.) was added to the above mixture at 0°C in several portions. The reaction mixture was stirred for 16 h at room temperature and diluted with brine (140 mL, 20 V). The mixture was extracted with ethyl acetate (2 x 280 mL, 40V). The combined organic layers were washed with brine (140 mL, 20 V), dried with anhydrous Na₂SO₄ and filtered. To the filtrate was added 15 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and evaporated to adsorb the material on silica gel. The crude product thus adsorbed was purified on a silica gel column (120 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with Hep/EA (volume ratio), (gradient from 100:0 to 70:30 and collected product eluent Hep/EA=85/15). After TLC analysis. (PE: DCM = 1:10, 0.2) fractions were pooled and evaporated under reduced pressure to afford **Lipid 52-6** (8 g, 91.06%) as a little yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.06 min, m/z (Calcd.) 1017.6, (found) 1040.6 (M+Na). HPLC Purity 95.4%.

[0681] **Synthesis of Lipid 52-7:** bis(4-(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy) methyl)propoxy)-4-oxobutyl) trifluoroacetate



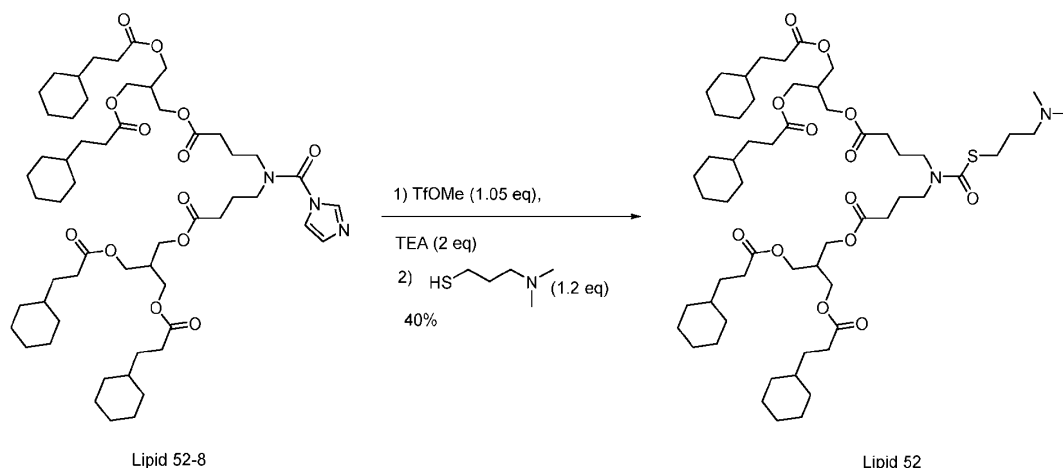
[0682] To a 50 mL round-bottle flask, was added Lipid 52-6 (3 g, 2.946 mmol, 1 equiv..) in DCM (30 mL) at room temperature and the solution was cooled to 0°C in an ice/water bath. To this was added TFA (8 mL) at 0°C. The ice/water bath was removed, and the mixture was stirred for 3 h at room temperature and concentrated under vacuum to get Lipid 52-7 as its trifluoroacetic acid salt (2.8 g, 88.76%) as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.46 min, m/z (Calcd.) 917.6, (found) 918.6 (M+H). HPLC Purity 94.9%.

[0683] Synthesis of Lipid 52-8: (((4,4'-((1H-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis (methylene))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



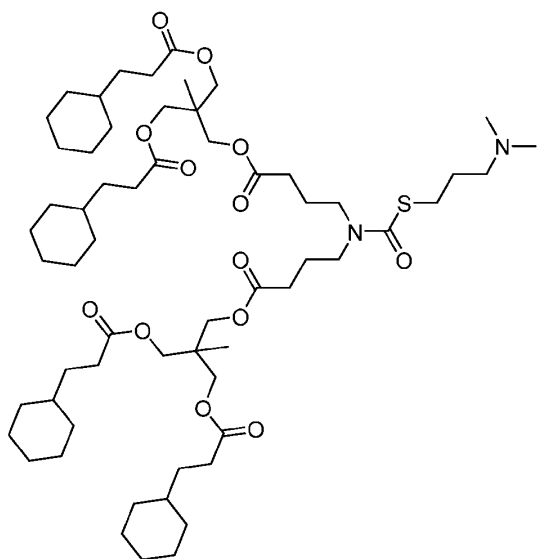
[0684] Into a 100 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution of Lipid 52-7 (2.8 g, 2.755 mmol, 1 equiv.) in DCM (56 mL). To this solution was added the CDI (3.57 g, 22.040 mmol, 8 equiv.) followed by TEA (1.39 g, 13.775 mmol, 5 equiv.) at 0°C. The mixture was stirred overnight at room temperature. The resulting mixture was diluted with water (50 mL) and extracted with CH₂Cl₂ (2 x 100 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous Na₂SO₄ and filtered. The filtrate was concentrated under reduced pressure to obtain Lipid 52-8 (2.6 g, 86%) as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.61 min, m/z (Calcd.) 1012.0, (found) 1013.0 (M+H). HPLC Purity 91.8%. This material was used in the next step without further purification.

[0685] Synthesis of LIPID 52: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)

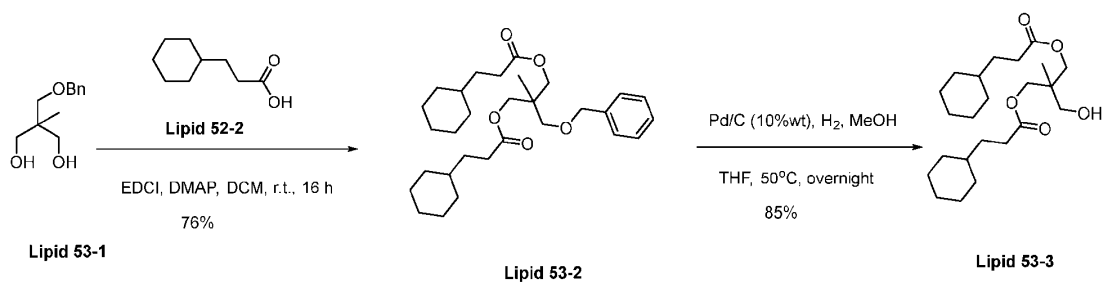


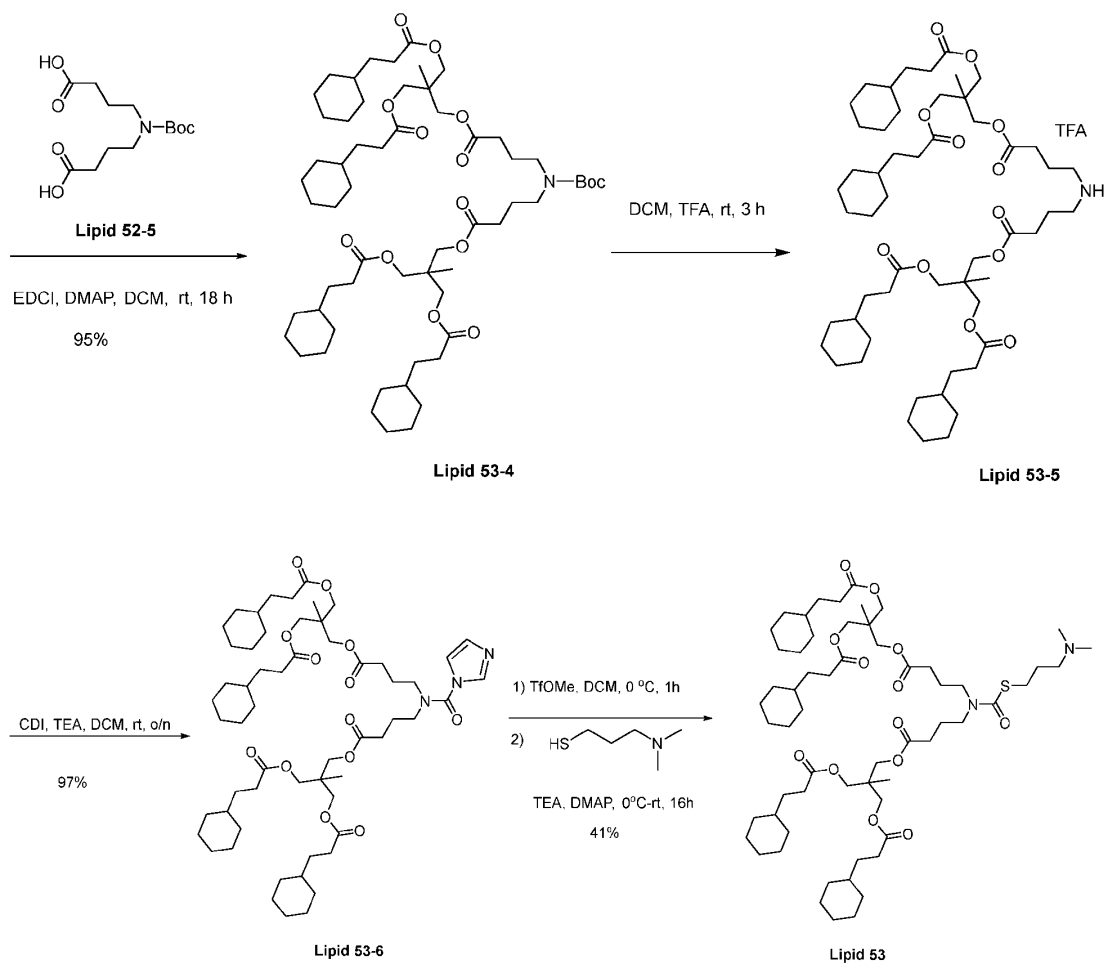
[0686] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed a solution Lipid 52-8 (3.0 g, 2.963 mmol, 1 equiv.) in DCM (60 mL). The solution was cooled to 0°C in an ice/water bath. Methyl trifluoromethanesulfonate (0.51 g, 3.111 mmol, 1.05 equiv.) was added and the mixture was stirred at 0°C for 1 hour. TEA (0.60 g, 5.926 mmol, 2 equiv.) was added to the reaction mixture followed by 3-(dimethylamino)propane-1-thiol (0.71 g, 5.926 mmol, 2 equiv.). The ice/water bath was removed, and the mixture was stirred overnight at room temperature. The resulting mixture was diluted with DCM (80 mL) and washed with water (2x60 mL). The organic phase was collected and dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography, eluted with DCM/MeOH (95/5) to afford 1.8 g (HPLC= 95.2%) as yellow oil. This was further purified on a chiral column CHIRAL ART Cellulose-SB using Mobile Phase A: CO₂, Mobile Phase B: MeOH (0.1% 2M NH₃-MeOH); Flow rate: 80 mL/min; Gradient: isocratic 40% B; Column Temperature(°C): 35; Back Pressure(bar): 100; Wave Length: 220 nm; RT1 (min): 4.3; RT2 (min): 4.6; Sample Solvent: MeOH: ACN=2: 1; Injection Volume: 1.5 mL; Number Of Runs: 45. Fractions were pooled and concentrated under reduced pressure to obtain a residue that was dissolved in n-heptane (40 mL, 30 V), washed with MeOH/H₂O (4:1) (2 x 40 mL, 30 V), dried over anhydrous Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure to afford (1.2725 g, 40%) as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.28 min, m/z (Calcd.) 1062.6, (found) 1063.6 (M+H). HPLC Purity 98.1%; ¹H NMR (400 MHz, Chloroform-d) δ 4.16-4.10 (m, 12H), 3.39 (s, 4H), 2.95-2.91

[0687] Example 43. LIPID 53: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl)tetrakis(3 cyclohexylpropanoate)

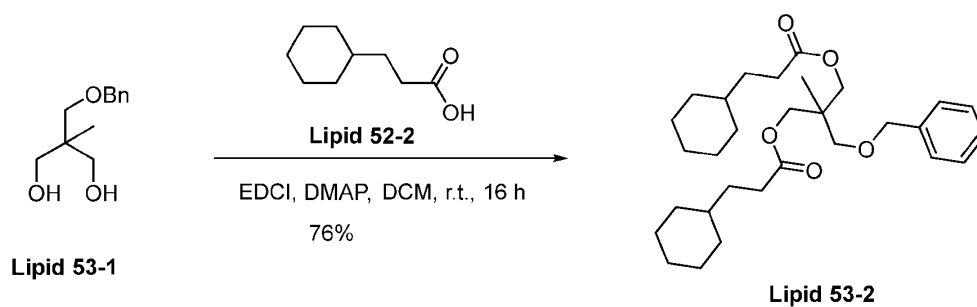


[0688] General scheme



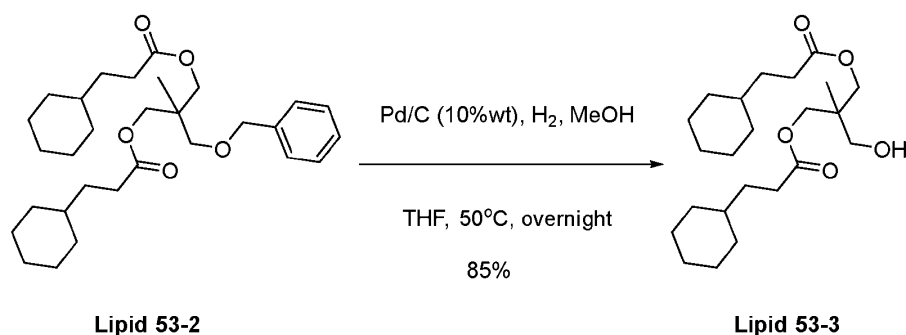


[0689] Synthesis of Lipid 53-2: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(3-cyclohexylpropanoate)



[0690] To a stirred solution of **Lipid 53-1** (10 g, 47.619 mmol, 1 equiv.) and **Lipid 52-2** (15.6 g, 100.00 mmol, 2.1 equiv.) in DCM (100 mL, 10 V) were added DMAP (1.163 g, 9.511 mmol, 0.2 equiv.) followed by EDCI (21.13 g, 11.893 mmol, 2.5 equiv.) in portions at room temperature. The reaction mixture was stirred for 16 h at room temperature. The resulting mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc (100 mL, 10 V) and the organic phase was washed with 10% acetic acid (1 x 100 mL, 10 V), water (1 x 100 mL, 10 V) and brine (1 x 100 mL, 10 V), dried over anhydrous Na₂SO₄. To the filtrate was added 40 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and evaporated to adsorb the material on silica gel. The crude product thus adsorbed was purified on a silica gel column (200 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with Hep/EA (volume ratio), (gradient from 100:0 to 90:10. After TLC analysis. (PE: EA = 4:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 53-2** (17.7 g, 76%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.76 min, m/z (Calcd.) 486.6, (found) 508.6 (M+Na). HPLC Purity 97.4%.

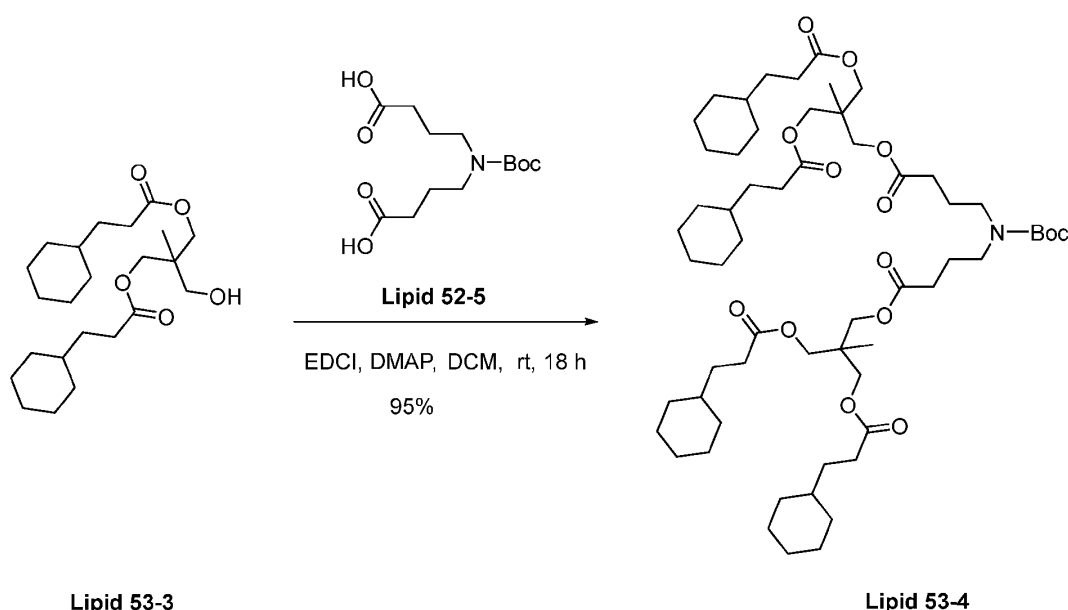
[0691] Synthesis of Lipid 53-3: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(3-cyclohexylpropanoate)



[0692] To a stirred solution of **Lipid 53-2** (17.7 g, 36.395 mmol, 1 equiv.) in MeOH (180 mL, 10 V) and THF (180 mL, 10 V) was added Pd/C (1.77 g, 10% w/w) at room temperature. The resulting mixture was stirred overnight at 50°C under hydrogen atmosphere. The resulting mixture was filtered, the filter cake was washed with DCM (2 x 180 mL, 20 V). The filtrate was

concentrated under reduced pressure. This resulted in **Lipid 53-3** (12.2 g, 30.785 mmol, yield: 85%) as a light- yellow oil that was used as such in the next reaction. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.25 min, m/z (Calcd.) 396.0, (found) 397.0 (M+H). HPLC Purity 98.8%.

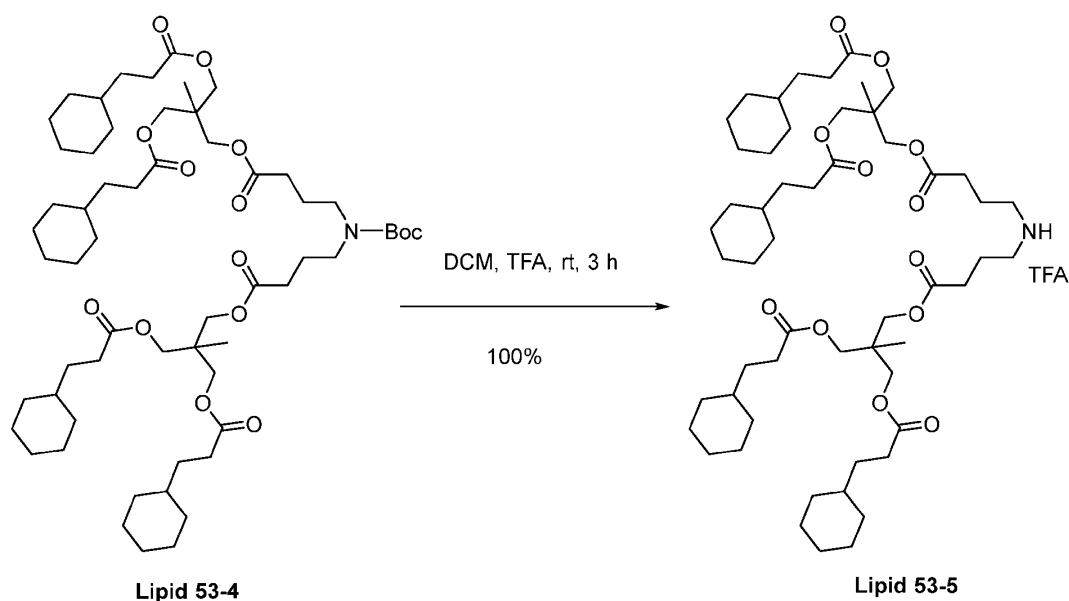
[0693] Synthesis of Lipid 53-4: (((4,4'-(((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))) bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate)



[0694] To a stirred solution of **Lipid 53-3** (8.000 g, 20.187 mmol, 2.00 equiv.) and **Lipid 52-5** (2.918 g, 10.093 mmol, 1.00 equiv.) in DCM (60 mL, 20V) were added DMAP (1.231 g, 10.093 mmol, 1.0 equiv.) and EDCI (4.837 g, 25.232 mmol, 2.50 equiv.) in portions at room temperature. The reaction mixture was stirred for 18 h at room temperature. The resulting mixture was concentrated under reduced pressure and the residue was dissolved in EtOAc (120 mL, 40V). The resulting mixture was washed with 10% acetic acid (aq.) (2x60 mL, 40V), dried over anhydrous Na₂SO₄ and filtered. To the filtrate was added 18 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and evaporated to adsorb the material on silica gel. The crude product thus adsorbed was purified on a silica gel column (180 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with Hep/EA (volume ratio), (gradient from 100:0 to 85:15. After

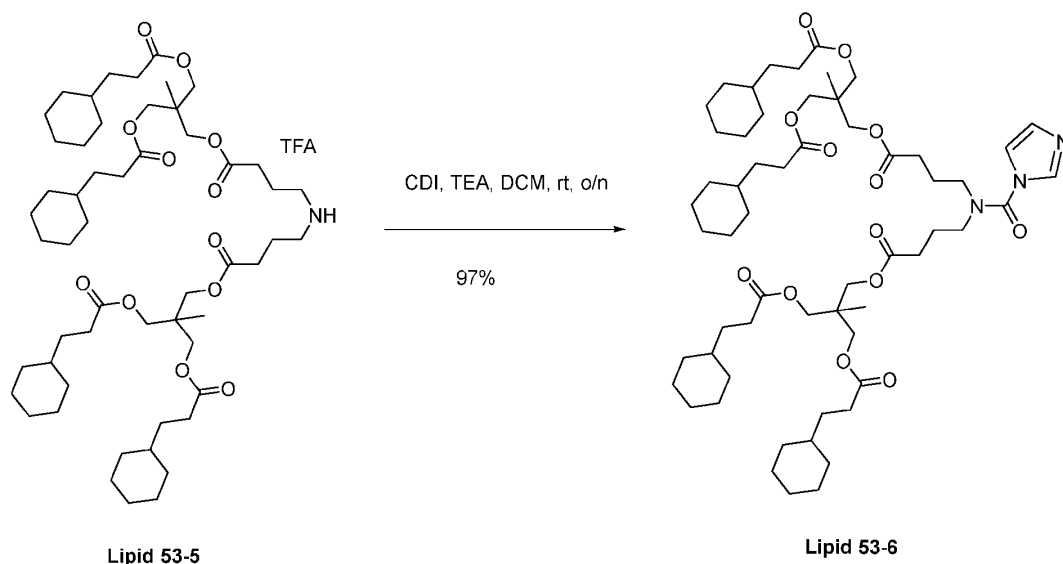
TLC analysis. (PE: EA = 4:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 53-4** (10 g, 95%) as a light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.13 min, m/z (Calcd.) 1045.6, (found) 1068.6 (M+Na). HPLC Purity 93.8%.

[0695] Synthesis of Lipid 53-5: (((4,4'-azanediylbis(butanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-cyclohexylpropanoate) trifluoroacetic acid salt



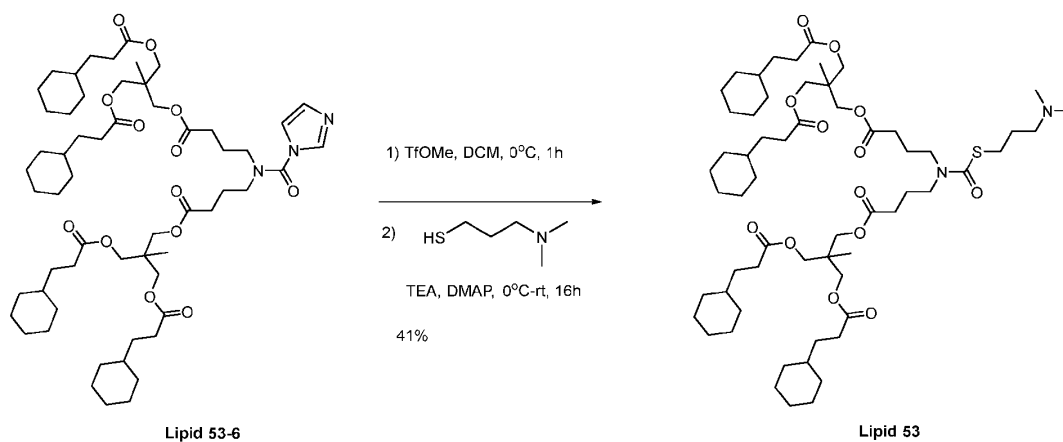
[0696] To a stirred solution of **Lipid 53-4** (4.0 g, 3.825 mmol, 1.00 equiv.) in DCM (40 mL, 10V) was added TFA (10 mL, 2.5V) at room temperature. The reaction mixture was stirred for 3 h at room temperature. The resulting mixture was washed with K₂HPO₄ (5.994 g, 34.426 mmol, 9.0 equiv.) dissolved in H₂O (40 mL, 10 V). The above mixture was extracted with CH₂Cl₂ (40 mL, 10 V). The combined organic layers were washed with water (40 mL, 10 V), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure to get **Lipid 53-5** (4.00 g) in quantitative yield as a yellow oil. The crude product was used in the next step directly without further purification. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.56 min, m/z (Calcd.) 945.6, (found) 946.6 (M+H). HPLC Purity 97.5%.

[0697] Synthesis of Lipid 53-6: (((4,4'-((1H-imidazole-1-carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl)tetrakis(3-cyclohexylpropanoate)



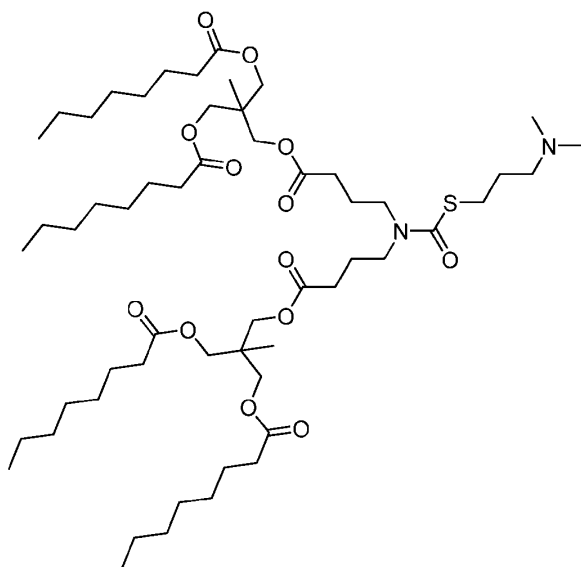
[0698] To a stirred solution of **Lipid 53-5** (4.0 g, 3.830 mmol, 1 equiv.) in DCM (80 mL, 20 V) was added CDI (6.21 g, 38.300 mmol, 10.0 equiv.) and TEA (3.10 g, 30.640 mmol, 8.0 equiv.) dropwise at room temperature. The reaction mixture was stirred overnight at room temperature. The resulting mixture was washed with 0.8 M HCl (2 x 80 mL, 40V). The organic layers were washed with 10% brine (160 mL, 40 V), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The residue was dissolved in n-heptane (80 mL, 20 V). The residue was washed with MeOH/H₂O (4:1) (2 x 80 mL, 40 V), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. This resulted in **Lipid 53-6** (4.0 g, yield: 97%) as a light yellow oil that was used immediately in the next step. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.58 min, m/z (Calcd.) 1040, (found) 1041 (M+H).

[0699] Synthesis of Lipid 53: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl)tetrakis(3-cyclohexylpropanoate)



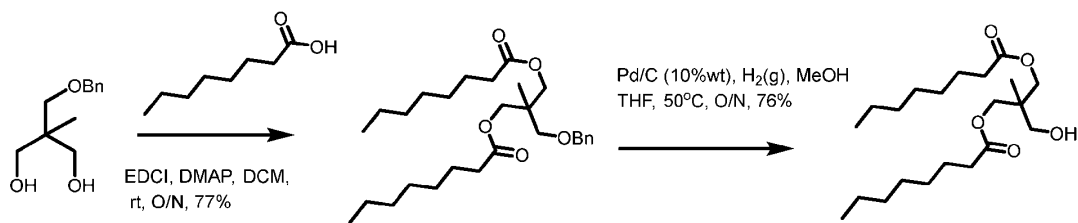
[0700] To a stirred solution of **Lipid 53-6** (3.5 g, 3.364 mmol, 1 equiv.) in DCM (35 mL, 10 V) was added methyl trifluoromethane sulfonate (579.6 mg, 3.532 mmol, 1.05 equiv.) dropwise at 0°C under nitrogen atmosphere. The reaction mixture was stirred for 1h at 0°C under nitrogen atmosphere. To the above mixture was added TEA (1.02 g, 10.092 mmol, 3.0 equiv.), 3-(dimethylamino)propane-1-thiol (0.60 g, 5.046 mmol, 1.5 equiv.) and DMAP (0.08 g, 0.673 mmol, 0.2 equiv.) at 0°C. The resulting mixture was stirred for an additional 16 h at room temperature. The resulting mixture was diluted with 10% citric acid (35 mL, 10 V) and 10% NaCl (35 mL, 10 V). The mixture was extracted with CH₂Cl₂ (2 x 70 mL, 40 V). The combined organic layers were washed with 10% Na₂CO₃ (2x70 mL, 40 V) and 10% NaCl (140 mL, 40 V), dried over anhydrous Na₂SO₄ and filtered. To the filtrate was added 7 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and evaporated to adsorb the material on silica gel. The crude product thus adsorbed was purified on a silica gel column (70 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with DCM/MeOH (volume ratio), (gradient from 100:0 to 95:5. After TLC analysis. (DCM : MeOH = 10:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 53** (1.5 g, 41%) as a light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 30 min.): RT 14.56 min, m/z (Calcd.) 1090.7, (found) 1091.8 (M+H). HPLC Purity 97.3%. ¹H NMR (400 MHz, Chloroform-*d*) δ 4.015 (d, *J* = 7.2 Hz, 12H), 3.390 (br, 4H), 2.936 (t, *J* = 7.2 Hz, 4H), 2.440-2.287 (m, 20H), 1.867-1.794 (m, 2H), 1.722-1.647 (m, 20H), 1.520 (q, *J* = 7.2 Hz, 8H), 1.338-1.096 (m, 19H), 1.027 (s, 6H), 0.947-0.858 (m, 9H).

[0701] **Example 44. LIPID 54: (((4,4'-((((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy)) bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetraoctanoate**



Lipid 54

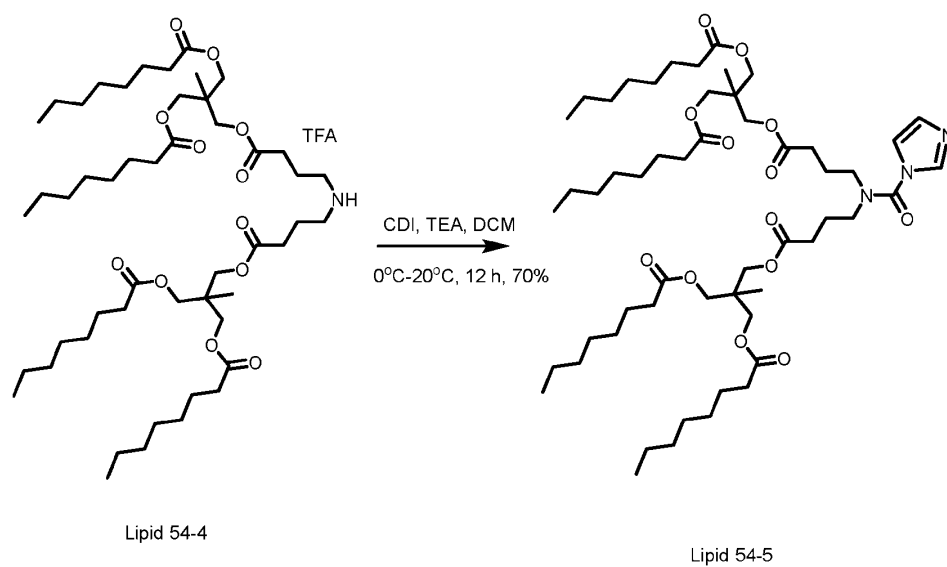
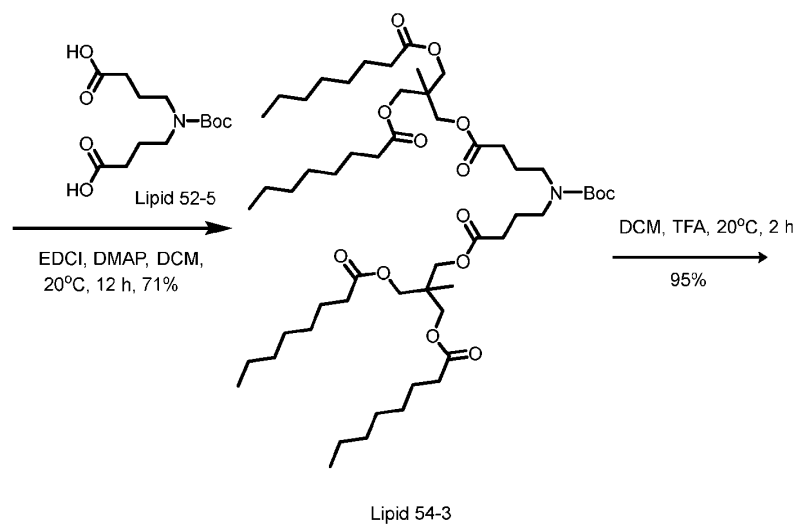
[0702] General scheme

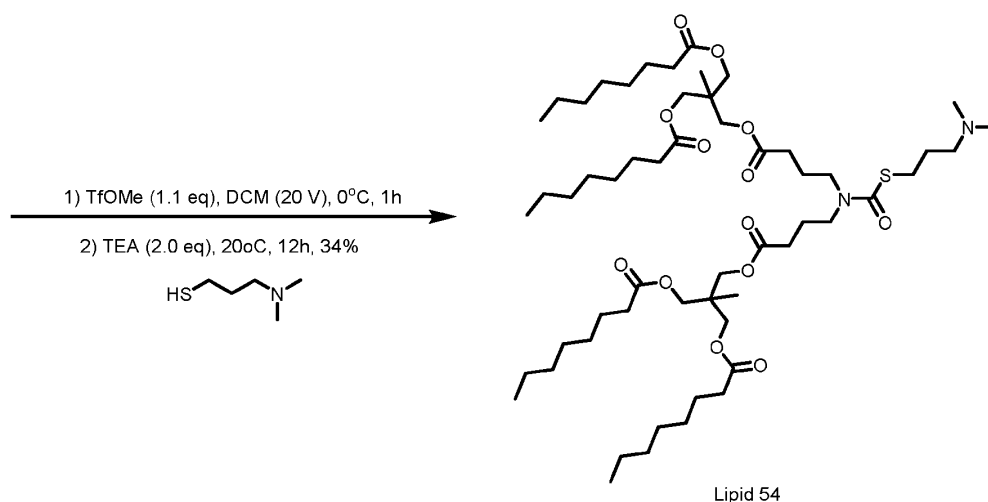


Lipid 53-1

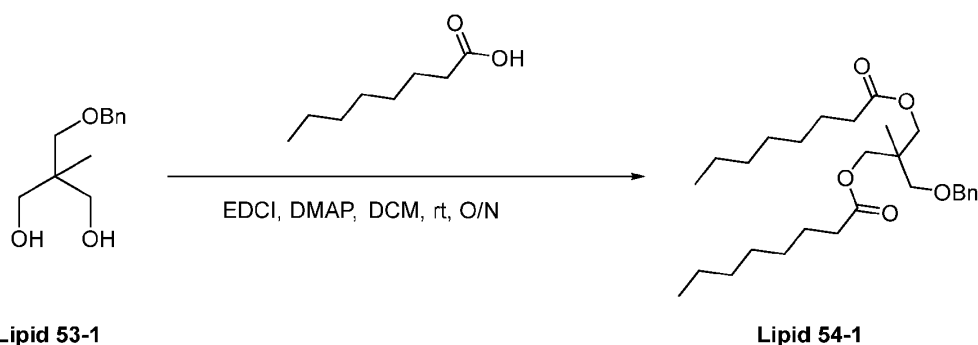
Lipid 54-1

Lipid 54-2





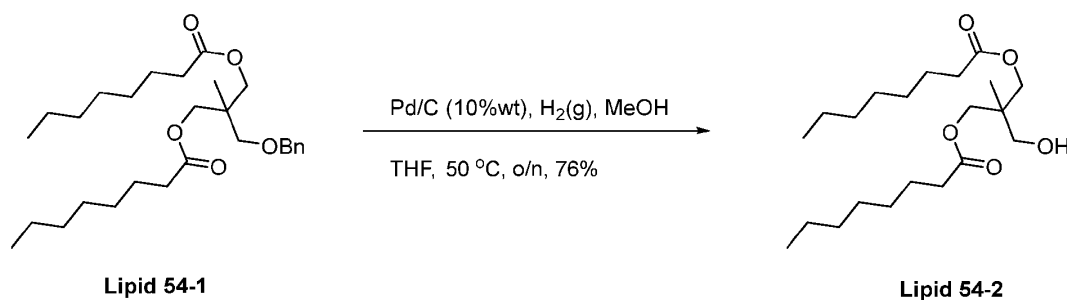
[0703] Synthesis of Lipid 54-1: 2-((benzyloxy)methyl)-2-methylpropane-1,3-dioldioctanoate



[0704] Into a 250 mL round-bottom flask, was placed Lipid 53-1 (6.0 g, 28.534 mmol, 1 equiv.), DCM (120 mL), caprylic acid (9.05 g, 62.775 mmol, 2.2 equiv.), DMAP (3.49 g, 28.534 mmol, 1.0 equiv.). The EDCI (13.68 g, 71.335 mmol, 2.5 equiv.) was added at 0 °C and stirred at room temperature overnight. The resulting mixture was diluted with DCM (480 mL, 80 v). The resulting mixture was washed with (2 x 240 mL, 80 v) of water. brine (120 mL, 20 v), dried over anhydrous Na₂SO₄. The resulting mixture was concentrated under reduced pressure. The crude product was adsorbed on 30 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (300 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with heptane/EA (volume ratio), (gradient from 100:0 to 90:10. After TLC analysis. (heptane : EA = 10:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 54-1** (10.2 g, 77%) as a light yellow oil. ELSD A: water/0.05% TFA: B:

CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.42 min, m/z (Calcd.) 462.6, (found) 463.6 (M+H). HPLC Purity 97.4%.

