

Lipid nanoparticle with nucleic acid cargo and ionizable lipid

The field of the present invention relates to lipid nanoparticles (LNPs) with nucleic acid cargo, in particular as used in messenger ribonucleic acid (mRNA) vaccines, as well as to ionizable lipids suitable for use in LNPs.

LNPs have recently come into focus because LNP-based mRNA vaccines against SARS-CoV-2 (primarily elasomeran marketed under Spikevax® by Moderna Inc., and tozinameran marketed under Comirnaty® by Biontech SE/Pfizer Inc) were administered to hundreds of millions of individuals. LNPs as delivery systems for RNA-based vaccines in general are reviewed in Aldosari et al., 2021. LNPs for mRNA delivery are discussed in detail also in Hou et al, 2021.

LNPs are not only usable for vaccines but also for the delivery of other therapeutic nucleic acids. For instance, patisiran is an LNP-based drug for RNA interference therapy of Transthyretin-Mediated Amyloidosis (see Zhang et al, 2019).

In general, LNPs with nucleic acid cargo (or payload) comprise a lipid layer as well as microdomains of lipid and encapsulated nucleic acid. They have a median diameter between 10 nm to 1000 nm (e.g. as determined by dynamic light scattering, DLS) and may adopt e.g. a spherical or polyhedral shape. They may be multilamellar, dependent on their specific lipid composition. The LNPs comprise ionizable lipids, in particular lipids which are protonated at endosomal pH (e.g. in the range of pH 4.5 to 6.5, preferably pH 4.5 to 6.0, especially pH 4.5 to 5.5), i.e., when in an endosome. Typically, the LNPs further comprise stabilizers such as polyethylenglycol (PEG) lipids which decrease LNP aggregation, enzymatic degradation, opsonisation and immunogenicity. In addition, LNPs usually comprise other types of lipids (often termed "helper lipids"), such as phosphatidylcholines or phosphatidylethanolamines, to improve properties such as delivery efficacy, tolerability or biodistribution. Finally, LNPs may contain cholesterol or other sterols to modulate membrane integrity and rigidity.

LNPs are for instance also disclosed in US patents US 7,404,969, US 8,058,069, US 9,364,435 and US 9,404,127 as well as in US 2013/0245107 A1.

WO 2017/099823 A1 discloses an accelerated-blood-clearance-insensitive LNP, comprising a cationic lipid, a polyethylene glycol (PEG)-lipid, a sterol, and a helper lipid, wherein the helper lipid does not comprise a phosphatidyl choline.

WO 2020/061284 A1 and WO 2019/089818 A1 also concern LNPs with PEG lipids.

Eygeris et al, 2021, is a review on the chemistry of LNPs for RNA delivery. It discloses PEG lipids in LNP formulations.

WO 2014/143806 A1 discloses lipid particles with PEG lipids.

WO 2020/219941 A1 discloses further LNPs and formulations containing LNPs.

WO 2021/123332 A1 relates to cationic lipids and to LNPs comprising said cationic lipids useful for the delivery of nucleic acids into living cells.

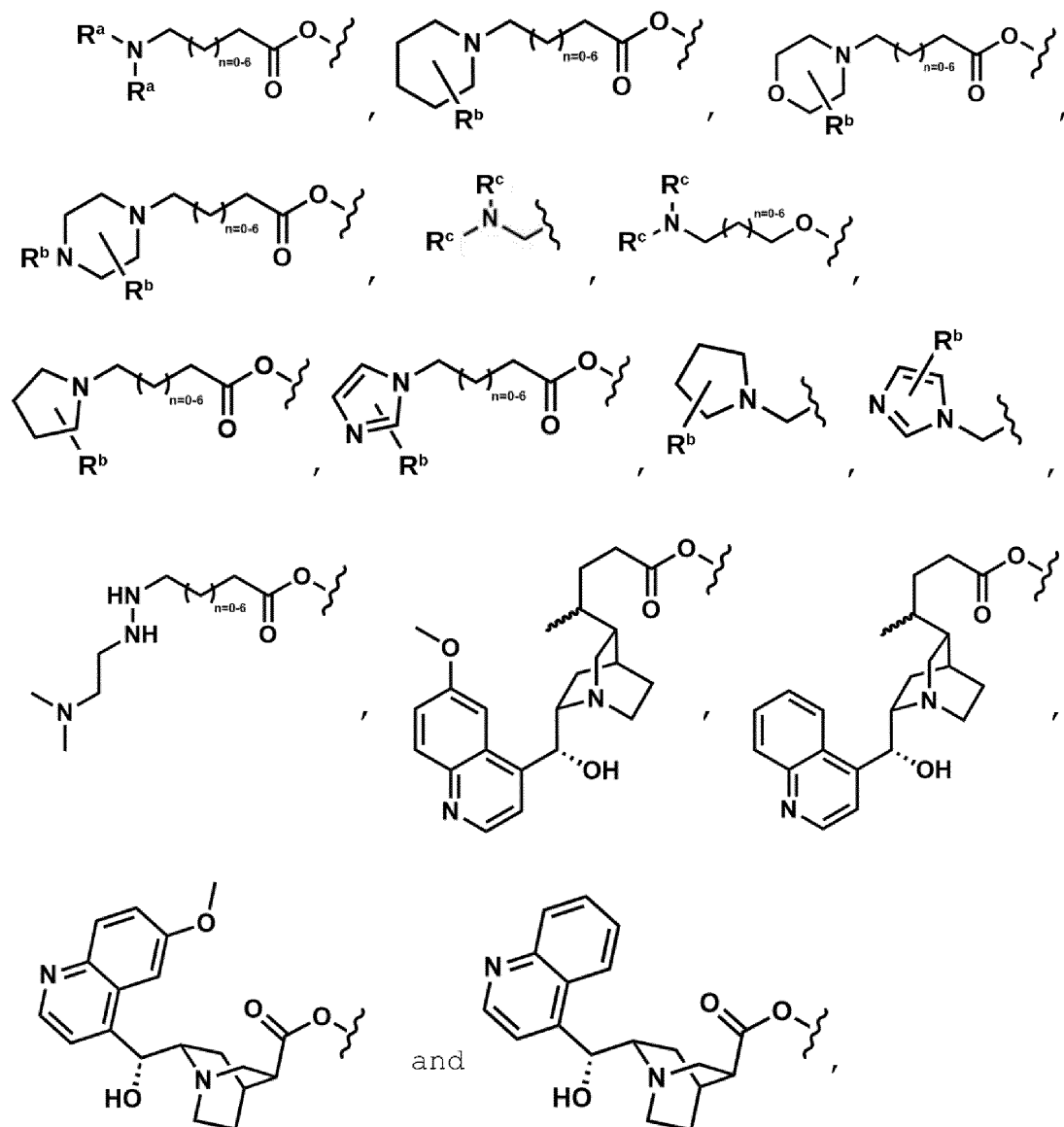
Despite the recent advances in the field, there is still a need for improved LNPs, in particular with regard to storage stability and/or transformation (transfection) efficiency. For instance, the LNP-based SARS-CoV-2 vaccine Comirnaty® generally has to be stored at -90°C to -60°C (Summary of product characteristics, version of 13 September 2022, EMEA/H/C/005735 - II/0143, European Medicines Agency - EMA). Furthermore, an increase in transformation efficiency would allow for lower dosage, thereby decreasing potential side effects.

It is thus an object of the present invention to provide an improved LNP with nucleic acid cargo (such as mRNA, siRNA or cDNA cargo), in particular an LNP that has increased transformation efficiency and/or higher storage stability, especially at non-refrigerated temperatures (e.g., room temperature). It is a further object of the present invention to provide novel ionizable lipids suitable for use in LNPs, preferably ionizable lipids which lead to increased transformation efficiency and/or higher storage stability, especially at non-refrigerated temperatures (e.g., room temperature).

The present invention provides an LNP encapsulating a nucleic acid cargo. The LNP comprises at least an ionizable lipid fraction and a stabilizer fraction (preferably comprising PEG lipids). The ionizable lipid fraction comprises at least one ionizable GDGT lipid. The LNP may also comprise neutral GDGT lipids as disclosed hereinbelow.

As used herein, the ionizable lipid is a lipid which carries an overall positive charge (i.e., is cationic) at endosomal pH, e.g. a pH of 5.0-6.5, in particular at a pH of 5.0, 5.5, 6 or 6.5

In another aspect, the present invention relates to a GDGT lipid comprising at least one ionizable head group S. This head group S is select from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide (preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine), each occurrence of R^b is independently selected from H, alkyl,

alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide and alkyl thiol (preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol), and each occurrence of R^c is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide (preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide). Preferably, this GDGT lipid is suitable for use as an ionizable lipid in an LNP, such as the LNP disclosed herein.

In a further aspect, the present invention provides an ether lipid fraction comprising this GDGT lipid. This ether lipid fraction is preferably obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture, followed by substitution (of the GDGTs present therein) with the ionizable head group S.

The present invention also relates to an LNP (as defined herein) comprising the GDGT lipid or ether lipid fraction disclosed above.

In yet another aspect, the present invention relates to a pharmaceutical composition (in particular a vaccine) comprising the LNP. This pharmaceutical composition typically comprises further excipients. It is preferably for use in prevention or treatment of a disease or condition in a (human) patient, in particular as a vaccine to prevent (or ameliorate) a disease such as an infectious disease or as a cancer vaccine.

In yet another aspect, the present invention provides a method for producing the GDGT lipid, comprising the steps of:

- obtaining a lipid fraction comprising one or more precursor GDGT lipids from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture;
- purifying the one or more precursor GDGT lipids; and

- contacting the one or more precursor GDGT lipids with one or more reagents to produce the GDGT lipid with the at least one ionizable head group S.

GDGT lipids (also called "GDGTs" herein) were discovered as membrane lipids of extremophilic archaea but more recently were also observed as membrane components of some bacteria (cf. Schouten et al, 2013). Many phylogenetic groups within the Archaea synthesize GDGTs. They form a monolayer instead of a bilayer in the cell membrane. Surprisingly, both ionizable and non-ionizable GDGT lipids are exceptionally well suited to improve the properties (such as storage stability and transformation efficiency) of LNPs with nucleic acid cargo.

Importantly, LNPs (as described in more detail above) are distinct from other lipid-containing delivery vehicles such as liposomes, lipo(poly)plexes or archaeosomes and present unique advantages but also challenges, especially in the context of packaging mRNA. For instance, Midoux & Pichon, 2014, reviews various lipid-based mRNA vaccine delivery systems, and distinguishes between lipoplexes, lipopolyplexes, LNPs and cationic nanoemulsions. More broadly, drug delivery for RNA therapeutics (such as small interfering RNAs and mRNAs) is enabled by lipid-based vehicles such as micelles, liposomes and LNPs (Paunovska et al, 2022).

In contrast to LNPs, liposomes are spherical lipid bilayer vesicles (lipid esters) surrounding an aqueous space. They are carriers for the administration of drugs, vaccines, genes, proteins, small molecules, antibiotics and nutrients. Liposomes are made of phospholipids, mainly phosphatidylcholine, and cholesterol, but may also include other lipids, like phosphatidylethanolamine. Liposomes are generated by a large number of different methods (reviewed e.g. by van Hoogevest, 2017; Szoka et al., 1980), for example by dispersing phospholipids in aqueous medium, e.g. using mechanical treatment (e.g. in a homogenizer, preferably by high pressure homogenization) or sonication. They vary between 0.02 to 10 μm in diameter.

Archaeosomes represent a special class of liposomes based on membrane lipids isolated from archaea. Archaeosomes are made of

lipid ethers, namely diether structures (such as archaeols) and tetraether structures (e.g. GDGTs, such as caldarchaeols); see e.g. Kaur et al., 2016; Patel et al., 1999. Diether structures are typically composed of a glycerol moiety carrying two phytanyl chains (20-40 carbons in length) on the sn-2,3 positions. Tetraether structures typically carry two di-phytanyl chains linked to two glycerol residues in either an antiparallel manner (caldarchaeol) or a parallel manner (iso-caldarchaeol). Furthermore, one or several cyclopentane rings may occur.

Benvegnu et al, 2009, relates to GDGTs with polar substituents. Vaz et al, 1985, discloses GDGTs with phosphoethanol-amine-containing substituents. Morii et al, 1994, discloses the structures of major polar lipids of *M. thermoautotrophicum*. Schwarzmann et al, 2015, discloses the polar GDGT caldarchaeol-bis-amine.

As used herein, "caldarchaeol" refers to the whole group of isoprenoid GDGT lipids with 0 up to 8 cyclopentane moieties. In particular, the nomenclature suggested by Schouten et al., 2013, is used herein: "GDGT-x", where x denotes the number of cyclopentane moieties, i.e. GDGT-0, GDGT-1, GDGT-2, GDGT-3, GDGT-4, GDGT-5, GDGT-6, GDGT-7 and GDGT-8. All of these belong to the group of caldarchaeols.

Depending on the composition (e.g. amount of archaeols vs. caldarchaeols), the lipid layer of archaeosomes is a mono- or a bi-layer or a mixture thereof.

WO 2020/187526 A1 discloses archaeosomes comprising archaeal lipids from a *Sulfolobus* cell culture, mainly intended for oral acute or oral retard delivery.

WO 02/053554 A2 relates to tetraether lipid derivatives and liposomes containing tetraether lipid derivatives and lipid agglomerates and the use thereof. In the example, the tetraether lipids were obtained from *Sulfolobus acidocaldarius*. The tetraether scaffold may contain e.g. four cyclopentane and may be substituted. WO 97/31927 A1, WO 99/10337 A1, US 2010/316657 A1 and US 6,316,260 B1 also disclose tetraether lipid derivatives. Further, WO 03/064360 A1 and WO 2004/037223 A2 relate to tetraether lipid derivatives and liposomes containing tetraether lipid derivatives.

Sateesh et al, 2008, concerns surface modification of medical-grade polyurethane by cyanurichloride-activated tetraether lipid, thereby providing a new approach for bacterial antiadhesion.

Engelhardt et al, 2017, concerns transfection studies with colloidal systems containing highly purified bipolar tetraether lipids from *Sulfolobus acidocaldarius*.

WO 2017/067642 A1 discloses liposomes containing cell penetrating peptides and tetraether lipids for the oral delivery of macromolecules.

Vishakarma et al, 2019, reviews various lipid-based carriers for lymphatic transportation, among them liposomes and archaeosomes.

GB 2463801 A discloses liposomes comprising isoprenoid lipids.

Further in relation to archaeosomes, Daswani et al, 2021, discloses that the polar lipid fraction E from *Sulfolobus acidocaldarius* can be used as liposomal drug stabilizing agents to reduce the leakage of the antivasular drug combretastatin a4 disodium phosphate from tetraether/diester hybrid archaeosomes.

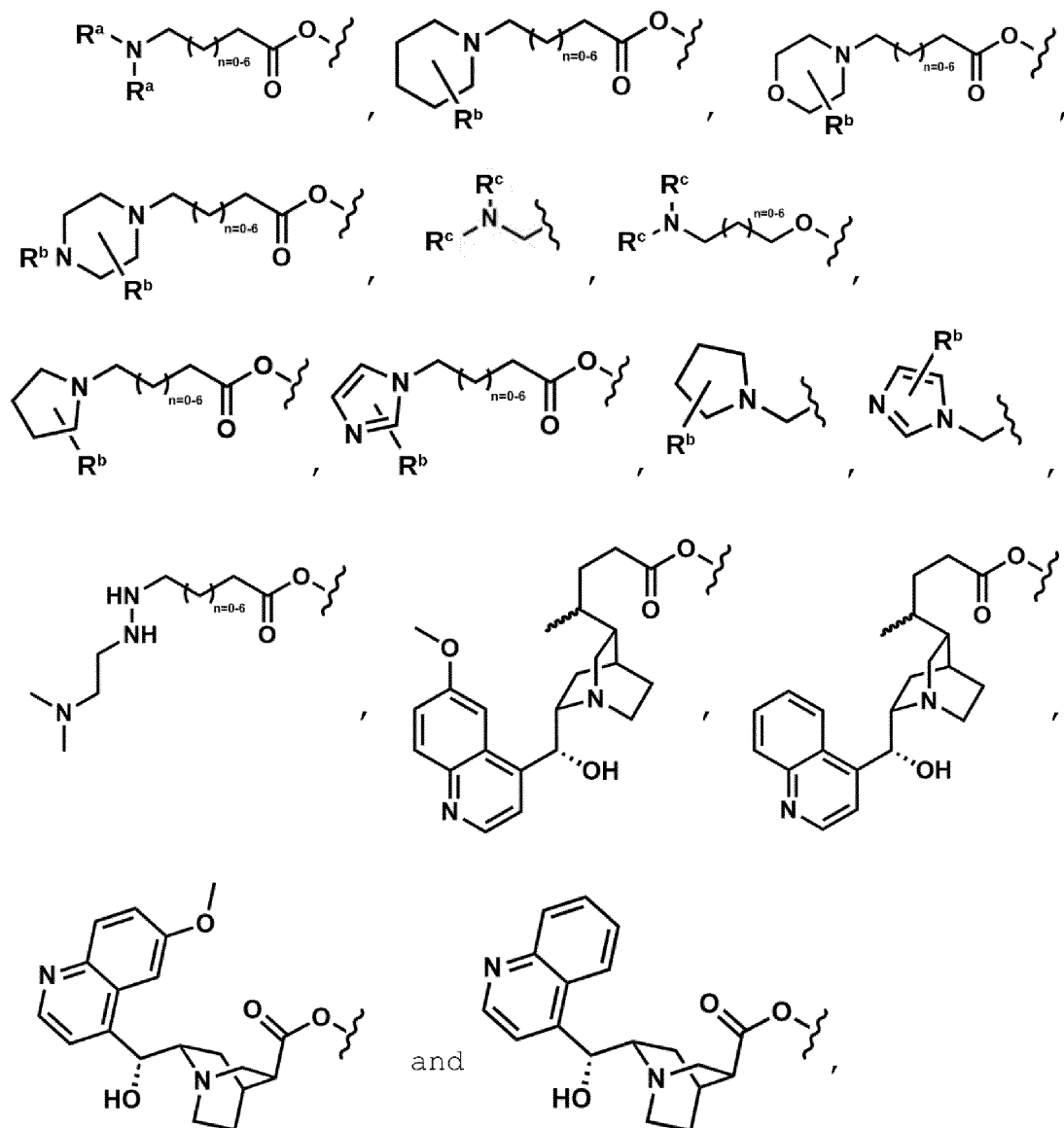
In contrast to archaeosomes, LNPs contain ionizable lipids. Such ionizable lipids are for instance discussed in Cornebise et al, 2022.

Tetraether lipids and their function in membranes in general are discussed in the following publications: Weijers et al., 2010, disclose the carbon isotopic composition of branched tetraether membrane lipids in soils. Schuster et al, 1998, concern voltage clamp studies on S-layer-supported tetraether lipid membranes. EP 1 777 520 A1 discloses a method of coating lipid membranes which may contain tetraether lipids. WO 2002/066012 A2 discloses liposomes which may comprise tetraether lipids. US 2014/0178462 A1 relates to amphoteric liposomes comprising neutral lipids which may be tetraether lipids.

Typically, the ionizable GDGT lipid comprises at least one ionizable head group S. Preferably, the GDGT lipid comprises two ionizable head groups S (which are typically both the same).

In a preferred embodiment of the present invention, the ionizable lipid is a lipid which carries an overall positive charge at endosomal pH, e.g. a pH between 5.0-6.5, such as pH 5.0, 5.5, 6 or 6.5, but is neutral at a higher pH such as pH 7.0. Accordingly, the ionizable head group S disclosed herein preferably carries an overall positive charge at endosomal pH, e.g. a pH between 5.0-6.5, such as pH 5.0, 5.5, 6 or 6.5, but is neutral at a higher pH such as pH 7.0.

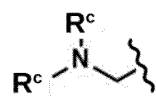
The following ionizable head groups have turned out to be particularly suitable for the present invention (in particular for use in the inventive LNP):



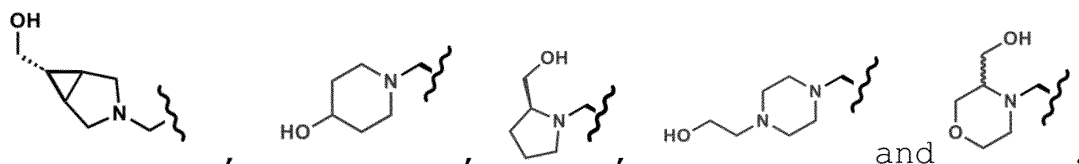
wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide (preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine), each occurrence of R^b is independently selected from H, alkyl,

alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide and alkyl thiol (preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol), and each occurrence of R^c is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide (preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide).

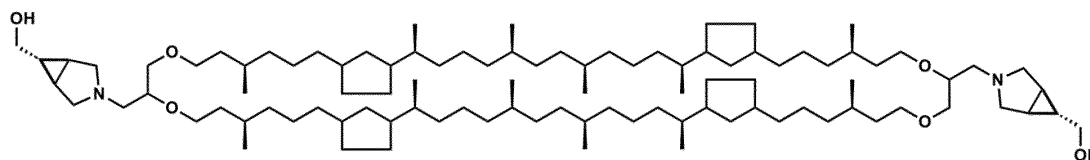
It goes without saying that each occurrence of R^a , or each occurrence of R^c , may be part of the same ring structure (in particular based on alkyl, alkenyl, hydroxyalkyl alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide). Accordingly, a preferred embodiment of the head group



may be selected from any one of



GDGT based on the first embodiment, EABM-GDGT, is particularly preferred. A representative structure (based on a GDGT-4 scaffold) is shown in the following:



Further preferred embodiments of this head group are shown e.g., in Example 12 below.

Preferably, the alkyl is selected from methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl, heptyl, and iso-heptyl.

Alternatively, or in addition thereto, the cycloalkyl is preferably selected from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl.

Alternatively, or in addition thereto, the alkenyl is preferably selected from prop-2-enyl, but-2-enyl, but-3-enyl, pent-2-enyl, pent-3-enyl, pent-4-enyl, hex-2-enyl, hex-3-enyl, hex-4-enyl, hex-5-enyl, hept-2-enyl, hept-3-enyl, hept-4-enyl, hept-5-enyl and hept-6-enyl.

Alternatively, or in addition thereto, the hydroxyalkyl is preferably selected from hydroxymethyl, hydroxyethyl, hydroxypropyl, hydroxybutyl, hydroxypentyl and hydroxyhexyl.

Alternatively, or in addition thereto, the alkylamine is preferably selected from methylamine, ethylamine, propylamine, butylamine, pentylamine, hexylamine and heptylamine.

Alternatively, or in addition thereto, the alkyl ether is preferably selected from methyl ether, ethyl ether, propyl ether, butyl ether, pentyl ether, hexyl ether and heptyl ether.

Alternatively, or in addition thereto, the alkyl thiol is preferably selected from methyl thiol, ethyl thiol, propyl thiol, butyl thiol, pentyl thiol, hexyl thiol and heptyl thiol.

Alternatively, or in addition thereto, the alkyl ester is preferably selected from methyl ester, ethyl ester, propyl ester, butyl ester, pentyl ester, hexyl ester and heptyl ester.

Alternatively, or in addition thereto, the alkyl amide is preferably selected from methyl amide, ethyl amide, propyl amide, butyl amide, pentyl amide, hexyl amide and heptyl amide.

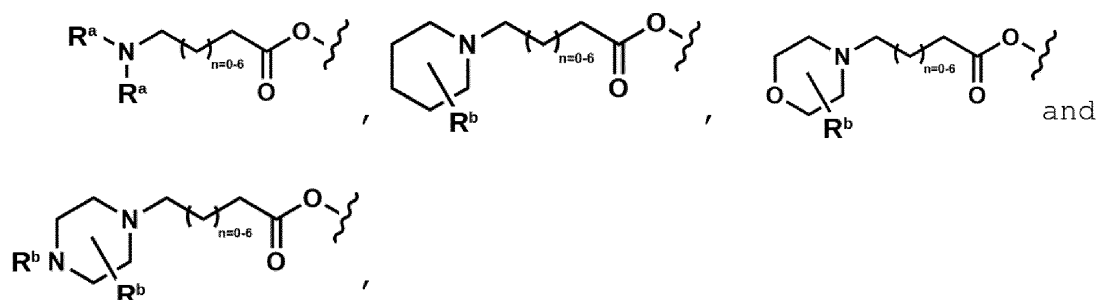
Alternatively, or in addition thereto, the alkyl carbamate is preferably selected from methyl carbamate, ethyl carbamate, propyl carbamate, butyl carbamate, pentyl carbamate, hexyl carbamate and heptyl carbamate.

Alternatively, or in addition thereto, the alkyl sulfonate is preferably selected from methyl sulfonate, ethyl sulfonate,

propyl sulfonate, butyl sulfonate, pentyl sulfonate, hexyl sulfonate and heptyl sulfonate.

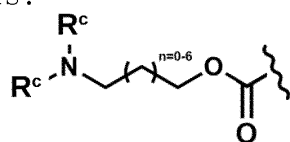
Alternatively, or in addition thereto, the alkyl sulfonamide is preferably selected from methyl sulfonamide, ethyl sulfonamide, propyl sulfonamide, butyl sulfonamide, pentyl sulfonamide, hexyl sulfonamide and heptyl sulfonamide.

The following head groups are particularly preferred in the context of the present invention:



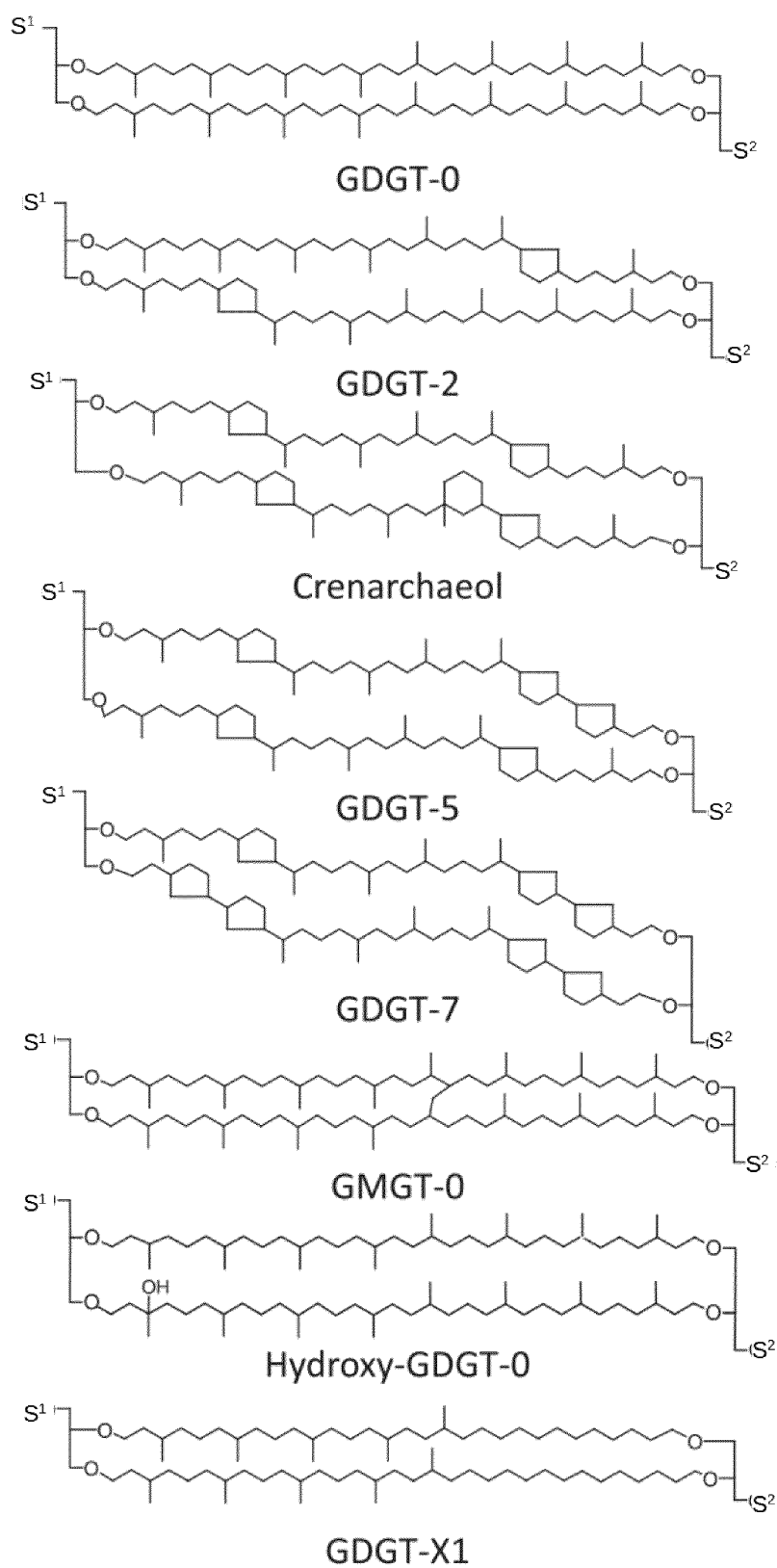
wherein each occurrence of R^a and R^b independently are as defined above.