[0705] Synthesis of Lipid 54-2: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl dioctanoate



[0706] Into a 250 mL pressure tank, was placed 3-(benzyloxy)-2-methyl-2-[(octanoyloxy)methyl]propyl octanoate (10 g, 21.614 mmol, 1 equiv.), THF (100 mL), MeOH (100 mL), Pd/C (1.15 g, 10.807 mmol, 0.5 equiv.). The mixture was hydrogenated at 50 °C under 20 atm of hydrogen pressure for overnight. The mixture was allowed to cool down to rt. The resulting mixture was filtered, the filter cake was washed with THF (100 mL x 3). The filtrate was concentrated under reduced pressure. The crude product was adsorbed on 30 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w/w.) and purified on a silica gel column (300 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w/w.) by eluting with heptane/EA (volume ratio), (gradient from 100:0 to 90:10. After TLC analysis. (heptane : EA = 10:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 54-2** (6.1 g, 76%) as a light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.18 min, m/z (Calcd.) 372.3, (found) 373.3 (M+H). HPLC Purity 97.3%.

[0707] Synthesis of Lipid 54-3: (((4,4'-((tert-butoxycarbonyl)azanediyl)bis(butanoyl))bis(oxy))bis (methylene))bis(2-methylpropane-2,1,3-triyl) tetraoctanoate

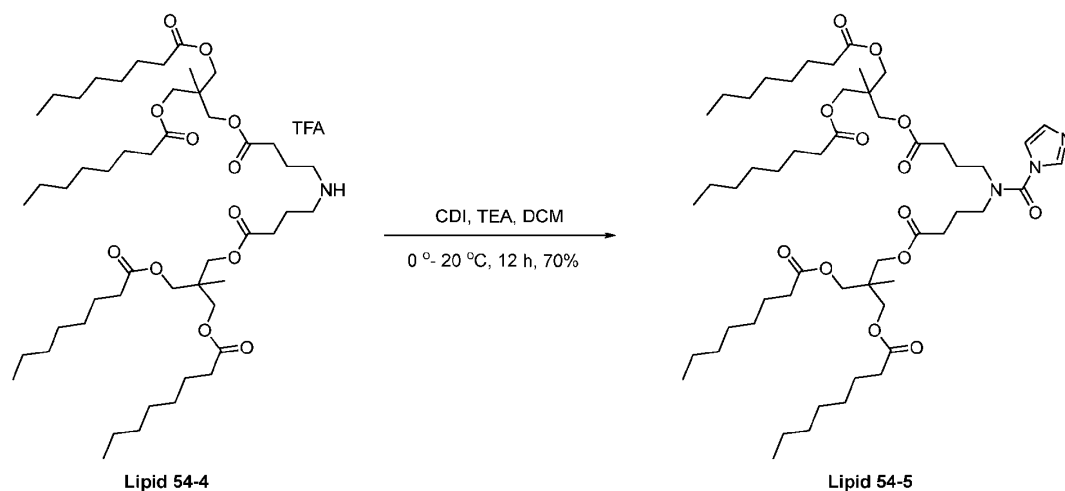


[0709] Synthesis of Lipid 54-4: (((4,4'-azanediy)bis(butanoyl))bis(oxy))bis(methylene))bis(2-methyl propane-2,1,3-triyl) tetraoctanoate



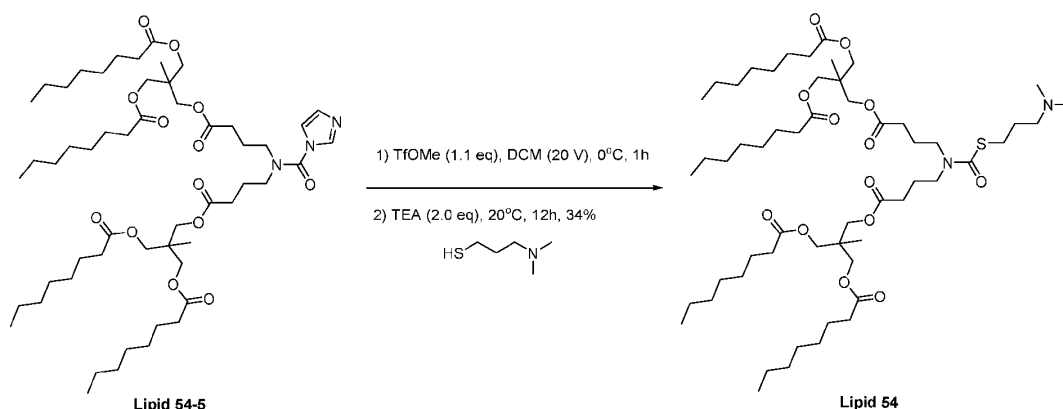
Lipid 54-4

285



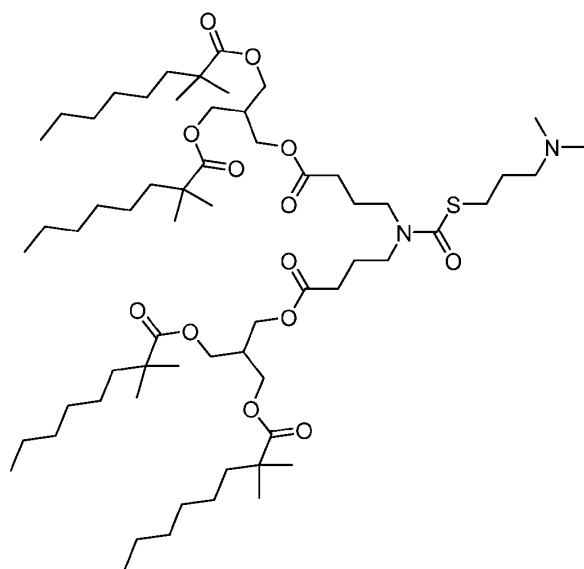
[0712] Into a 250 mL round-bottom flask, was placed Lipid 54-4 (5.0 g, 5.019 mmol, 1 equiv), DCM (100 mL, 20 v), CDI (8.14 g, 50.190 mmol, 10 equiv). The TEA (4.06 g, 40.152 mmol, 8 equiv) was added at 0 °C. The reaction mixture was stirred at 20 °C for 12 h. The resulting mixture was diluted with DCM (250 mL, 50 v). The resulting mixture was washed with (3 x 50 mL, 30 v) of water, brine (50 mL, 10 v), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. The crude mixture was diluted with n-Heptane (250 mL) and washed with 3 x 50 mL of MeOH/H₂O (4/1, 30 v), water (50 mL, 10 v), brine (50 mL, 10 v), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford Lipid 54-5 (3.48 g, 3.51 mmol, yield: 70%) as a light yellow oil. This was used in the next reaction as such. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.58 min, m/z (Calcd.) 991.7, (found) 992.7 (M+H). HPLC Purity 94.4%.

[0713] **Synthesis of LIPID 54:** ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy)) bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetraoctanoate



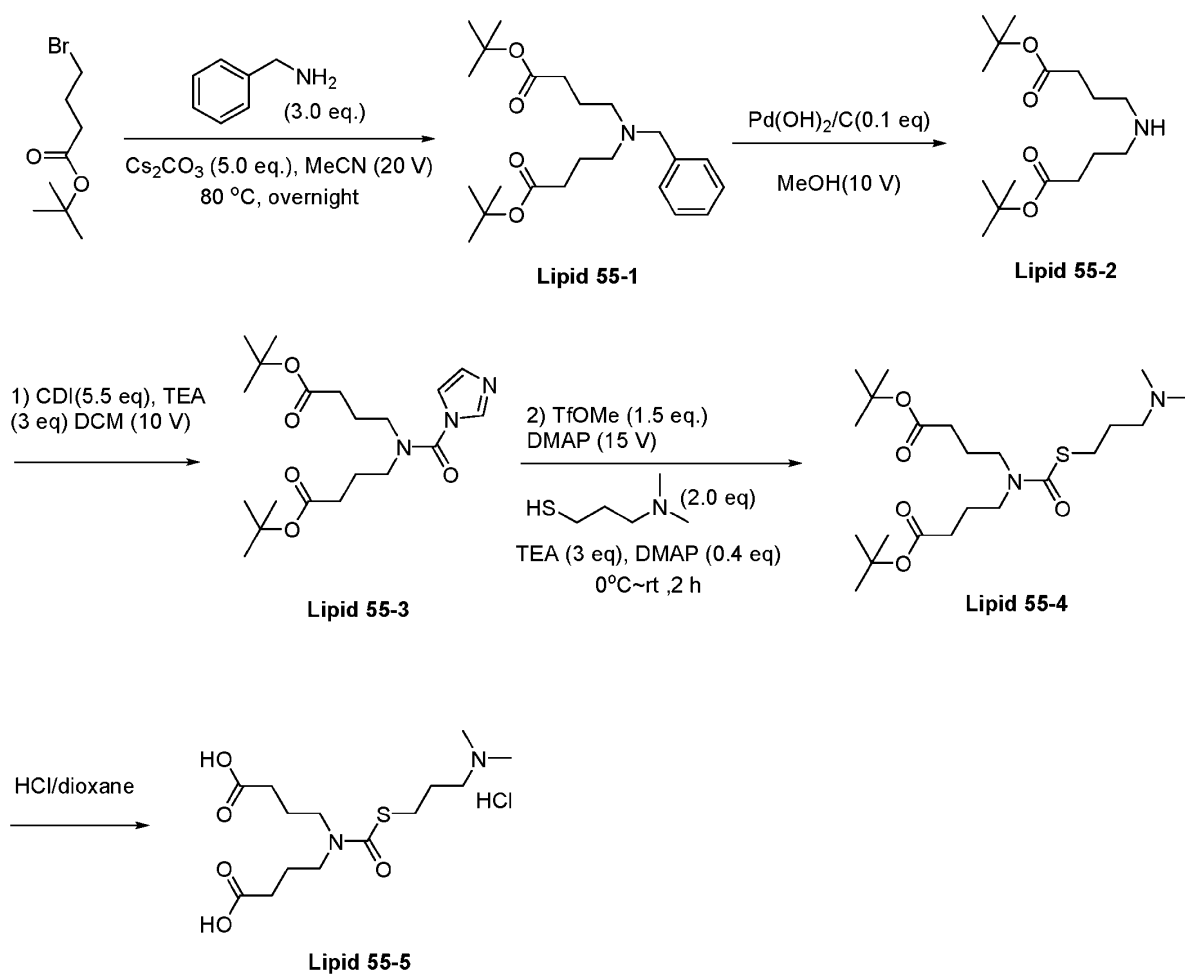
[0714] Into a 250 mL round-bottom flask, was placed Lipid 54-5 (3.4 g, 3.426 mmol, 1 equiv.), DCM (68 mL, 20 v). The triflate ester (618.47 mg, 3.769 mmol, 1.1 equiv.) was added at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. The TEA (693.42 mg, 6.852 mmol, 2.0 equiv.) and 3-(dimethylamino)propane-1-thiol (612.76 mg, 5.139 mmol, 1.5 equiv.) was added at 0 °C. The reaction mixture was stirred at 20 °C for 12 h. The resulting mixture was diluted with DCM (170 mL, 50 v), The resulting mixture was washed with 3 x 34 mL (30 v) of water, brine (34 mL, 10 v), dried over anhydrous Na₂SO₄. The resulting mixture was concentrated under reduced pressure. The crude product was adsorbed on 34 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (340 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with DCM/MeOH (volume ratio), (gradient from 100:0 to 95:5. After TLC analysis. (DCM : MeOH = 20:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 54** (1.2 g, 34%) as a light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.06 min, m/z (Calcd.) 1042.7, (found) 1043.7 (M+H). HPLC Purity 98.1%. H-NMR-PH-ARC-ATX-278-0: (300 MHz, Chloroform-*d*, ppm) δ 4.088-3.918 (m, 12H), 3.373 (s, 4H), 2.911 (t, *J* = 7.2 Hz, 2H), 2.393-2.251 (m, 14H), 2.215 (s, 6H), 1.968-1.839 (m, 4H), 1.832-1.711 (m, 2H), 1.666-1.538 (m, 8H), 1.357-1.225 (m, 33H), 1.010 (s, 6H), 0.877 (t, *J* = 6.6 Hz, 12H).

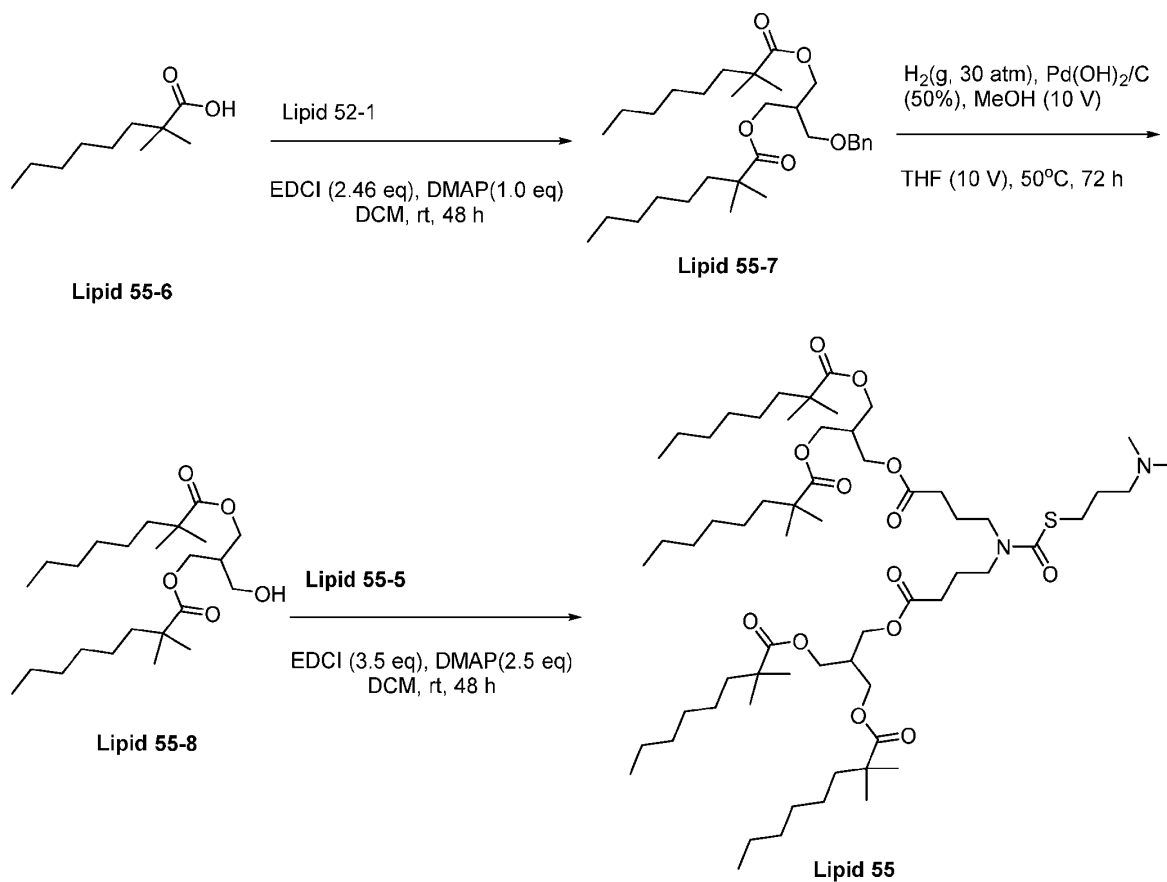
[0715] **Example 55. LIPID 55: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis (butanoyl))bis(oxy)) bis(methylene))bis(propane-2,1,3-triyl) tetrakis(2,2-dimethyloctanoate)**



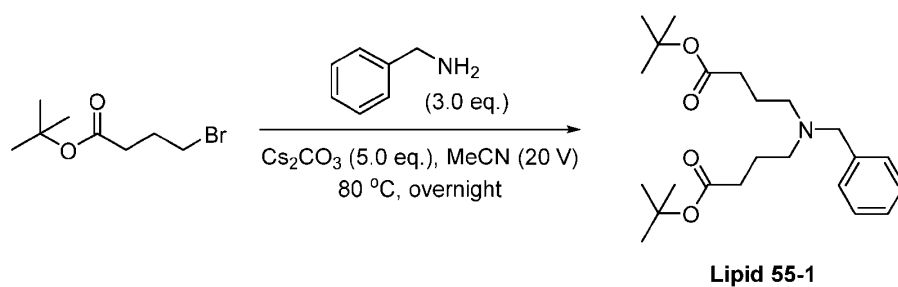
Lipid 55

[0716] General scheme





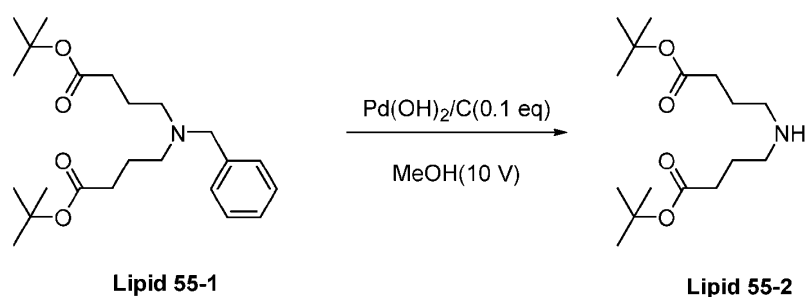
[0717] Synthesis of Lipid 55-1: di-tert-butyl 4,4'-(benzylazanediyl)dibutyrates



[0718] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was charged tert-butyl 4-bromobutanoate (62.2 g, 280.37 mmol, 3.0 equiv.), Cs_2CO_3 (152.3 mL, 467.29 mmol, 5.0 equiv.) and phenylmethanamine (10 g, 93.46 mmol, 1.00 equiv.) in MeCN (200 mL, 20 V) at 20 °C. The reaction solution was stirred

overnight at 80 °C. The resulting mixture was quenched with sat. NaHCO_3 (aq.) (150 mL, 15 V) and extracted with CH_2Cl_2 (2 x 300 mL, 60V). The combined organic layers were washed with brine (300 mL, 30 V), dried over anhydrous Na_2SO_4 . After filtration, the filtrate was concentrated under reduced pressure. The crude product was adsorbed on 100 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (800 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with PE/EA (volume ratio), (gradient from 100:0 to 40:60. After TLC analysis. (EA : PE = 1:2) fractions were pooled and evaporated under reduced pressure to afford **Lipid 55-1** (31.7 g, 87%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.15 min, m/z (Calcd.) 392.3, (found) 393.3 (M+H).

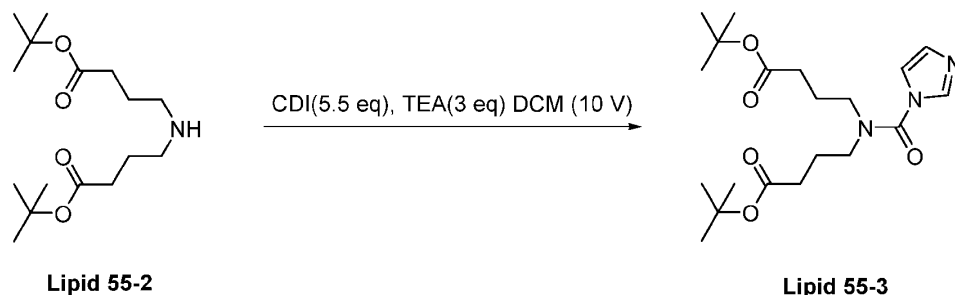
[0719] Synthesis of Lipid 55-2: di-tert-butyl 4,4'-azanediylidibutyrates



[0720] Into a 1L round-bottom flask were added **Lipid 55-1** (23.7 g, 63.082 mmol, 1 equiv.) and MeOH (237 mL) at room temperature followed by $\text{Pd}(\text{OH})_2/\text{C}$ (11 g, 0.5 w/w) in portions at room temperature under nitrogen atmosphere. The mixture was hydrogenated for 16 h at room temperature.

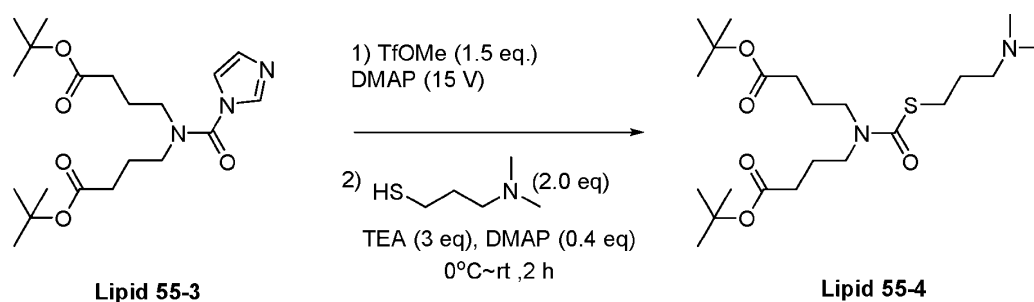
[0721] The reaction mixture was filtered through a pad of Celite. The crude product was adsorbed on 70 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (600 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with DCM/MeOH (volume ratio), (gradient from 100:0 to 90:10. After TLC analysis. (DCM : MeOH = 5:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 55-2** (15.5 g, 67%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.01 min, m/z (Calcd.) 301.2, (found) 303.2 (M+2H). This material was used as such in the next reaction.

[0722] **Synthesis of Lipid 55-3:** di-tert-butyl 4,4'-(((1H-imidazole-1-carbonyl)azanediyl)dibutyrat



[0723] Into a 250 mL 3-necked round-bottom flask were added **Lipid 55-2** (14.5 g, 48.105 mmol, 1 equiv.), CDI (42.90 g, 264.577 mmol, 5.5 equiv.) in DCM (145 mL) at room temperature. To the above mixture was added TEA (14.60 g, 144.315 mmol, 3.0 equiv.) dropwise at 0 °C. The resulting mixture was stirred for 24 h at room temperature. The resulting mixture was diluted with water (100 mL). The residue was acidified to pH 7 with HCl (aq., 4 M). The resulting mixture was extracted with CH₂Cl₂ (2 x 200 mL). The combined organic layers were washed with brine (2x100 mL), dried over anhydrous Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure. The resulting **Lipid 55-3** (20.4 g, 77%) as a yellow oil was used as such in the reaction.

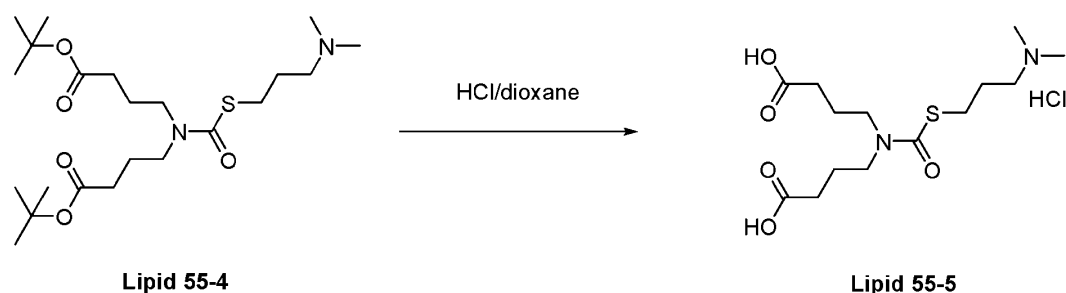
[0724] **Synthesis of Lipid 55-4:** di-tert-butyl 4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl dibutyrat



[0725] Into a 1000 mL round-bottom flask were added **Lipid 55-3** (20.4 g, 51.580 mmol, 1.00 equiv.) in DCM (305.99 mL) at 0°C followed by TfOMe (13.08 g, 77.370 mmol, 1.5 equiv.)

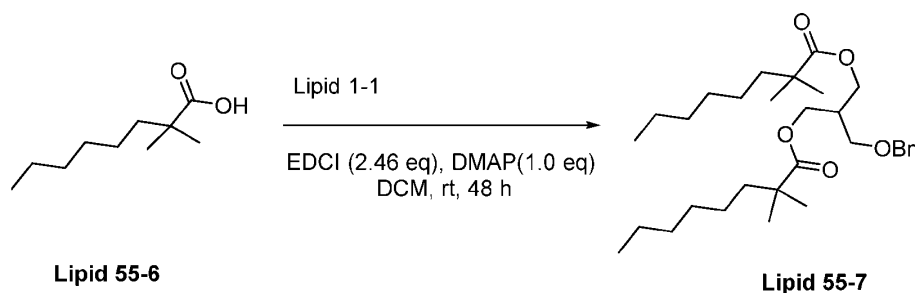
dropwise. The resulting mixture was stirred for an additional 2h at 0 °C. To this mixture was added TEA (15.66 g, 154.740 mmol, 3 equiv.) and DMAP (2.52 g, 20.632 mmol, 0.4 equiv.) at 0 °C followed by 3-(dimethylamino)propane-1-thiol (12.30 g, 103.160 mmol, 2 equiv.) dropwise. The resulting mixture was stirred for 2h at room temperature. The resulting mixture was washed with 5% 400 mL of citric acid and 5% 400 mL of sodium carbonate. The organic phase was collected and dried over anhydrous Na₂SO₄, the solid was filtered out. The crude product was adsorbed on 40 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (400 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with PE/EA (volume ratio), (gradient from 100:0 to 50:50. After TLC analysis. (EA : PE = 2:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 55-4** (10 g, 54%) as yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 0.77 min, m/z (Calcd.) 334.2, (found) 335.3 (detected as corresponding dicarboxylic acid). HPLC purity 95.5%.

[0726] Synthesis of Lipid 55-5: 4,4'-((((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)dibutyric acid hydrochloride



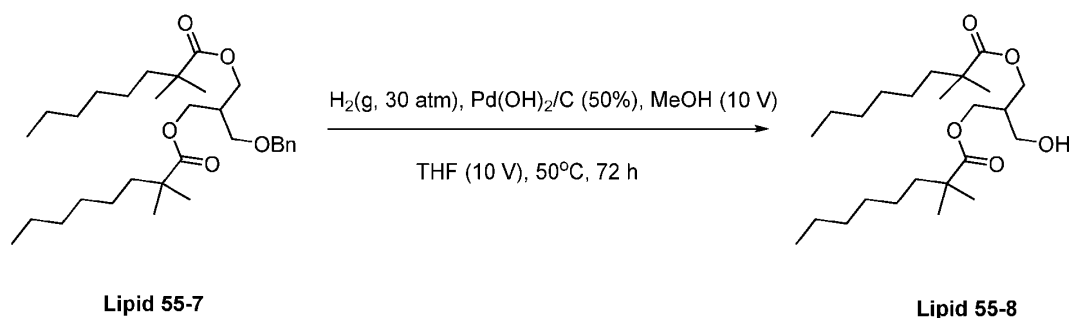
[0727] Into a 100 mL 3-necked round-bottom flask were added **Lipid 55-4** (9 g, 18.86 mmol, 1 equiv.) in HCl/dioxane (4M, 90 mL) at room temperature. The resulting mixture was stirred for 4 h at room temperature and concentrated under reduced pressure. This resulted in **Lipid 55-5** (6.0 g, 84.6%) and used in the next step directly without purification. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 0.91 min, m/z (Calcd.) 334.2, (found) 335.3.

[0728] Synthesis of Lipid 55-7: 2-((benzyloxy)methyl)propane-1,3-diyl bis(2,2-dimethyloctanoate)



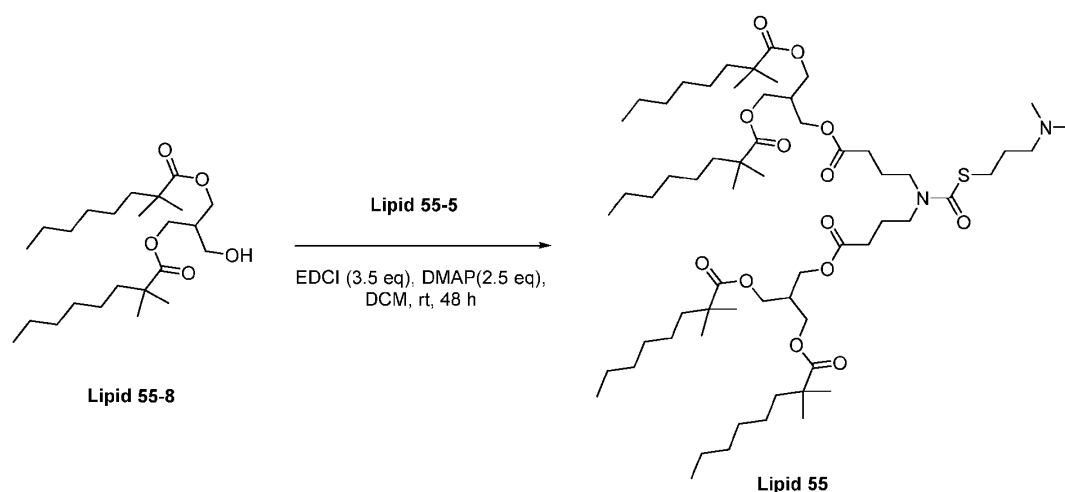
[0729] Into a 2 L round-bottom flask were added Lipid 52-1 (10.9 g, 55.543 mmol, 1.00 equiv) and DCM (400 mL) at room temperature. Followed by **Lipid 55-6** (Commercially available, 20 g, 116.1 mmol, 2.09 equiv) and DMAP (6.8 g, 55.7 mmol, 1.00 equiv) in portions at room temperature. To this was added EDCI (26.2 g, 136.672 mmol, 2.46 equiv) in portions at room temperature. The resulting mixture was stirred for 48 h at 25 °C. The reaction was quenched with Water at 25 °C. The resulting mixture was extracted with DCM (2 x 1 L). The combined organic layers were washed with brine (2x500 mL), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. The crude product was adsorbed on 30 g of silica gel using DCM as solvent (type: ZCX-2, 100-200 mesh, 2.00 w./w.) and purified on a silica gel column (300 g of silica gel type: ZCX-2, 100-200 mesh, 10.00 w./w.) by eluting with PE/EA (volume ratio), (gradient from 100:0 to 80:20. After TLC analysis. (EA : PE = 4:1) fractions were pooled and evaporated under reduced pressure to afford **Lipid 55-7** (14 g, 50%) as yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.82 min, m/z (Calcd.) 504.3, (found) 527.3 (M+Na). HPLC purity 96.5%.

[0730] **Synthesis of Lipid 55-8:** 2-(hydroxymethyl)propane-1,3-diyl bis(2,2-dimethyloctanoate)



[0731] To a solution of **Lipid 55-7** (10 g, 19.81 mmol, 1 equiv) in THF (80 mL) and MeOH (80 mL) was added Pd(OH)₂/C (10 g, 1 w/w) in a pressure tank. The mixture was hydrogenated at room temperature under 30 psi of hydrogen pressure for 72h. The reaction was filtered through a Celite pad and concentrated under reduced pressure. This resulted in **Lipid 55-8** (7 g, 85.22%) as a light-yellow oil which was used in the next reaction without further purification. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.62 min, m/z (Calcd.) 414.5, (found) 415.5 (M+H). HPLC purity 91.8%.

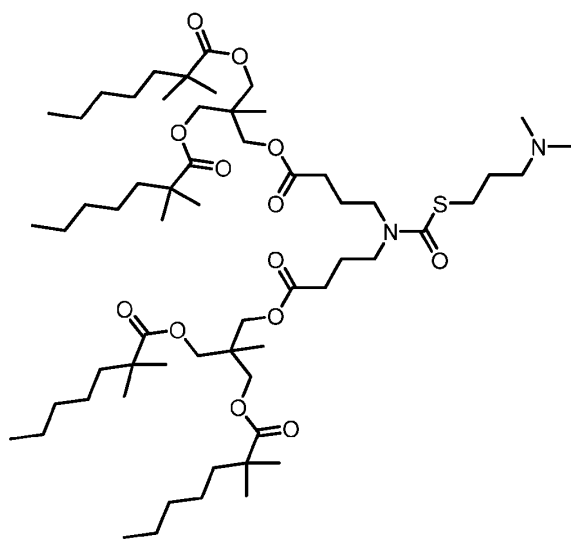
[0732] **Synthesis of Lipid 55:** ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis (oxy))bis(methylene))bis(propane-2,1,3-triyl) tetrakis(2,2-dimethyloctanoate)



[0733] Into a 250 mL round-bottom flask were added DCM (90 mL) **Lipid 55-8** (3 g, 7.235 mmol, 2.5 equiv.), DMAP (0.884 g, 7.236 mmol, 2.5 equiv.), Lipid 4-5 (0.968 g, 2.894 mmol, 1.0 equiv.) and EDCI (1.94 g, 10.129 mmol, 3.5 equiv.) at 25°C. The resulting mixture was stirred for 48 h at 25 °C. The resulting mixture added water (50 mL) and extracted with heptane (2 x 50 mL). The combined organic layers were washed with water (2x50 mL), dried over anhydrous Na₂SO₄, filtered and the filtrate was concentrated under reduced pressure. The residue was purified by Prep-HPLC with the following conditions: column, WelFlash XSelect CSH Prep C18 5um OBD, Regular 30*150mm Column; Flow: 55ml/min; mobile phase, MeCN/i-PrOH

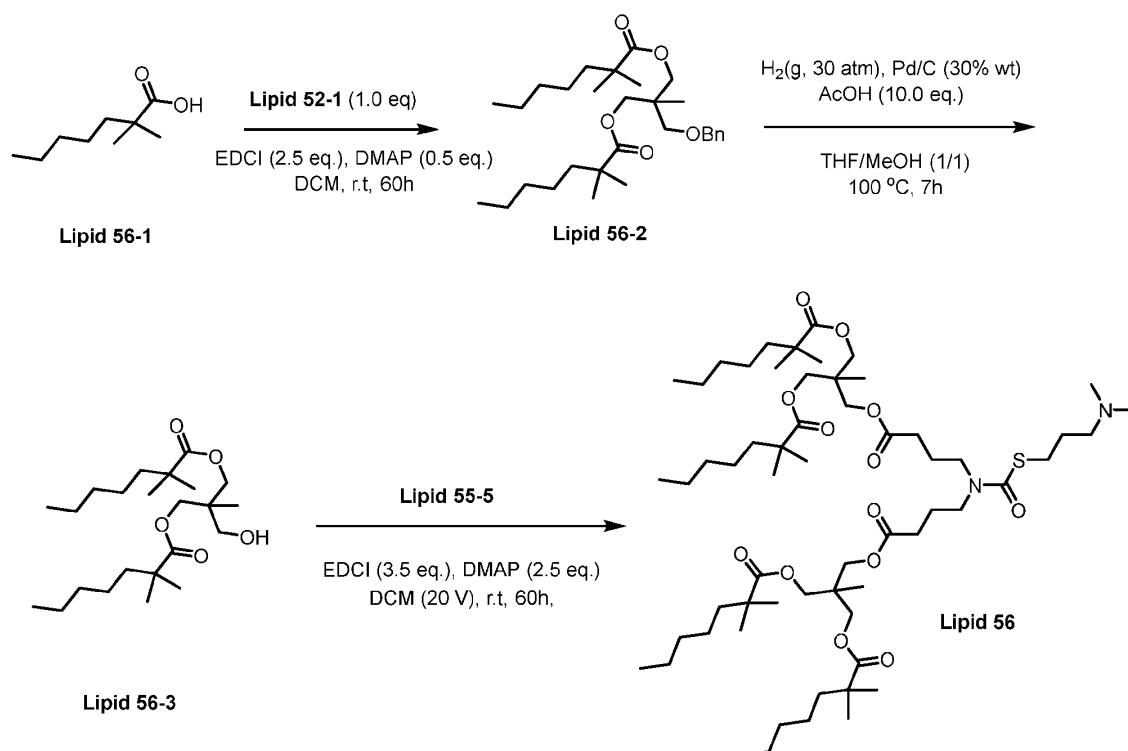
(1/1) in Water (0.1% TFA), 50% to 90% gradient in 20 min; detector, ELSD. The product containing fractions were pooled and concentrated under vacuum. The resulting material was diluted with 30 mL of n-heptane washed with MeOH/H₂O (2:1) (1 x 30 mL) and the n-heptane layer was collected and washed with H₂O (1 x 30 mL), dried over anhydrous Na₂SO₄, filtered, the filtrate was concentrated under vacuum to obtained **Lipid 55** (1.0 g, 12.2%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.47 min, m/z (Calcd.) 1126.8, (found) 1127.8 (M+H). HPLC purity 98.6%. ¹H NMR-PH-ARC-ATX-299-0: (300 MHz, CDCl₃, *ppm*) δ 4.147-4.101 (m, 12H), 3.374 (s, 4H), 2.941-2.893 (m, 2H), 2.426-2.249 (m, 14H), 1.995-1.764 (m, 7H), 1.529-1.476 (m, 8H), 1.314-1.247 (m, 31H), 1.199-1.160 (m, 28H), 1.958-1.809 (m, 12H).

[0734] Example 46. LIPID 56: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl) bis(butanoyl))bis(oxy)) bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2,2-dimethylheptanoate)

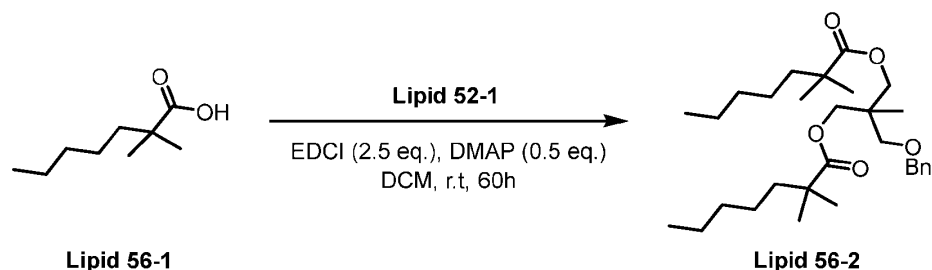


Lipid 56

[0735] General scheme

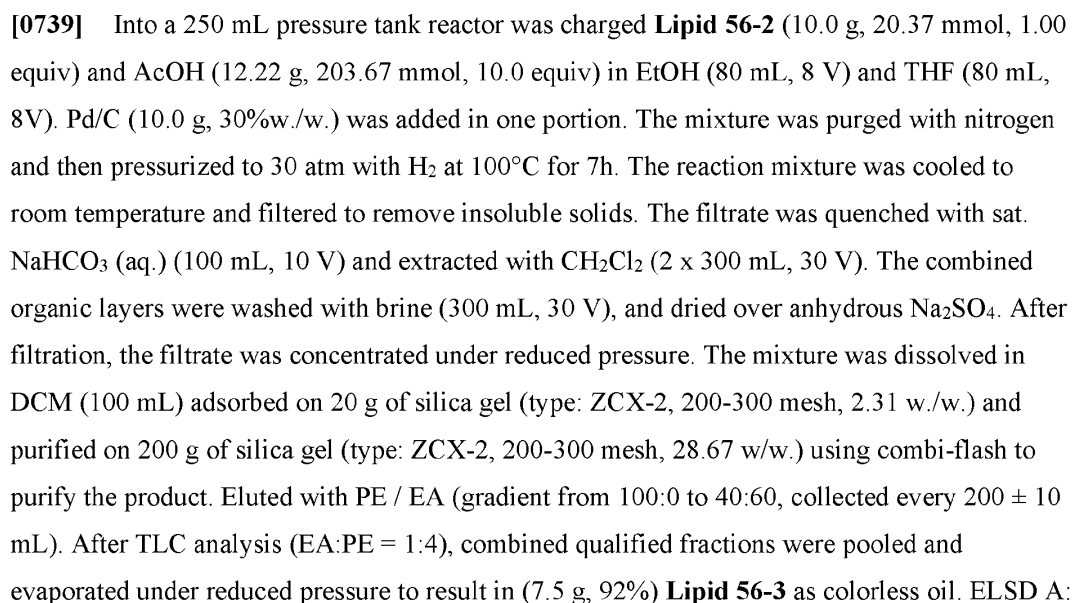


[0736] **Synthesis of Lipid 56-2:** 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(2,2-dimethyl heptanoate)



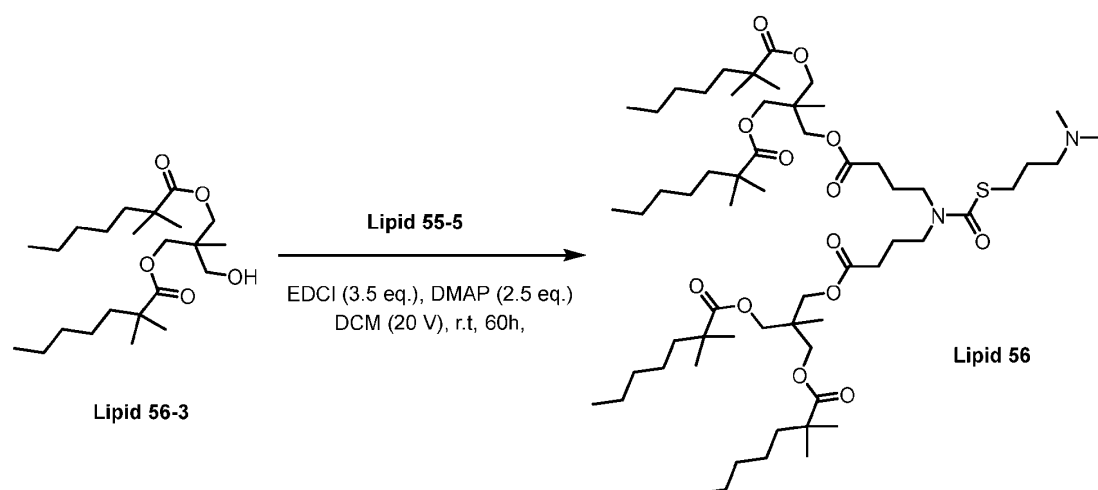
[0737] Into a 500 L single-neck round-bottom flask was charged **Lipid 56-1** (6.90 g, 32.90 mmol, 1.0 equiv.), EDCI (15.80 g, 82.28 mmol, 2.50 equiv.), DMAP (2.01 g, 16.46 mmol, 0.5 equiv.) and **Lipid 52-1** (13 g, 82.28 mmol, 2.50 equiv.) in DCM (200 mL). The reaction solution was stirred for 60 h at room temperature. The resulting mixture was washed with of sat. NaHCO₃ (aq.) (150 mL, 11.5 V) and extracted with CH₂Cl₂ (2 x 300 mL, 46V). The combined organic layers were washed with brine (300 mL, 23V), and dried over anhydrous Na₂SO₄, filtered and

[0738] Synthesis of Lipid 56-3: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(2,2-dimethyl heptanoate)



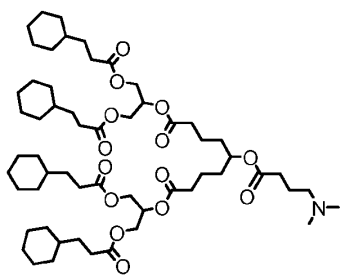
water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.62 min, m/z (Calcd.) 400.3, (found) 401.3 (M+H). HPLC purity 96.8%.

[0740] Synthesis of Lipid 56: ((4,4'-(((3-(Dimethylamino)propyl)thio)carbonyl)azanediyl)bis(butanoyl))bis(oxy)) bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(2,2-dimethylheptanoate)

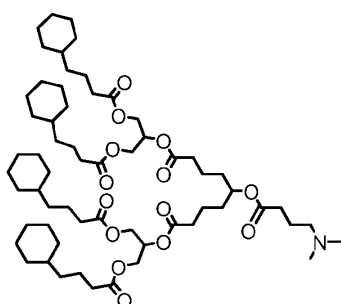


[0741] To a stirred solution of **Lipid 56-3** (2.5 g, 6.24 mmol, 2.5 equiv.) and **Lipid 55-5** (0.93 g, 2.49 mmol, 1 equiv.) in DCM (25 mL) added EDC.HCl (1.36 g, 8.73 mmol, 3.5 equiv.) and DMAP (0.76 g, 6.24 mmol, 2.5 eq) in portions at room temperature under nitrogen atmosphere. The reaction mixture was stirred for 60h at room temperature. The resulting mixture added water (50 mL) and extracted with heptane (2 x 50 mL). The combined organic layers were washed with water (2x50 mL), and dried over anhydrous Na₂SO₄, filtered and evaporated. The residue was dissolved in DCM (10 mL) adsorbed on 5 g of silica gel (type: ZCX-2, 200-300 mesh, 2.31 w./w.) and purified on 50 g of silica gel (type: ZCX-2, 200-300 mesh, 28.67 w/w.) using combi-flash to purify the product. Eluted with PE / EA (gradient from 100:0 to 50:50, collected every 200 ± 10 mL). After TLC analysis (EA:PE = 1:4), combined qualified fractions were pooled and evaporated under reduced pressure. The product was dissolved in n-heptane (45 mL) and washed with MeOH/H₂O (4:1) (2 x 45 mL), and dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure to afford **Lipid 56** (1.0 g, 15%) as a light yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.48

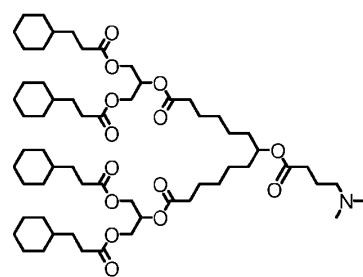
min, m/z (Calcd.) 1098.7, (found) 1099.7 (M+H). HPLC purity 96.4%. ¹H-NMR-PH-ARC-ATX-300-0: (400 MHz, CDCl₃, ppm): δ 3.998 (d, *J* = 17.4 Hz, 12H), 3.381 (s, 4H), 2.925 (t, *J* = 7.3 Hz, 2H), 2.360 (d, *J* = 6.7 Hz, 6H), 2.249 (d, *J* = 2.7 Hz, 6H), 1.908 (d, *J* = 9.2 Hz, 4H), 1.837-1.740 (m, 2H), 1.571-1.463 (m, 6H), 1.168 (s, 50H), 1.035 (s, 6H), 0.876 (t, *J* = 6.9 Hz, 12H).



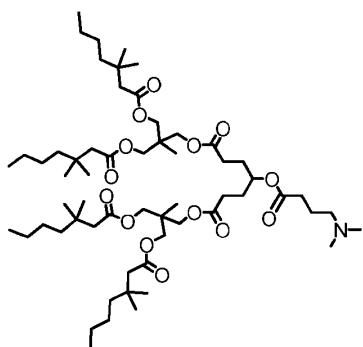
LIPID 57



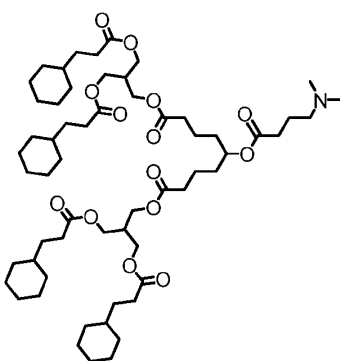
LIPID 58



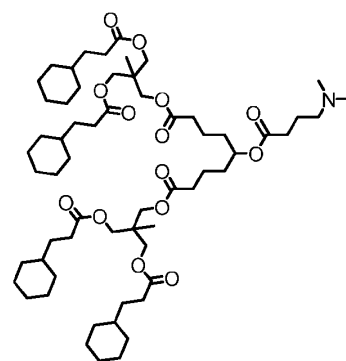
LIPID 59



LIPID 60

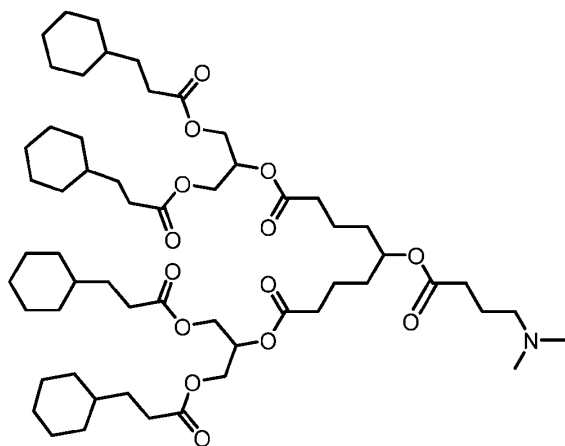


LIPID 61



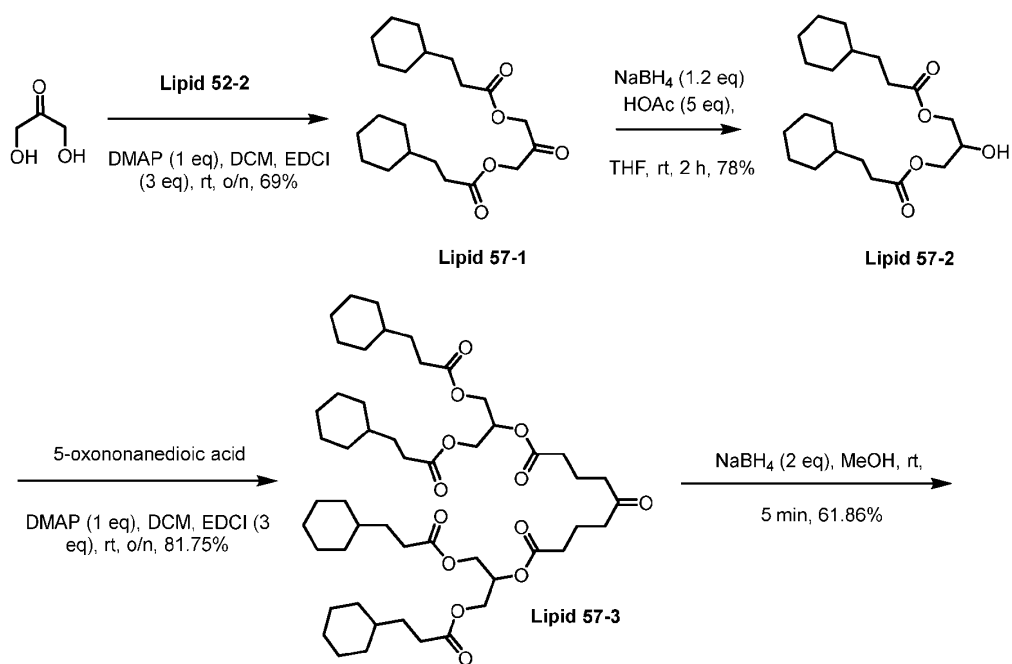
LIPID 62

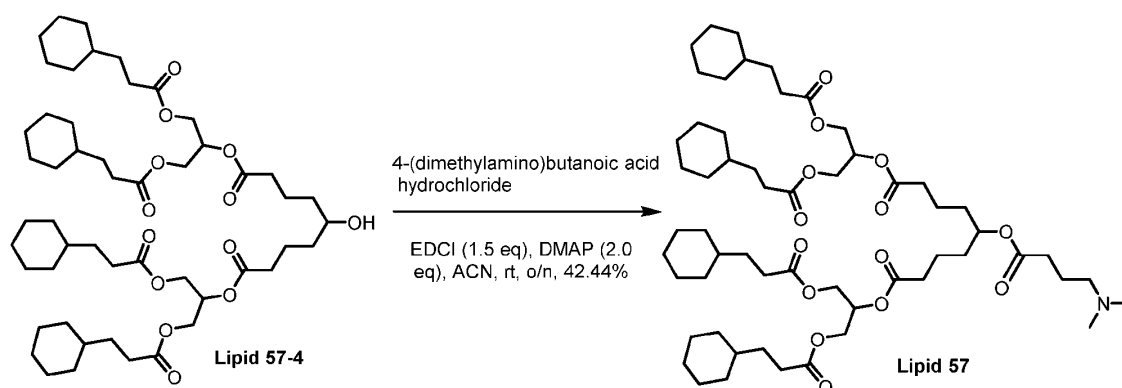
[0742] **Example 47. Lipid 57: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 5-((4-(dimethyl amino)butanoyl)oxy)nonanedioate**



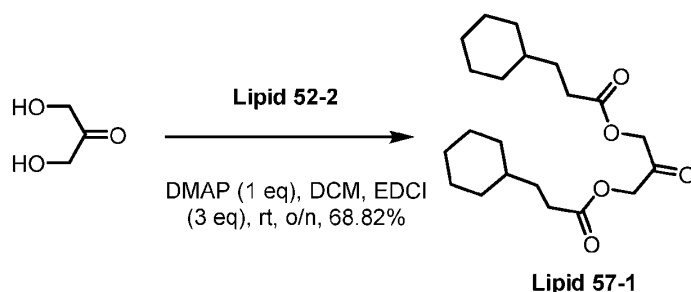
Lipid 57

[0743] General scheme





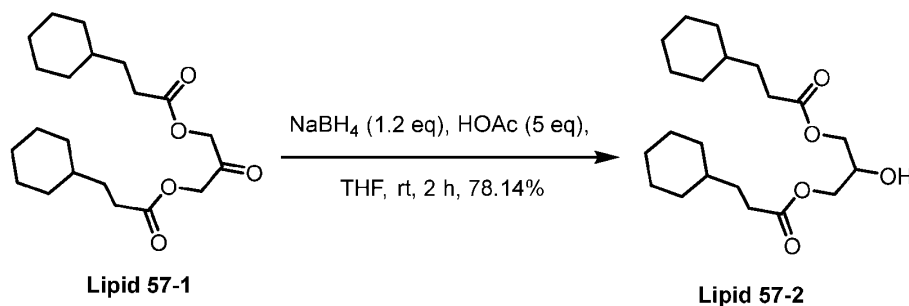
[0744] Synthesis of Lipid 57-1: 2-oxopropane-1,3-diyl bis(3-cyclohexylpropanoate)



[0745] Into a 1-L round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 1,3-dihydroxypropan-2-one (10 g, 111.02 mmol, 1 equiv), **Lipid 52-2** (38.16 g, 244.23 mmol, 2.2 equiv), DMAP (13.56 g, 111.02 mmol, 1 equiv), DCM (500 mL) and EDCI (63.84 g, 333.05 mmol, 3 equiv) at room temperature. The resulting solution was stirred overnight at room temperature. The reaction was then quenched by the addition of 100 mL of 5% citric acid. The resulting solution was extracted with 2 x 500 mL of DCM and the organic layers combined and dried over anhydrous sodium sulfate (200 g) and concentrated. The residue was dissolved in DCM (200 mL) and adsorbed onto 90 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w/w.), and purified on a silica gel column (450 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w/w.) by eluting with n-heptane/EA (gradient from 100:0 to 95:5 and collect product eluent (gradient: n-heptane/EA=95/5), collected every 200 ± 20 mL). After TLC (EA/n-heptane=1/8, R_f = 0.3) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 57-1** (28 g, 76.5 mmol, yield: 69%) as a white solid. ELSD A:

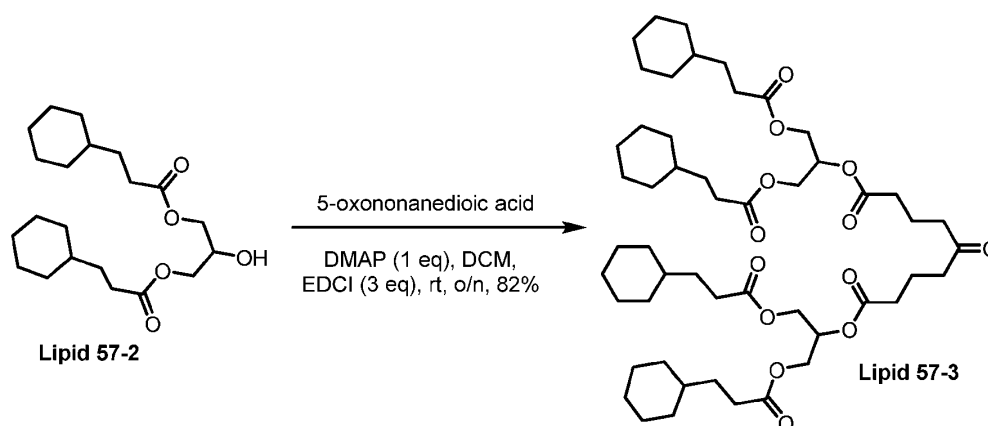
water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.55 min, m/z (Calcd.) 366.3, (found) 367.3 (M+H). HPLC purity 89%.

[0746] Synthesis of Lipid 57-2: 2-hydroxypropane-1,3-diyl bis(3-cyclohexylpropanoate)



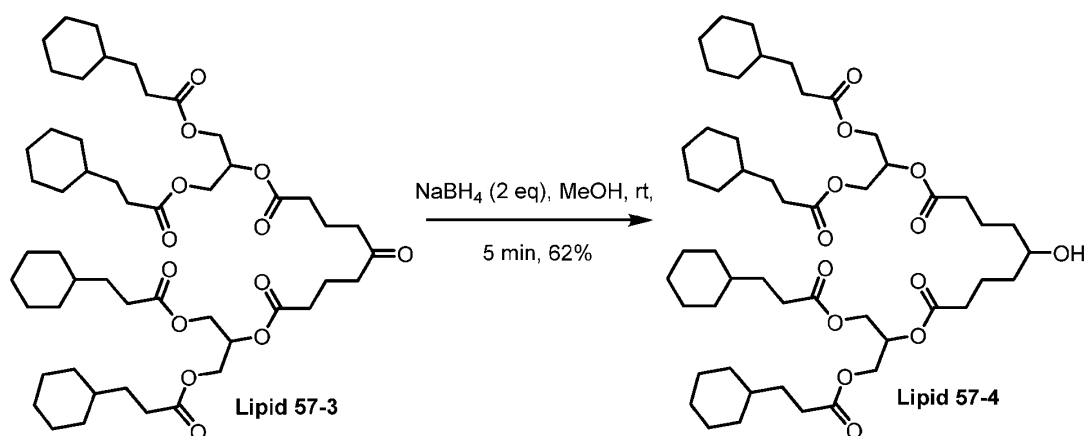
[0747] Into a 500 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **Lipid 57-1** (28 g, 76.4 mmol, 1 equiv), THF (280 mL) and HOAc (22.94 g, 382 mmol, 5 equiv) at room temperature. To the above mixture was added NaBH₄ (3.47 g, 91.68 mmol, 1.2 equiv) with stirring at room temperature. The resulting solution was stirred at room temperature for 2 h. The resulting mixture was quenched by the addition of 100 mL of 5% citric acid at 0°C. The resulting mixture was extracted with EA (2 x 500 mL). The combined organic layer was washed with sat. aqueous NaHCO₃ (100 mL), water (200 mL), brine (200 mL), and dried over anhydrous Na₂SO₄ (200 g). After filtration, the filtrate was concentrated under reduced pressure. The residue was dissolved in DCM (200 mL) and adsorbed onto 90 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (450 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with n-heptane/EA (gradient from 100:0 to 95:5 and collect product eluent (gradient: n-heptane/EA=90:10), collected every 200 ± 20 mL). After TLC (EA/n-heptane=1/8, R_f = 0.3) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 57-2** (28 g, 76.5 mmol, yield: 69%) as a white solid. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.45 min, m/z (Calcd.) 368.3, (found) 351.3 (M-OH). HPLC purity 94.1%.

[0748] **Synthesis of Lipid 57-3:** bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 5-oxononanedioate



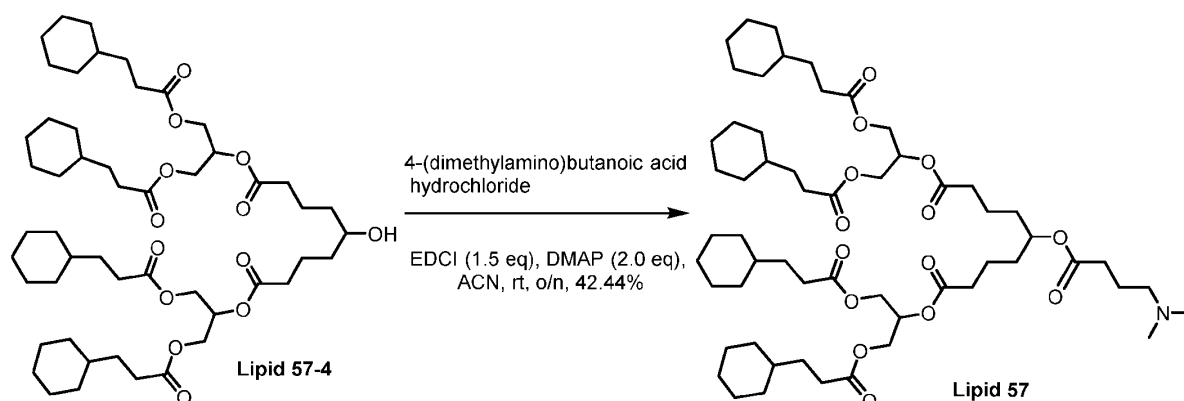
[0749] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 5-oxononanedioic acid (1.12 g, 5.55 mmol, 1 equiv), **Lipid 57-2** (4.50 g, 12.22 mmol, 2.2 equiv), DMAP (0.68 g, 5.55 mmol, 1 equiv) and DCM (20 mL) at room temperature. To the above mixture was added EDCI (3.19 g, 16.66 mmol, 3 equiv) while stirring at room temperature. The resulting solution was stirred overnight at room temperature. The reaction was then quenched by the addition of 50 mL of 5% citric acid, the resulting solution was extracted with 2 x 50 mL of DCM and the organic layers combined and dried over anhydrous sodium sulfate (30 g) and concentrated. The residue was dissolved in DCM (200 mL) and adsorbed onto 90 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (450 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with n-heptane/EA (gradient from 100:0 to 95:5 and collect product eluent (gradient: n-heptane/EA=90:10), collected every 200 ± 20 mL). After TLC (EA/n-heptane=1/8, R_f = 0.3) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 57-3** (4.1 g, yield: 82%). ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.85 min, m/z (Calcd.) 903.6, (found) 925.6 (M-H+Na). HPLC purity 94.8%.

[0750] **Synthesis of Lipid 57-4:** bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 5-hydroxynonanedioate



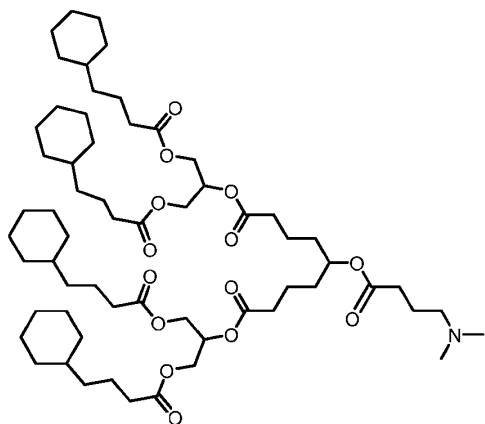
[0751] Into a 250 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **Lipid 57-3** (4 g, 4.42 mmol, 1 equiv) and MeOH (60 mL) at room temperature. To the above mixture was added NaBH₄ (0.34 g, 8.86 mmol, 2 equiv) with stirring at room temperature. The resulting solution was stirred at room temperature for 5 min. The resulting mixture was quenched by the addition of 5% citric acid at 0°C. The resulting mixture was extracted with EA (2 x 100 mL). The combined organic layer was washed with sat. aqueous NaHCO₃ (100 mL), water (100 mL), brine (50 mL), and dried over anhydrous Na₂SO₄ (50 g). After filtration, the filtrate was concentrated under reduced pressure. The residue was dissolved in DCM (200 mL) and adsorbed onto 8 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w/w.), and purified on a silica gel column (80 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w/w.) by eluting with n-heptane/EA (gradient from 100:0 to 80:20 and collect product eluent (gradient: n-heptane/EA=90:10), collected every 200 ± 20 mL). After TLC (EA/n-heptane=1/6, R_f = 0.3) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 57-4** (2.48 g, yield: 62%). ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.82 min, m/z (Calcd.) 905.6, (found) 927.6 (M-H+Na). HPLC purity 93.9%.

[0752] Synthesis of Lipid 57: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl)5-((4-(dimethyl amino)butanoyl)oxy)nonanedioate



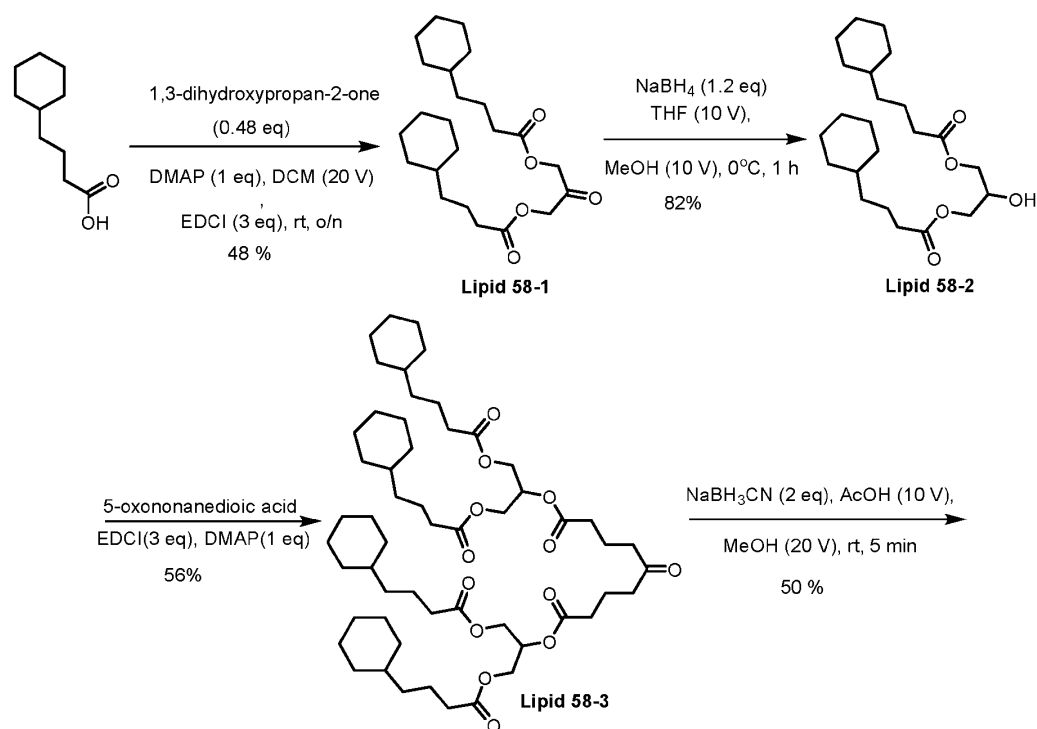
[0753] Into a 100 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed **Lipid 57-4** (2.48 g, 2.74 mmol, 1 equiv), 4-(dimethylamino)butanoic acid hydrochloride (0.55 g, 3.29 mmol, 1.2 equiv), DMAP (0.67 g, 5.48 mmol, 2 equiv) and ACN (50 mL). This was followed by the addition of EDCI (0.79 g, 4.11 mmol, 1.5 equiv) with stirring at room temperature. The resulting solution was stirred overnight at room temperature. The reaction was then quenched by the addition of 50 mL of 5% NaHCO₃, the resulting solution was extracted with 2 x 100 mL of EA and the organic layers combined and dried over anhydrous sodium sulfate and concentrated. The crude product was dissolved in n-heptane (200 mL) and washed with 2 x 5 mL of ACN/H₂O = 3/1 and 2 x 5 mL of ACN, and the n-heptane layer was dried by over anhydrous sodium sulfate and concentrated to obtain **Lipid 57** (1.08 g, yield: 42%) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.24 min, m/z (Calcd.) 1017.6, (found) 1018.6 (M+H). HPLC purity 96.5%. ¹H-NMR-PH-ARC-ATX-301-0 (300 MHz, CDCl₃) δ: 5.285-5.218 (m, 2H), 4.900 (brs, 1H), 4.338-4.276 (m, 4H), 4.174-4.110 (m, 4H), 2.377-2.231 (m, 22H), 1.849-1.776 (m, 2H), 1.722-1.608 (m, 28H), 1.561-1.486 (m, 8H), 1.261-1.082 (m, 16H), 0.954-0.844 (m, 8H).

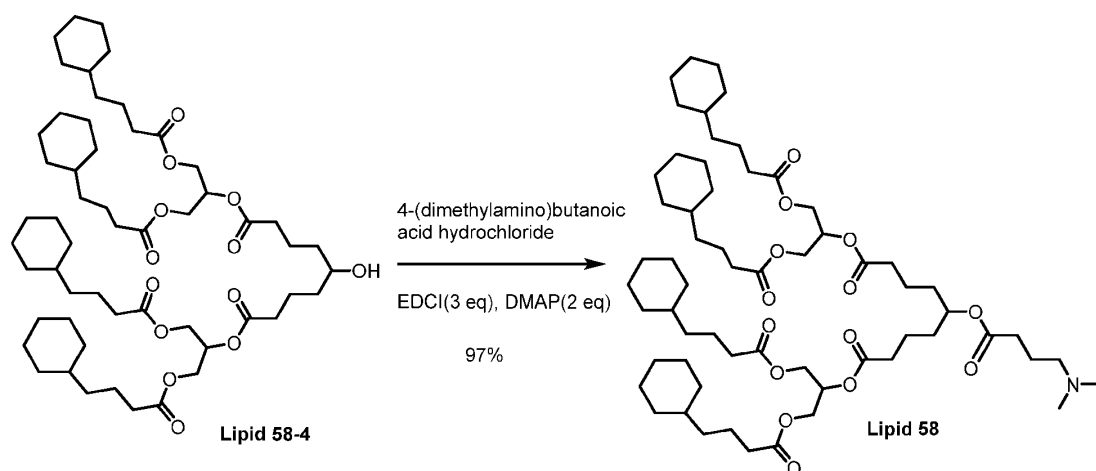
[0754] **Example 48. Lipid 58: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 5-((4-(dimethyl amino)butanoyl)oxy)nonanedioate**



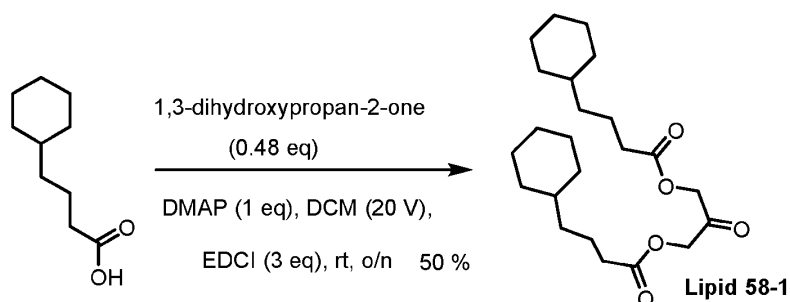
Lipid 58

[0755] General scheme





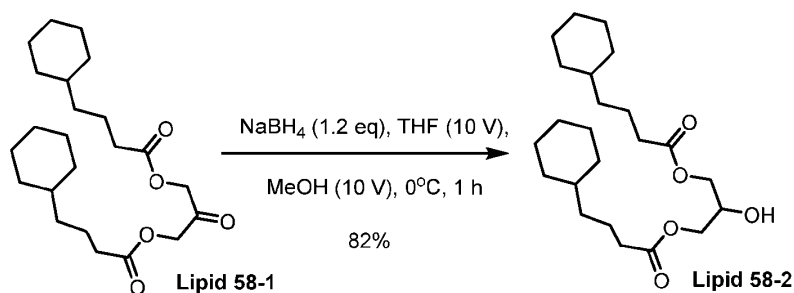
[0756] Synthesis of Lipid 58-1: 2-oxopropane-1,3-diyl bis(4-cyclohexylbutanoate)



[0757] Into a 500 ml 3-necked round bottom flask was added 4-cyclohexylbutanoic acid (25 g, 73.42 mmol, 2.2 equiv), 1,3-dihydroxypropan-2-one (5.61 g, 33.37 mmol, 1 equiv), DMAP (4.08 g, 33.37 mmol, 1 equiv), EDCI (19.19 g, 100.12 mmol, 3 equiv) and DCM (250 mL) at room temperature. The mixture was stirred overnight at room temperature. The resulting solution was diluted with 250 ml of DCM. The resulting solution was washed with 1 x 300 mL of 5 % citric acid in water, 2 x 300 mL of water, and 1 x 500 mL of brine, and dried with anhydrous Na₂SO₄ and filtered. The residue was adsorbed onto 100 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (600 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with n-heptane/EA (gradient from 100:0 to 80:20 and collect product eluent (gradient: n-heptane/EA=90:10), collected every 200 ± 20 mL). After TLC (EA/n-heptane=1/6, R_f = 0.3) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 58-1** (7.2 g, yield: 50%). ELSD A: water/0.05% TFA: B:

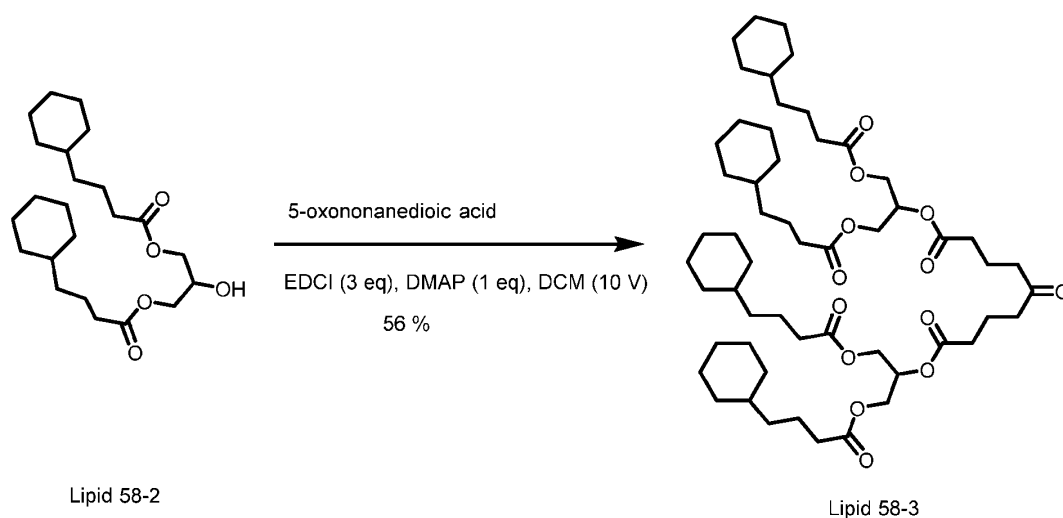
CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 0.94 min, m/z (Calcd.) 394.3, (found) 395.3 (M+H). HPLC purity 97.1%.