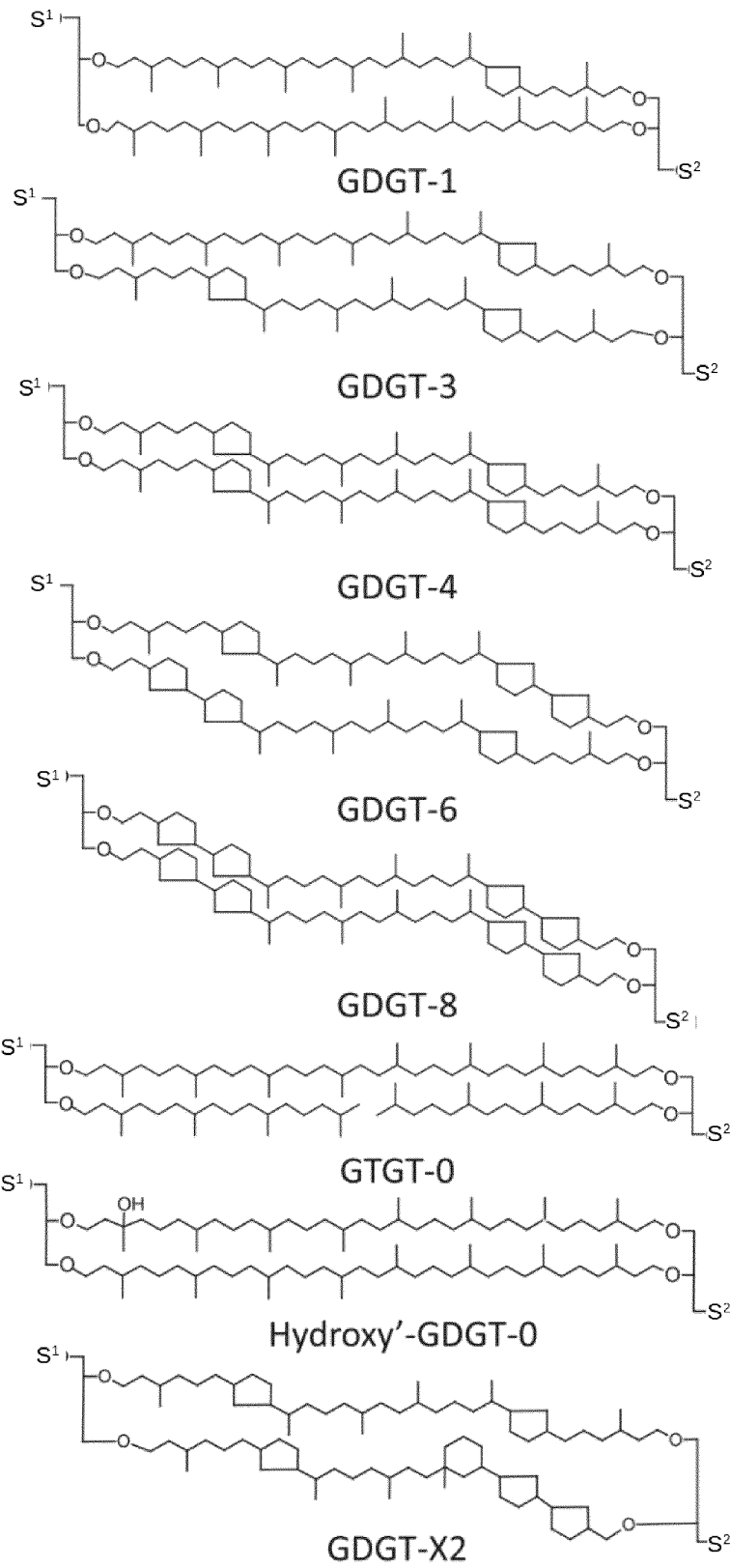
According to another preferred embodiment, the head group is:

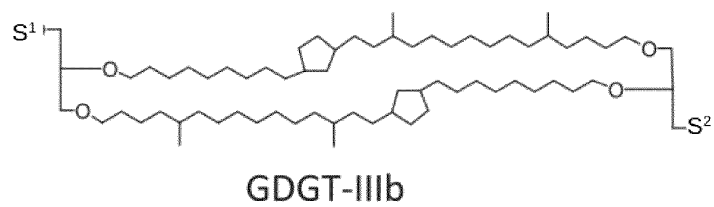
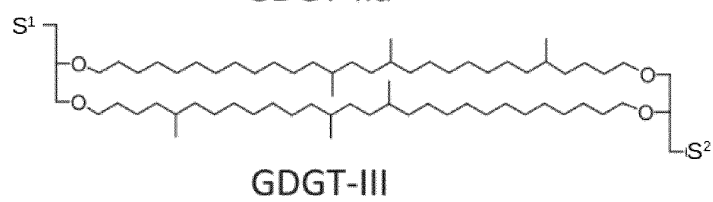
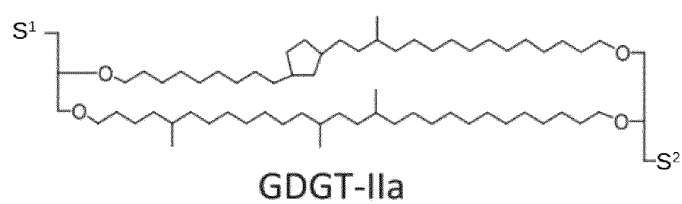
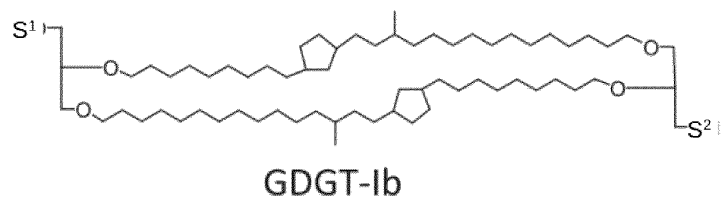
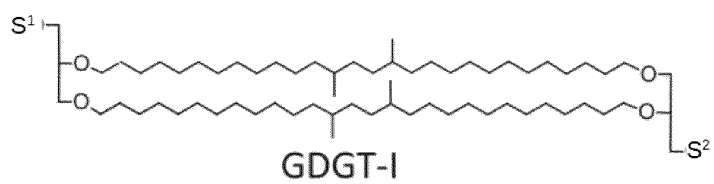


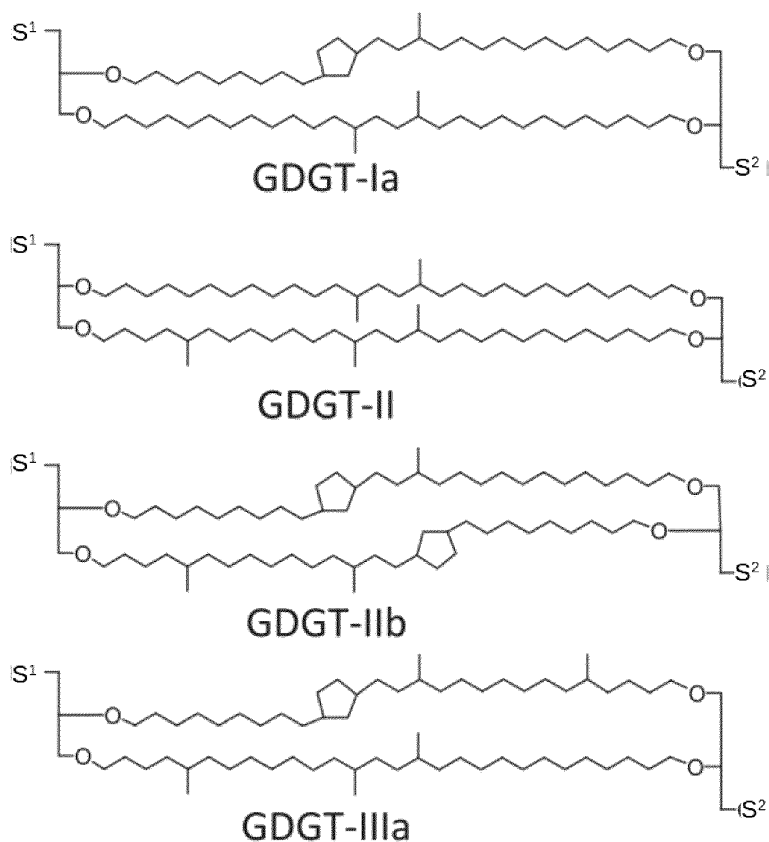
wherein each occurrence of R^c independently is as defined above. Particularly preferred examples of this head group are disclosed in Example 11.

The following ionizable GDGT lipids (i.e. containing the ionizable head group S as defined herein) are particularly suitable for use in the inventive LNP:









wherein S^1 is S and S^2 is OH, or S^1 is OH and S^2 is S, or each of S^1 and S^2 independently is S. Preferably, S^1 and S^2 are the same head group S as defined herein.

From the GDGT scaffolds above, the following scaffolds are particularly preferred: GDGT-0, GDGT-1, GDGT-2, GDGT-3, GDGT-4, GDGT-5, GDGT-6, GDGT-7 and/or GDGT-8. The ionizable lipid fraction of the LNP (or the inventive ether lipid fraction) preferably contains a mixture containing at least two, preferably at least three, more preferably at least four, even more preferably at least five, yet even more preferably at least six, especially at least seven or even at least eight, or all of these scaffolds.

Figures 1-10 depict particularly preferred ionizable GDGT lipids. While GDGT-4 scaffolds are depicted, other GDGT scaffolds (in particular GDGT-0, GDGT-1, GDGT-2, GDGT-3, GDGT-5, GDGT-6, GDGT-7 and GDGT-8) are also preferred in this connection. For substitutions such as "alkyl", "cycloalkyl",

etc., the above preferred embodiments preferably apply (e.g. that alkyl is preferably selected from methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl, heptyl, and iso-heptyl; that cycloalkyl is preferably selected from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl, etc.). Each instance of "R₁" or "R₂" may independently be selected from the definitions given directly below the structure.

In another preferred embodiment, the ionizable lipid fraction comprises at least one further ionizable lipid selected from the group consisting of [(4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) (ALC-0315), heptadecan-9-yl 8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate (SM-102), 3-(didodecylamino)-N1,N1,4-tridodecyl-1-piperazineethanamine (KL10), N1-[2-(didodecylamino)ethyl] N1,N4,N4-tridodecyl-1,4-piperazinediethanamine (KL22), 14,25-ditridecyl-15,18,21,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (Dlin-DMA), 2,2-dilinoleyloxy-4-dimethylaminomethyl-[1,3]-dioxolane (Dlin-K-DMA), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (Dlin-MC3-DMA), 2,2-dilinoleyloxy-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (Dlin-KC2-DMA), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 2-[(8-[(3β)-cholest-5-en-3-yloxy]octyl)oxy] N,N dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA), (2R)-2-[(8-[(3β)-cholest-5-en-3-yloxy]octyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2R)), (2S) 2-[(8-[(3β)-cholest-5-en-3-yloxy]octyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2S)) and mixtures thereof. Alternatively, or in addition thereto, the ionizable lipid is preferably selected from the group consisting of (20Z,23Z)-N,N-dimethylnonacos-20,23-dien-10-amine, (17Z,20Z)-N,N-dimethylhexacos-17,20-dien-9-amine, (1Z,19Z)-N5N-dimethylpentacos-16,19-dien-8-amine, (13Z,16Z)-N,N-dimethyldocos-13,16-dien-5-amine, (12Z,15Z)-N,N-dimethylhenicos-12,15-dien-4-amine, (14Z,17Z)-N,N-dimethyltricos-14,17-dien-6-amine, (15Z,18Z)-N,N-

dimethyltetracos-15,18-dien-7-amine, (18Z,21Z)-N,N-dimethylheptacos-18,21-dien-10-amine, (15Z,18Z)-N,N-dimethyltetracos-15,18-dien-5-amine, (14Z,17Z)-N,N-dimethyltricos-14,17-dien-4-amine, (19Z,22Z)-N,N-dimethyloctacos-19,22-dien-9-amine, (18Z,21 Z)-N,N-dimethylheptacos-18,21-dien-8-amine, (17Z,20Z)-N,N-dimethylhexacos-17,20-dien-7-amine, (16Z,19Z)-N,N-dimethylpentacos-16,19-dien-6-amine, (22Z,25Z)-N,N-dimethylhentriacont-22,25-dien-10-amine, (21 Z,24Z)-N,N-dimethyltriacont-21,24-dien-9-amine, (18Z)-N,N-dimethylheptacos-18-en-10-amine, (17Z)-N,N-dimethylhexacos-17-en-9-amine, (19Z,22Z)-N,N-dimethyloctacos-19,22-dien-7-amine, N,N-dimethylheptacos-10-amine, (20Z,23Z)-N-ethyl-N-methylnonacos-20,23-dien-10-amine, 1-[(11Z,14Z)-1-nonylicosa-11,14-dien-1-yl]pyrrolidine, (20Z)-N,N-dimethylheptacos-20-en-10-amine, (15Z)-N,N-dimethyl eptacos-15-en-10-amine, (14Z)-N,N-dimethylnonacos-14-en-10-amine, (17Z)-N,N-dimethylnonacos-17-en-10-amine, (24Z)-N,N-dimethyltritriacont-24-en-10-amine, (20Z)-N,N-dimethylnonacos-20-en-10-amine, (22Z)-N,N-dimethylhentriacont-22-en-10-amine, (16Z)-N,N-dimethylpentacos-16-en-8-amine, (12Z,15Z)-N,N-dimethyl-2-nonylhenicos-12,15-dien-1-amine, (13Z,16Z)-N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl] eptadecan-8-amine, 1-[(1S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan-10-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]nonadecan-10-amine, N,N-dimethyl-21-[(1S,2R)-2-octylcyclopropyl]henicosan-10-amine, N,N-dimethyl-1-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]nonadecan-10-amine, N,N-dimethyl-1-[(1 S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyl-[(1R,2S)-2 undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3-{7-[(1S,2R)-2-octylcyclopropyl]heptyl} dodecan-1-amine, 1-[(1R,2S)-2-heptylcyclopropyl]-N,N-dimethyloctadecan-9-amine, 1-[(1S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]pentadecan-8-amine, R-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}pyrrolidine, (2 S)-N,N-dimethyl-1-

[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct-5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(nonyloxy)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine; (2S)-N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)-N,N-dimethyl-H(1-metoyloctyl)oxy]-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(octyloxy)-3-({8-[(1S,2S)-2-[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl)octyl}oxy)propan-2-amine, N,N-dimethyl-1-{{8-(2-octylcyclopropyl)octyl}oxy}-3-(octyloxy)propan-2-amine, and (11E,20Z,23Z)-N,N-dimethylnonacosa-11,20,2-trien-10-amine, and pharmaceutically acceptable salts and stereoisomers thereof, and mixtures thereof.

Further suitable ionizable lipids are disclosed e.g. in Hou et al, 2021, US patents US 7,404,969, US 8,058,069, US 9,364,435 and US 9,404,127, WO 2017/099823 A1, WO 2020/061284 A1, WO 2020/219941 A1 and WO 2021/123332 A1. These documents are included herein by reference in their entirety.

Even further suitable ionizable lipids are disclosed e.g. in WO 2017/049245 A1, WO 2017/112865 A1, WO 2012/040184, WO 2011/153120 A1, WO 2011/149733 A1, WO 2011/090965 A1, WO 2011/043913 A1, WO 2011/022460 A1, WO 2012/061259 A1, WO

2012/054365 A1, WO 2012/044638 A1, WO 2010/080724 A1, WO 2010/21865 A1, WO 2008/103276 A1, WO 2013/086373 A1 and WO 2013/086354 A1, US patent nos. 7,893,302, 7,404,969, 8,283,333, and 8,466,122 and US patent publication no. US20100036115, US20120202871, US20130064894, US20130129785, US20130150625, US20130178541, and US20130225836. These documents are incorporated herein by reference in their entirety.

The stabilizer fraction of the inventive LNP is typically suitable for achieving one or more of the following: decreasing LNP aggregation, increasing average particle size or hydrodynamic radius, increasing the half-life of the LNP *in vivo* (e.g. in a human, in particular in blood circulation) and modifying zeta potential.

According to a particular preference, the stabilizer fraction comprises at least one PEG lipid. Suitable PEG lipids are for instance 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), pegylated diacylglycerol lipid (PEG-DAG), a pegylated ceramide lipid (PEG-Cer), a pegylated phosphatidylethanolamine lipid (PEG-PE), a pegylated succinate diacylglycerol lipid (PEG-S-DAG), a pegylated dialkoxypropylcarbamate lipid, 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol ("PEG-DMG" or "DMG-PEG"), in particular PEG2000-DMG, 1,2-dicapryl-rac-glycero-3-methylpolyoxyethylene glycol (C₁₀-diacylglycerol PEG), N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (comprising N-octanoyl-D-erythro-sphingosine (d18:1/8:0), also named PEG-Ceramide8), or a PEG lipid as disclosed in WO 2018/126084 A1, WO 2020/093061 A1, or WO 2020/219941 A1 (all three references are incorporated by reference in their entirety); and any combination thereof.