[0758] Synthesis of Lipid 58-2: 2-hydroxypropane-1,3-diyl bis(4-cyclohexylbutanoate)



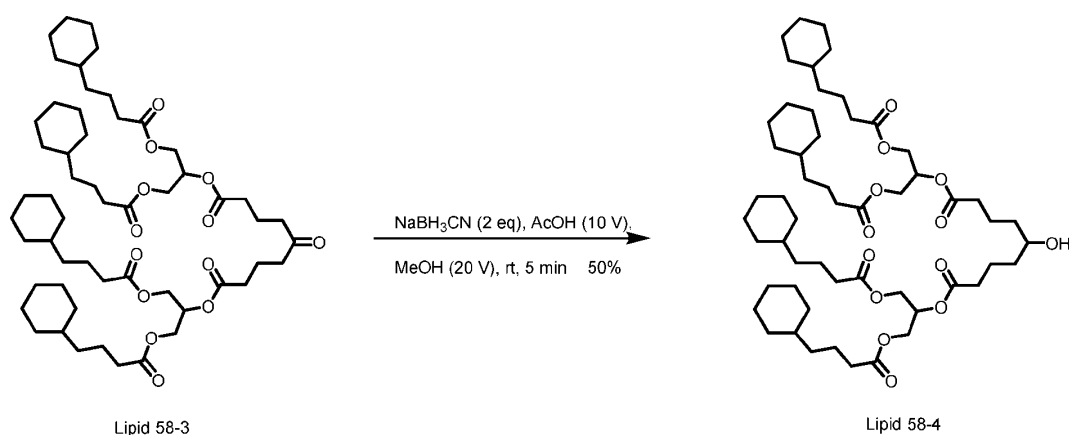
[0759] Into a 500 ml 3-necked round bottom was added **Lipid 58-1** (7.1 g, 17.995 mmol, 1 equiv), HOAc (70 mL), THF (100 mL) at room temperature. To the above mixture was added NaBH₄ (0.82 g, 21.594 mmol, 1.2 equiv) in portions over 10 min at 0 °C. The mixture was stirred for 1 h at 0 °C. The reaction was then quenched by the addition of 100 mL of saturated NH₄Cl. The resulting solution was extracted with 3 x 200 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate and concentrated. The residue was adsorbed onto 100 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (600 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with DCM/MeOH (gradient from 100:0 to 95:5. After TLC (EA/n-heptane=1:1) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 58-2** (5.87 g, yield: 82%). ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.55 min, m/z (Calcd.) 396.3, (found) 379.3 (M-OH). HPLC purity 86.5%.

[0760] Synthesis of Lipid 58-3: bis(1,3-bis((4-cyclohexylbutanoyl)oxy)propan-2-yl) 5-oxononanedioate



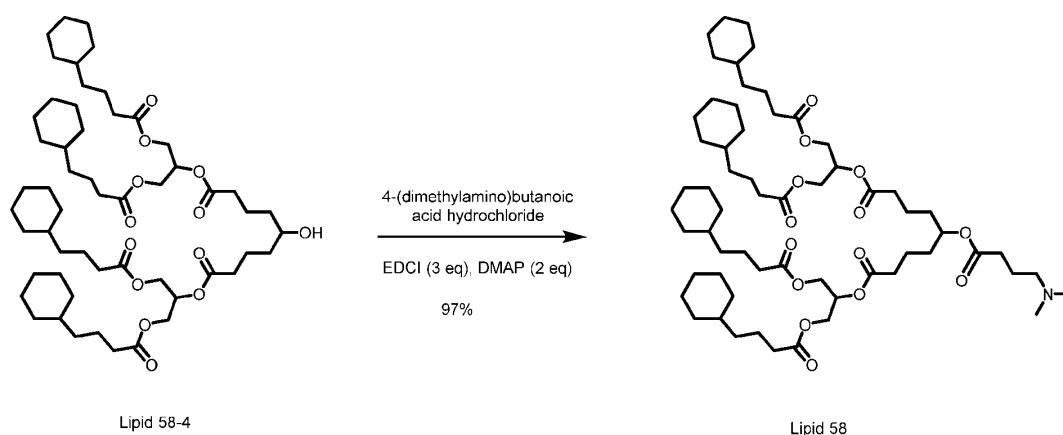
[0761] Into a 250 mL round bottom were added **Lipid 58-3** (5.87 g, 14.802 mmol, 2.2 equiv), **Lipid 57-4** (1.36 g, 6.728 mmol, 1 equiv), DMAP (0.82 g, 6.728 mmol, 1 equiv), DCM (80 mL), and EDCI (19.19 g, 100.120 mmol, 3 equiv) at room temperature. The resulting mixture was stirred overnight at room temperature. The resulting solution was diluted with 100 mL of DCM. The resulting solution was washed with 1 x 100 mL of 5 % citric acid in water, 2 x 100 mL of water, and 1 x 200 mL of brine, dried with anhydrous Na_2SO_4 . The residue was adsorbed onto 20 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (100 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with PE/EA (gradient from 100:0 to 12:88). After TLC (PE:EA 12:88) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 58-3** (4 g, yield: 56%). ELSD A: water/0.05% TFA: B: CH_3CN /0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 2.02 min, m/z (Calcd.) 958.6, (found) 981.6 (M+Na). HPLC purity 86.5%.

[0762] **Synthesis of Lipid 58-4:** bis(1,3-bis((4-cyclohexylbutanoyl)oxy)propan-2-yl) 5-hydroxynonanedioate



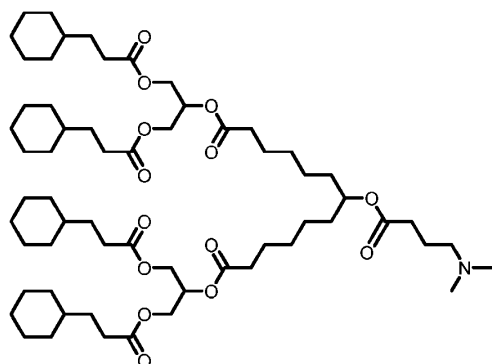
[0763] Into a 250 mL three necked round bottom flask were added **Lipid 58-3** (4.0 g, 4.17 mmol, 1 equiv) , AcOH (40 mL, 698.06 mmol, 167.41 equiv), MeOH (80 mL) at 0°C. To this was added the NaBH₄ (0.32 g, 8.34 mmol, 2 equiv) in portion with stirred at 0 °C. The resulting mixture was stirred for 7 min at 0 °C. The reaction was then quenched by the addition of 100 mL of saturated NH₄Cl. The resulting solution was extracted with 3 x 200 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate and concentrated. The residue was adsorbed onto 30 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (180 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with heptane/EA (gradient from 100:0 to 60:40. After TLC (PE:EA 12:88) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 58-4** (2 g, yield: 50%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.99 min, m/z (Calcd.) 960.6, (found) 983.6 (M+Na). HPLC purity 91.4%.

[0764] Synthesis of Lipid 58: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 5-((4-(dimethyl amino)butanoyl)oxy)nonanedioate

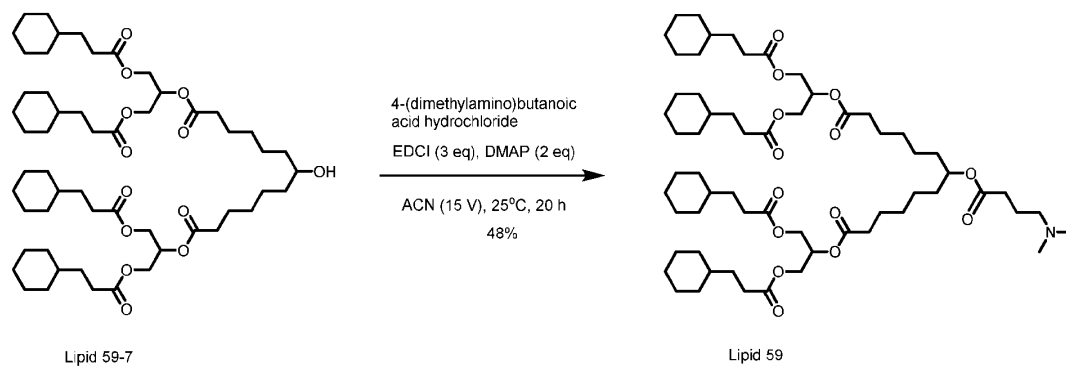
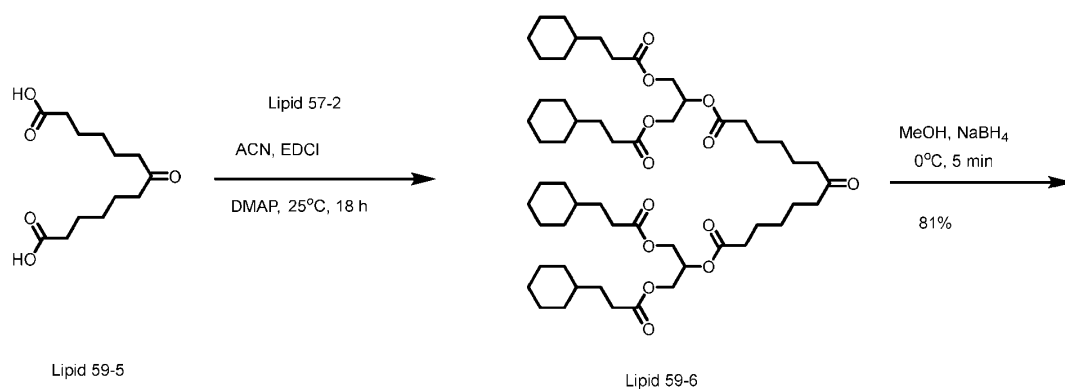


[0765] Into a 250 mL three necked round bottom flask were added **Lipid 58-4** (2.0 g, 2.080 mmol, 1 equiv), 4-(dimethylamino)butanoic acid hydrochloride (0.42 g, 2.496 mmol, 1.2 equiv), DMAP (0.56 g, 4.576 mmol, 2.2 equiv), EDCI (1.20 g, 6.240 mmol, 3 equiv) and ACN (40 mL) at room temperature. The resulting mixture was stirred overnight at room temperature. To the mixture was added 100 ml ethyl acetate and washed with water (3 x 50 mL) and brine (1 x 50 mL), dried with anhydrous Na₂SO₄, filtered and concentrated under vacuum. The residue was adsorbed onto 20 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w/w.), and purified on a silica gel column (180 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w/w.) by eluting with DCM/MeOH (gradient from 100:0 to 95:5. After TLC (heptane:EA 5:1) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 58** (1.3 g, yield: 97%) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.99 min, m/z (Calcd.) 960.6, (found) 983.6 (M+Na). HPLC purity 91.4%. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.21 min, m/z (Calcd.) 1073.6, (found) 1074.6 (M+H). HPLC purity 97.1%. ¹H NMR-PH-ARC-ATX-302-4 (300 MHz, CDCl₃) δ: 5.260-5.194 (m, 2H), 4.892 (brs, 1H), 4.331-4.269 (m, 4H), 4.167-4.105 (m, 4H), 2.715-2.540 (m, 8H), 2.411-2.344 (m, 2H), 2.318-2.268(m, 12H), 2.033-1.987 (m, 2H), 1.708-1.592 (m, 34H), 1.455-1.407(m, 26H), 1.355-1.331 (m, 8H).

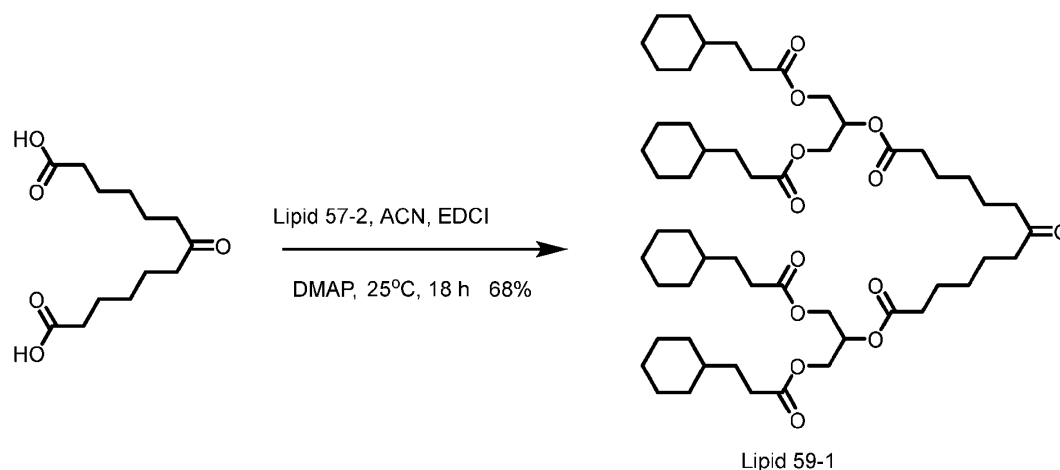
[0766] **Example 49. Lipid 59: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 7-((4-(dimethyl amino)butanoyl)oxy)tridecanedioate**



Lipid 59

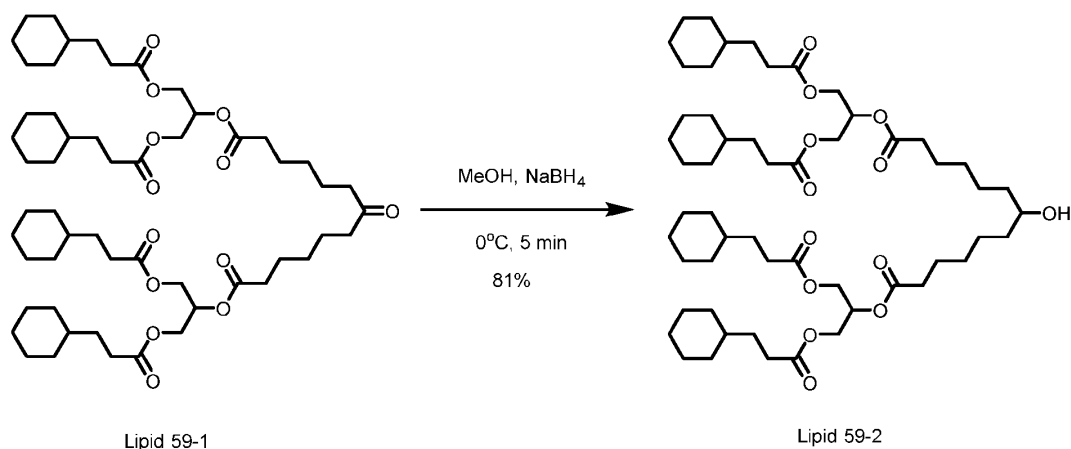
[0767] General scheme

[0768] Synthesis of Lipid 59-1: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl)-7-oxotridecanedioate



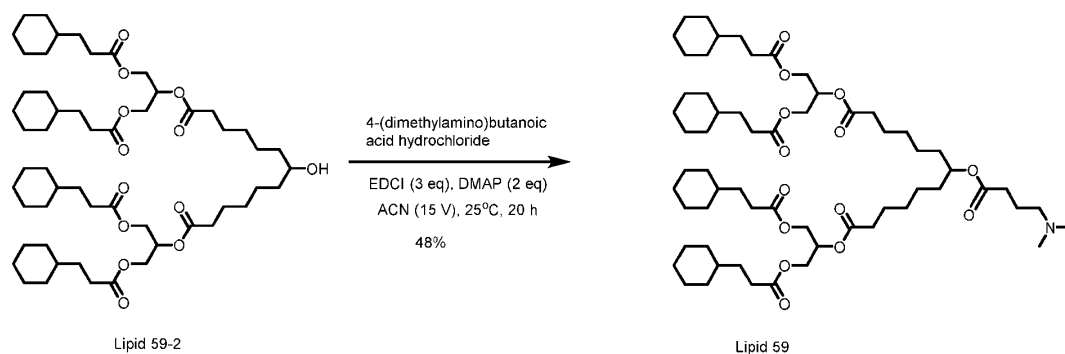
Into a 100 mL round-bottom flask were added **7-oxotridecanedioic acid** (1.5 g, 5.807 mmol, 1 equiv) and ACN (100 mL) at 25°C. To the above mixture was added EDCI (3.12 g, 16.260 mmol, 2.8 equiv) and DMAP (0.85 g, 6.968 mmol, 1.2 equiv) in portions at 25°C. After being stirred for 10 min, **Lipid 57-2** (4.92 g, 13.356 mmol, 2.3 equiv) were added at 25°C. The resulting mixture was stirred for 18 h at 25°C. The resulting mixture was diluted with EA (200 mL). The organic layers were washed with brine (2 x 100 mL), dried over anhydrous Na₂SO₄. The residue was adsorbed onto 15 g of silica gel (type: ZCX-2, 100-200 mesh, 9.00 w./w.), and purified on a silica gel column (180 g of silica gel, type: ZCX-2, 100-200 mesh, 45.00 w./w.) by eluting with heptane/EA (gradient from 100:0 to 85:15. After TLC (heptane:EA 9:1) analysis, fractions were pooled and concentrated under vacuum to obtain the desired product **Lipid 59-1** (3.8 g, yield: 68%) as a light-yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.; RT 1.98 min, m/z (Calcd.) 958.6, (found) 984.6 (M+Na+H). HPLC purity 82.6%. This material was used without further purification.

[0769] Synthesis of Lipid 59-2: bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl)-7-hydroxytridecanedioate



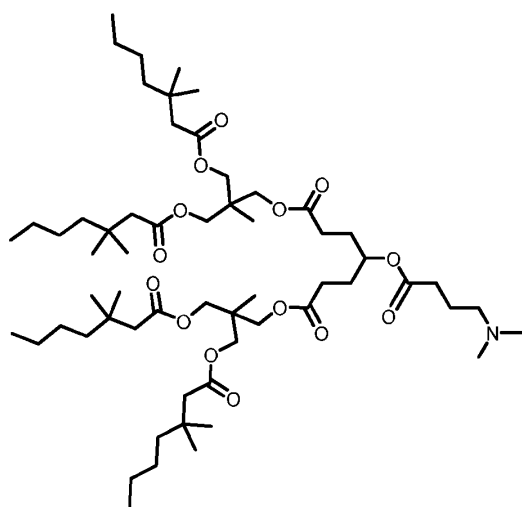
[0770] Into a 100 mL round-bottom flask were added **Lipid 59-1** (2.6 g, 2.71 mmol, 1 equiv) and MeOH (30 mL) at 25°C. To the above mixture was added NaBH₄ (205.06 mg, 5.42 mmol, 2 equiv) in portions over 5 min at 0 °C. The resulting mixture was stirred for 5 min at 0°C. The reaction was quenched by the addition of 5% citric acid (3mL) at 0 °C. The resulting mixture was extracted with EtOAc (1 x 100 mL). The combined organic layers were washed with brine (1x50 mL), dried over anhydrous Na₂SO₄ and filtered. After filtration, the filtrate was adsorbed onto 15 g of silica gel (type: ZCX-2, 100-200 mesh, 2.09 w./w.) and purified on 180 g of silica gel (type: ZCX-2, 100-200 mesh, 16.0 w/w.) and purified on combi-flash. Product was eluted with Hep / EA (gradient from 10:1 to 4:1, collected every 100 ± 10 mL). After TLC analysis (Hep: EA = 5.5:1-5:1), fractions were pooled and concentrated under vacuum to afford **Lipid 59-2** (2.1 g, yield 81%) as a colorless oil. HPLC purity 96.7%. This material was used without further characterization.

[0771] **Synthesis of Lipid 59:** bis(1,3-bis((3-cyclohexylpropanoyl)oxy)propan-2-yl) 7-((4-(dimethyl amino)butanoyl)oxy)tridecanedioate



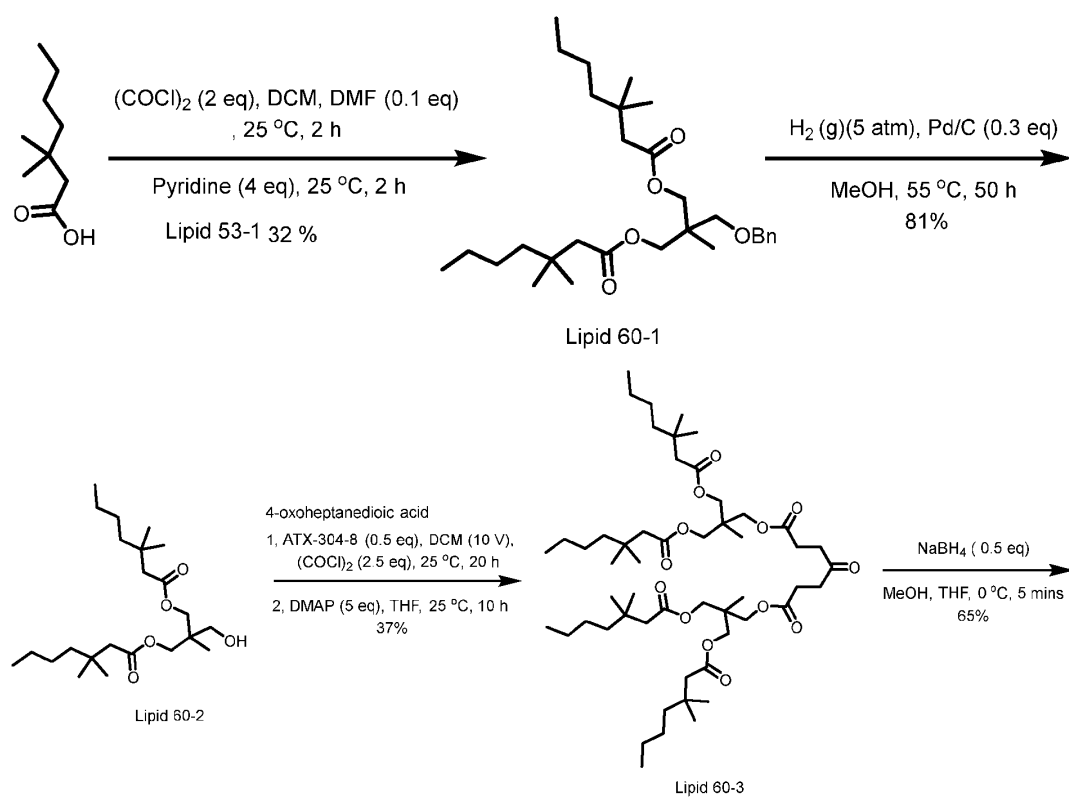
[0772] Into a 250mL round-bottom flask were added **Lipid 59-2** (0.46 g, 2.747 mmol, 1.2 equiv) and ACN (80 mL) at 25°C. To the above mixture was added DMAP (0.28 g, 2.289 mmol, 1 equiv) and 4-(dimethylamino)butanoic acid hydrochloride (2.2 g, 2.289 mmol, 1 equiv). The resulting mixture was stirred for 20 h at 25°C and diluted with EA (150 mL). The combined organic layers were washed with brine (2x150 mL), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure. Heptane (50 mL) and 5% citric acid (20 mL) was added in and stirred for 10 min, collected the heptane phase and washed with 2x50 mL of 5% NaHCO₃. The heptane phase was washed with ACN:H₂O (3:1, 30 mL) twice, and washed with ACN (20 mL) once and concentrated under reduced pressure. This resulted in **Lipid 59** (1.18 g, 1.10 mmol, yield: 48%) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.64 min, m/z (Calcd.) 1073.7, (found) 1074.7 (M+H). HPLC purity 97.1%. ¹H-NMR-PH-ARC-ATX-303-0: (400 MHz, CDCl₃, ppm) δ 5.304- 5.205 (m, 2H), 4.910-4.807 (m, 1H), 4.335-4.236 (m, 4H), 4.187-4.093 (m, 4H), 2.389- 2.252 (m, 16H), 2.213 (s, 6H), 1.825-1.743 (m, 2H), 1.742-1.560 (m, 22H), 1.558-1.430 (m, 12H), 1.394-1.071 (m, 25H), 0.946-0.799 (m, 9H).

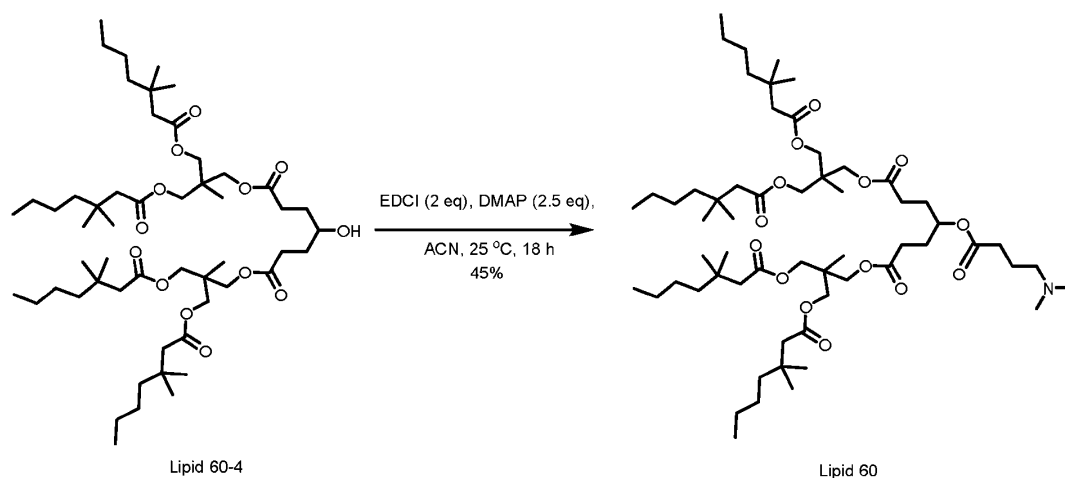
[0773] **Example 50. Lipid 60: bis(3-((3,3-dimethylheptanoyl)oxy)-2-(((3,3-dimethylheptanoyl)oxy) methyl)-2-methylpropyl) 4-((4-(dimethylamino)butanoyl)oxy)heptanedioate**



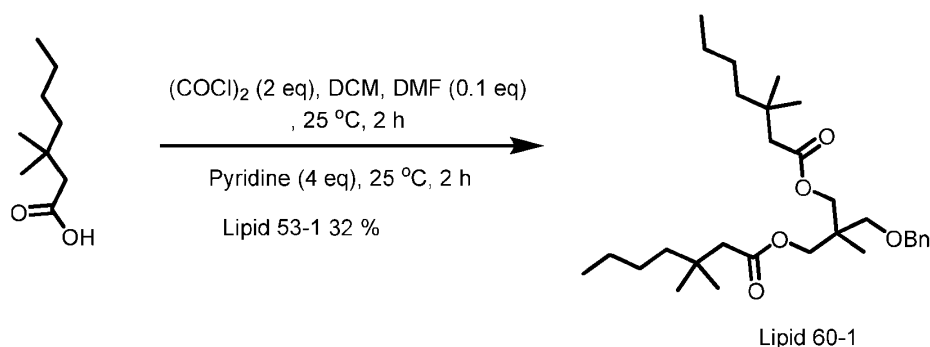
Lipid 60

[0774] General scheme





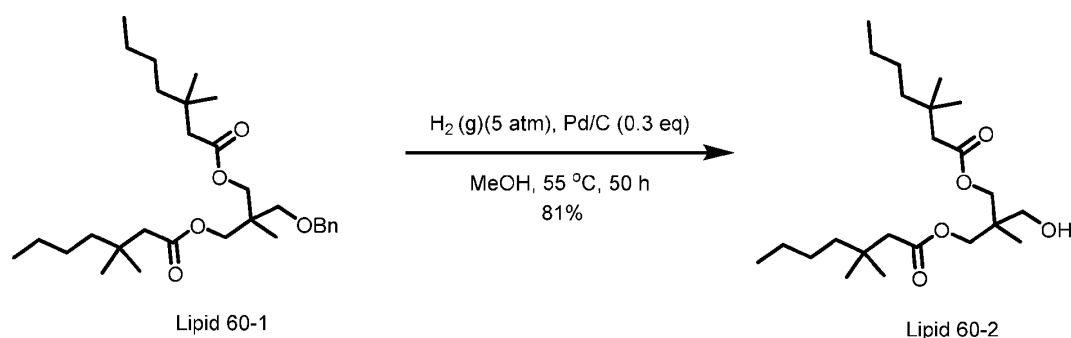
[0775] Synthesis of Lipid 60-1: 2-((benzyloxy)methyl)-2-methylpropane-1,3-diyl bis(3,3-dimethylheptanoate)



[0776] Into a 250 mL round-bottom flask were added 3,3-dimethylheptanoic acid (10 g, 63.19 mmol, 1 equiv), DMF (0.46 g, 6.32 mmol, 0.1 equiv) and DCM (150 mL) at 25 °C under N₂ atmosphere. To the above mixture was added (COCl)₂ (16.04 g, 126.39 mmol, 2 equiv) dropwise over 30 min at 25 °C. The resulting mixture was stirred for 2 h at 25 °C. The resulting mixture was concentrated under reduced pressure and dissolved in DCM (100 mL). To a stirred solution of **Lipid 53-1** (6.51 g, 30.966 mmol, 0.49 equiv) and pyridine (19.99 g, 252.78 mmol, 4 equiv) in DCM (100 mL) were added the above acyl chloride in DCM dropwise at 25 °C under nitrogen atmosphere. The resulting mixture was stirred for 2 h at 25 °C under nitrogen atmosphere. The reaction was monitored by TLC. The resulting mixture was diluted with DCM (300 mL). The resulting mixture was washed with 1 x 200 mL of 0.5 M HCl and brine (2 x 200 mL). The organic phase was dried over anhydrous Na₂SO₄. After

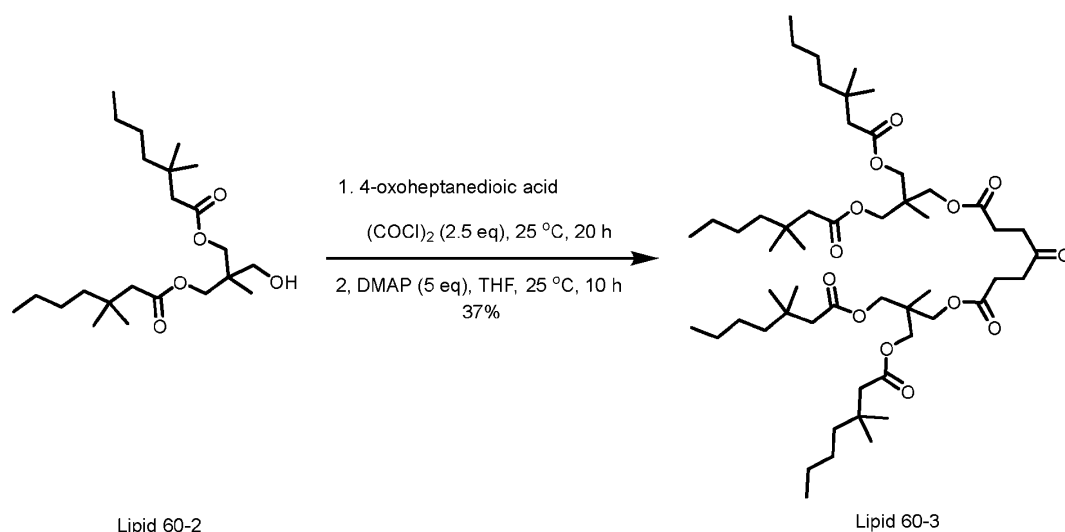
filtration, the crude product was adsorbed on 20 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.), and purified on 100 g of silica gel (type: ZCX-2, 100-200 mesh, 10.00 w./w.) using a Combi-flash column by eluting with n-heptane / EA (100:0 to 15:1 v/v). After TLC analysis. (n-heptane / EA = 1:10, R_f=0.3) fractions were pooled and concentrated under reduced pressure to afford **Lipid 60-1** (10 g, 20.4 mmol, yield: 32 %) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.76 min, m/z (Calcd.) 490.3, (found) 513.4 (M+Na). HPLC purity 99.4%.

[0777] Synthesis of Lipid 60-2: 2-(hydroxymethyl)-2-methylpropane-1,3-diyl bis(3,3-dimethylheptanoate)



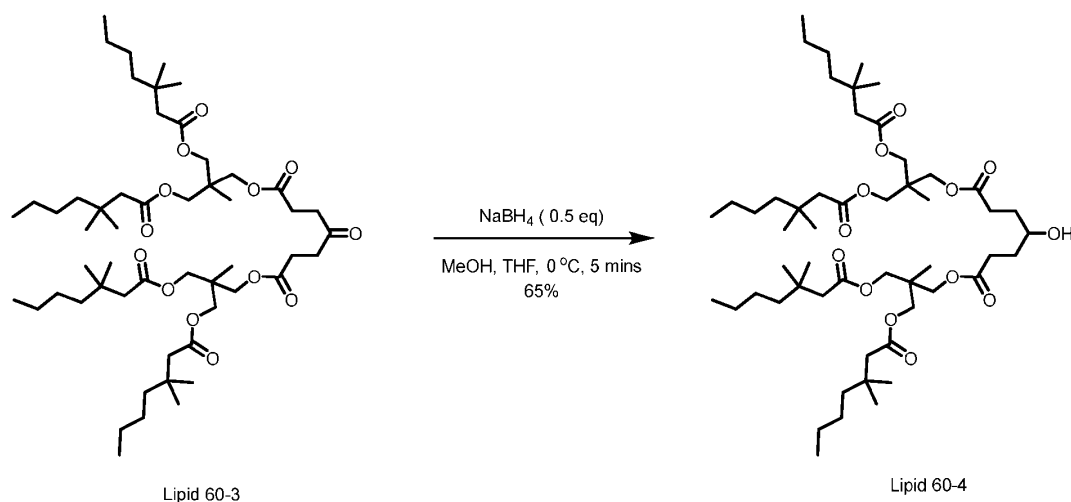
[0778] Into a 250 mL pressure tank reactor were added **Lipid 60-1** (10 g, 20.378 mmol, 1 equiv) and MeOH (120 mL) at 25 °C. To the above mixture was added Pd/C (650.59 mg, 6.113 mmol, 0.3 equiv) in portions at 25°C. The reactor was evacuated and flushed three times with nitrogen, followed by flushing with hydrogen. The mixture was stirred for additional 50 h at 55°C under hydrogen atmosphere (5 atm). The resulting mixture was filtered, the filter cake was washed with MeOH (1 x 200 mL). The filtrate was concentrated under reduced pressure. The residue was dissolved in EA, washed with brine (2 x 200 mL), dried over anhydrous Na₂SO₄. After filtration, the filtrate was concentrated under reduced pressure to get **Lipid 60-2** (6.6 g, 16.5 mmol, yield: 81 %) as a colorless oil that was used as such without further purification.

[0779] Synthesis of Lipid 60-3: bis(3-((3,3-dimethylheptanoyl)oxy)-2-(((3,3-dimethylheptanoyl)oxy)methyl)-2-methylpropyl 4-oxoheptanedioate



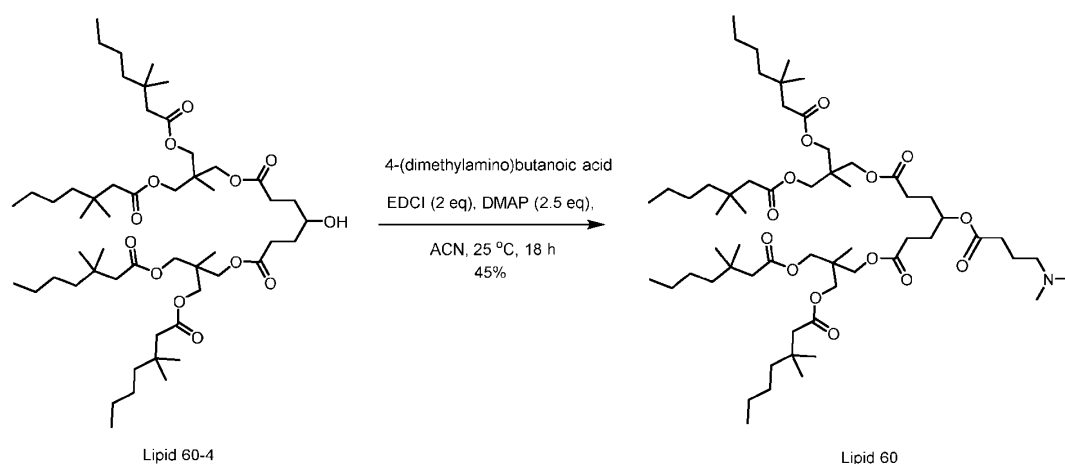
[0780] Into a 250 mL round-bottom flask were **Lipid 60-2** (0.87 g, 4.992 mmol, 0.5 equiv), DMF (0.04 g, 0.499 mmol, 0.05 equiv) and DCM (80 mL) at 25 °C. To the above mixture was added (COCl)₂ (3.17 g, 24.962 mmol, 2.5 equiv) dropwise at 25 °C. The resulting mixture was stirred for 20 h at 25 °C. The resulting mixture was concentrated under reduced pressure and dissolved in DCM (100 mL). To a stirred solution of 4-oxoheptanedioic acid (4 g, 9.985 mmol, 1 equiv) and DMAP (6.10 g, 49.925 mmol, 5 equiv) in THF (80 mL) were added the above acyl chloride dropwise at 40 °C under nitrogen atmosphere. The resulting mixture was stirred for 10 h at 25 °C under nitrogen atmosphere. The resulting mixture was diluted with EA (300 mL), washed with 0.5N-HCl (2 x 200 mL) and brine (2 x 200 mL), dried over anhydrous Na₂SO₄. After filtration, to the filtrate was added 10 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.), and the gained mixture was concentrated to no solvent under vacuum while maintaining the temperature below 30 °C. 50 g of silica gel (type: ZCX-2, 100-200 mesh, 10.00 w./w.) was charged to the column, followed by the prepared dry silica gel which absorbed the reaction mixture of the last step. Combi-flash was used to purify the product. Eluted with n-heptane / EA (volume ratio). (gradient from 100:0 to 10:1 and collected product eluent n-heptane / EA = 10:1). Took sample for TLC analysis. (n-heptane / EA = 1:10, R_f=0.2) to afford **Lipid 60-3** (3.5 g, 3.73 mmol, yield: 37 %) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.99 min, m/z (Calcd.) 938.6, (found) 961.6 (M+Na). HPLC purity 99.9%.

[0781] Synthesis of Lipid 60-4: bis(3-((3,3-dimethylheptanoyl)oxy)-2-(((3,3-dimethylheptanoyl)oxy) methyl)-2-methylpropyl) 4-hydroxyheptanedioate



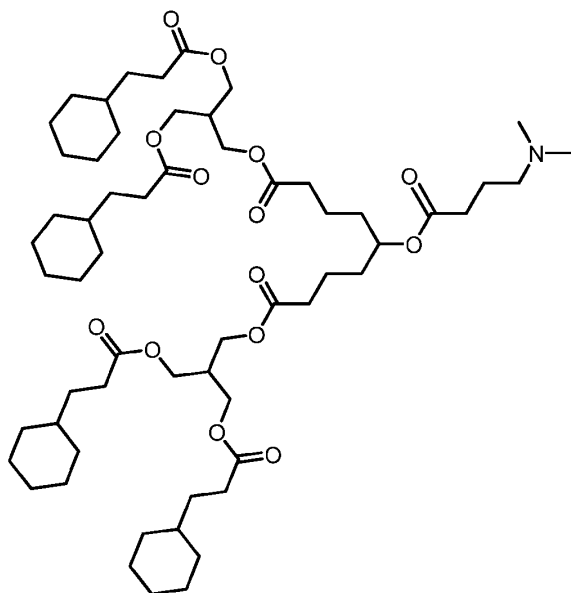
[0782] Into a 100 mL 3-necked round-bottom flask were added **Lipid 60-3** (3.4 g, 3.620 mmol, 1 equiv), THF (20 mL), MeOH (10 mL) at 0 °C. To the above mixture was added NaBH₄ (0.07 g, 1.810 mmol, 0.5 equiv) at 0 °C. The resulting mixture was stirred for 5 mins at 0 °C under nitrogen atmosphere and quenched with citric acid (10 %) at 0 °C. The mixture was extracted with EtOAc (2x50 mL). The organic phase was dried over anhydrous Na₂SO₄. After filtration, to the filtrate was added 10 g of silica gel (type: ZCX-2, 100-200 mesh, 2.00 w./w.), and concentrated under vacuum while maintaining the temperature below 30 °C and purified on 50 g of silica gel (type: ZCX-2, 100-200 mesh, 10.00 w./w.) using Combi-flash by eluting with n-heptane / EA (volume ratio). (gradient from 100:0 to 100:20 and collected product eluent n-heptane / EA = 100:10). Took sample for TLC analysis. (n-heptane / EA = 1:10, R_f=0.2) to afford **Lipid 60-4** (2.2 g, yield: 64 %) as a light yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.97 min, m/z (Calcd.) 940.7, (found) 963.7 (M+Na). HPLC purity 98.9%.

[0783] Synthesis of Lipid 60: bis(3-((3,3-dimethylheptanoyl)oxy)-2-(((3,3-dimethylheptanoyl)oxy) methyl)-2-methylpropyl) 4-((4-(dimethylamino)butanoyl)oxy)heptanedioate



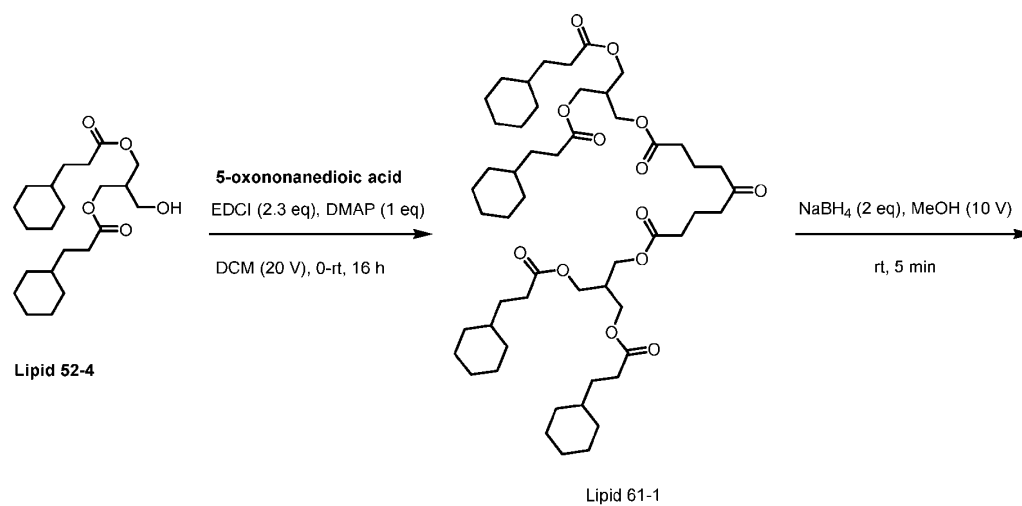
[0784] To a 100 mL round-bottom flask were added **Lipid 60-4** (2 g, 2.125 mmol, 1 equiv), ACN (30 mL), 4-(dimethylamino)butanoic acid hydrochloride (0.43 g, 2.550 mmol, 1.2 equiv), DMAP (0.65 g, 5.313 mmol, 2.5 equiv) and EDCI (0.81 g, 4.250 mmol, 2 equiv) at 25 °C. The resulting mixture was stirred for 18 h at 25 °C under nitrogen atmosphere. The resulting mixture was diluted with EA (200 mL). The resulting mixture was washed with 1x100 mL of brine. The organic phase was dried over anhydrous Na₂SO₄, filtered and the organic layer was concentrated under reduced pressure. The residue was purified by reversed-phase flash chromatography with the following conditions: (A: water; B: ACN, Gradient: 20-90% 20 min, Flow: 50ml/min; Column: WelFlash XSelect CSH Prep C18 5um OBD, Regular 30*150mm Column). The product eluent was collected and concentrated to afford **Lipid 60** (1.0082 g, 0.956 mmol, HPLC: 99.0 %, yield: 45.00 %) as a light-yellow oil. ELSD A: water/0.05% TFA; B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.18 min, m/z (Calcd.) 1053.7, (found) 1054.7 (M+Na). HPLC purity 98.9%. ¹H-NMR-PH-ARC-ATX-304-0: (300 MHz, CDCl₃, ppm): δ 4.979-4.898 (m, 1H), 3.984 (d, *J* = 11.4 Hz, 12H), 2.373-2.285 (m, 8H), 2.215 (d, *J* = 7.5 Hz, 14H), 1.926-1.770 (m, 6H), 1.313-1.216 (m, 24H), 1.021 (s, 6H), 0.970 (s, 24H), 0.898 (t, *J* = 6.3 Hz, 12H).

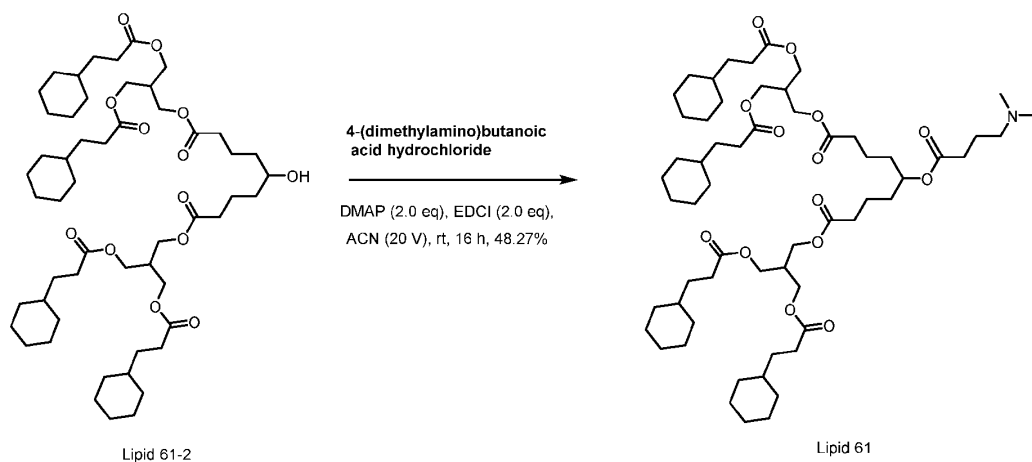
[0785] Example 51. Lipid 61: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy)methyl)propyl)5-((4-(dimethylamino)butanoyl)oxy)nonanedioate



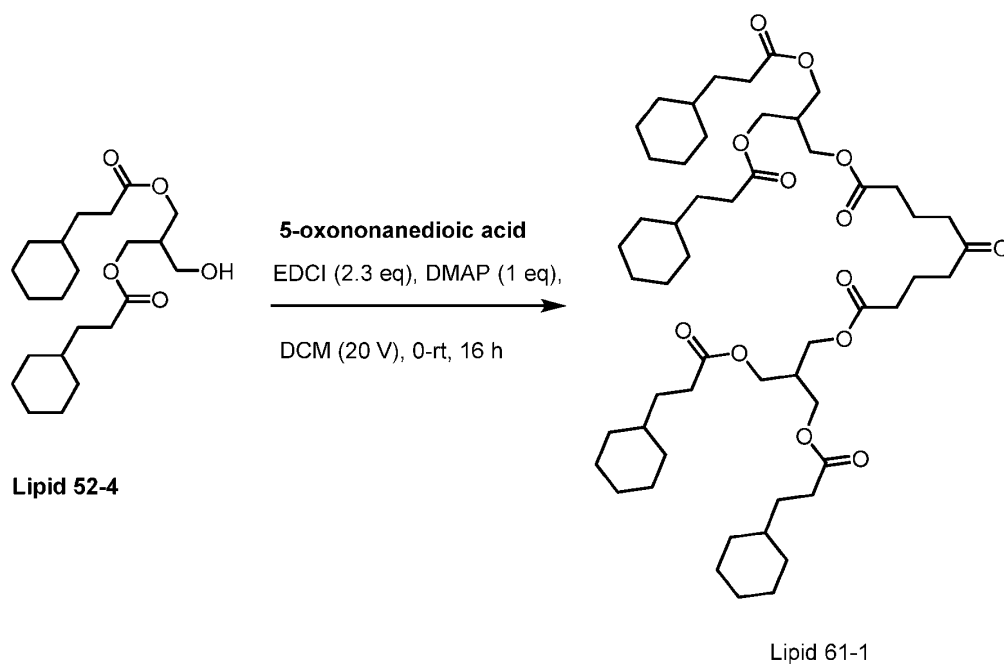
Lipid 61

General scheme





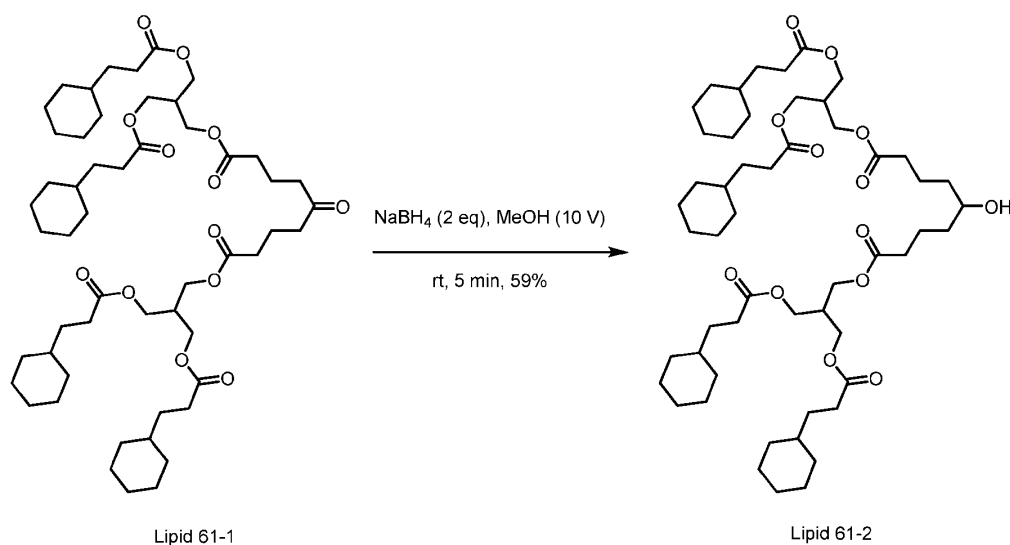
[0786] Synthesis of Lipid 61-1: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy)methyl)propyl) 5-oxononanedioate



[0787] Into a 250-mL 3-necked round-bottom flask, was placed a solution of 5-oxononanedioic acid (1.3 g, 6.429 mmol, 1 equiv), **Lipid 52-4** (5.41 g, 14.144 mmol, 2.2 equiv), DMAP (0.79 g, 6.429 mmol, 1 equiv) and DCM (100 mL) at room temperature. To the

above mixture EDCI (3.08 g, 16.073 mmol, 2.5 equiv) was added in portion over 10 mins. The resulting solution was stirred 16 h at room temperature. The resulting mixture was diluted with brine (100 mL, 20 V). The mixture was extracted with ethyl acetate (2 x 200 mL, 40 V). The combined organic layers were washed with brine (100 mL, 20 V). The organic phase was dried with anhydrous Na₂SO₄ and then filtration. To the filtrate was added 15 g of silica gel (type: ZCX-2, 100-200 mesh, 3.00 w./w.), to adsorb the material on silica gel. This was purified on 60 g of silica gel (type: ZCX-2, 100-200 mesh, 12.00 w./w.) using COMBIFLASH[®] (flash HPLC system) and eluting with n-heptane/ethyl acetate (gradient from 100:0 to 90:10). After TLC analysis. (Hep:ethyl acetate = 5:1), fractions were combined and concentrated under reduced pressure to afford **Lipid 61-1** (5.3 g, yield: 84%) as colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.88 min, m/z (Calcd.) 930.6, (found) 953.6 (M+Na). HPLC purity 95.4%.

[0788] Synthesis of Lipid 61-2: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy) methyl)propyl) 5-hydroxynonanedioate



[0789] Into a 100-mL 3-necked round bottom flask, was placed **Lipid 61-1** (3.5 g, 3.585 mmol, 1 equiv, 95.4%) and MeOH (35 mL) at room temperature. To the above mixture was added NaBH₄ (0.27 g, 7.170 mmol, 2 equiv) while stirring at room temperature. The resulting solution was stirred at room temperature for 5 min and quenched by the addition of 5% citric acid at 0 °C. The resulting mixture was extracted with ethyl acetate (2 x 80 mL). The combined

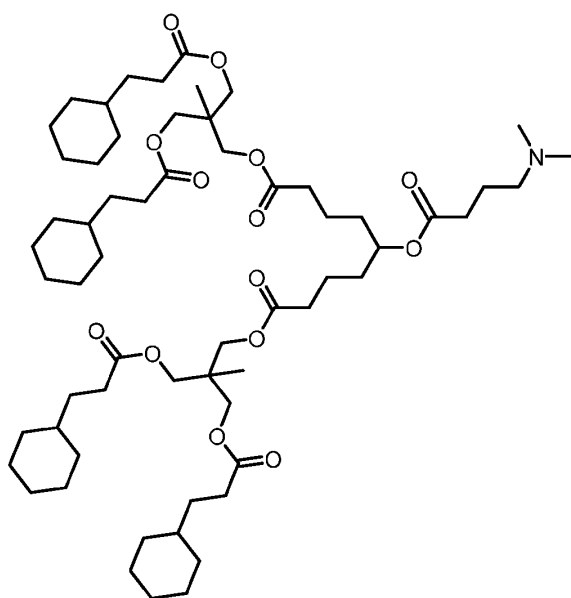
[0790] Synthesis of Lipid 61: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy)methyl)propyl)5-((4-(dimethylamino)butanoyl)oxy)nonanedioate



326

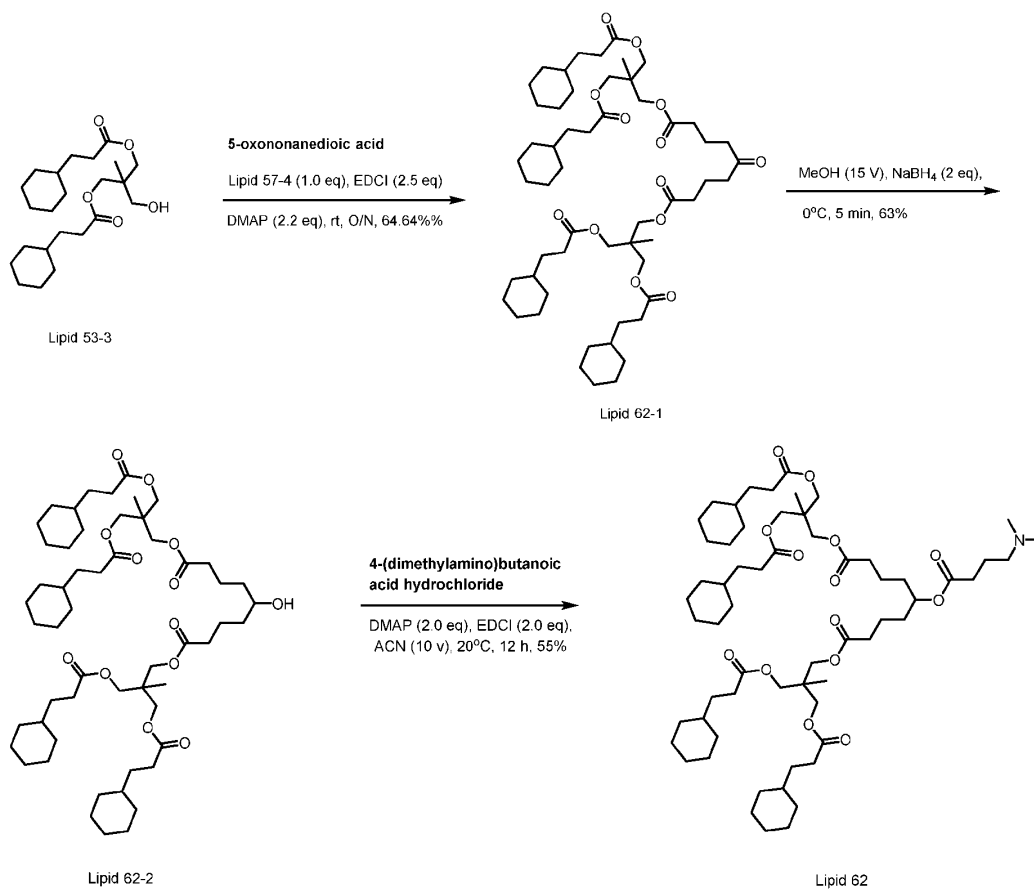
61 (1.05 g, 0.965 mmol, 48%, HPLC: 97.4%) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.66 min, m/z (Calcd.) 1045.7, (found) 1046.7 (M+H). HPLC purity 97.4%. ¹H NMR (400 MHz, Chloroform-d) δ 4.918-4.905 (m, 1H), 4.147-4.103 (m, 12H), 2.446-2.301 (m, 24H), 1.884-1.811 (m, 2H), 1.724 -1.503 (m, 34H), 1.342-1.106 (m, 18H), 0.948-0.919 (m, 8H).

[0792] **Example 52. Lipid 62: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy) methyl)-2-methylpropyl) 5-((4-(dimethylamino)butanoyl)oxy)nonanedioate**

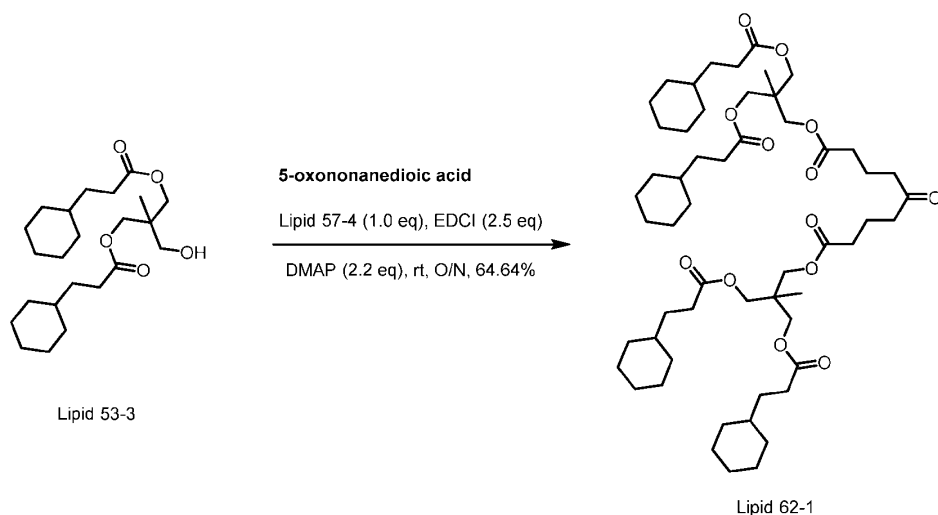


Lipid 62

[0793] General scheme

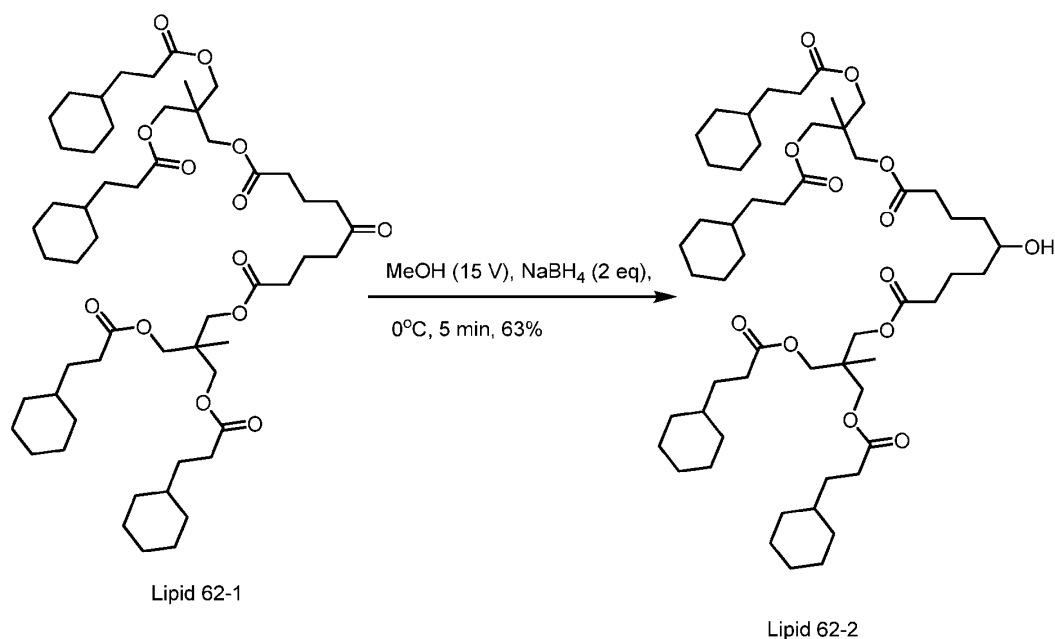


[0794] Synthesis of Lipid 62-2: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy)methyl)-2-methylpropyl) 5-oxononanedioate



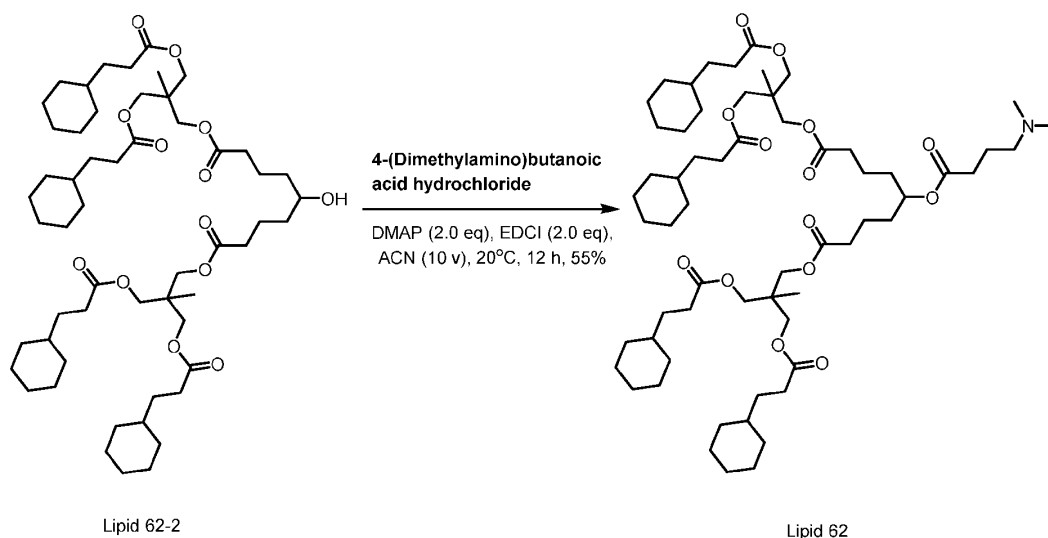
[0795] Into a 250 mL round-bottom flask, was placed 5-oxononanedioic acid (1.5 g, 7.418 mmol, 1 equiv), DCM (110 mL), **Lipid 53-3** (6.18 g, 15.584 mmol, 2.10 equiv), DMAP (0.91 g, 7.418 mmol, 1.0 equiv), EDCI (3.56 g, 18.545 mmol, 2.5 equiv). The reaction mixture was stirred at room temperature overnight. The resulting mixture was diluted with DCM (400 mL). The resulting mixture was washed with 3 x 200 mL of water. The resulting solution was dried over anhydrous Na₂SO₄ and filtered. The resulting mixture was concentrated under reduced pressure. The mixture was adsorbed onto 30 g of silica gel (type: ZCX-2, 100-200 mesh, 20 w./w.) and purified 90 g of silica gel (type: ZCX-2, 100-200 mesh, 60.0 w./w.) using COMBIFLASH[®] (flash HPLC system) and eluting with n-heptane/ethyl acetate (gradient from 100:0 to 92:8, collected every 200 mL). After TLC analysis (n-heptane/ethyl acetate = 20:1, R_f = 0.30), qualified fractions were pooled and concentrated at 35 °C under reduced pressure to obtain **Lipid 62-1** (4.6 g, yield:65%) as a light-yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.96 min, m/z (Calcd.) 958.6, (found) 981.6 (M+Na). HPLC purity 99.5%.

[0796] Synthesis of Lipid 62-2: bis(3-((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy) methyl)-2-methylpropyl) 5-hydroxynonanedioate

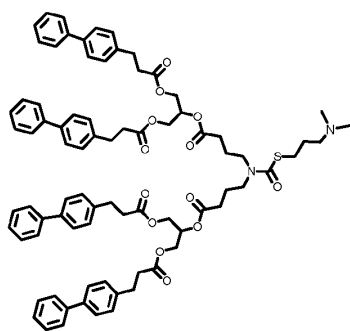


[0797] Into a 250 round-bottom flask, was placed **Lipid 62-1** (4.4 g, 4.587 mmol, 1 equiv), MeOH (88 mL), NaBH₄ (0.35 g, 9.174 mmol, 2 equiv) was added at 0°C. The reaction mixture was stirred at 0 °C for 5 min. The reaction was poured into ice water. The aqueous layer was extracted with ethyl acetate (3 x 88 mL), brine (88 mL). The resulting solution was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The mixture was adsorbed onto 44 g of silica gel (type: ZCX-2, 100-200 mesh, 10 w./w.) and purified on 88 g of silica gel (type: ZCX-2, 100-200 mesh, 20.0 w./w.) column using COMBIFLASH[®] (flash HPLC system) and eluting with n-heptane/ethyl acetate (gradient from 100:0 to 86:14, collected every 200 mL). After TLC analysis (n-heptane/ ethyl acetate = 5:1, R_f = 0.50), qualified fractions were pooled and concentrated at 35°C under reduced pressure to obtain **Lipid 62-2** (2.8 g, yield: 63 %) as a colorless oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.94 min, m/z (Calcd.) 960.6, (found) 983.6 (M+Na). HPLC purity 97.8%.

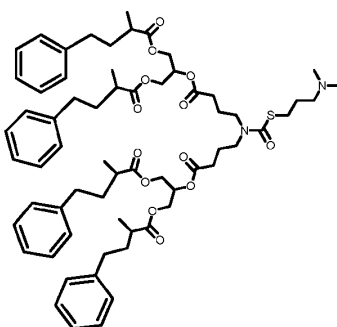
[0798] Synthesis of Lipid 62: bis(3-(((3-cyclohexylpropanoyl)oxy)-2-(((3-cyclohexylpropanoyl)oxy) methyl)-2-methylpropyl) 5-((4-(dimethylamino)butanoyl)oxy)nonanedioate



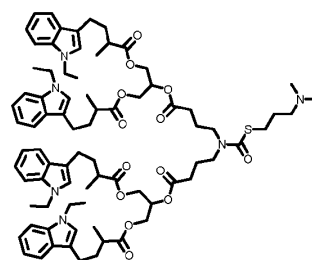
[0799] Into a 100 mL round-bottom flask, was placed **Lipid 62-2** (2.2 g, 2.289 mmol, 1 equiv), MeCN (44 mL), 4-(dimethylamino)butanoic acid hydrochloride (460.35 mg, 2.747 mmol, 1.2 equiv), DMAP (559.18 mg, 4.578 mmol, 2.0 equiv), EDCI (877.41 mg, 4.578 mmol, 2.0 equiv). The reaction mixture was stirred at 12 h at 20°C. The resulting mixture was diluted with ethyl acetate (176 mL) and washed with 5% NaHCO₃ and 3 x 44 mL of water. Solvent was removed under reduced pressure. The crude product was diluted with n-heptane (176 mL), washed with 3 x 88 mL of MeOH/H₂O (3/1), 3 x 88 mL of ACN/H₂O (3/1), water (88 mL), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford **Lipid 62** (1.3431 g, yield: 55%) as a yellow oil. ELSD A: water/0.05% TFA: B: CH₃CN/0.05% TFA 95:5 to 5:95 A/B at 3 min.): RT 1.70 min, m/z (Calcd.) 1073.7, (found) 1074.7 (M+H). HPLC purity 97.1%. ¹H NMR (400 MHz, Chloroform-d) δ 4.993-4.832 (m, 1H), 4.002 (s, 12H), 2.401-2.283 (m, 16H), 2.253 (s, 6H), 1.854-1.769 (m, 2H), 1.745-1.486 (m, 36H), 1.297-1.146 (m, 16H), 1.023 (s, 6H), 0.964-0.853 (m, 8H).