Further suitable PEG lipids are e.g. disclosed in Hou et al, 2021, WO 2017/099823, WO 2020/061284 A1, WO 2020/219941 A1 and WO 2021/123332 A1. All of these documents are incorporated by reference herein in their entirety.

Alternatively, or in addition thereto, the stabilizer fraction may comprise at least one non-PEG moiety such as an XTEN peptide that may or may not be conjugated to a lipid. The XTEN peptide is capable of forming a hydrated shell around the

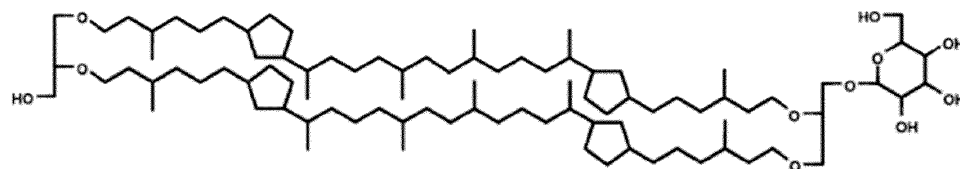
LNP due to its hydrophilic nature. It further serves to increase the half-life of the LNP, compared to an LNP lacking (or free of) a stabilizer fraction. XTEN amino acid sequences are known in the art, including for example those reported in U.S. patent no. 9,062,299 (incorporated herein by reference in its entirety). Alternatively, or in addition thereto, in some embodiments, the stabilizer fraction may comprise non-PEG moiety such as a PAS peptide (that may or may not be conjugated to a lipid). A PAS peptide is a peptide comprising primarily if not exclusively proline, alanine and serine. Like PEG and XTEN peptides, the PAS peptide is capable of forming a hydrated shell around the LNP. It too serves to increase the half-life of an LNP, compared to an LNP lacking (or free of) a stabilizer fraction. PAS amino acid sequences are known in the art, including for example, those reported in WO 2008/155134 A1 (incorporated herein by reference in its entirety).

The inventive LNP preferably comprises at least one neutral (or non-ionizable, or, in other words, neutral at endosomal pH) GDGT lipid. This GDGT lipid may e.g. be an isoprenoid GDGT lipid such as a GDGT-0, a GDGT-1, a GDGT-2, a GDGT-3, a GDGT-4, a GDGT-5, a GDGT-6, a GDGT-7 and a GDGT-8, or crenarchaeol (unsubstituted, cf. Schouten et al, 2013, Fig. 1, or substituted), or a branched GDGT such as GDGT-I, GDGT-II or GDGT-III, or any mixture thereof. In particular, the GDGT lipid may comprise any of the GDGTs disclosed in Schouten et al, 2013 (in particular Fig. 1), Kaur et al, 2016 (in particular Fig. 2, Fig. 3 and Fig. 4), and WO 2020/187526 A1 (all of which are incorporated herein by reference in their entirety). The GDGTs may be substituted (e.g. with hexose moieties or phosphatidylinositol moieties) or unsubstituted.

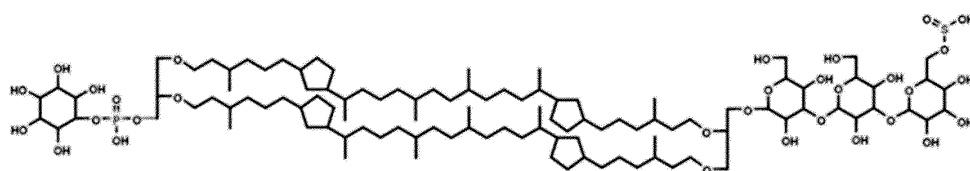
Caldarchaeols (as e.g. obtained from *Sulfolobus*) turned out to be particularly suitable for the present invention. According to a preferred embodiment, at least one GDGT lipid (of the ether lipid fraction of the LNP) thus comprises at least one neutral (or non-ionizable, or, in other words, neutral at endosomal pH) caldarchaeol, preferably selected from the group consisting of unsubstituted caldarchaeol, phosphatidylinositol (PI)-caldarchaeol, dihexose(2Hex)-caldarchaeol and 2Hex-PI-caldarchaeol (in particular as disclosed in WO 2020/187526 A1,

Fig. 4), hexose(Hex)-caldarchaeol and sulfono-trihexose(3Hex)-caldarchaeol, sulfono-3Hex-PI-caldarchaeol, and any mixture thereof.

An especially preferred Hex-caldarchaeol is:



An especially preferred sulfono-3Hex-PI-caldarchaeol is:



The inventive LNPs are particularly suitable for RNA cargo. Accordingly, in a preferred embodiment, the nucleic acid cargo comprises at least one (therapeutic) RNA. Examples of suitable RNA payloads are for instance disclosed in Paunovska et al, 2022 (in particular Fig. 1). In embodiments, the cargo may be a small interfering RNA (siRNA), an antisense oligonucleotide, an adenosine deaminase acting on RNA (ADAR) oligonucleotide or an mRNA.

The RNA may be chemically modified, e.g. to improve its chemical stability. For instance, it may comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone modifications. In some embodiments, the RNA may comprise nucleoside analogs (e.g, 2- aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5- methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5- propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g, methylated bases); intercalated bases; modified sugars (e.g, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose);

and/or modified phosphate groups (e.g ., phosphorothioates and 5'-N- phosphoramidite linkages). Further modifications are known to the person skilled in the art. Suitable modifications are e.g. disclosed in WO 2017/099823 A1 and WO 2020/061284 A1 (each incorporated by reference in their entirety).

In the course of the present invention, it was found that GDGT lipids improve LNP properties in particular for mRNA payload. According to an especially preferred embodiment, the nucleic acid cargo of the LNP hence comprises an mRNA. The mRNA may e.g. be a therapeutic mRNA or an mRNA encoding a vaccine antigen. The mRNA may be codon-optimized. Examples of suitable mRNAs are for instance given in WO 2017/099823 A1 and WO 2020/061284 A1 (each incorporated by reference in their entirety), in particular paragraphs [00178]-[00180] of the latter.

According to a further preferred embodiment, the LNP comprises at least one further (non-ionizable or neutral) ether lipid, preferably a diether lipid (in particular an archaeol). In particular, the diether lipid may comprise any of the diether lipid disclosed in Kaur et al, 2016, WO 2017/212197 A1 and WO 2020/187526 A1 (all of which are incorporated herein by reference in their entirety). The diether lipid (in particular the archaeol) may be substituted (e.g. with hexose moieties or phosphatidylinositol moieties) or unsubstituted. A particularly preferred substituted archaeol is phosphatidyl inositol-archaeol (PI-Arc).

According to another preference, the LNP comprises an ether lipid fraction comprising at least one (non-ionizable or neutral) GDGT lipid (in particular a caldarchaeol) and preferably at least one further ether lipid (in particular the diether lipid as disclosed above).

It is particularly preferred when the ionizable lipid fraction (or ether lipid fraction) comprises ether lipids obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture, followed by substitution with the ionizable head group S. Suitable growth conditions and extraction methods are e.g. disclosed in WO 2020/187526 A1 (incorporated herein by reference

in its entirety) also published as EP 3 708 151 A1. In particular, the entire ether lipid fraction is obtainable by extraction from said culture. Suitable substitutions are e.g. disclosed in the Examples.

Alternatively, or in addition thereto, it is particularly preferred when the neutral ether lipid fraction comprises ether lipids obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture. Suitable growth conditions and extraction methods are e.g. disclosed in WO 2020/187526 A1 (incorporated herein by reference in its entirety). In particular, the entire ether lipid fraction is obtainable by extraction from said culture.

In embodiments, the archaeal culture may e.g. also be a culture of *S. acidocaldarius*, *M. hungatei*, *M. voltae*, *M. concilii*, *M. smithii*, *M. espanolae*, *T. acidophilum*, *M. mazei*, *M. espanole*, *T. acidophilum*, *H. salinarum*, *M. smithii*, *M. stadtmanae*, *H. halobium*, *H. morrhuae*, *M. jannaschii*, *S. islandicus*, *S. solfataricus*, *S. shibatae*, *S. tokodaii*, *S. Metallicus*, *M. sedula*, *H. hispanica*, or *H. volcanii*, or mixtures (co-cultures) thereof.

By way of example, total archaeal lipids may be extracted from e.g. lyophilized or spray-dried biomass by organic solvent extraction using chloroform/methanol/water. Then, the polar and the neutral lipids may be separated by precipitation using acetone. The resulting lipid extracts may be used directly for the preparation of the ether lipid fraction used for LNP production or may be further purified by chromatography to isolate ether lipids of a particular class to be used for LNP production. They may also be formed into ionizable GDGT lipids by substituting head groups, e.g. as disclosed herein, in particular in the Examples.

Methods for preparing lipids from archaea or for cultivating archaea are also disclosed e.g. in US 2017/0152533 A1, US 6,316,260 B1, EP 1 999 137 B1, EP 0 883 624 B1, EP 2 109 459 B1, Siliakus et al., 2017, WO 2020/187526 A1, Jain et al., 2014; each incorporated herein by reference in its entirety.

The LNPs themselves may be produced by microfluidic mixing of the LNP components (fractions), including the GDGT lipids (e.g. present in isolated form or in an ether lipid fraction comprising several ether lipids). Suitable LNP production methods are e.g. disclosed in WO 2017/099823 A1, WO 2020/061284 A1, and WO 2021/123332 A1; each incorporated herein by reference in its entirety. Further LNP production methods are available to the person skilled in the art.

According to another preferred embodiment, the LNP further comprises a sterol lipid fraction. Incorporation of sterol lipids in the LNP mitigates aggregation of other lipids in the LNP. Sterol lipids are preferably selected from the group consisting of cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, phytosterols and mixtures thereof. According to a preferable definition, "sterol lipids" comprise the entire subgroup of steroids which consists of steroid alcohols.

In particular, the sterol lipid fraction comprises cholesterol.

According to yet another preferred embodiment, the LNP further comprises a helper lipid fraction. Helper lipids useful in the present invention comprise (preferably consist of) non-ionizable lipids. In particular, the helper lipid may be a phospholipid.

Preferably, the helper lipid is selected from the group consisting of distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), Dipalmitoylphosphatidylcholine (DOPC), phosphatidylcholine (PC) and mixtures thereof. Further suitable helper lipids are e.g. 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OchemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-

glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin, and mixtures thereof.

Further suitable helper lipids and sterol lipids are e.g. disclosed in WO 2017/099823 A1 and WO 2020/061284 A1 which are incorporated herein by reference.

Certain (molar) ratios in the LNP composition have turned out to be particularly suitable for achieving improved LNPs, in particular with regard to storage stability and/or transformation efficiency:

It is thus preferred that the ether lipid fraction (as defined above) accounts for 1 mol% - 20 mol% of total lipids, preferably 2 mol% - 15 mol% of total lipids, even more preferably 4 mol% - 12 mol% of total lipids, especially 6 mol% - 10 mol% of total lipids or even 7 mol% - 9 mol% of total lipids of the LNP.

Alternatively, or in addition thereto, it is preferred that the molar ratio of (neutral) ether lipid fraction to helper lipid fraction is from 20:1 to 1:20, preferably from 15:1 to 1:10, more preferably from 12:1 to 1:3, even more preferably from 8:1 to 4:1, especially from 7:1 to 5:1.

Further, alternatively, or in addition thereto, it is preferred that the molar ratio of (neutral) ether lipid fraction to sterol lipid fraction is from 0.25:1 to 1:30, preferably from 0.5:1 to 1:20, more preferably from 1:1 to 1:10, even more preferably from 1:2 to 1:6, especially from 1:3 to 1:5.

According to a particular preference, at least 50 mol% of the ionizable lipid fraction of the LNP is made up by the ionizable GDGT lipids, preferably at least 60 mol%, more preferably at least 70 mol% or even at least 80 mol%, especially at least 90mol%.

Particularly good results are achieved with the following LNP, which thus forms another preferred embodiment of the invention (total lipids of the LNP, i.e. 100 mol%):

- (a) 40 mol% - 70 mol% ionizable lipid fraction (which comprises the ionizable GDGT lipids, wherein in particular at least 50 mol% of the fraction is made up by ionizable GDGT lipids, preferably at least 60 mol%, more preferably at least 70 mol% or even at least 80 mol%, especially at least 90mol%),
- (b) 5 mol% - 20 mol% helper lipid fraction,
- (c) 20 mol% - 40 mol% sterol lipid fraction,
- (d) 0.1 mol% - 4 mol% stabilizer fraction, and preferably
- (e) 1 mol% - 20 mol% neutral ether lipid fraction (in particular 2 mol% - 15 mol%, even more preferably 4 mol% - 12 mol%, especially 6 mol% - 10 mol% or even 7 mol% - 9 mol%).

As evident to the skilled person upon reading the present application, these ratios and mol% given above may be observed as mean values over an entire population of LNPs (e.g. all LNPs present in a pharmaceutical composition).

The pharmaceutical composition of the present invention (which comprises a plurality of the inventive LNP) is preferably provided with at least one excipient. Excipients suitable for the pharmaceutical composition of the present invention are known to the person skilled in the art, upon having read the present specification, for example water (especially water for injection), saline, Ringer's solution, dextrose solution, buffers, Hank solution, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. This pharmaceutical composition can (as a drug) be administered via appropriate procedures known to the skilled person (upon having read the present specification) to a patient or individual in need thereof (i.e. a patient or individual having or having the risk of developing the diseases or conditions mentioned herein). The preferred route of administration of said pharmaceutical composition is parenteral administration, in particular through intraperitoneal, subcutaneous, intramuscular and/or intravenous administration. The dosage and method of administration depends on the individual patient or individual to be treated. Said

pharmaceutical composition can be administered in any suitable dosage known from other biological dosage regimens or specifically evaluated and optimized for a given individual. For example, the nucleic acid cargo may be present in the pharmaceutical composition in an amount from 0.1 µg to 1000 µg, preferably 5 µg to 200 µg, in particular 10 µg to 100 µg. Usual dosages can also be determined on the basis of kg body weight of the patient, for example preferred dosages are in the range of 0.01 µg to 100 µg/kg body weight, especially 0.1 to 10 µg/kg body weight (per administration session). The administration may occur e.g. once daily, once every other day, once per week or once every two weeks. Alternatively, the administration may occur for instance a single time or multiple times (e.g. on a weekly, monthly or yearly basis). As the preferred mode of administration of the inventive pharmaceutical composition is parenteral administration, the pharmaceutical composition according to the present invention is preferably liquid or ready to be dissolved in liquid such sterile, de-ionized or distilled water or sterile isotonic phosphate-buffered saline (PBS). Preferably, 1000 µg (dry-weight) of such a composition comprises 0.1-990 µg, preferably 1-900µg, more preferably 10- 200µg compound, and optionally 1-500 µg, preferably 1-100 µg, more preferably 5-15 µg (buffer) salts (preferably to yield an isotonic buffer in the final volume), and optionally 0.1-999.9 µg, preferably 100-999.9 µg, more preferably 200-999 µg other excipients. Preferably, 100 mg of such a dry composition is dissolved in sterile, de-ionized/distilled water or sterile isotonic phosphate-buffered saline (PBS) to yield a final volume of 0.1-100 ml, preferably 0.5-20 ml, more preferably 1-10 ml.

According to a particular preference, the LNP of the present invention has a z-average diameter between 10 nm and 900 nm, preferably between 20 nm and 750 nm, more preferably between 30 nm and 500 nm, especially between 40 nm and 250 nm or even between 50 nm and 150 nm as determined by DLS, in particular according to ISO 22412:2017. The z-average diameter as defined in ISO 22412-2017 is determined by the cumulants method and yields a scattered light intensity-weighted harmonic mean particle diameter. For instance, Markova et al, 2022, discloses in detail how to measure the z-average diameter of LNPs.

It is evident to the skilled person upon having read the above disclosure, that the inventive GDGT lipids can also be provided as pharmaceutically acceptable salts. Accordingly, the scope of the present invention shall also encompass all pharmaceutically acceptable salts of the inventive ionizable GDGT lipids. In particular, the ionizable GDGT lipids may be provided in cationic form (particularly as ammonium cations), e.g., as hydrochloride, sodium bisulfate, potassium bisulfate, monosodium phosphate, monopotassium phosphate, citrate or acetate salts.

The present invention also relates to the following embodiments:

Embodiment 1. A lipid nanoparticle (LNP) encapsulating a nucleic acid cargo, wherein the LNP comprises at least

- an ionizable lipid fraction and
- a stabilizer fraction;

wherein the ionizable lipid fraction comprises at least one ionizable glycerol dialkyl glycerol tetraether (GDGT) lipid.

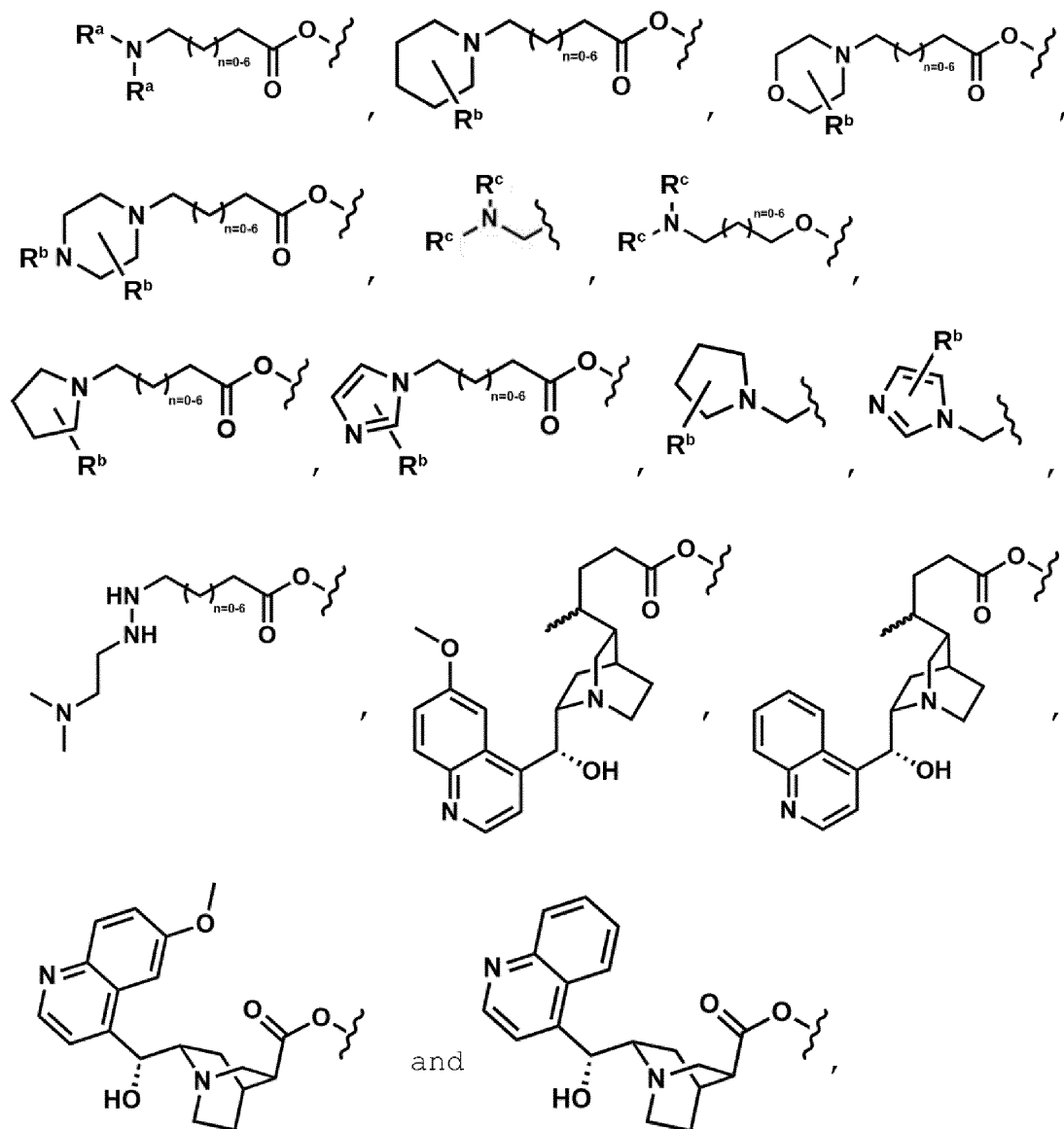
Embodiment 2. The LNP of embodiment 1, wherein the nucleic acid cargo comprises a messenger ribonucleic acid (mRNA).

Embodiment 3. The LNP of embodiment 1 or 2, wherein the stabilizer fraction comprises at least one polyethylene glycol (PEG) lipid.

Embodiment 4. The LNP of any one of embodiments 1 to 3, wherein the LNP further comprises a sterol lipid fraction, preferably comprising cholesterol.

Embodiment 5. The LNP of any one of embodiments 1 to 4, wherein the LNP further comprises a helper lipid fraction.

Embodiment 6. The LNP of any one of embodiments 1 to 5, wherein the at least one ionizable GDGT lipid comprises at least one ionizable head group S; preferably wherein S is selected from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine,

each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide

and alkyl thiol, preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol, and each occurrence of R^o is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide.

Embodiment 7. The LNP of any one of embodiments 1 to 6, wherein the LNP comprises at least one further ether lipid, preferably a tetraether lipid, preferably a GDGT, or a diether lipid, in particular an archaeol.

Embodiment 8. The LNP of any one of embodiments 1 to 7, wherein the LNP comprises an ether lipid fraction comprising at least one further GDGT lipid (preferably at least two different GDGT lipids, more preferably at least three different GDGT lipids, especially at least four different GDGT lipids) and preferably at least one further ether lipid; in particular comprising archaeols and caldarchaeols, preferably with a composition as given in Table 1 above.

Embodiment 9. The LNP of embodiment 8, wherein the ether lipid fraction comprises ether lipids obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture.

Embodiment 10. The LNP of embodiment 9, wherein the entire ether lipid fraction is obtainable by extraction from said culture.

Embodiment 11. The LNP of any one of embodiments 1 to 10, wherein the ionizable lipid fraction accounts for 40 mol% - 70 mol% of total lipids.

Embodiment 12. The LNP of any one of embodiments 1 to 11, wherein the molar ratio of neutral ether lipid fraction to helper lipid fraction (in particular to DSPC, if present) is from 20:1 to 1:20, preferably from 15:1 to 1:10, more preferably from 12:1 to 1:3, even more preferably from 8:1 to 4:1, especially from 7:1 to 5:1.

Embodiment 13. The LNP of any one of embodiments 1 to 12, wherein the molar ratio of neutral ether lipid fraction to sterol lipid fraction (in particular to cholesterol, if present) is from 0.25:1 to 1:30, preferably from 0.5:1 to 1:20, more preferably from 1:1 to 1:10, even more preferably from 1:2 to 1:6, especially from 1:3 to 1:5.

Embodiment 14. The LNP of any one of embodiments 1 to 13, wherein the total lipids of the LNP comprise (preferably consist of):

- (a) 40 mol% - 70 mol% ionizable lipid fraction,
- (b) 5 mol% - 20 mol% helper lipid fraction,
- (c) 20 mol% - 40 mol% sterol lipid fraction,
- (d) 0.1 mol% - 4 mol% stabilizer fraction, and/or
- (e) 1 mol% - 20 mol% neutral ether lipid fraction.

Embodiment 15. The LNP of any one of embodiments 1 to 14, wherein the ionizable lipid fraction comprises at least one further ionizable lipid selected from the group consisting of [(4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) (ALC-0315), heptadecan-9-yl 8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate (SM-102), 3-(didodecylamino)-N1,N1,4-tridodecyl-1-piperazineethanamine (KL10), N1-[2-(didodecylamino)ethyl] N1,N4,N4-tridodecyl-1,4-piperazinediethanamine (KL22), 14,25-ditridecyl-15,18,21,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), 2,2-dilinoley-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoley-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 1,2-

dioleyloxy-N,N-dimethylaminopropane (DODMA), 2-({8-[(3 β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA), (2R)-2-({8-[(3 β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2R)), (2S) 2-({8-[(3 β)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2S)) and mixtures thereof; and/or wherein the ionizable lipid is selected from the group consisting of (20Z,23Z)-N,N-dimethylnonacos-20,23-dien-10-amine, (17Z,20Z)-N,N-dimethylhexacos-17,20-dien-9-amine, (1Z,19Z)-N,N-dimethylpentacos-16,19-dien-8-amine, (13Z,16Z)-N,N-dimethyldocos-13,16-dien-5-amine, (12Z,15Z)-N,N-dimethylhenicos-12,15-dien-4-amine, (14Z,17Z)-N,N-dimethyltricos-14,17-dien-6-amine, (15Z,18Z)-N,N-dimethyltetracos-15,18-dien-7-amine, (18Z,21Z)-N,N-dimethylheptacos-18,21-dien-10-amine, (15Z,18Z)-N,N-dimethyltetracos-15,18-dien-5-amine, (14Z,17Z)-N,N-dimethyltricos-14,17-dien-4-amine, (19Z,22Z)-N,N-dimethyloctacos-19,22-dien-9-amine, (18Z,21Z)-N,N-dimethylheptacos-18,21-dien-8-amine, (17Z,20Z)-N,N-dimethylhexacos-17,20-dien-7-amine, (16Z,19Z)-N,N-dimethylpentacos-16,19-dien-6-amine, (22Z,25Z)-N,N-dimethylhentriacont-22,25-dien-10-amine, (21Z,24Z)-N,N-dimethyltriacont-21,24-dien-9-amine, (18Z)-N,N-dimethylheptacos-18-en-10-amine, (17Z)-N,N-dimethylhexacos-17-en-9-amine, (19Z,22Z)-N,N-dimethyloctacos-19,22-dien-7-amine, N,N-dimethylheptacos-10-amine, (20Z,23Z)-N-ethyl-N-methylnonacos-20,23-dien-10-amine, 1-[(11Z,14Z)-1-nonylicosa-11,14-dien-1-yl]pyrrolidine, (20Z)-N,N-dimethylheptacos-20-en-10-amine, (15Z)-N,N-dimethylheptacos-15-en-10-amine, (14Z)-N,N-dimethylnonacos-14-en-10-amine, (17Z)-N,N-dimethylnonacos-17-en-10-amine, (24Z)-N,N-dimethyltritriacont-24-en-10-amine, (20Z)-N,N-dimethylnonacos-20-en-10-amine, (22Z)-N,N-dimethylhentriacont-22-en-10-amine, (16Z)-N,N-dimethylpentacos-16-en-8-amine, (12Z,15Z)-N,N-dimethyl-2-nonylhenicos-12,15-dien-1-amine, (13Z,16Z)-N,N-dimethyl-3-nonyldocos-13,16-dien-1-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]heptadecan-8-amine, 1-[(1S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan-10-

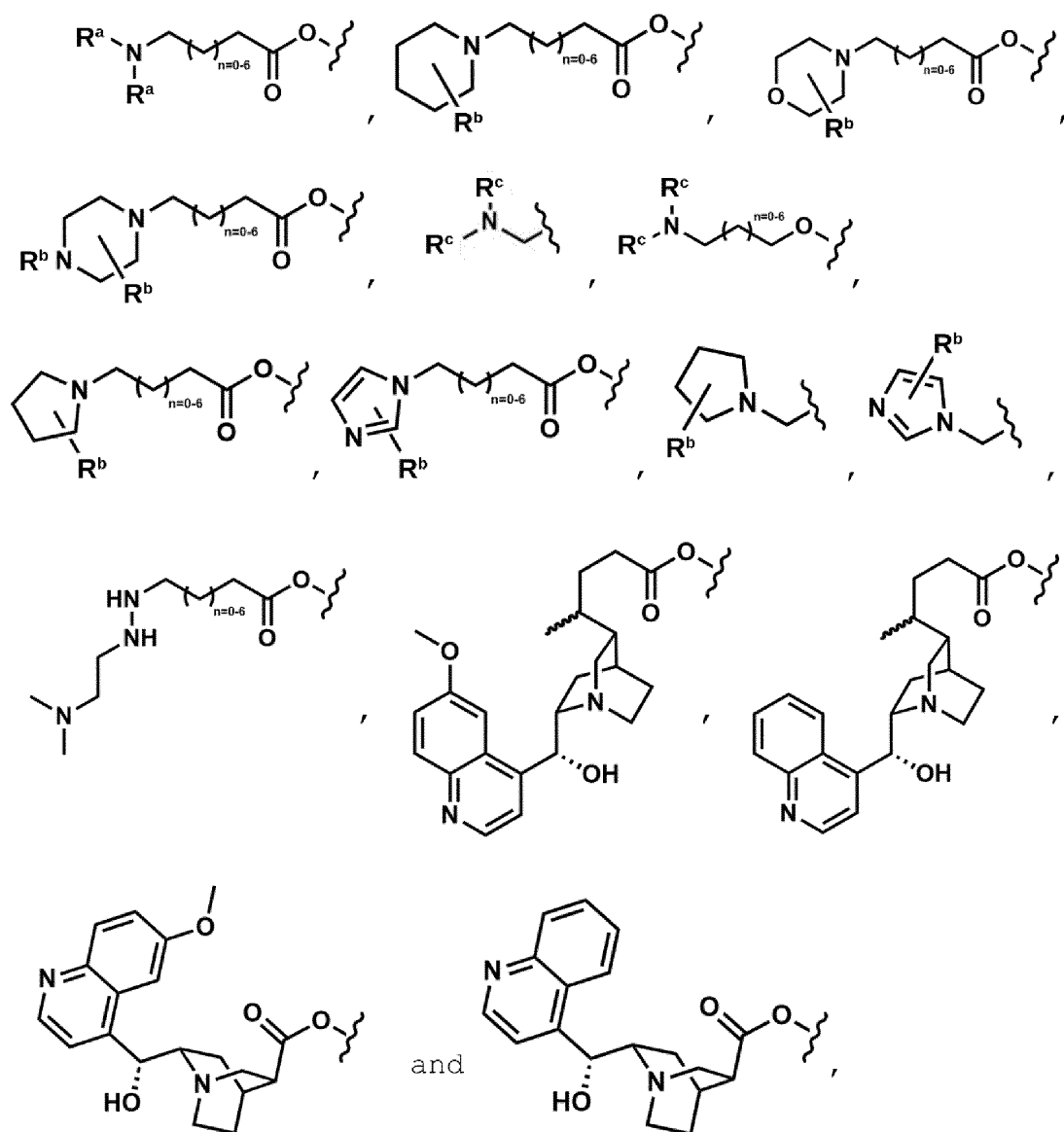
amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]nonadecan-10-amine, N,N-dimethyl-21-[(1S,2R)-2-octylcyclopropyl]heneicosan-10-amine, N,N-dimethyl-1-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]nonadecan-10-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyl-[(1R,2S)-2-undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3-{7-[(1S,2R)-2-octylcyclopropyl]heptyl}dodecan-1-amine, 1-[(1R,2S)-2-heptylcyclopropyl]-N,N-dimethyloctadecan-9-amine, 1-[(1S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]pentadecan-8-amine, R-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}pyrrolidine, (2S)-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct-5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(nonyloxy)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine; (2S)-N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)-N,N-dimethyl-H(1-metoyloctyl)oxy]-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(octyloxy)-3-({8-[(1S,2S)-2-[(1R,2R)-2-

pentylcyclopropyl)methyl}cyclopropyl]octyl}oxy)propan-2-amine, N,N-dimethyl-1-{[8-(2-octylcyclopropyl)octyl]oxy}-3-(octyloxy)propan-2-amine, and (11E,20Z,23Z)-N,N-dimethylnonacos-11,20,2-trien-10-amine, and pharmaceutically acceptable salts and stereoisomers thereof, and mixtures thereof.