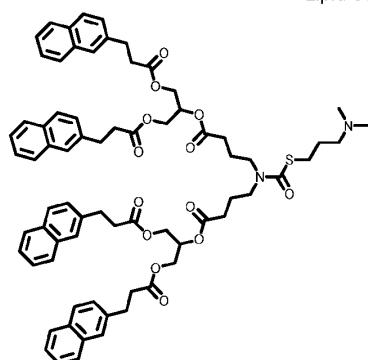
Lipid 42



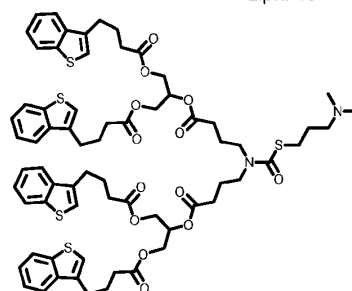
Lipid 63



Lipid 43



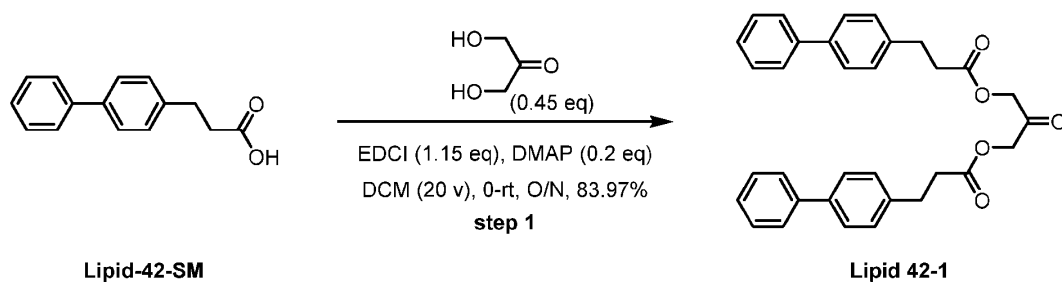
Lipid 44

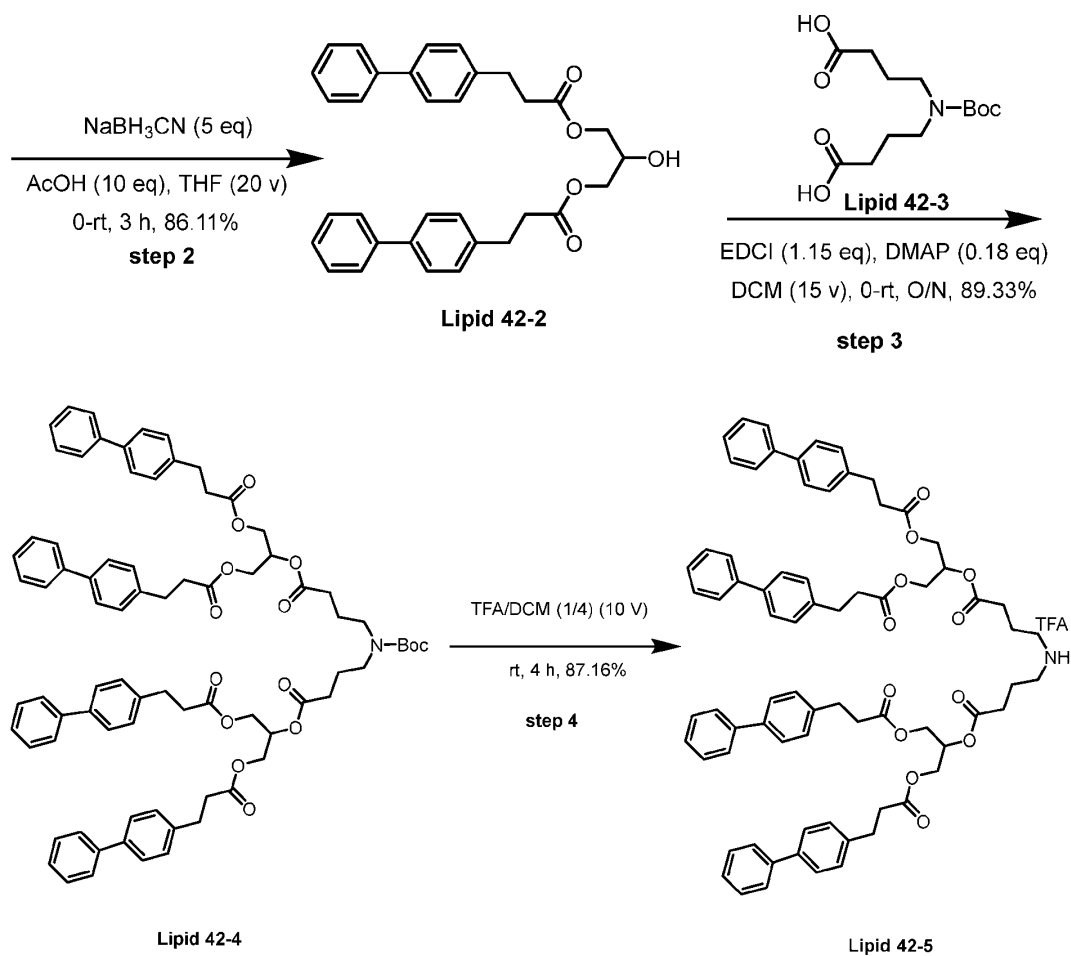


Lipid 45

[0800] **Example 53 Lipid 42:** ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-([1,1'-biphenyl]-4-yl)propanoate)

Synthesis of Lipid 42: General synthesis scheme



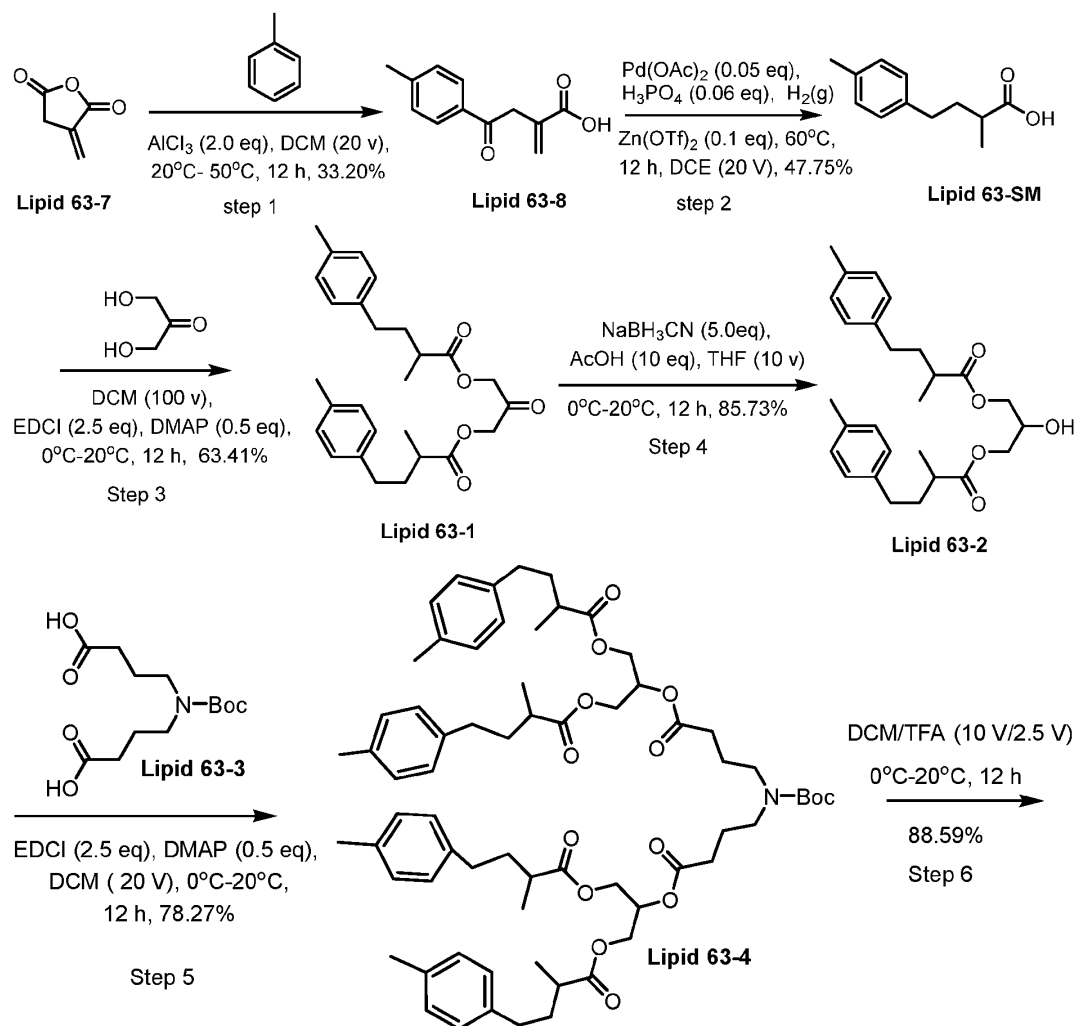


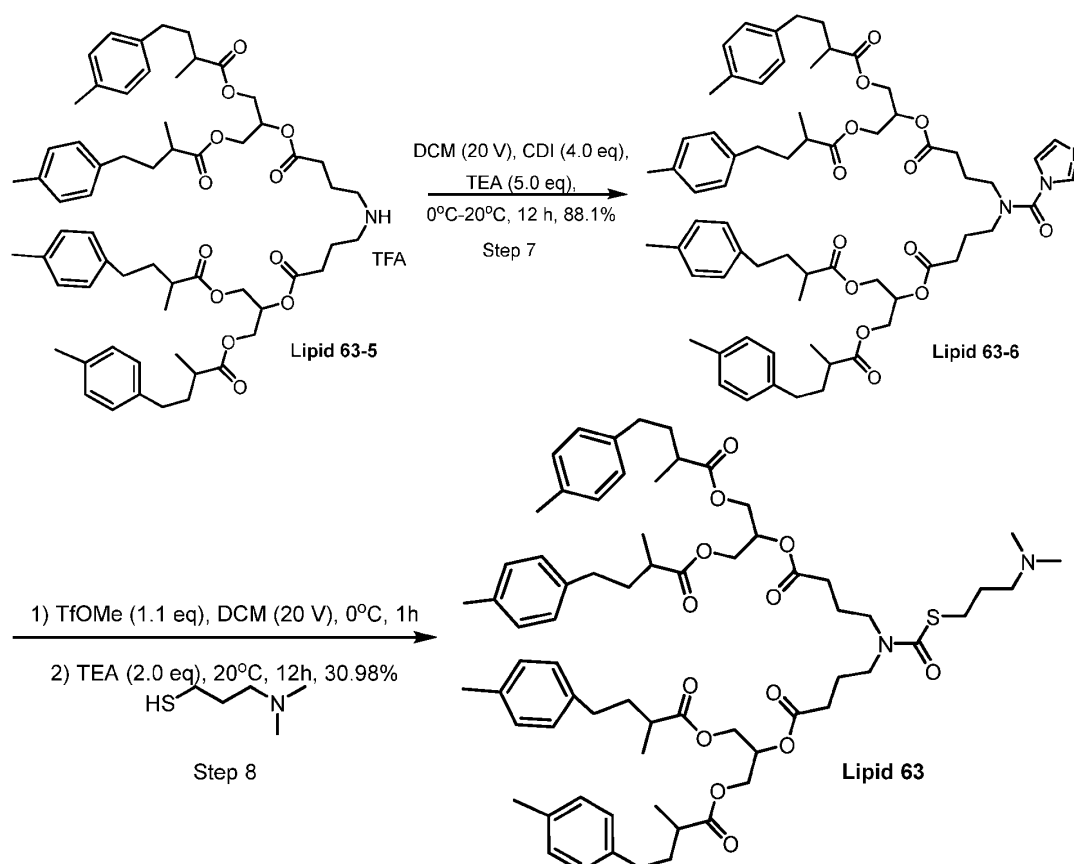
[0801] Synthesis was executed following well-established protocols known in the art for similar compounds as in detailed examples above.

[0802] **Example 53. Lipid 63:** ((4,4'-((((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyl-4-(p-tolyl)butanoate)

[0803] Synthesis of Lipid 63: General synthesis scheme

[0804] Synthesis of Lipid 63: General synthesis scheme

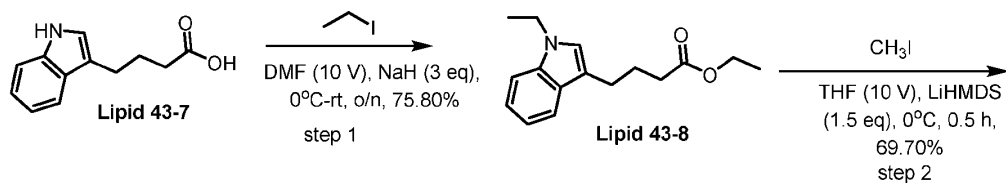


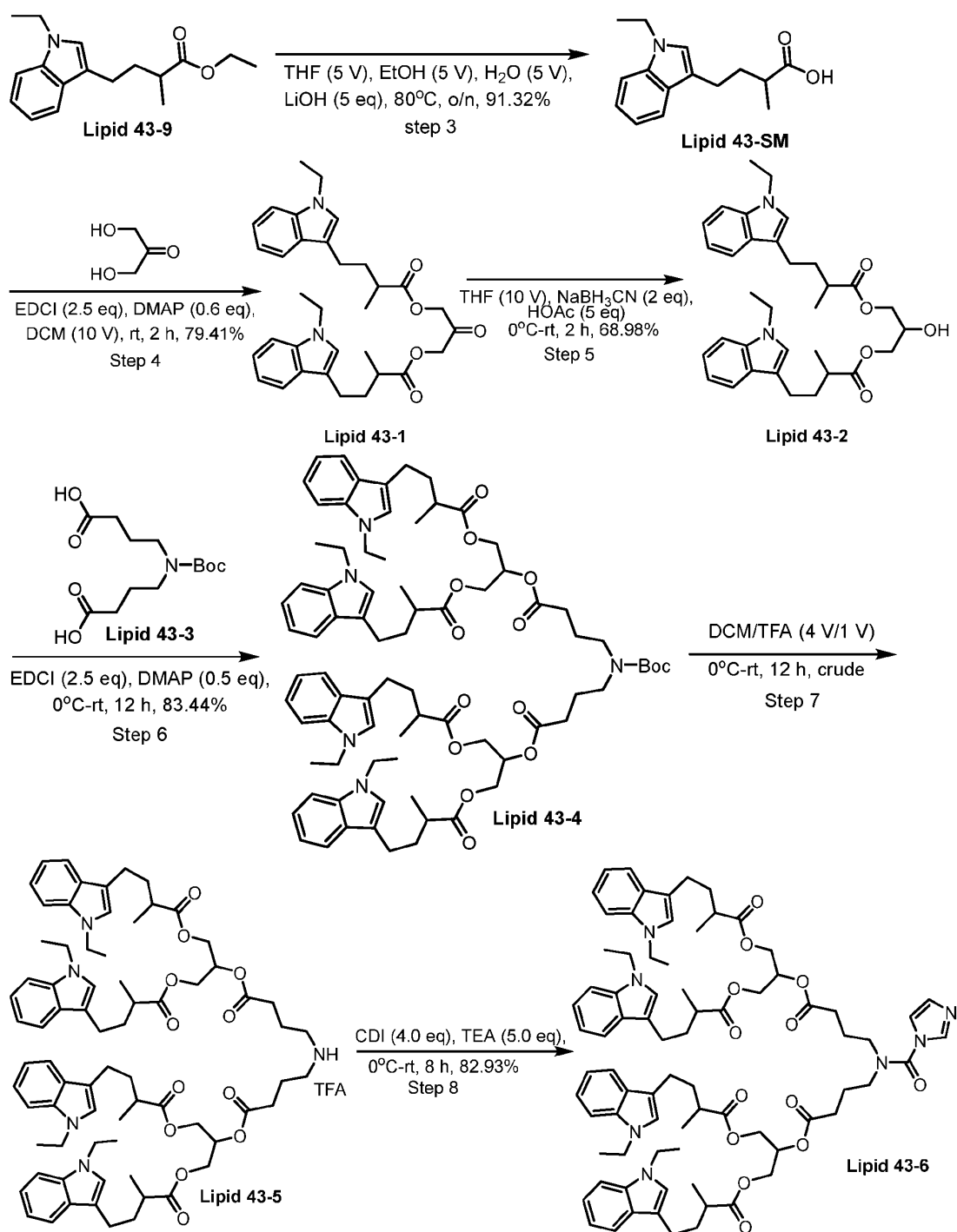


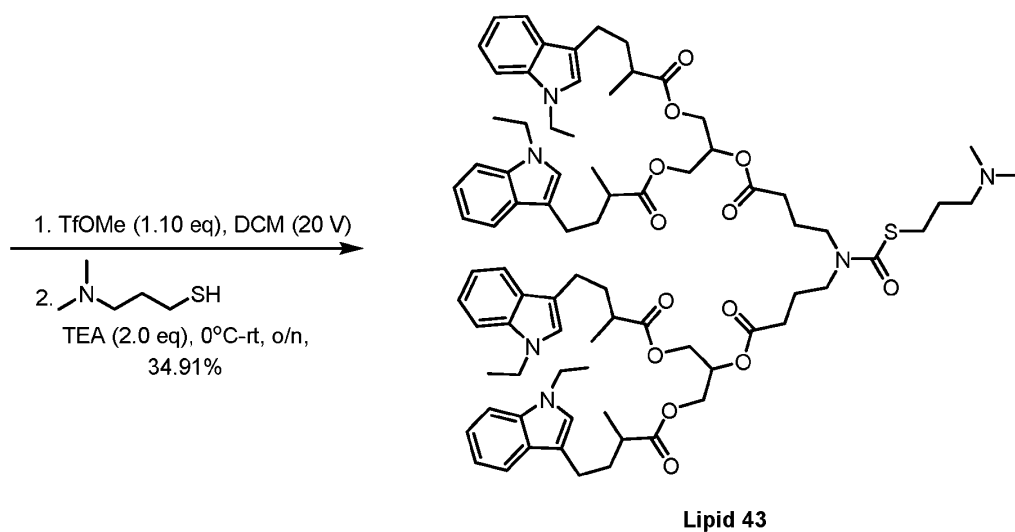
[0805] Synthesis of Lipid 63 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0806] **Example 54. LIPID 43: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-(1-ethyl-1H-indol-3-yl)-2-methylbutanoate)**

[0807] Synthesis of Lipid 43: General synthesis scheme



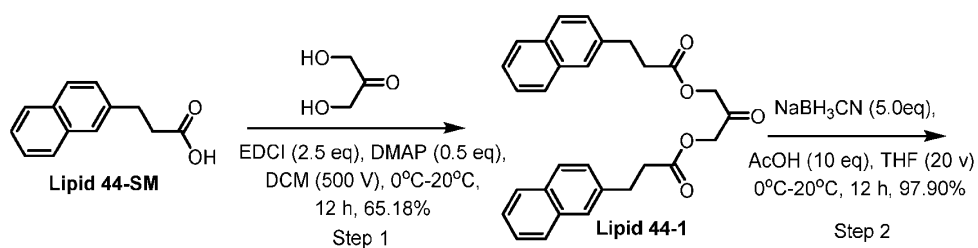


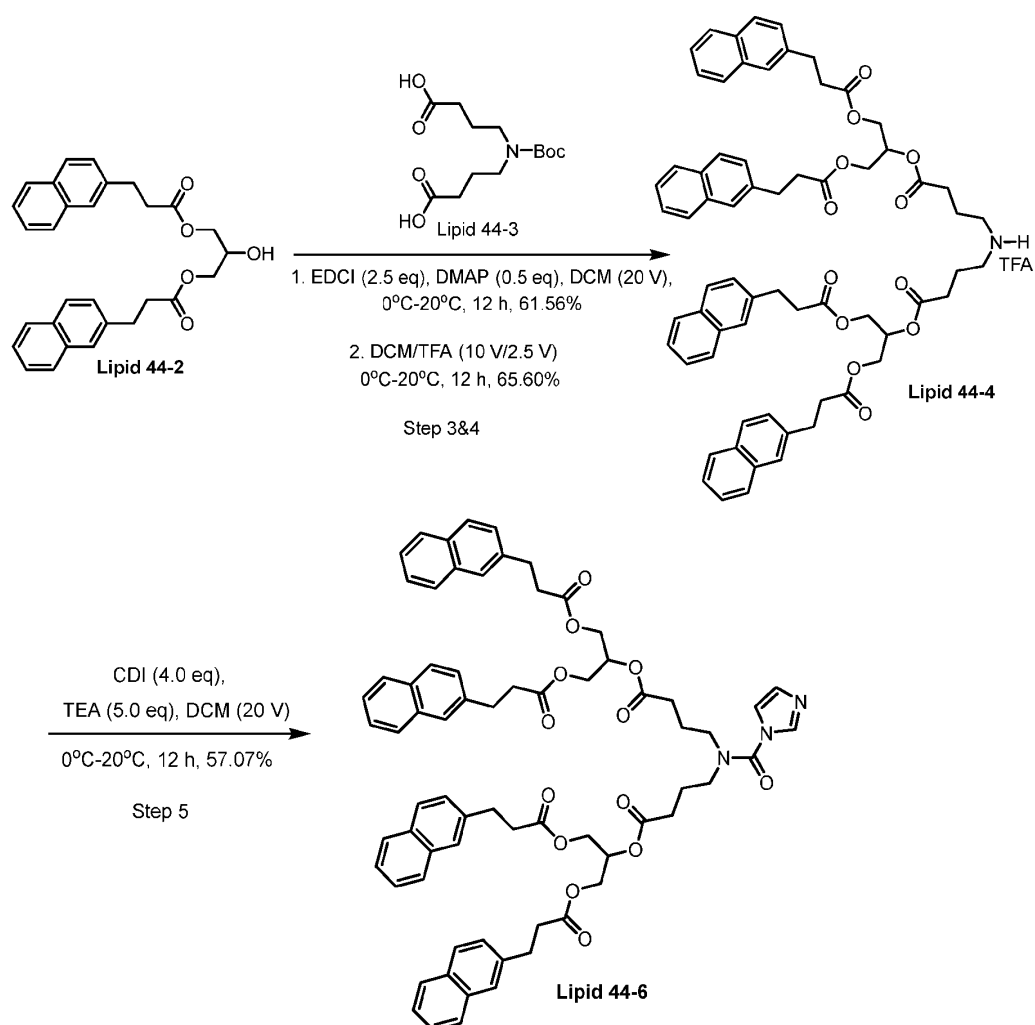


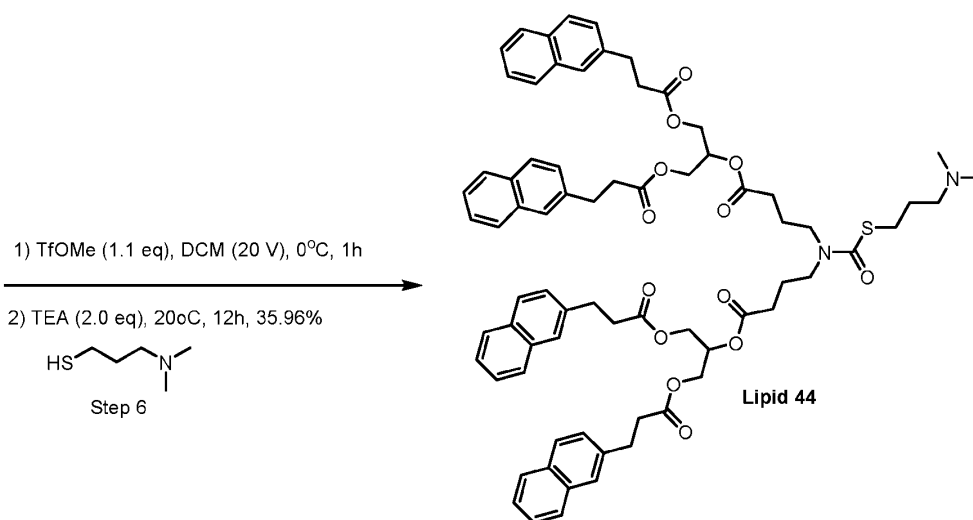
[0808] Synthesis of Lipid 43 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0809] **Example 55. LIPID 44: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(3-(naphthalen-2-yl)propanoate)**

[0810] Synthesis of Lipid 44: General synthesis scheme



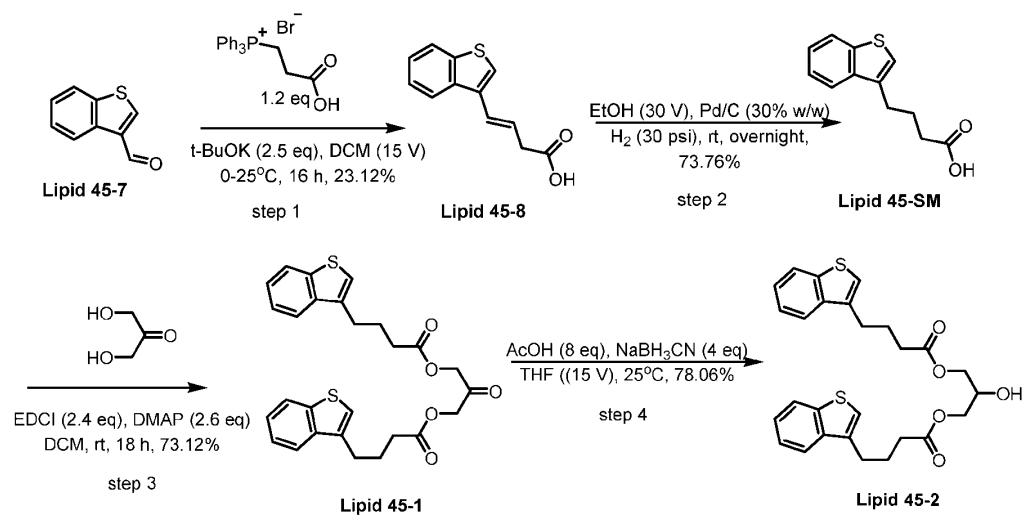


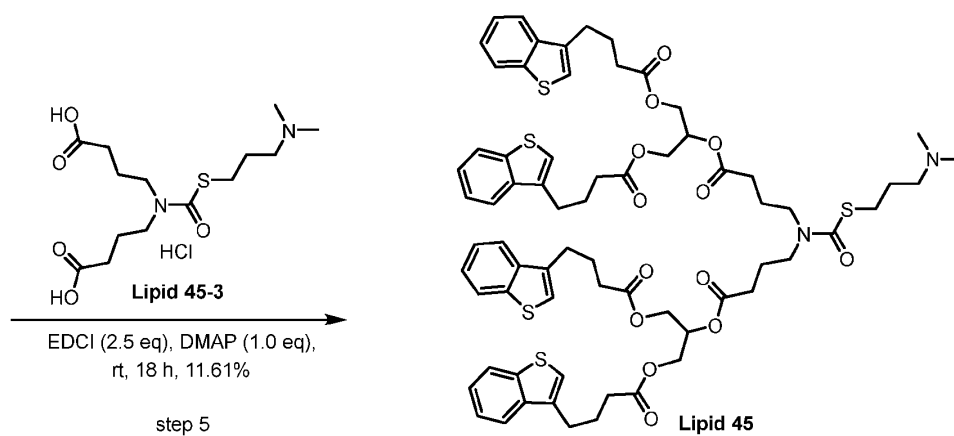


[0811] Synthesis of Lipid 44 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

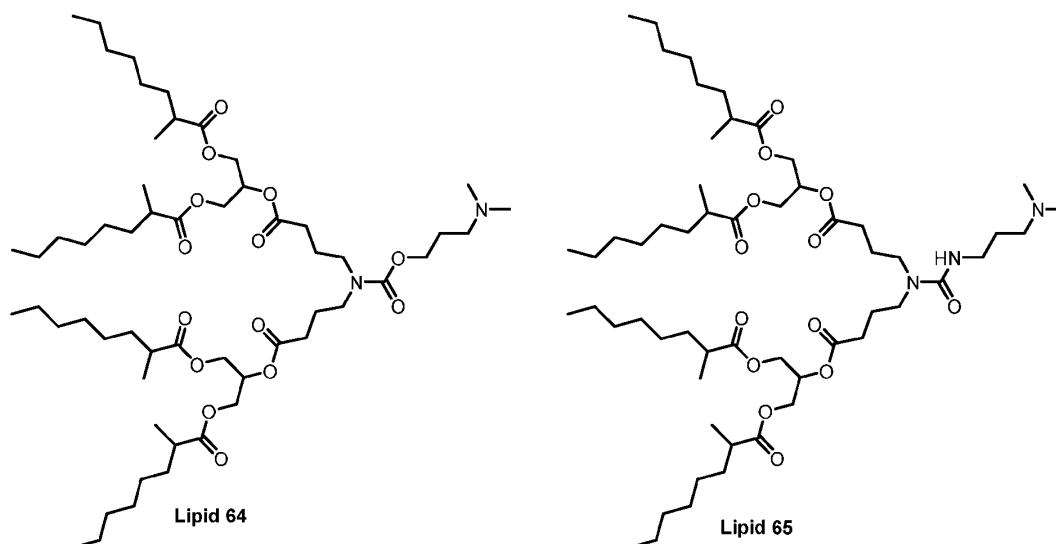
[0812] **Example 56. LIPID 45: ((4,4'-(((3-(dimethylamino)propyl)thio)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(4-(benzo[b]thiophen-3-yl)butanoate)**

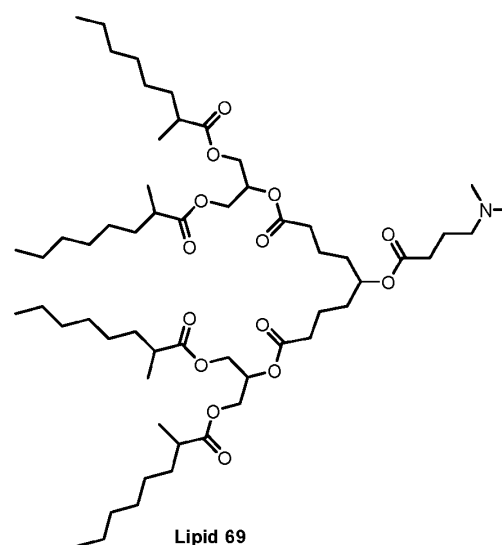
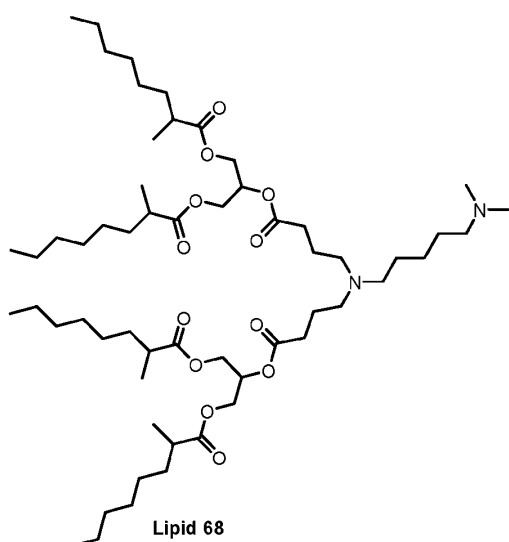
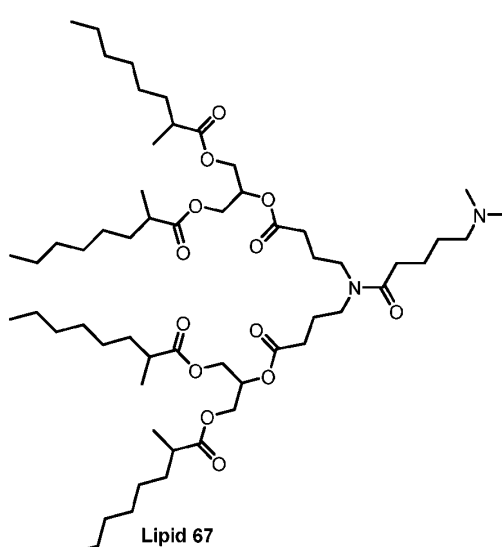
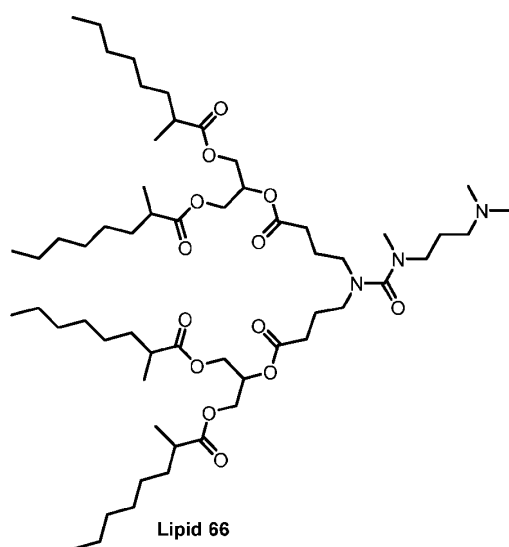
[0813] Synthesis of Lipid 45: General synthesis scheme





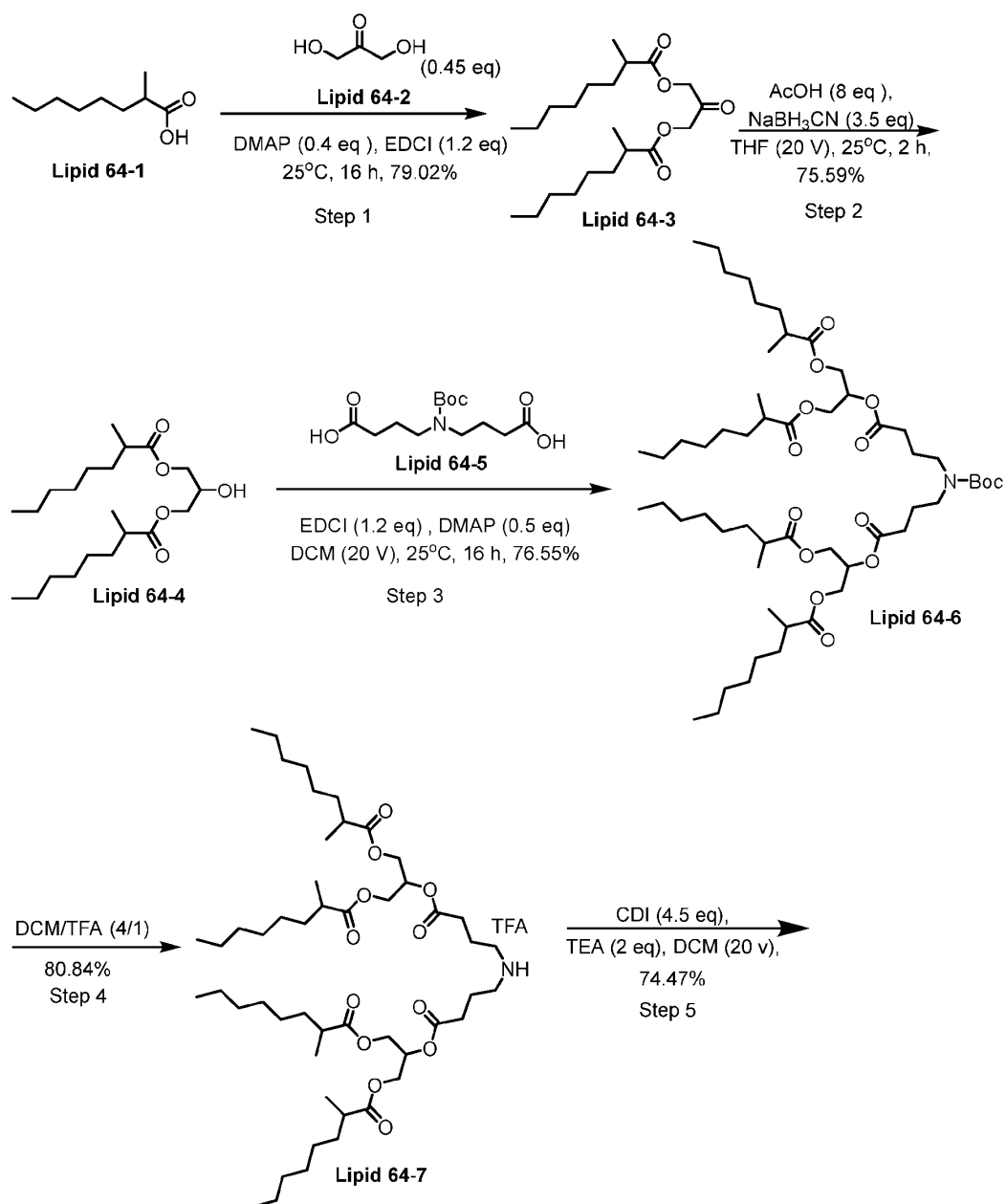
[0814] Synthesis of Lipid 45 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

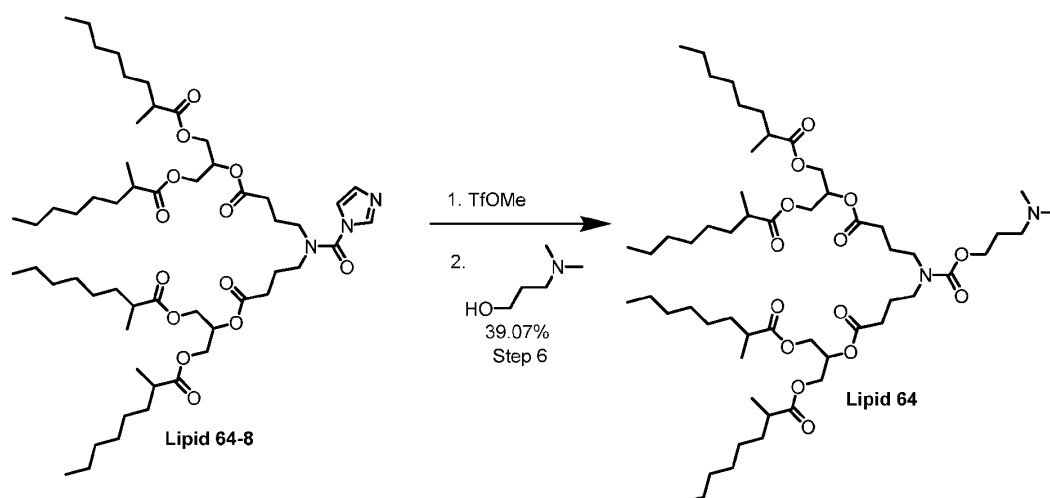




[0815] Example 57. LIPID 64: ((4,4'-(((3-(dimethylamino)propoxy)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)