Embodiment 16. The LNP of any one of embodiments 1 to 15, wherein the helper lipid fraction comprises a helper lipid selected from the group consisting of distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), Dipalmitoylphosphatidylcholine (DOPC), phosphatidylcholine (PC), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemSPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosaheptaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosaheptaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin and mixtures thereof.

Embodiment 17. The LNP of any one of embodiments 1 to 16, wherein the LNP has a z-average diameter between 10 nm and 900 nm, preferably between 20 nm and 750 nm, more preferably between 30 nm and 500 nm, especially between 40 nm and 250 nm or even between 50 nm and 150 nm (as determined by DLS, in particular according to ISO 22412:2017).

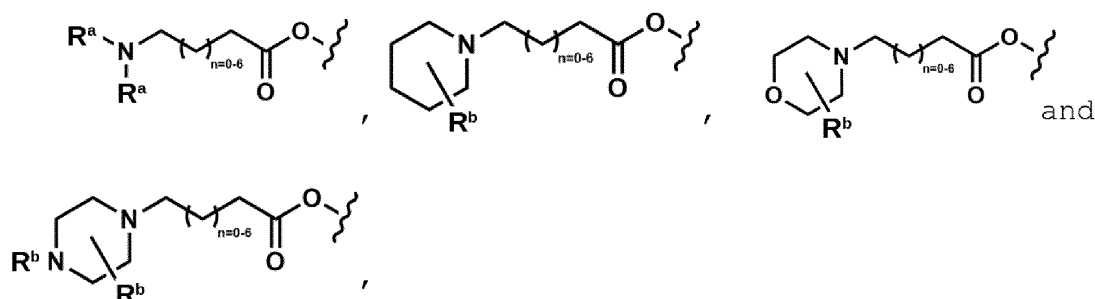
Embodiment 18. A GDGT lipid suitable for use as an ionizable lipid in an LNP, wherein the GDGT lipid comprises at least one ionizable head group S, wherein S is selected from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate,

alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine, each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide and alkyl thiol, preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol, and each occurrence of R^c is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide.

Embodiment 19. The GDGT lipid of embodiment 18, wherein S is selected from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine, and each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol.

Embodiment 20. The GDGT lipid of any one of embodiments 18 or 19, wherein said alkyl is selected from methyl, ethyl, propyl, preferably iso-propyl, butyl, preferably iso-butyl, pentyl, preferably iso-pentyl, hexyl, preferably iso-hexyl and heptyl, preferably iso-heptyl.

Embodiment 21. The GDGT lipid of any one of embodiments 18 to 20, wherein said cycloalkyl is selected from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl.

Embodiment 22. The GDGT lipid of any one of embodiments 18 to 21, wherein said alkenyl is selected from prop-2-enyl, but-2-enyl, but-3-enyl, pent-2-enyl, pent-3-enyl, pent-4-enyl, hex-2-enyl, hex-3-enyl, hex-4-enyl, hex-5-enyl, hept-2-enyl, hept-3-enyl, hept-4-enyl, hept-5-enyl and hept-6-enyl.

Embodiment 23. The GDGT lipid of any one of embodiments 18 to 22, wherein said hydroxyalkyl is selected from hydroxymethyl, hydroxyethyl, hydroxypropyl, hydroxybutyl, hydroxypentyl and hydroxyhexyl.

Embodiment 24. The GDGT lipid of any one of embodiments 18 to 23, wherein said alkylamine is selected from methylamine, ethylamine, propylamine, butylamine, pentylamine, hexylamine and heptylamine.

Embodiment 25. The GDGT lipid of any one of embodiments 18 to 24, wherein said alkyl ether is selected from methyl ether, ethyl ether, propyl ether, butyl ether, pentyl ether, hexyl ether and heptyl ether.

Embodiment 26. The GDGT lipid of any one of embodiments 18 to 25, wherein said alkyl thiol is selected from methyl thiol, ethyl thiol, propyl thiol, butyl thiol, pentyl thiol, hexyl thiol and heptyl thiol.

Embodiment 27. The GDGT lipid of any one of embodiments 18 to 26, wherein said alkyl ester is selected from methyl ester, ethyl ester, propyl ester, butyl ester, pentyl ester, hexyl ester and heptyl ester.

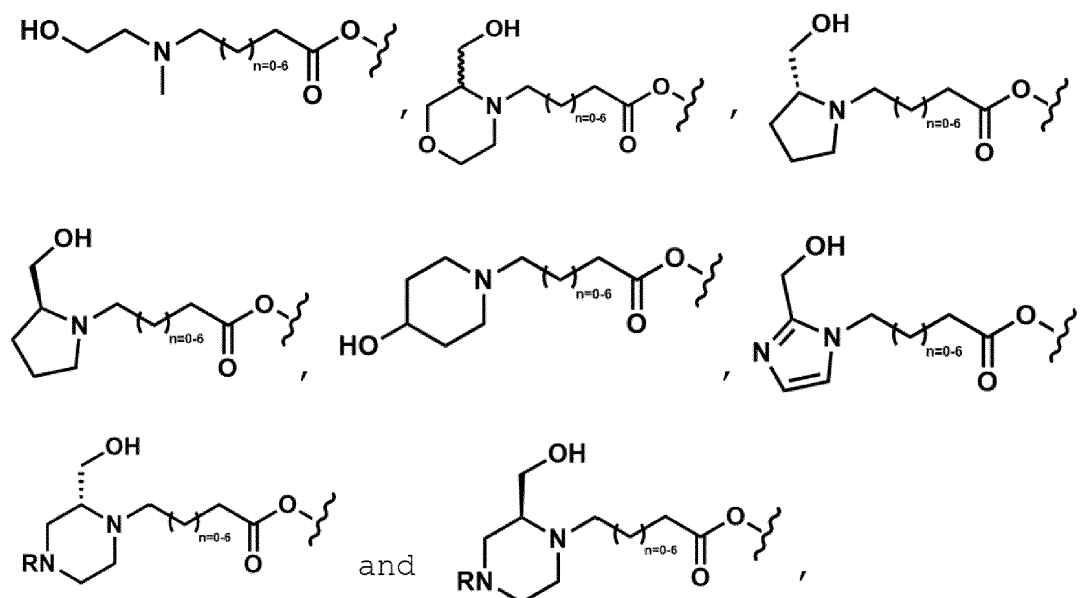
Embodiment 28. The GDGT lipid of any one of embodiments 18 to 27, wherein said alkyl amide is selected from methyl amide, ethyl amide, propyl amide, butyl amide, pentyl amide, hexyl amide and heptyl amide.

Embodiment 29. The GDGT lipid of any one of embodiments 18 to 28, wherein said alkyl carbamate is selected from methyl carbamate, ethyl carbamate, propyl carbamate, butyl carbamate, pentyl carbamate, hexyl carbamate and heptyl carbamate.

Embodiment 30. The GDGT lipid of any one of embodiments 18 to 29, wherein said alkyl sulfonate is selected from methyl sulfonate, ethyl sulfonate, propyl sulfonate, butyl sulfonate, pentyl sulfonate, hexyl sulfonate and heptyl sulfonate.

Embodiment 31. The GDGT lipid of any one of embodiments 18 to 30, wherein said alkyl sulfonamide is selected from methyl sulfonamide, ethyl sulfonamide, propyl sulfonamide, butyl sulfonamide, pentyl sulfonamide, hexyl sulfonamide and heptyl sulfonamide.

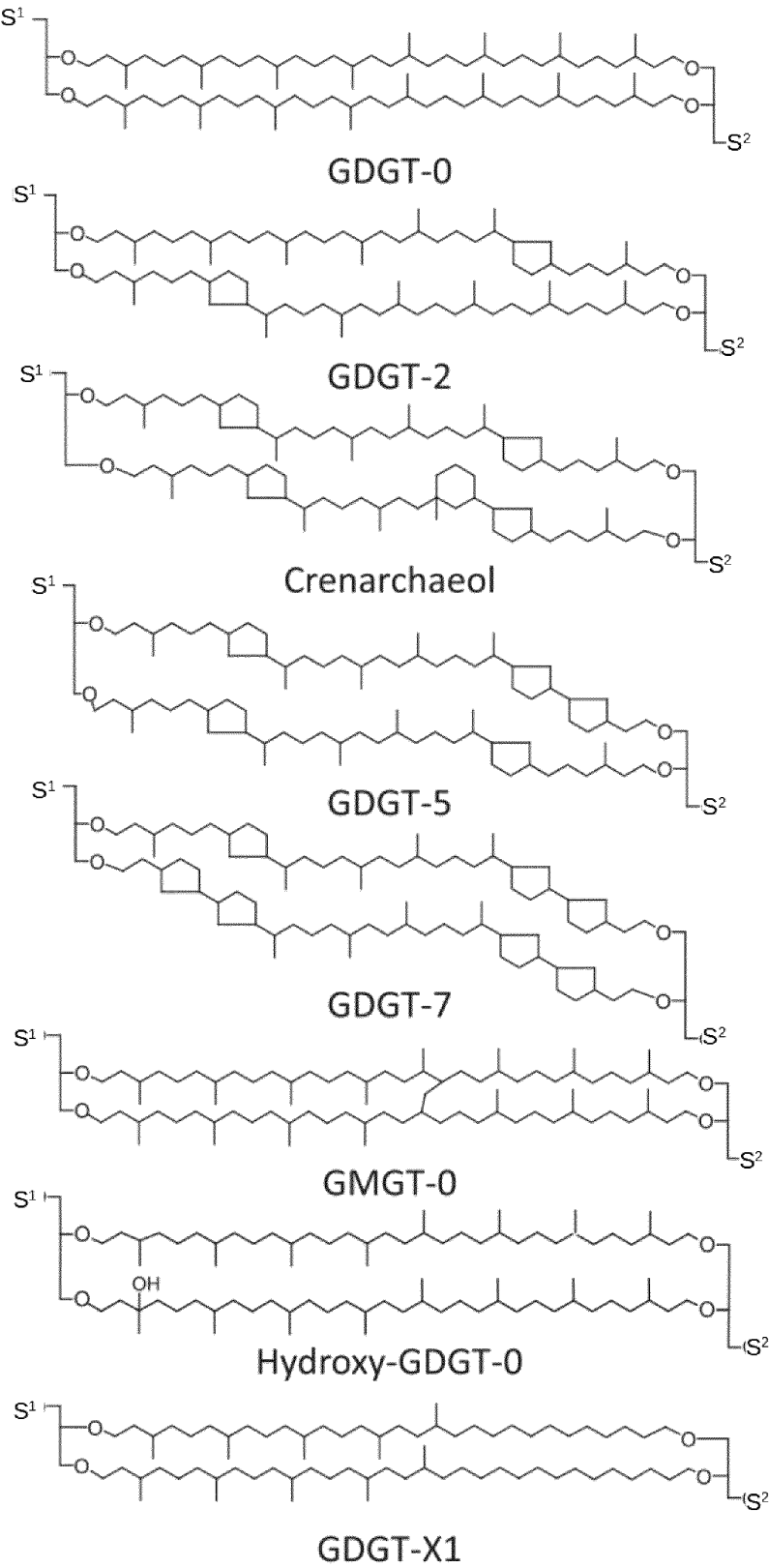
Embodiment 32. The GDGT lipid of any one of embodiments 18 to 31, wherein the head group S is selected from:

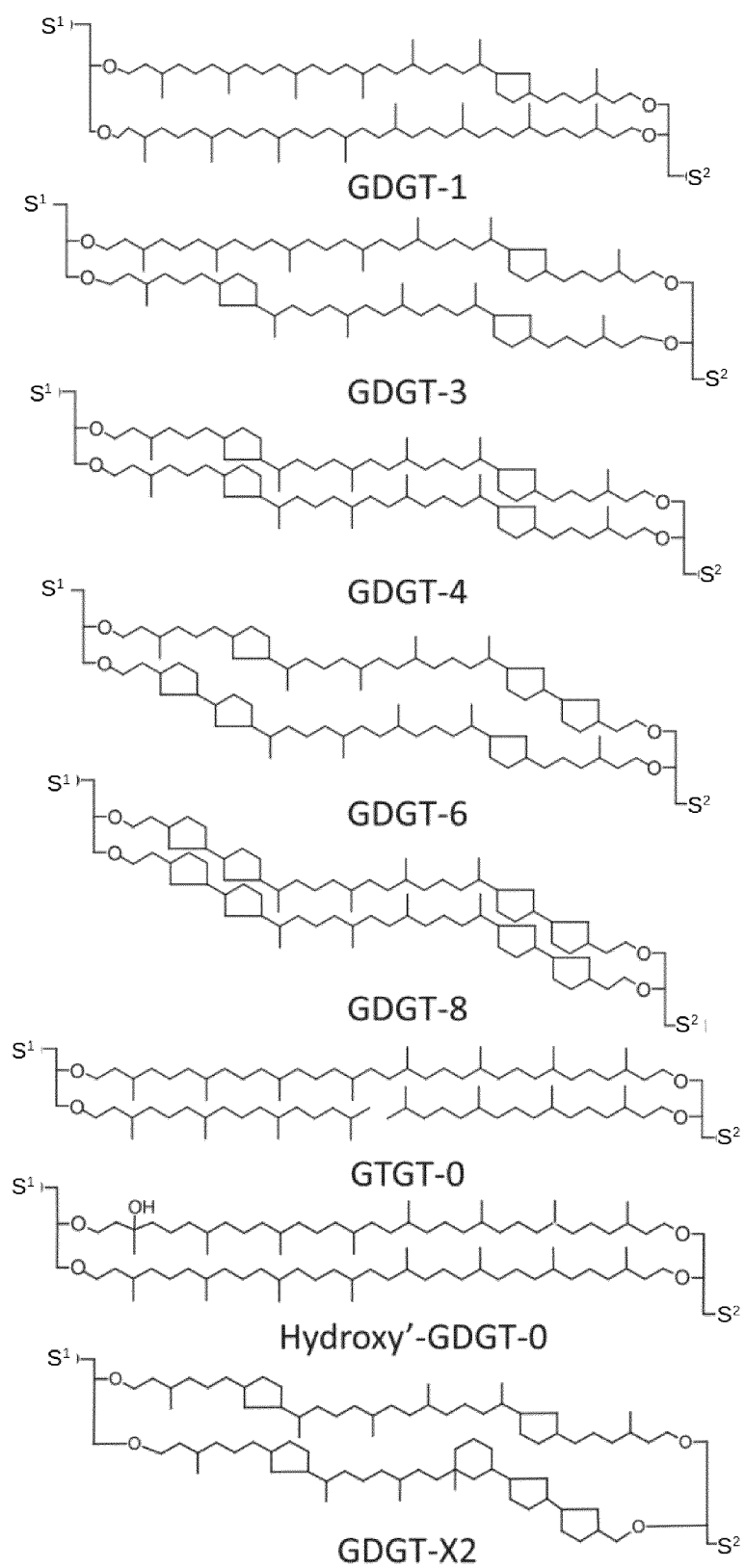


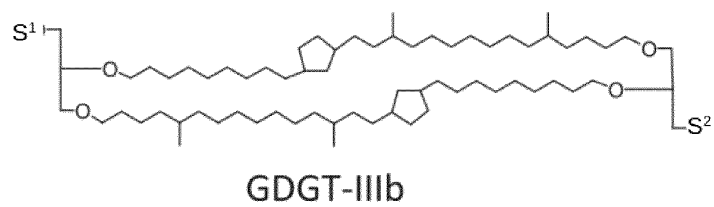
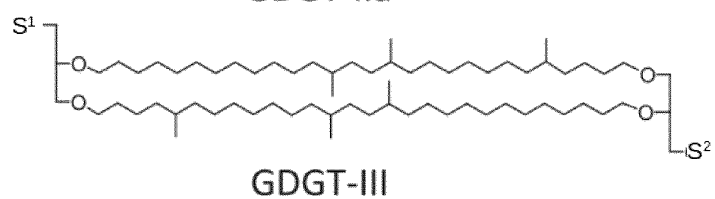
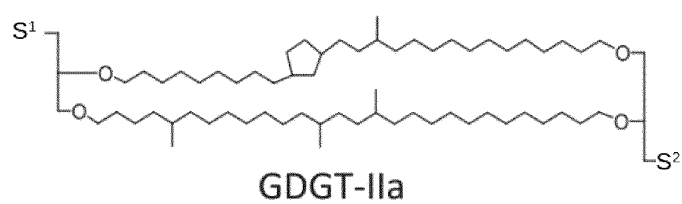
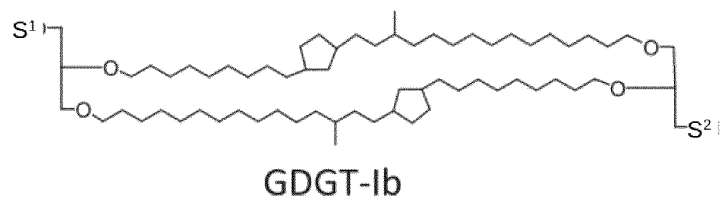
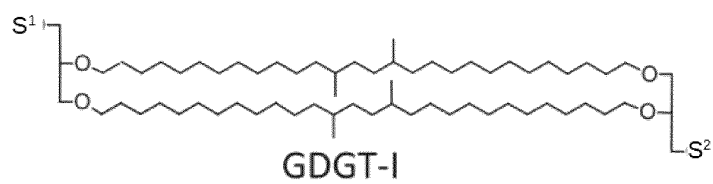
wherein each occurrence of R is independently selected from H and alkyl; preferably wherein the alkyl is selected from methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl, heptyl, and iso-heptyl.

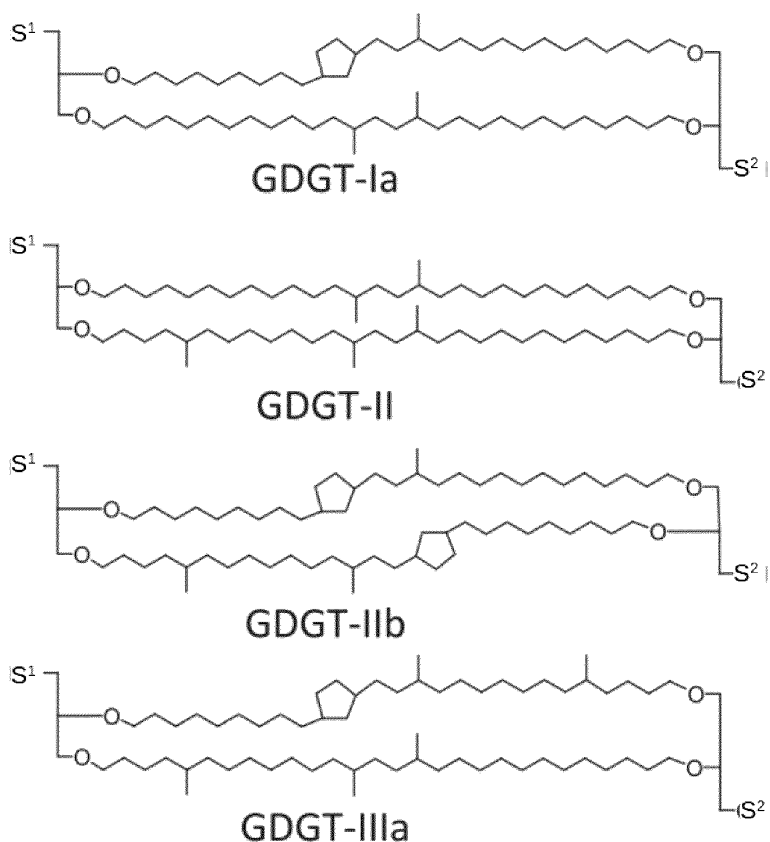
Embodiment 33. The GDGT lipid of any one of embodiments 18 to 32, wherein the GDGT lipid comprises two ionizable head groups S.

Embodiment 34. The GDGT lipid of any one of embodiments 18 to 33, wherein the GDGT lipid is selected from:









wherein S^1 is S and S^2 is OH, or S^1 is OH and S^2 is S, or each of S^1 and S^2 independently is S.

Embodiment 35. The GDGT lipid of embodiment 34, wherein each of S^1 and S^2 is the same S.

Embodiment 36. The GDGT lipid of any one of the previous embodiments, wherein the GDGT lipid carries an overall positive charge at endosomal pH, in particular at a pH between 5.0 and 6.5, such as pH 5.0, 5.5, 6 or 6.5; preferably wherein the GDGT lipid carries an overall neutral charge at a pH higher than endosomal pH, such as pH 7.0 or pH 7.4.

Embodiment 37. The GDGT lipid of any one of embodiments 18 to 36, wherein the ionizable head group S carries an overall positive charge at endosomal pH, in particular at a pH between 5.0 and 6.5, such as pH 5.0, 5.5, 6 or 6.5; preferably wherein the ionizable head group S carries an overall neutral charge at a pH higher than endosomal pH, such as pH 7.0 or pH 7.4.

Embodiment 38. The GDGT lipid of any one of embodiments 18 to 37, wherein the GDGT lipid is a caldarchaeol with the at least one ionizable head group S.

Embodiment 39. The GDGT lipid of any one of embodiments 18 to 38, wherein the GDGT lipid is selected from any one of the GDGT lipids of Figs. 1-10 and stereoisomers thereof, and variants thereof with a GDGT-0, GDGT-1, GDGT-2, GDGT-3, GDGT-5, GDGT-6, GDGT-7 or GDGT-8 scaffold instead of a GDGT-4 scaffold.

Embodiment 40. An ether lipid fraction comprising the GDGT lipid of any one of embodiments 18 to 39; preferably obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture, followed by substitution with the ionizable head group S.

Embodiment 41. An LNP comprising the GDGT lipid of any one of embodiments 18 to 39 or ether lipid fraction of embodiment 40, preferably wherein the LNP is as defined in embodiments 1 to 17.

Embodiment 42. A pharmaceutical composition comprising the LNP of any one of embodiments 1 to 17 and 41 and preferably at least one excipient.

Embodiment 43. The pharmaceutical composition of embodiment 42, wherein the pharmaceutical composition is a vaccine, preferably an mRNA vaccine.

Embodiment 44. The pharmaceutical composition of embodiment 42 or 43, for use in prevention or treatment of a disease or condition in a patient.

Embodiment 45. A method for producing the GDGT lipid of any one of embodiments 18 to 39, comprising the steps of:

- obtaining a lipid fraction comprising one or more precursor GDGT lipids from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture;
- purifying the one or more precursor GDGT lipids; and
- contacting the one or more precursor GDGT lipids with one or more reagents to produce the GDGT lipid with the at least one ionizable head group S.

The present invention is further illustrated by the following figures and examples, without being restricted thereto.

Figures 1-10 show particularly preferred ionizable GDGT lipids. The parentheses indicate that the length of the segment in parentheses may be from 0 to 6 carbon atoms ($n=0-6$). Each instance of " R_1 " or " R_2 " may independently be selected from the definitions given directly below the structure. Curly bonds indicate that both R and S enantiomers are preferred.

Examples

Example 1 - Purification of precursor GDGT lipid

Extraction of biomass: 240 g of dried biomass of *Sulfolobus acidocaldarius* were mixed with 360 g diatomaceous earth (Dionex ASE Prep DE) and the resulting 600g of solids were homogenously dispersed via shaking. 18 extraction cells (100mL Dionex ASE 350 stainless steel), equipped with a cellulose filter, were loaded with roughly equal amounts of dispersed solids (30-35g each) and placed in a Dionex ASE 350 Accelerated Solvent Extractor. The cells were extracted with 3 L of CHCl_3 :MeOH mixture (2:1) at 80°C and 1150 psi for 30 minutes each. The extracted liquid fractions were combined and the mixture was concentrated (Heidolph Hei-Vap Core, 50°C, 100mbar) to 200 mL. 50 mL of aqueous 2N HCl were added and the heterogenous, biphasic mixture was heated to 65°C for 24h. After cooling to room temperature, the mixture was neutralized with 50 mL NaHCO_3 , the phases were separated and the aqueous phase was extracted with Et_2O (3x 90mL), before the combined organic extracts were dried with Na_2SO_4 , filtered and the solvents evaporated, yielding 4.4 g (1.8% w/w) of crude extract as a brown solid.

Isolation of GDGT and *Sulfolobus* main polar lipid: The brown solid was dissolved in 40 mL dichloromethane (DCM) and subjected in two portions to column chromatography (Büchi Pure Chromatography System equipped with a light scattering detector

and an automated fraction collector). Column chromatography was conducted by liquid injection of 20 mL crude material onto a FlashPure EcoFlex 25g SiO₂ column (32mL/min flowrate), prior equilibrated with light petrol (LP). The yield was 193.4 mg (4.3%) of GDGT containing minor aromatic lipid impurities as a brown oil. Alongside GDGTs, 2.38g (54%) *Sulfolobus* main lipid was isolated within the same chromatography.

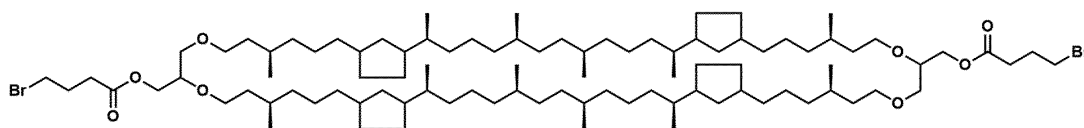
End purification of GDGT: 106.5 mg of said GDGTs were dissolved in 1 mL LP and separated on a Büchi FlashPure Ecoflex column 20g with LP:DCM (DCM=20% to 40%). All fractions displaying no UV-signal were collected and evaporated to yield 92.8 mg pure GDGTs (87%) as colorless oil.

These GDGT lipids were used in the following examples as precursor GDGT lipids to produce the GDGT lipids with ionizable head groups.

Example 2 - Preparation of bis-(4-bromobutanoic acid)-GDGT ester

Dimethylaminopyridine (DMAP) (5.4mg, 44 µmol, 0.4 equiv.) was added to GDGT as obtained according to Example 1 (142.7mg, 110 µmol, 1 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The vial was evacuated and backfilled with N₂ (three times) before 2.2 mL dry dichloromethane were added via canula. Subsequently the solution was cooled to 0°C by means of an ice/water bath before 4-bromobutyric acid chloride (45 µL, 240 µmol, 2.4 equiv.) were added dropwise. The mixture was stirred for 5 minutes at 0°C and then allowed to warm to room temperature and stirred for 18h. After complete consumption of the starting material (checked via thin-layer chromatography), the mixture was quenched with 20 mL sat. NaHCO₃ and extracted with Et₂O (3x 50 mL), once washed with brine, dried over Na₂SO₄ and evaporated to dryness. After column chromatography (12g SiO₂), 126.5 mg (72%) of bis-(4-bromobutanoic acid)-GDGT ester were isolated as white solid.

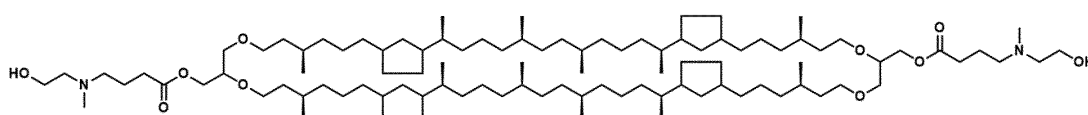
The product was a mixture of several different GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 3 - Preparation of bis-(4-((2-hydroxyethyl) (methyl)amino)butanoic acid)-GDGT ester

To bis-(4-bromobutanoic acid)-GDGT ester as obtained according to Example 2 (126.5 mg, 79 μmol , 1 equiv.) were added K_2CO_3 (32.9mg, 238 μmol , 3 equiv.) and KI (5.3mg, 31 μmol , 0.4 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The vial was evacuated and backfilled with N_2 (three times) before 2 mL HPLC-grade MeCN, followed by N-methylaminoethanol (20 μL , 238 μmol , 3 equiv.) were added via canula. The turbid mixture was heated to 75°C for 16 hours (full consumption of the starting material, checked via thin-layer chromatography), cooled to room temperature and the MeCN was evaporated. The solids were taken up in 20 mL NaHCO_3 and extracted with 20mL dichloromethane (three times), once washed with brine, filtered and evaporated yielding 91.8mg of crude yellow oil. The crude material was purified via flash chromatography (Biotage column, 5g) to obtain 65.8mg (52%) of bis-(4-((2-hydroxyethyl) (methyl)amino)butanoic acid)-GDGT ester as a colorless oil.

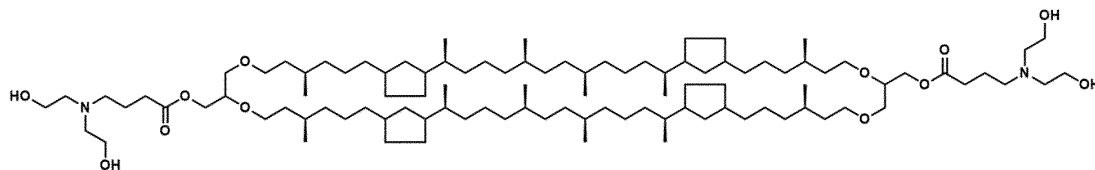
The product was a mixture of several different substituted GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 4 - Preparation of bis-(4-(diethanolamine)butanoic acid)-GDGT ester

To bis-(4-bromobutanoic acid)-GDGT as obtained according to Example 2 (1 equiv.) K_2CO_3 (3 equiv.) and KI (0.4 equiv.) are added in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before 2 mL HPLC-grade MeCN, followed by addition of diethanolamine (3 equiv.) via canula. The turbid mixture is heated to 75°C for 16 hours and subsequently cooled to room temperature before the MeCN is evaporated. The solids are taken up in 20 mL $NaHCO_3$ and extracted with 20 mL dichloromethane (three times), once washed with brine, filtered and evaporated. y. The crude material is purified via flash chromatography to isolate bis-(4-(bis-(2-hydroxyethyl)amino)butanoic acid)-GDGT ester.