[0816] Synthesis of Lipid 64: General synthesis scheme

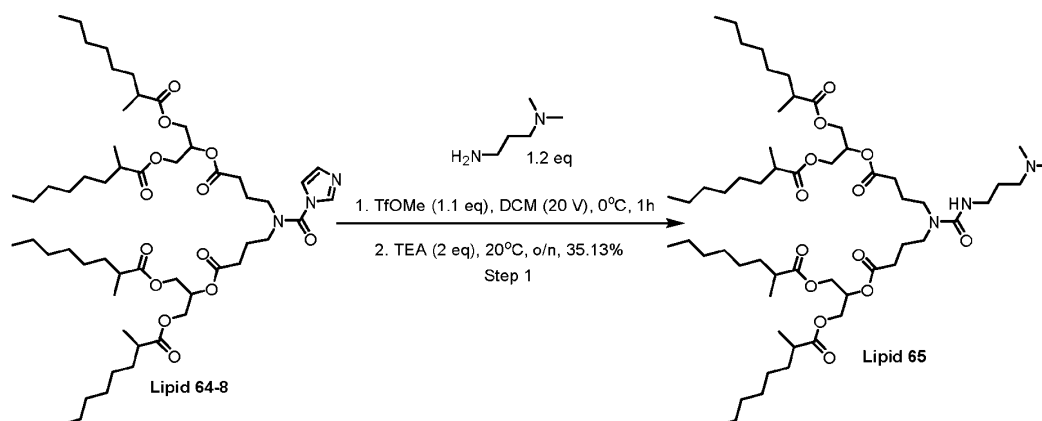




[0817] Synthesis of Lipid 64 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0818] **Example 58. LIPID 65: ((4,4'-(((3-(dimethylamino)propyl)carbonyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

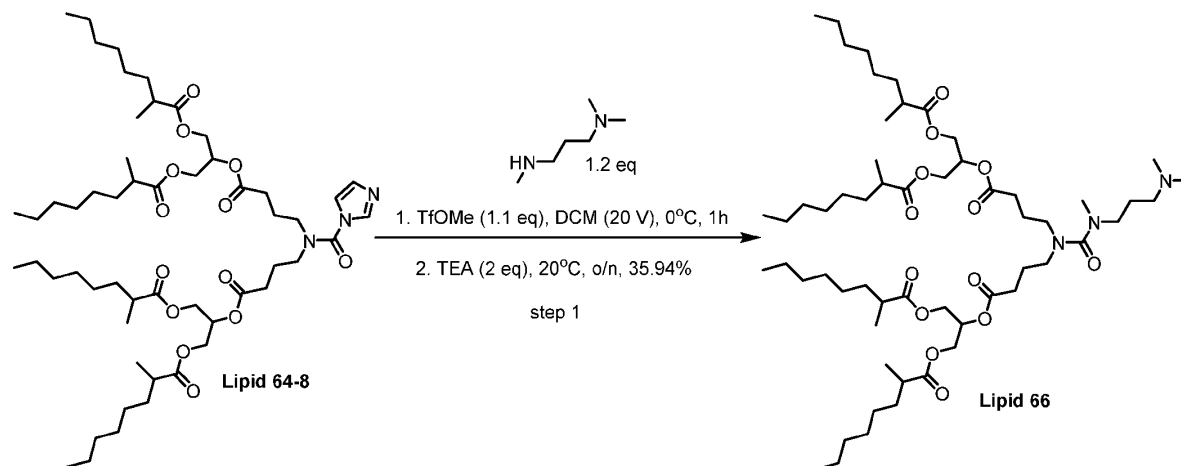
[0819] Synthesis of Lipid 65: General synthesis scheme



[0820] Synthesis of Lipid 65 from the intermediate Lipid 64-8 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0821] **Example 59. LIPID19: ((4,4'-(((3-(dimethylamino)propyl)(methyl)carbamoyl)azanediyl)bis (butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

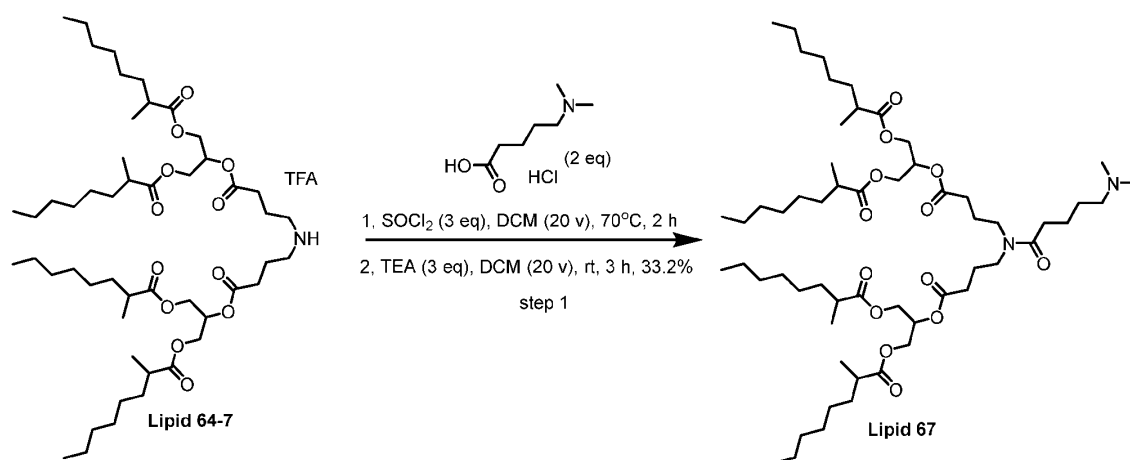
[0822] Synthesis of ATX-282: General synthesis scheme



[0823] Synthesis of Lipid 66 from the intermediate Lipid 64-8 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0824] **Example 60. LIPID 67: ((4,4'-(((5-(dimethylamino)pentanoyl)azanediyl)bis(butanoyl))bis (oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

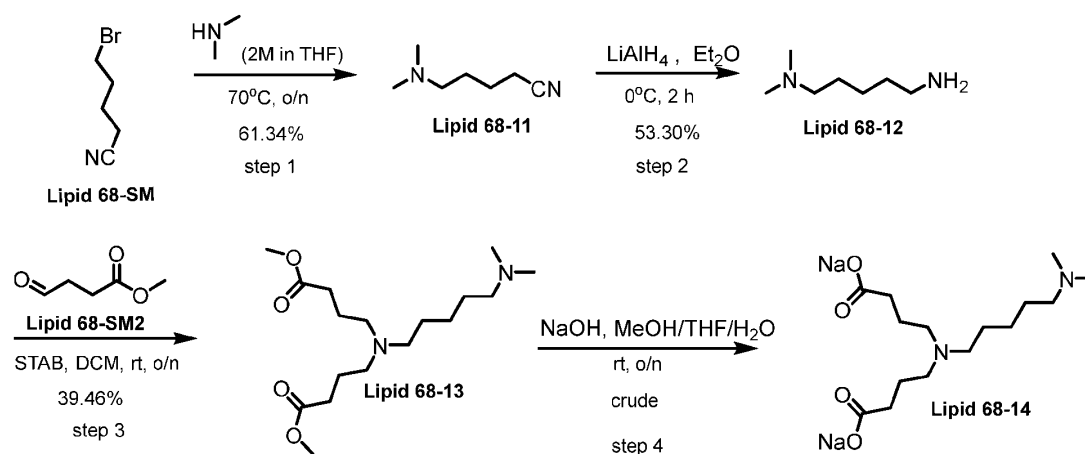
[0825] Synthesis of Lipid 67: General synthesis scheme

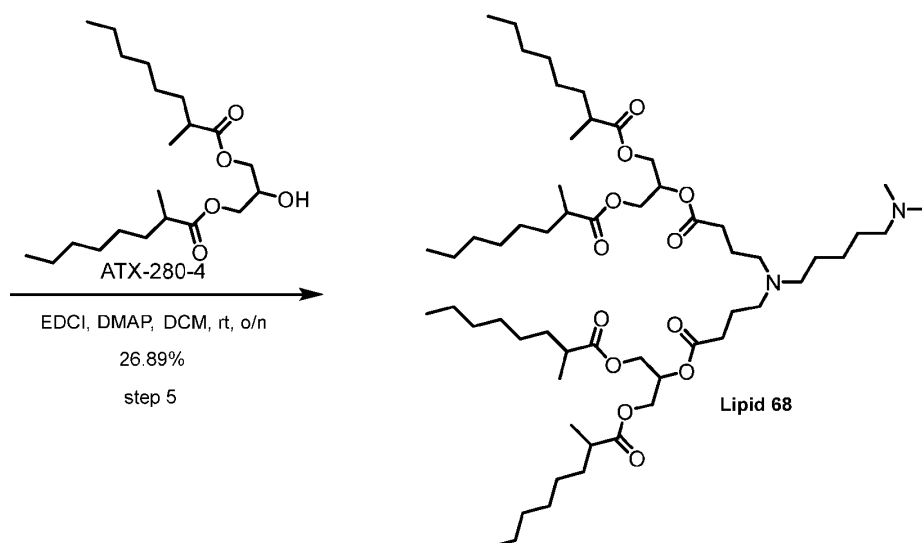


[0826] Synthesis of Lipid 67 from the intermediate Lipid 64-7 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0827] **Example 61. LIPID 68: ((4,4'-((5-(dimethylamino)pentyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

[0828] Synthesis of Lipid 68: General synthesis scheme

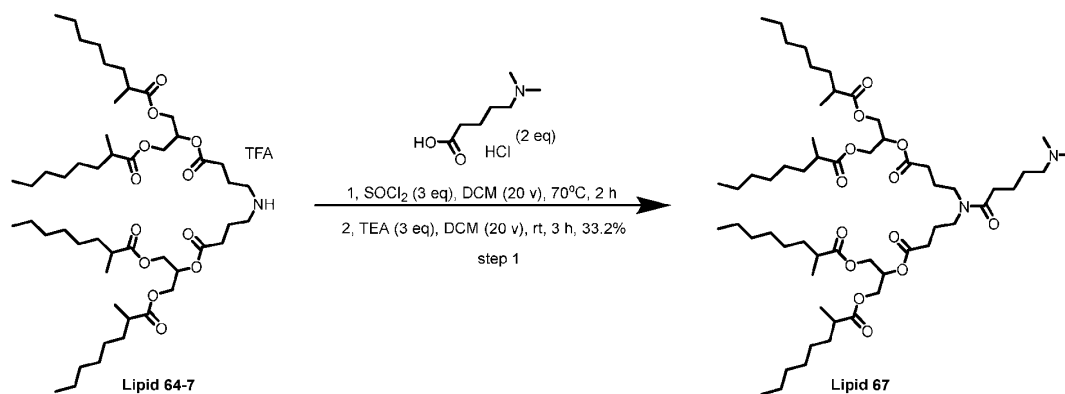




[0829] Synthesis of Lipid 68 from basic starting materials was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0830] **Example 62. LIPID 69: bis(1,3-bis((2-methyloctanoyl)oxy)propan-2-yl) 5-((4-(dimethylamino) butanoyl)oxy)nonanedioate**

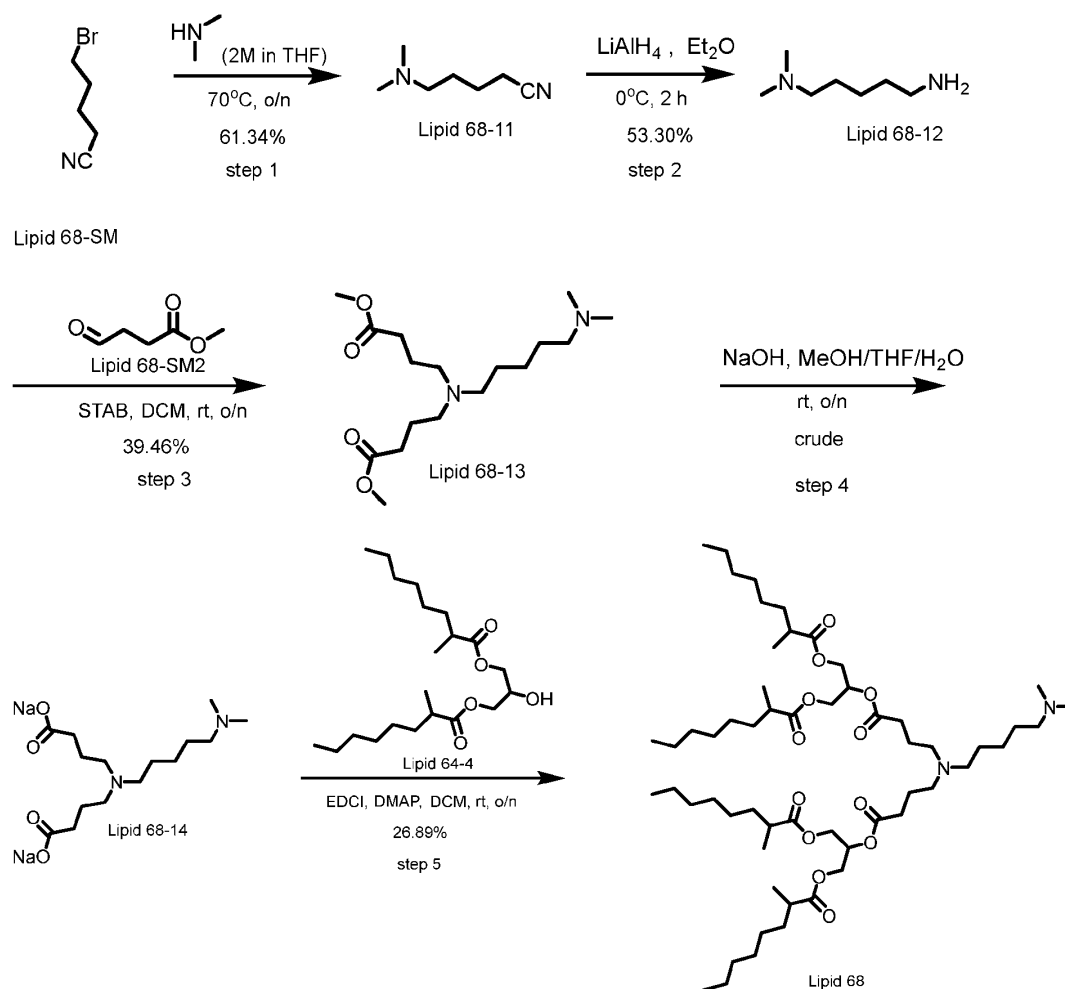
[0831] Synthesis of Lipid 69: General synthesis scheme



[0832] Synthesis of Lipid 67 from the intermediate Lipid 64-7 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0833] **Example 63. Lipid 68: ((4,4'-((5-(dimethylamino)pentyl)azanediyl)bis(butanoyl))bis(oxy))bis(propane-2,1,3-triyl) tetrakis(2-methyloctanoate)**

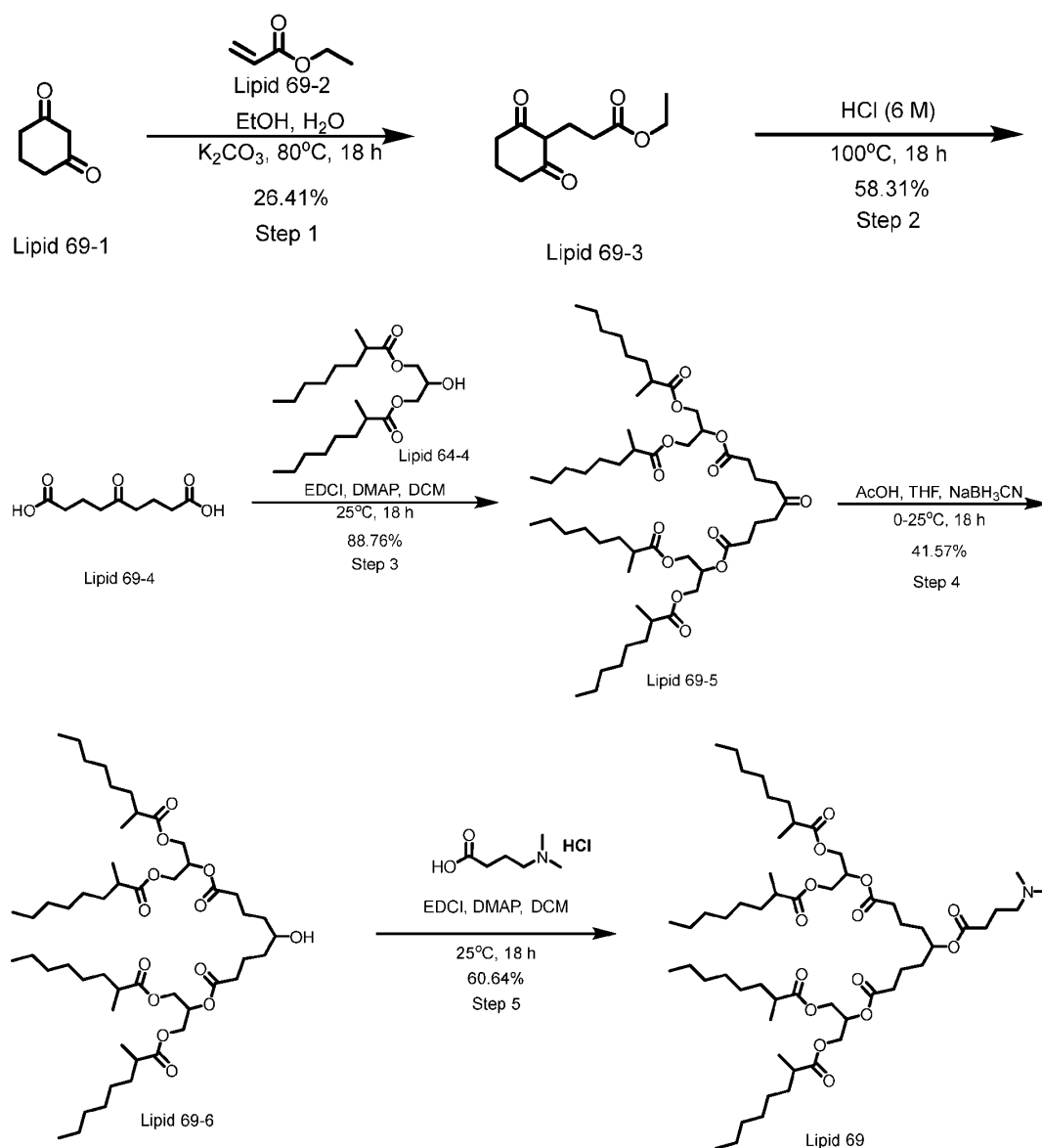
[0834] Synthesis of Lipid 68: General synthesis scheme



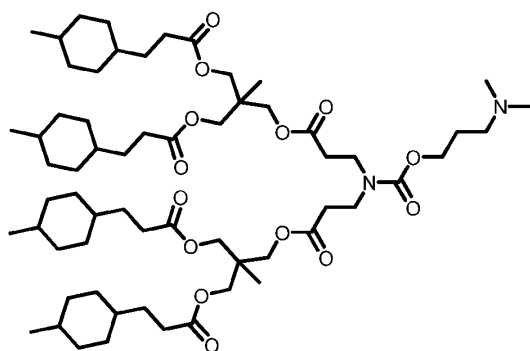
[0835] Synthesis of Lipid 68 from basic starting materials was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0836] **Example 64. Lipid 69: bis(1,3-bis((2-methyloctanoyl)oxy)propan-2-yl) 5-((4-(dimethylamino) butanoyl)oxy)nonanedioate**

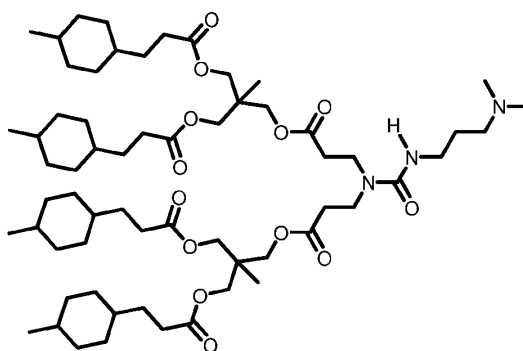
[0837] Synthesis of Lipid 69: General synthesis scheme



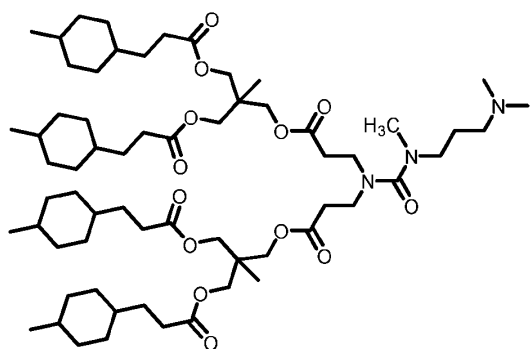
[0838] Synthesis of Lipid 69 from basic starting materials was executed following well established protocols known in the art for similar compounds as in detailed examples above.



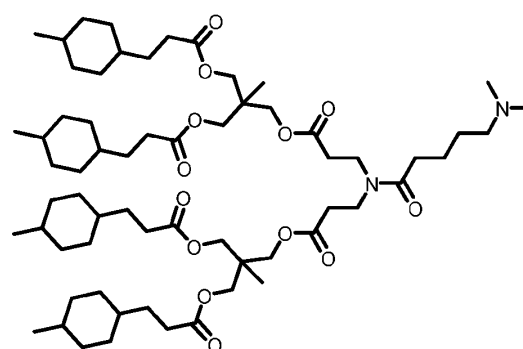
Lipid-46



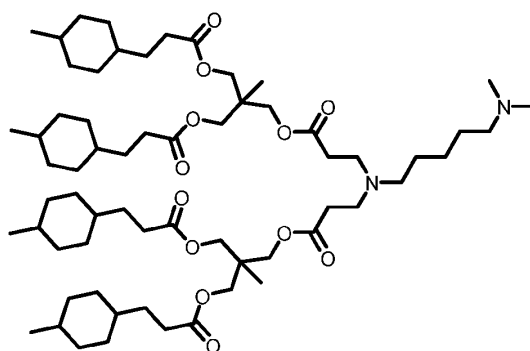
Lipid-47



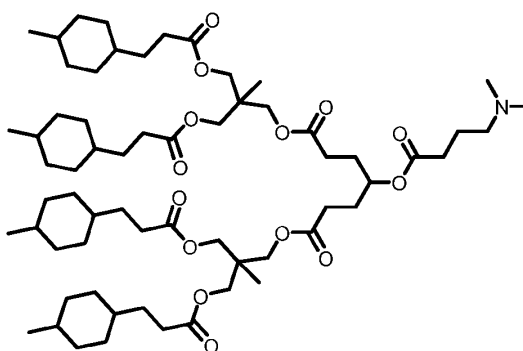
Lipid-48



Lipid-49



Lipid-50

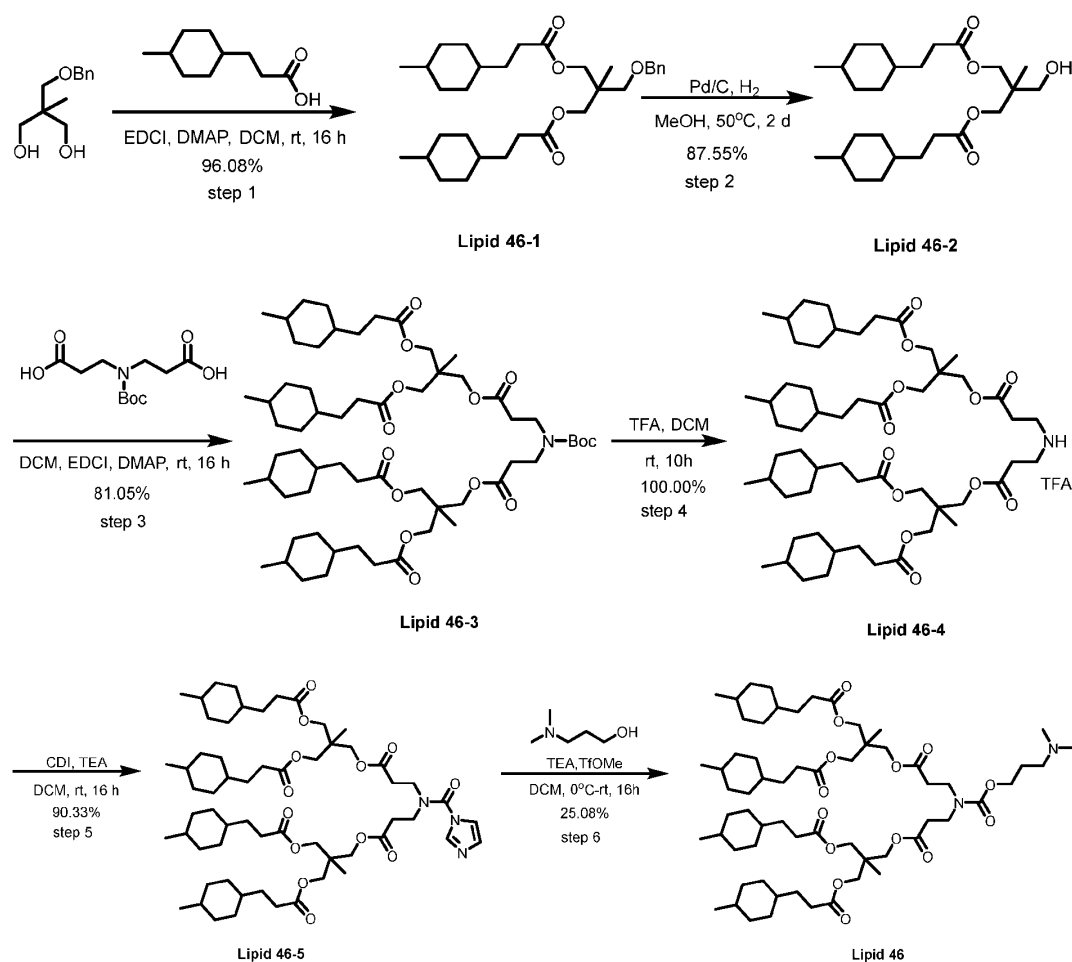


Lipid-51

[0839] **Example 65. Lipid 46: (((3,3'-(((3-dimethylamino)propyl)oxy)carbonyl)azanediyl)bis**

((propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)

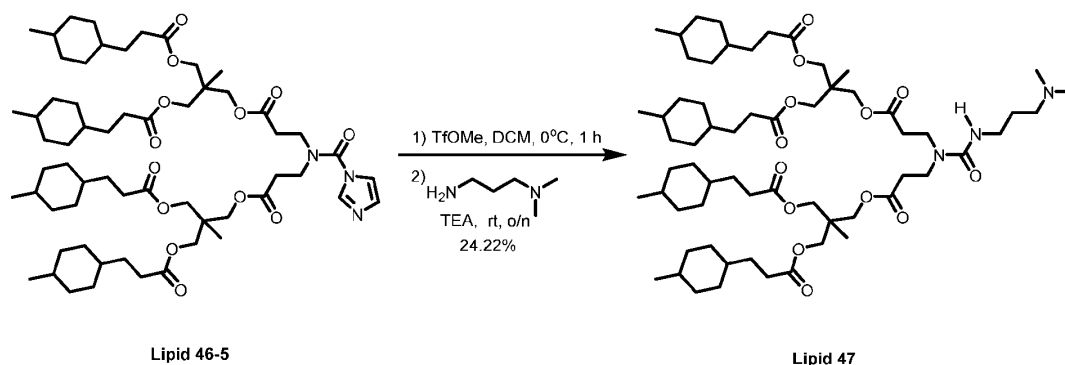
[0840] Synthesis of Lipid 46: General synthesis scheme



[0841] Synthesis of Lipid 46 from basic starting materials was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0842] Example 66. Lipid 47: (((3,3'-(((3-dimethylamino)propyl)carbamoyl)azanediyl))bis ((propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)

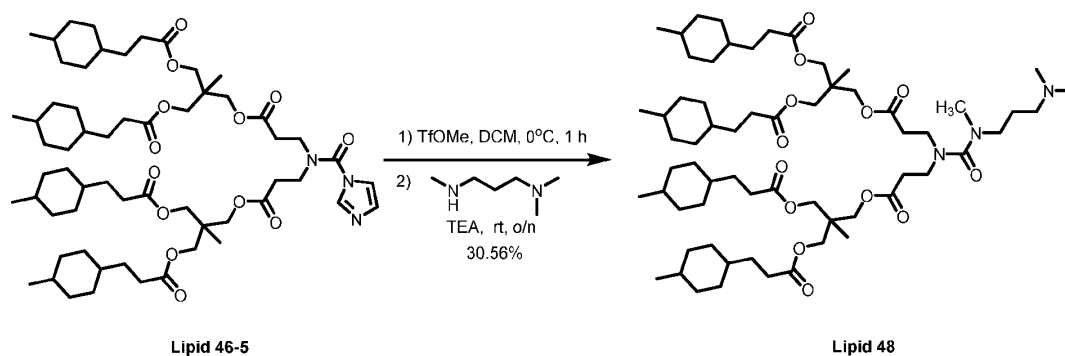
[0843] Synthesis of Lipid 47: General synthesis scheme



[0844] Synthesis of Lipid 47 from Lipid 46-5 was executed in one step following well established protocols known in the art for similar compounds as in detailed examples above.

[0845] **Example 67. Lipid 48: (((3,3'-(((3-dimethylamino)propyl)methylcarbamoyl)azanediyl)bis (propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**

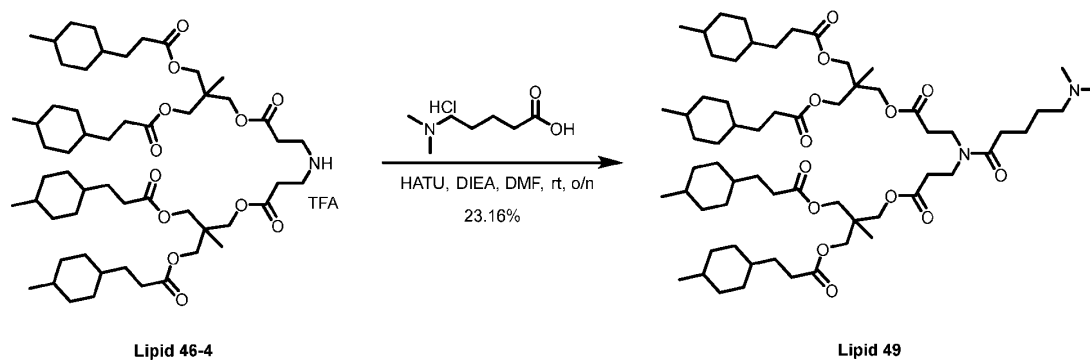
[0846] Synthesis of Lipid 48: General synthesis scheme



[0847] Synthesis of Lipid 48 from Lipid 46-5 was executed in one step following well established protocols known in the art for similar compounds as in detailed examples above.

[0848] **Example 68. Lipid 49: (((3,3'-((5-(dimethylamino)pentanoyl)azanediyl)bis (propanoyl))bis(oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl)propanoate)**

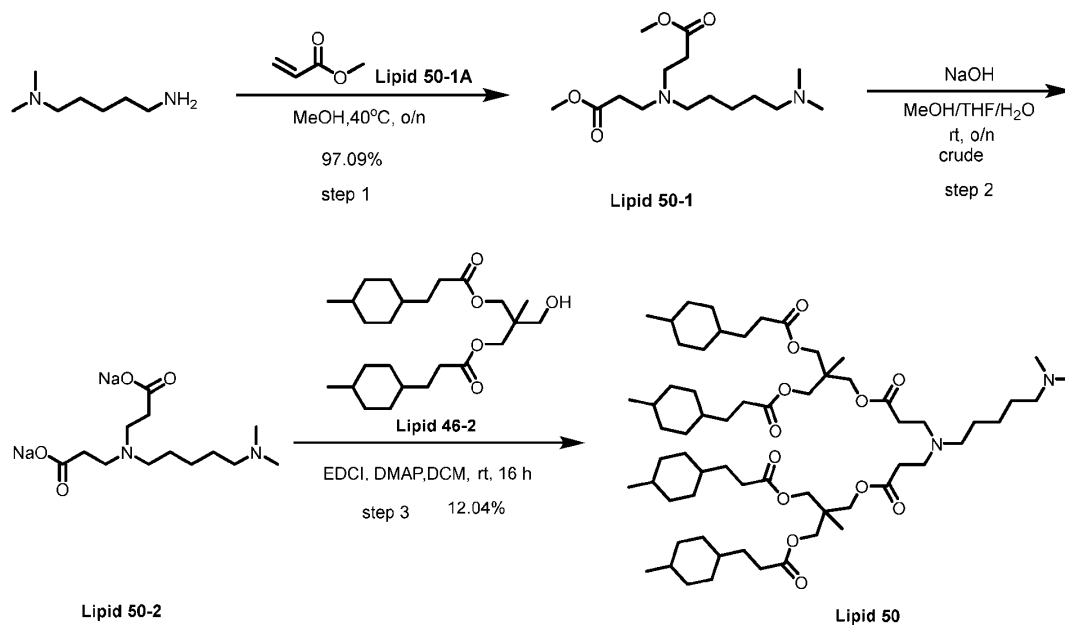
[0849] Synthesis of Lipid 49: General synthesis scheme



[0850] Synthesis of Lipid 49 from Lipid 46-4 was executed in one step following well established protocols known in the art for similar compounds as in detailed examples above.

[0851] **Example 69. Lipid 50: (((3,3'-((5-(dimethylamino)pentyl)azanediyl)bis(propanoyl))bis (oxy))bis(methylene))bis(2-methylpropane-2,1,3-triyl) tetrakis(3-(4-methylcyclohexyl) propanoate)**

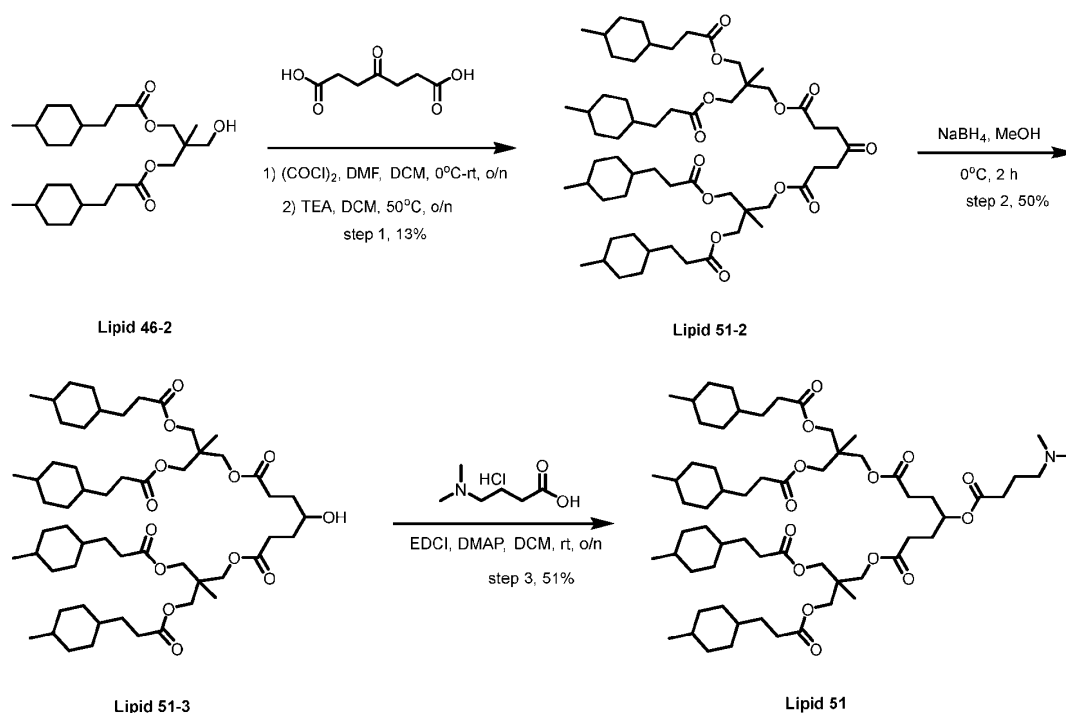
[0852] Synthesis of Lipid 50: General synthesis scheme



[0853] Synthesis of Lipid 50 from Lipid 46-2 and Lipid 50-2 was executed in one step following well established protocols known in the art for similar compounds as in detailed examples above. Lipid 50-2 was prepared from basic starting materials using chemical transformations well known in the art.

[0854] **Example 70. Lipid 51: Bis(2-methyl-3-((3-(4-methylcyclohexyl)propanoyl)oxy)-2-(((3-(4-methylcyclohexyl)propanoyl)oxy)methyl)propyl) 4-((4-(dimethylamino)butanoyl)oxy)heptanedioate**

[0855] Synthesis of Lipid 51: General synthesis scheme



[0856] Synthesis of Lipid 51 from Lipid 46-2 was executed following well established protocols known in the art for similar compounds as in detailed examples above.

[0857] **Table 1. Biological Assays, Half-Life, Degradability, and cpKa and cLogD Data**

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
1	100	84	432	1413	14	BL.OQ	BL.OQ	9.34 (6.4/ 6.64)	13.9
2	ND	ND	ND	ND	ND	ND	ND	9.36	11.9
3	87	51	191	471	15	BLOQ	BLOQ	9.36 (6.41/ 6.49)	13.95
4	33	71	392	1108	13	BLOQ	BLOQ	9.31 (6.22 /ND)	12.58
5	62	90	87	533	40	BLOQ	BLOQ	9.36 (6.37/ 6.54)	13.09
6	64	87	46	324	6	BLOQ	BLOQ	9.34 (7.12/ 7.02)	13.9
7	ND	ND	ND	ND	ND	ND	ND	9.31 (ND)	ND
8	ND	ND	169	701	81	BLOQ	BLOQ	9.29 (6.50/ ND)	13.23
9	ND	ND	ND	ND	ND	ND	ND	9.30 ND	ND

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
10	83	92	387	1179	36	BL.OQ	BL.OQ	9.35 (6.46/ 6.67)	11.59
11	22	84	169	548	40	BLOQ	BLOQ	9.35 (6.6/ 6.68)	13.63
12	69	91	286	768	57	BLOQ	BLOQ	9.35 (6.46/ 6.61)	13.08
13	87	60	ND	ND	74	<340	<340	9.34 (6.40/ 6.61)	13.29
14	90	65	ND	ND	116	<340	<340	9.34 (7.19/ 6.86)	13.63
15	ND	ND	ND	ND	256	<340	<340	9.34 (6.57/ 6.45)	12.72
16	88	58	2088	6358	40	<340	<340	9.34 (6.50/ 6.45)	12.72
17	81	55	1082	1555	ND	BLOQ	BLOQ	9.34 (6.35/ 6.35)	13.01
18	88	60	ND	1081	100	<340	639	9.34 (6.44/ 6.49)	13.27

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
19	ND	ND	ND	ND	ND	<340	847	9.34 (6.27/ ND)	14.92
20	ND	ND	ND	ND	ND	ND	ND	9.34 (ND)	14.29
21	ND	ND	ND	ND	ND	ND	ND	9.34 (ND)	12.26
22	ND	ND	ND	ND	ND	ND	ND	9.34	12.26
23	ND	ND	ND	ND	ND	ND	ND	9.34	13.43
24	ND	ND	ND	ND	ND	ND	ND	9.34	12.41
25	ND	ND	ND	ND	ND	ND	ND	9.34	14.23
26	ND	ND	ND	ND	ND	ND	ND	8.66	13.95
27	ND	ND	ND	ND	ND	ND	ND	9.34	13.47

LIPID / Attri- butes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
28	ND	ND	ND	ND	ND	ND	ND	9.34	12.02
29	ND	ND	ND	ND	ND	ND	ND	9.34	9.91
30	ND	ND	ND	ND	ND	ND	ND	9.34	12.02
31	ND	ND	ND	ND	ND	ND	ND	9.34	14.13
32	ND	ND	ND	ND	ND	ND	ND	ND	ND
33	ND	ND	ND	ND	ND	ND	ND	ND	ND
34	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: not determined

BLOQ: below level of quantification

[0858] Table 2. Biological Assays, Half-Life, Degradability, and cpKa and cLogD Data

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
1	100	84	432	1413	14	BL.OQ	BL.OQ	9.34 (6.4/6.6 4)	13.9
2	ND	ND	ND	ND	ND	ND	ND	9.36	11.9
3	87	51	191	471	15	BLOQ	BLOQ	9.36 (6.41/ 6.49)	13.95
4	33	71	392	1108	13	BLOQ	BLOQ	9.31 (6.22/ ND)	12.58
5	62	90	87	533	40	BLOQ	BLOQ	9.36 (6.37/ 6.54)	13.09
6	64	87	46	324	6	BLOQ	BLOQ	9.34 (7.12/ 7.02)	13.9
7	ND	ND	ND	ND	ND	ND	ND	9.31 (ND)	ND
8	ND	ND	169	701	81	BLOQ	BLOQ	9.29 (6.50/N D)	13.23
9	ND	ND	ND	ND	ND	ND	ND	9.30 ND	ND

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
10	83	92	387	1179	36	BL.OQ	BL.OQ	9.35 (6.46/6. 67)	11.59
11	22	84	169	548	40	BLOQ	BLOQ	9.35 (6.6/6.6 8)	13.63
12	69	91	286	768	57	BLOQ	BLOQ	9.35 (6.46/6. 61)	13.08
13	87	60	ND	1488.1	74	<340	<340	9.34 (6.40/6. 61)	13.29
14	90	65	ND	1152	100	<340	<340	9.34 (7.19/6. 86)	13.63
15	89.7	66.8	ND	1899	100	<340	<340	9.34 (6.57/6. 45)	12.72
16	88	58	ND	2088	40	<340	<340	9.34 (6.50/6. 45)	12.72
17	81	55	ND	1082	100	BLOQ	BLOQ	9.34 (6.35/6. 35)	13.01
18	88	60	ND	1081	100	<340	639	9.34 (6.44/6. 49)	13.27

LIPID / Attri- butes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
19	ND	ND	ND	958	100	<340	847	9.34 (6.27/ ND)	14.92
20	74	36	ND	1148	100	<340	<340	9.34 (ND)	14.29
21	90	57	ND	1028	100	<340	<340	9.34 (ND)	12.26
22	87	56	ND	1104	100	<340	<340	9.34 (ND)	12.26
23	61.7	17	ND	730	100	<340	<340	9.34 (ND)	13.43
24	87.4	60.5	ND	1420	100	BLOQ	BLOQ	9.34	12.41
25	95	63	ND	2945	100	BLOQ	BLOQ	9.34	14.23
26	50	8	ND	70	100	BLOQ	BLOQ	8.66	13.95
27	84.9	48.1	ND	69	100	BLOQ	BLOQ	9.34	13.47

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remain- ing after 2h	In vivo degrad- ability (ng/ tissue) at 48h 0.03 mpk	In vivo degrad- ability (ng/ tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/ FVII)	cLogD
28	75.3	26	ND	216	100	BL.OQ	BL.OQ	9.34	12.02
29	ND	ND	ND	ND	ND	ND	ND	9.34	9.91
30	62.1	3.2	ND	95	100	BLOQ	BLOQ	9.34	12.02
31	ND	ND	ND	ND	ND	ND	ND	9.34	14.13
32	ND	ND	ND	ND	ND	ND	ND	ND	ND
33	ND	ND	ND	ND	ND	ND	ND	ND	ND
34	ND	ND	ND	ND	ND	ND	ND	ND	ND
35	84	68.5	ND	678	ND	ND	ND	9.27	13.03
36	0	0	ND	0	ND	ND	ND	9.58	11.82

LIPID / Attributes	FVII KD% 0.03 mpk	FVII KD% 0.01 mpk	EPO exprssn (ng/mL) 0.03 mpk	EPO exprssn (ng/mL) 0.1 mpk	Plasma half life % remaining after 2h	In vivo degradability (ng/tissue) at 48h 0.03 mpk	In vivo degradability (ng/tissue) at 48h 0.1 mpk	c-pKa (pKa: EPO/FVII)	cLogD
37	ND	ND	ND	ND	ND	ND	ND	9.58	12.7
38	ND	ND	ND	ND	ND	ND	ND	9.68	12.28
39	ND	ND	ND	ND	ND	ND	ND	ND	11.83
40	ND	ND	ND	ND	ND	ND	ND	9.36	13.31
41	76	56	ND	1209	ND	ND	ND	ND	ND

ND: not determined

BLOQ: below level of quantification

[0859] Table 3. Biological Assays, Half-Life, Degradability, and cpKa and cLogD Data

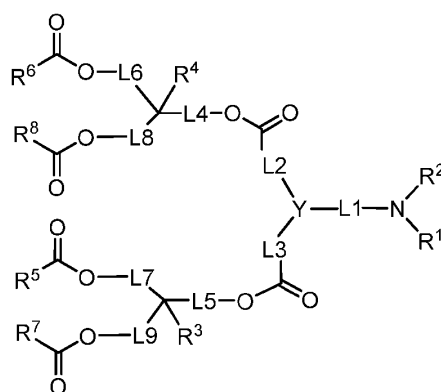
Lipid No.	Size (nm)	PDI	EE%	EPO expression in ng/mL @ 0.3 mg/kg	pKa	cLogD	Invivo lipid degradability ng/g of Liver tissue @ 0.3 mg/kg dose	FVII KD% @ 0.03 mg/kg
42	100.4	0.086	88.2			15.85		
63	82.65	0.046	85.9	4601	6.2	10.96	603 ± 57.6	76.17
43	92.35	0.05	90	14.3	6.33	12.06	1970 ± 379	15.24
44	113.1	0.134	85.7			12.93		

46	81.62	0.114	91.5	958.2	7.08	14.05		82.9
47	98.26	0.089	98.9			12.46		
48	103.2	0.065	96.2		7.18	13.74		
49	97.23	0.028	98.9		7.5	13.25		3.57
50	97.08	0.13	94.7	579.2	7.04	13.71	28100	93.5
51	65.71	0.059	90.4	868.5	6.53	14.09	36000	65.23
52	77.62	0.038	90.8	17888.62	7.20	11.93	BLOQ	69.66
53	85.94	0.076	93.1	15844.04	6.93	12.75	14200	85.45
54	76.03	0.072	90.9	25548.45	7.30	13.02	BLOQ	86.88
55	71.78	0.103	93.0	10312.89	6.59	15.25	63300	72.17
56	67.34	0.081	91.5	5463.76	6.55	14.04	81900	46.3
57	78.98	0.065	94.3	21737.69	6.52	11.63	BLOQ	
58	70.09	0.081	93.4	19337.84	6.49	13.67	BLOQ	
59	74.77	0.068	92.1	14723.72	6.37	13.44	BLOQ	
60	65.95	0.087	93.9	1631.03	6.28	13.23	53300	
61	70.54	0.064	95.3	4238.52	6.84	11.97	BLOQ	
62	72.43	0.056	95.3	2930.66	6.64	12.79	6880	

[0860] Although the foregoing disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference. Where a conflict exists between the instant application and a reference provided herein, the instant application shall dominate.

WHAT IS CLAIMED IS:

1. A compound of Formula I, or a pharmaceutically acceptable salt thereof:



(I)

wherein:

R^1 and R^2 are each independently H or C_{1-6} alkyl; or

R^1 and R^2 are joined to form a saturated heterocyclic ring, wherein:

R^1 is a linear C_{1-4} alkylene; and

R^2 is $-(CH_2)_m(X)_n-$, wherein

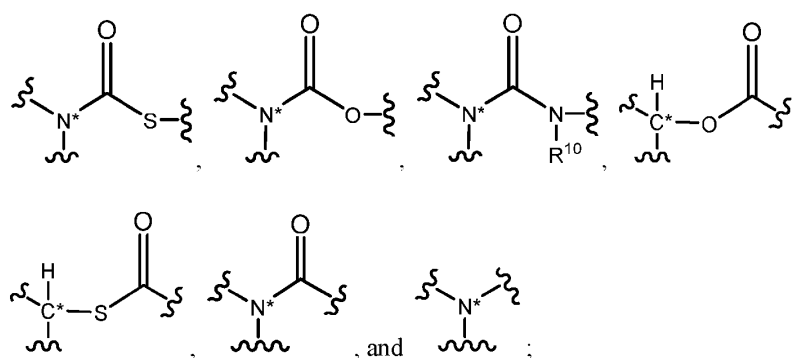
X is O, S, or NR^9 , wherein R^9 is H or C_{1-6} alkyl;

m is 1, 2, 3 or 4, and

n is 0 or 1;

L1 is a linear C_{1-6} alkylene optionally substituted with one to three methyl groups;

Y is selected from the group consisting of:



wherein:

each asterisk (*) indicates the atom attached to L2 and L3; and

R¹⁰ is H or C₁₋₆ alkyl;

L2 and L3 are each independently a linear C₁₋₈ alkylene;

L4, L5, L6, L7, L8 and L9 are each independently absent or -CH₂-, provided that:

at least two of L4, L6 and L8 are -CH₂-; and

at least two of L5, L7 and L9 are -CH₂-;

R³ and R⁴ are each independently H, methyl or ethyl; and

R⁵, R⁶, R⁷ and R⁸ are each independently selected from the group consisting of:

linear C₁₋₂₀ alkyl, wherein each said linear C₁₋₂₀ alkyl is optionally substituted with one or more substituents selected from the group consisting of:

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl;

6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl;

C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl; and

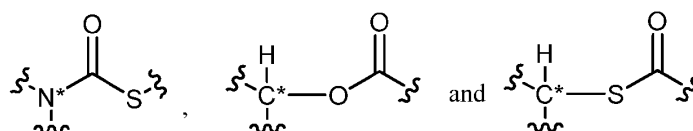
6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

2. The compound of claim 1, wherein:

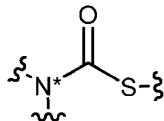
R¹ is H or C₁₋₆ alkyl, and R² is C₁₋₆ alkyl; or

R¹ and R² are joined to form said saturated heterocyclic ring.

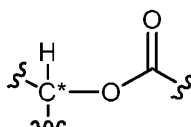
3. The compound of claim 1 or 2, wherein Y is selected from the group consisting of:



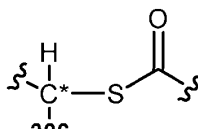
4. The compound of claim 1 or 2, wherein Y is:



5. The compound of claim 1 or 2, wherein Y is:



6. The compound of claim 1 or 2, wherein Y is:

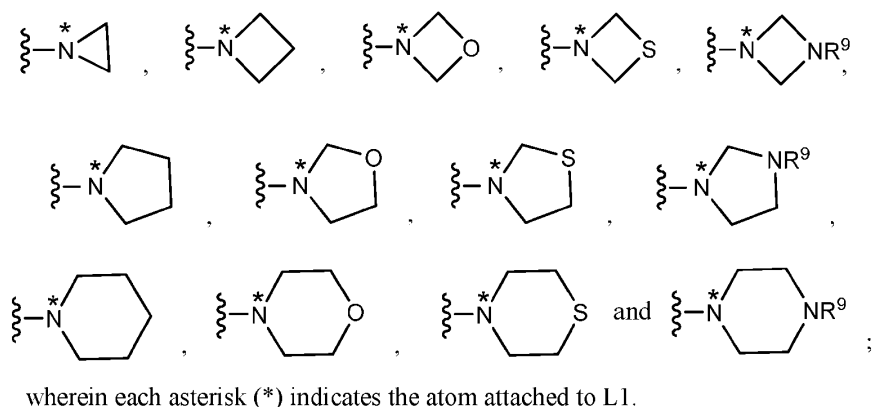


7. The compound of claim 5 or 6, wherein:

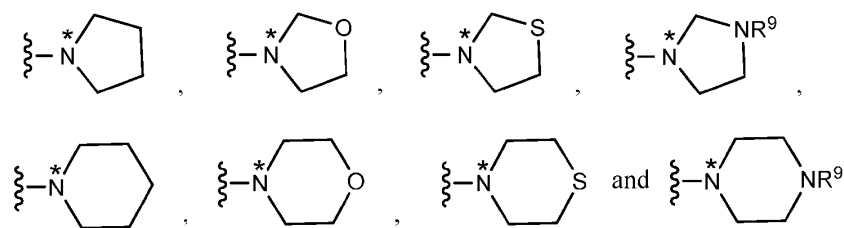
at least one of R^1 and R^2 is H; and

$L1$ is $-CH_2-$ or $-CH_2CH_2-$.

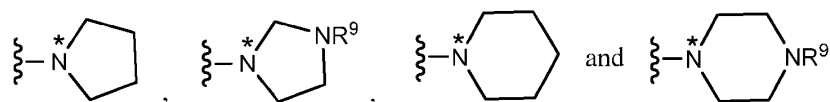
8. The compound of any one of claims 1 to 6, wherein R^1 and R^2 are each independently C_{1-6} alkyl.
9. The compound of claim 8, wherein R^1 and R^2 are each independently C_{1-3} alkyl.
10. The compound of claim 9, wherein R^1 and R^2 are each methyl.
11. The compound of any one of claims 1 to 6, wherein R^1 and R^2 are joined to form said heterocyclic ring.
12. The compound of claim 11, wherein the heterocyclic ring is selected from the group consisting of:



13. The compound of claim 12, wherein the heterocyclic ring is selected from the group consisting of:



14. The compound of claim 13, wherein the heterocyclic ring is selected from the group consisting of:



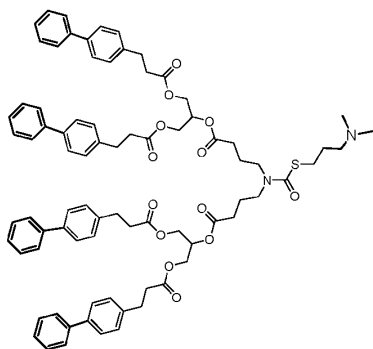
15. The compound of any one of the preceding claims, wherein R^5 , R^6 , R^7 and R^8 are each independently linear C_{1-8} alkyl, wherein each said linear C_{1-8} alkyl is optionally substituted with one or more substituents selected from:

C_{6-10} aryl, wherein each said C_{6-10} aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C_{6-10} aryl that is optionally substituted with one or more C_{1-6} alkyl; and

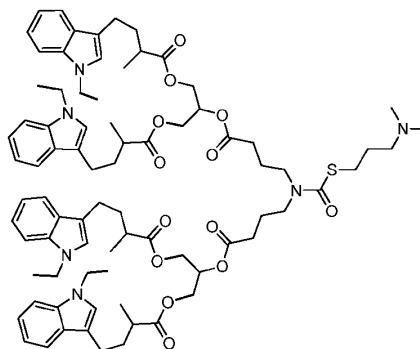
6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.

16. The compound any one of claims 1-14, wherein R⁵, R⁶, R⁷ and R⁸ are each independently C₆₋₁₀ aryl, wherein each said C₆₋₁₀ aryl is a monocyclic or bicyclic aromatic hydrocarbon optionally substituted with one or more C₆₋₁₀ aryl that is optionally substituted with one or more C₁₋₆ alkyl.
17. The compound of any one of claims 1-14, wherein R⁵, R⁶, R⁷ and R⁸ are each independently a 6-10 membered heteroaryl, wherein each said 6-10 membered heteroaryl is a monocyclic or bicyclic aromatic system optionally substituted with one or more C₁₋₆ alkyl.
18. The compound of any of the preceding claims, wherein R⁵ and R⁶ are the same.
19. The compound of any of the preceding claims, wherein R⁷ and R⁸ are the same.
20. The compound of any one of the preceding claims, wherein L1 is linear unsubstituted alkylene.
21. The compound of any one of the preceding claims, wherein L1 is propylene.
22. The compound of any one of the preceding claims, wherein L2 and L3 are each independently linear C₁₋₅ alkylene.
23. The compound of any one of the preceding claims, wherein L2 and L3 are the same.
24. The compound of any one of the preceding claims, wherein L4 and L5 are the same.
25. The compound of any one of the preceding claims, wherein L6 and L7 are the same.
26. The compound of any one of the preceding claims, wherein L8 and L9 are the same.

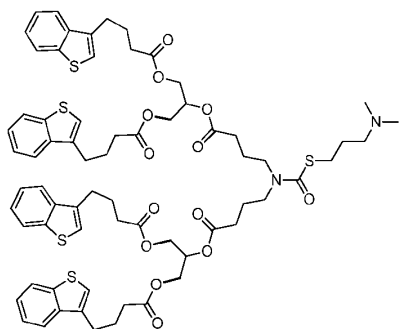
27. The compound of any one of the preceding claims, wherein L4, L5, L6, L7, L8 and L9 are each -CH₂-.
28. The compound of any one of claims 1 to 26, wherein L6, L7, L8 and L9 are each -CH₂-; and L4 and L5 are absent.
29. The compound of any one of claims 1 to 26, wherein L4, L5, L8 and L9 are each -CH₂-; and L6 and L7 are absent.
30. The compound of any one of claims 1 to 26, wherein L4, L5, L6 and L7 are each -CH₂-; and L8 and L9 are absent.
31. The compound of any one of the preceding claims, wherein R³ and R⁴ are each independently H or methyl.
32. The compound of any one of the preceding claims, wherein R³ and R⁴ are each H.
33. The compound of any one of the preceding claims, wherein R³ and R⁴ are each methyl.
34. The compound of claim 1, selected from the group consisting of:



Lipid 42



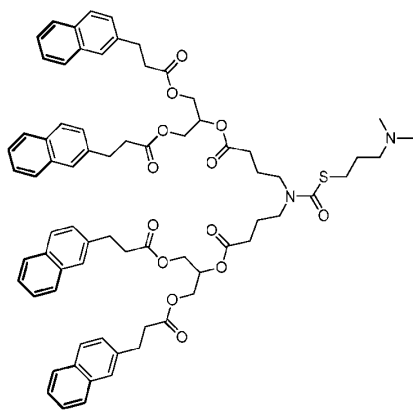
Lipid 43, and



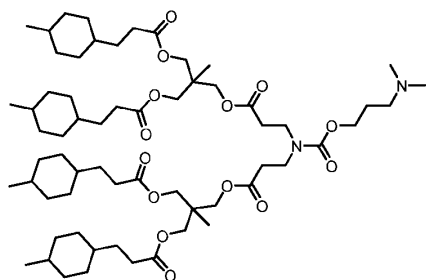
Lipid 45,

or pharmaceutically acceptable salts thereof.

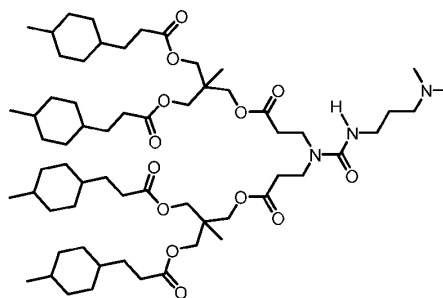
35. A compound selected from the group consisting of:



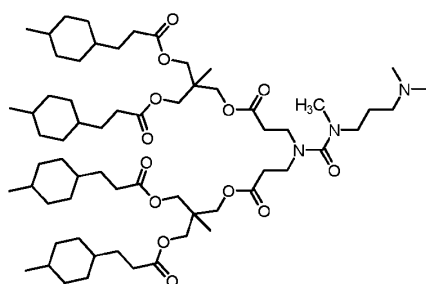
Lipid 44



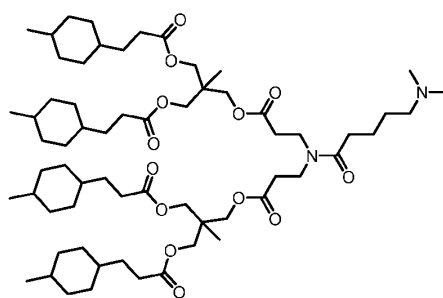
Lipid 46



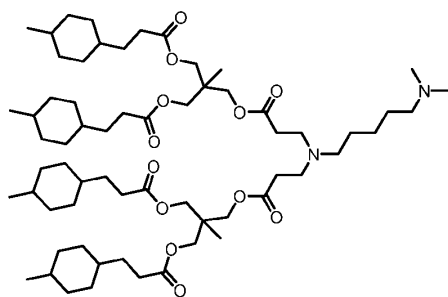
Lipid 47



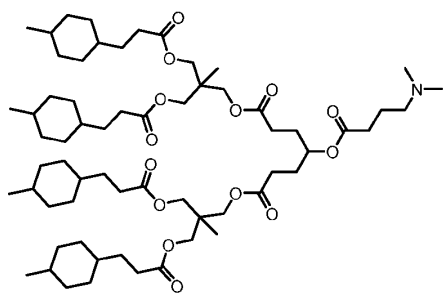
Lipid 48



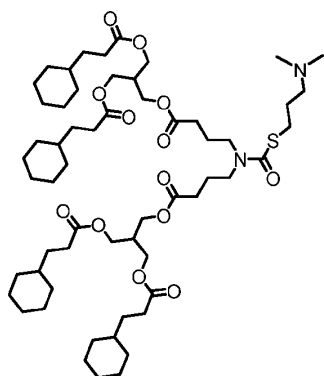
Lipid 49



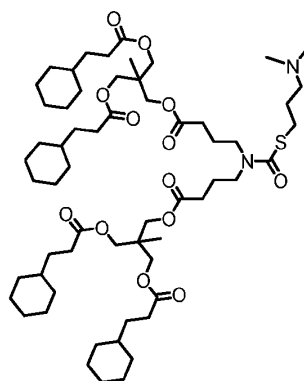
Lipid 50



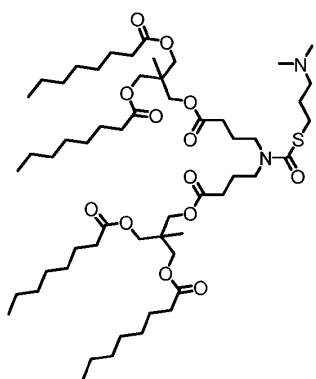
Lipid 51



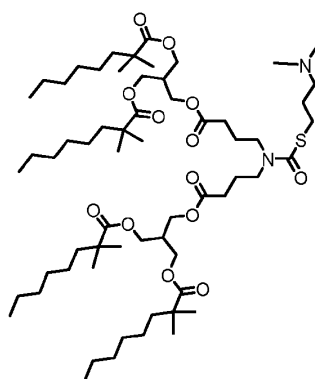
Lipid 52



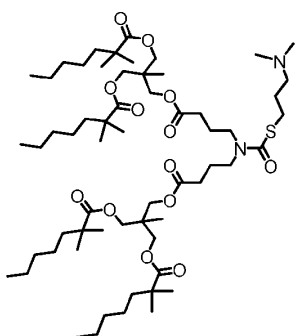
Lipid 53



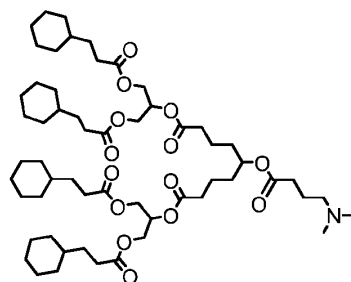
Lipid 54



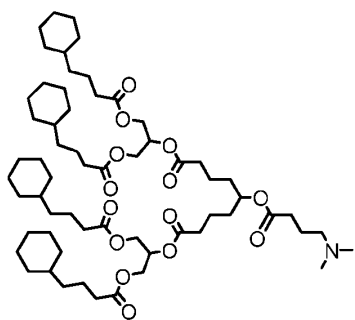
Lipid 55



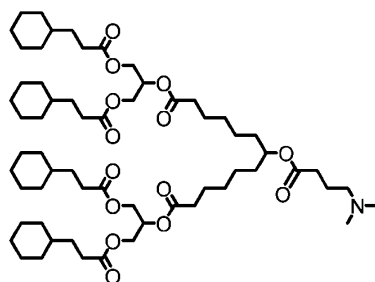
Lipid 56



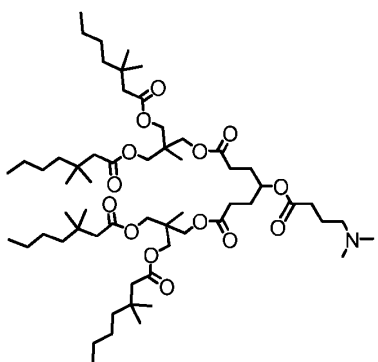
Lipid 57



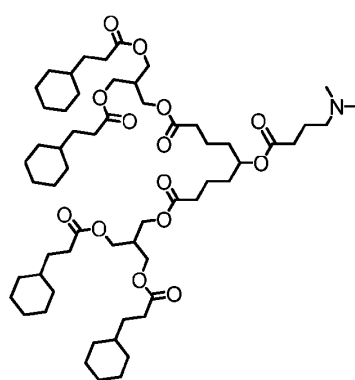
Lipid 58



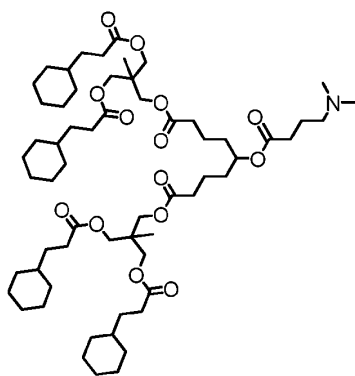
Lipid 59



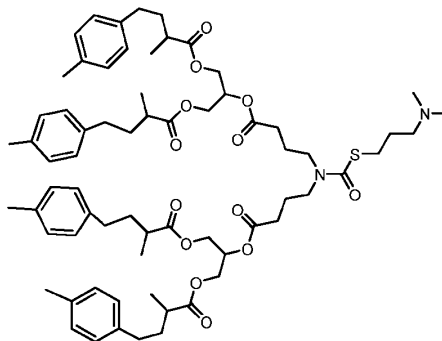
Lipid 60



Lipid 61



Lipid 62, and



Lipid 63;

or pharmaceutically acceptable salts thereof.

36. A lipid composition comprising a nucleic acid and a compound of any one of the preceding claims.
37. The lipid composition of claim 36, wherein the nucleic acid is selected from an siRNA, an mRNA, a self-replicating RNA, a DNA plasmid, and an antisense oligonucleotide.
38. The lipid composition of claim 36 or 37, wherein the nucleic acid is a mRNA or a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest.
39. The lipid composition of claim 38, wherein the therapeutic protein of interest is an enzyme, and antibody, an antigen, a receptor, or a transporter.
40. The lipid composition of claim 38 or 39, wherein the therapeutic protein of interest is a gene-editing enzyme.
41. The lipid composition of claim 40, wherein the gene-editing enzyme is selected from a TALEN, a CRISPR, a meganuclease, or a zinc finger nuclease.
42. The lipid composition of any one of claims 36 to 41, wherein the lipid composition comprises liposomes, lipoplexes, or lipid nanoparticles.
43. A lipid nanoparticle, comprising a plurality of ligands, wherein each ligand is independently a compound of any one of claims 1 to 35, wherein the plurality of ligands self-assembles to form the lipid nanoparticle comprising an interior and exterior.
44. The lipid nanoparticle of claim 43, wherein the average particle size of the lipid nanoparticle is less than about 100 nm.
45. The lipid nanoparticle of claim 43 or 44, wherein the average particle size of the lipid nanoparticle is about 55 nm to about 85 nm.
46. The lipid nanoparticle of any one of claims 43 to 45, wherein the lipid nanoparticle further comprises a nucleic acid encapsulated in the interior.
47. The lipid nanoparticle of claim 46, wherein the nucleic acid is selected from an siRNA, an mRNA, a self-replicating RNA, a DNA plasmid, and an antisense oligonucleotide.

48. The lipid nanoparticle of claim 46 or 47, wherein the nucleic acid is a mRNA or a self-replicating RNA comprising a coding region that encodes a therapeutic protein of interest.
49. The lipid nanoparticle of claim 48, wherein the therapeutic protein of interest is an enzyme, and antibody, an antigen, a receptor, or a transporter.
50. The lipid nanoparticle of claim 48 or 49, wherein the therapeutic protein of interest is a gene-editing enzyme.
51. The lipid nanoparticle of claim 50, wherein the gene-editing enzyme is selected from a TALEN, a CRISPR, a meganuclease, or a zinc finger nuclease.
52. The lipid nanoparticle of any one of claims 43 to 51, wherein the lipid nanoparticle further comprises a helper lipid selected from: dioleoylphosphatidyl ethanolamine (DOPE), dimyristoylphosphatidyl choline (DMPC), distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidyl glycerol (DMPG), dipalmitoyl phosphatidylcholine (DPPC), and phosphatidylcholine (PC).
53. The lipid nanoparticle of claim 52, wherein the helper lipid is distearoylphosphatidylcholine (DSPC).
54. The lipid nanoparticle of any one of claims 43 to 53, further comprising cholesterol.
55. The lipid nanoparticle of any one of claims 43 to 54, further comprising a polyethylene glycol(PEG)-lipid conjugate.
56. The lipid nanoparticle of claim 55, wherein PEG-lipid conjugate is PEG-DMG.
57. The lipid nanoparticle of claim 56, wherein the PEG-DMG is PEG2000-DMG.
58. The lipid nanoparticle of any one of claims 43 to 57, wherein the lipid nanoparticle comprises about 45 mol% to 65 mol% of the compound of any one of claims 1 to 35, about 2 mol% to about 15 mol% of a helper lipid, about 20 mol% to about 42 mol% of cholesterol, and about 0.5 mol% to about 3 mol% of a PEG-lipid conjugate.
59. The lipid nanoparticle of claim 58, wherein the lipid nanoparticle comprises about 50 mol% to about 61 mol% of the compound of any one of claims 1 to 35, about 5 mol% to

about 9 mol% of the helper lipid, about 29 mol% to about 38 mol% of cholesterol, and about 1 mol% to about 2 mol% of the PEG-lipid conjugate.

60. The lipid nanoparticle of claim 59, wherein the lipid nanoparticle comprises about 56 mol% to about 58 mol% of the compound of any one of claims 1 to 35, about 6 mol% to about 8 mol% of DSPC, about 31 mol% to about 34 mol% of cholesterol, and about 1.25 mol% to about 1.75 mol% of the PEG-lipid conjugate.

61. The lipid nanoparticle of any one of claims 43 to 60, wherein the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 50:1 to about 10:1.

62. The lipid nanoparticle of claim 61, wherein the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 40:1 to about 20:1.

63. The lipid nanoparticle of claim 61, wherein the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 35:1 to about 25:1.

64. The lipid nanoparticle of claim 61, wherein the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 32:1 to about 28:1.

65. The lipid nanoparticle of claim 61, wherein the lipid nanoparticle has a total lipid:nucleic acid weight ratio of about 31:1 to about 29:1.

66. A pharmaceutical composition comprising the compound of any one of claims 1 to 35, or the lipid nanoparticle of any one of claims 43 to 65, and a pharmaceutically acceptable excipient.

67. The pharmaceutical composition of claim 66, wherein the pharmaceutical composition is a lyophilized composition.

68. The pharmaceutical composition of claim 66 or 67, wherein the pharmaceutical composition comprises a HEPES buffer at a pH of about 7.4.

69. The pharmaceutical composition of claim 68, wherein the HEPES buffer is at a concentration of about 7 mg/mL to about 15 mg/mL.

70. The pharmaceutical composition of any one of claims 66 to 69, wherein the pharmaceutical composition further comprises about 2.0 mg/mL to about 4.0 mg/mL of NaCl.
71. The pharmaceutical composition of any one of claims 66 to 70, wherein the pharmaceutical composition further comprises one or more cryoprotectants.
72. The pharmaceutical composition of claim 71, wherein the one or more cryoprotectants are selected from sucrose, glycerol, or a combination of sucrose and glycerol.
73. The pharmaceutical composition of claim 72, wherein the pharmaceutical composition comprises a combination of sucrose at a concentration of about 70 mg/mL to about 110 mg/mL and glycerol at a concentration of about 50 mg/mL to about 70 mg/mL.
74. A method of treating a disease in a subject in need thereof, comprising administering a therapeutically effective amount to the subject, the lipid nanoparticle of any one of claims 43 to 65, or the pharmaceutical composition of any one of claims 66 to 73.
75. The method of claim 74, wherein the pharmaceutical composition or lipid nanoparticle is administered intravenously or intramuscularly.
76. A method of expressing a protein or polypeptide in a target cell, comprising contacting the target cell with a lipid nanoparticle of any one of claims 43 to 65, or the pharmaceutical composition of any one of claims 66 to 73.
77. The method of claim 76, wherein the protein or polypeptide is an antigen, and expression of the antigen elicits an in vivo immunogenic response.
78. A method of delivering a nucleic acid to a subject in needed thereof, comprising encapsulating a therapeutically effective amount of the nucleic acid in the lipid nanoparticle of any one of 43 to 65, and administering the lipid nanoparticle to the subject.