The product typically is a mixture of several different substituted GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:

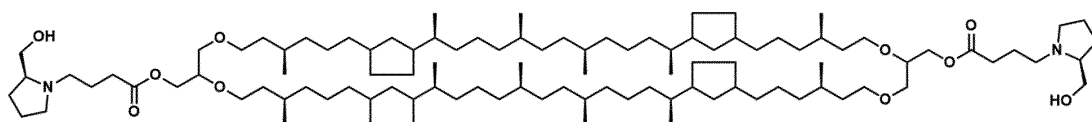


Example 5 - Preparation of bis-(S)-(4-2-(hydroxymethyl)pyrrolidin-1-yl)butanoic acid)-GDGT ester

To bis-(4-bromobutanoic acid)-GDGT ester as obtained according to Example 2 (1 equiv.) K_2CO_3 (3 equiv.) and KI (0.4 equiv.) are added in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before 2 mL HPLC-grade MeCN, followed by addition of (S)-prolinol via canula. The turbid mixture is heated to 75°C for 16 hours and subsequently cooled to room temperature before the MeCN is evaporated. The solids are taken up in 20 mL $NaHCO_3$ and extracted with 20 mL dichloromethane (three times), once washed

with brine, filtered and evaporated. The crude material is purified via flash chromatography to isolate bis-(4-(prolinol)butanoic acid)-GDGT ester.

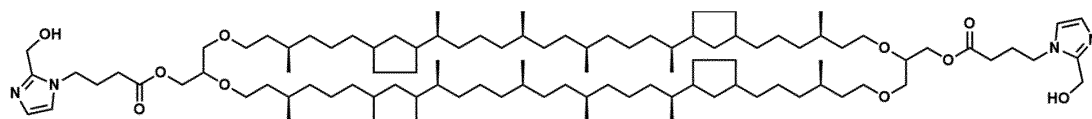
The product typically is a mixture of several different substituted GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 6 - Preparation of bis-(4-(2-(hydroxymethyl)-1H-imidazolyl)-GDGT ester

To bis-(4-bromobutanoic acid)-GDGT ester as obtained according to Example 2 (1 equiv.) K_2CO_3 (3 equiv.) and KI (0.4 equiv.) are added in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before 2 mL HPLC-grade MeCN, followed by addition of 2-(hydroxymethyl)-1H-imidazole via canula. The turbid mixture is heated to 75°C for 16 hours and subsequently cooled to room temperature before the MeCN is evaporated. The solids are taken up in 20 mL $NaHCO_3$ and extracted with 20 mL dichloromethane (three times), once washed with brine, filtered and evaporated. The crude material is purified via flash chromatography to bis-(4-(2-(hydroxymethyl)-1H-imidazolyl)-GDGT ester.

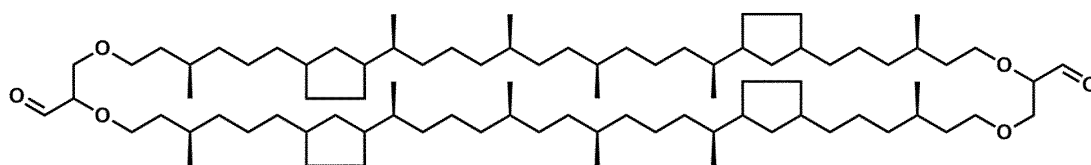
The product typically is a mixture of several different substituted GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 7 - Preparation of GDGT aldehyde

To an 8 mL vial containing GDGT as obtained according to Example 1 (1 equiv.) and Trichlorocyanuric acid (TCCA) (2.2 equiv) dichloromethane is added and cooled to 0°C. 2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO) (0.2 equiv.) are added and the mixture is stirred at 0°C for 15 minutes. The mixture is quenched with saturated aqueous NaHCO₃ solution and extracted with Et₂O (3x 50 mL). The combined organic extracts are once washed with 2N HCl and brine before dried with Na₂SO₄, filtered and evaporated. This yields crude GDGT aldehyde, which is used without further purification.

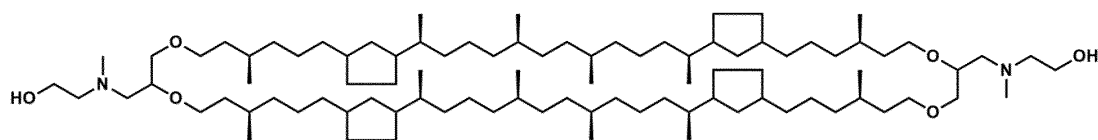
The product typically is a mixture of several different GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 8 - Preparation of bis-((methyl)amino)ethanol GDGT

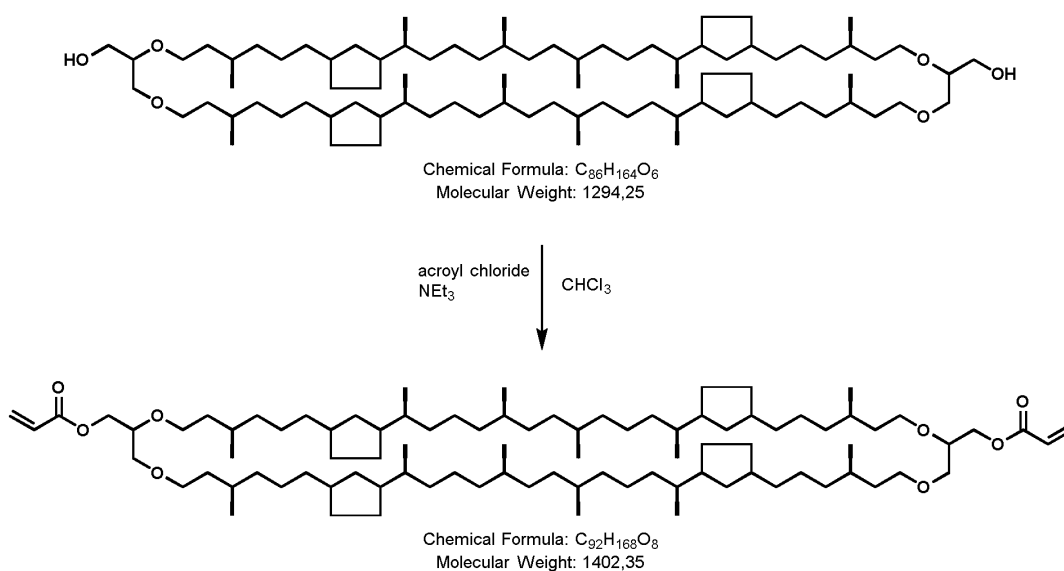
In an 8 mL vial under N₂ atmosphere GDGT aldehyde as obtained according to Example 7 (1 equiv.) is dissolved in tetrahydrofuran and N-methylaminoethanol is added (2.2 equiv.) and stirred for 10 minutes. Sodium triacetoxyborohydride (3 equiv.) is added and the mixture is stirred at room temperature for 24h. The reaction mixture is quenched via addition of 3N NaOH and the product is extracted with Et₂O and dried with MgSO₄. Treatment of the cooled ethereal extract with ethereal HCl yields bis-((methyl)amino)ethanol GDGT hydrochloride salt after filtration.

The product typically is a mixture of several different substituted GDGT scaffolds. A representative structure (with a GDGT-4 scaffold) is shown in the following:



Example 9 - Synthesis of ionizable GDGTs from GDGT acrylate

Preparation of bis-GDGT-acrylate

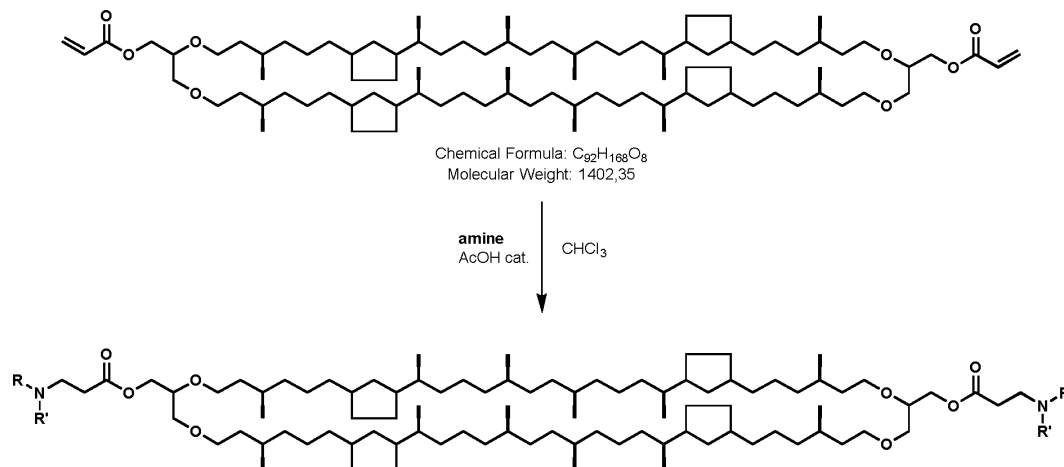


GDGT as preferably obtained according to Example 1 (1 equiv.) is placed in a 20mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before dry $CHCl_3$ and triethylamine (2 equiv.) are added via canula. Subsequently the solution is cooled to 0°C by means of an ice/water bath before acryloyl chloride in $CHCl_3$ (0.1M, 2.2 equiv.) are added dropwise. The mixture is stirred for 5 minutes at 0°C and then allowed to warm to room temperature and stirred for 5h. After complete consumption of the starting material (checked via TLC), the mixture is quenched with 20 mL sat. $NaHCO_3$ and extracted with $CHCl_3$, once washed with brine, dried over $MgSO_4$ and evaporated to dryness. After column chromatography (12g

SiO₂) 83% of the Bis-acrylic-GDGT ester is isolated as white solid.

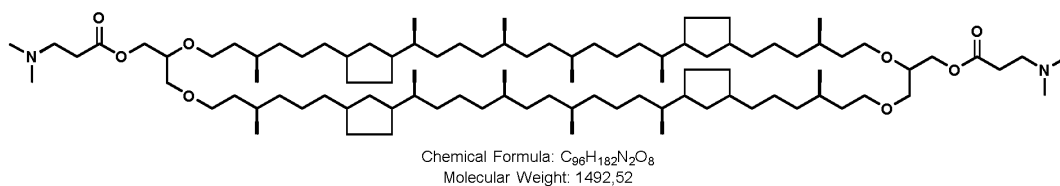
Further steps

Bis-acrylic-GDGT ester (1 equiv.) is added to an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N₂ (three times) before dry CHCl₃ was added, followed by amine (2.2 equiv.) and a catalytic amount of acetic acid (0.1 eq.) is added via canula or in one portion if the amine is a solid. The homogenous mixture is heated to 40°C for 3 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and quenched with saturated aqueous NaHCO₃ and extracted with CHCl₃ (three times), once washed with brine, dried with MgSO₄, filtered and evaporated. The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain bis-(aminopropanoic acid)-GDGT ester as a colorless oil.



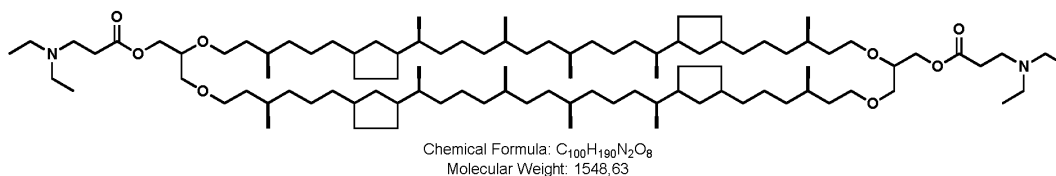
Crude materials obtained depend on the amine used. The products typically are mixtures of several different substituted GDGT scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

bis-(3-dimethylamino)propanoic acid)-GDGT ester



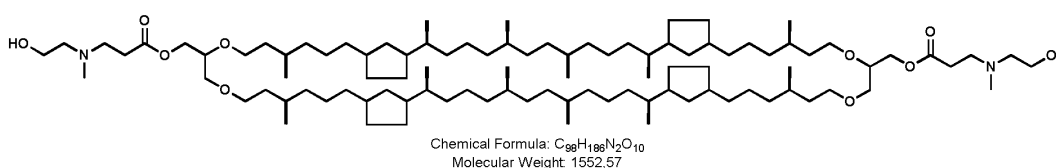
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 67% of bis-(4-(dimethylamino)butanoic acid)-GDGT ester as a colorless oil.

bis-(3-diethylamino)propanoic acid)-GDGT ester



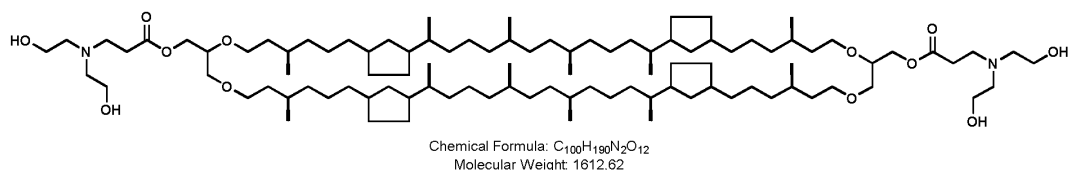
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 63% of bis-(3-(diethylamino)propanoic acid)-GDGT ester as a colorless oil.

bis-(3-(2-hydroxyethyl)(methyl)amino) propanoic acid)-GDGT ester



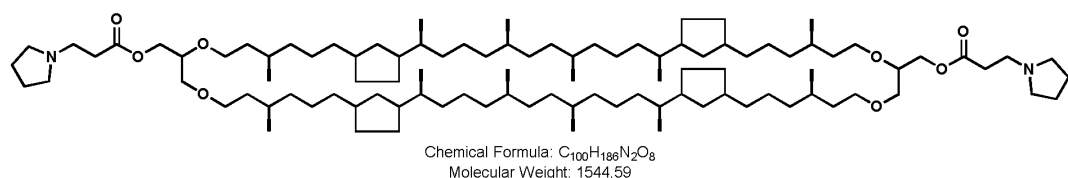
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 54% of bis-(3-(2-hydroxyethyl)(methyl)amino) propanoic acid)-GDGT ester as a colorless oil.

bis 3-(bis(2-hydroxyethyl)amino)propanoic acid)-GDGT ester



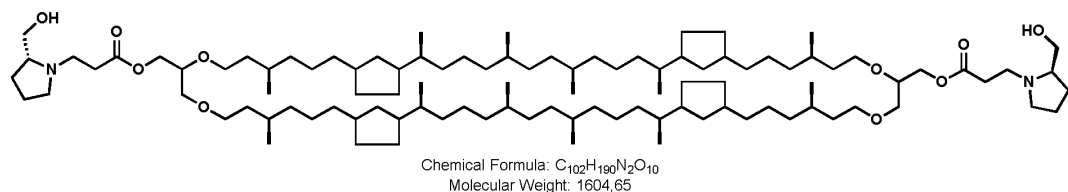
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 57% 3-(bis(2-hydroxyethyl)amino)propanoic acid)-GDGT ester as a colorless oil.

bis-(3-pyrrolidin-1-yl) propanoic acid)-GDGT ester



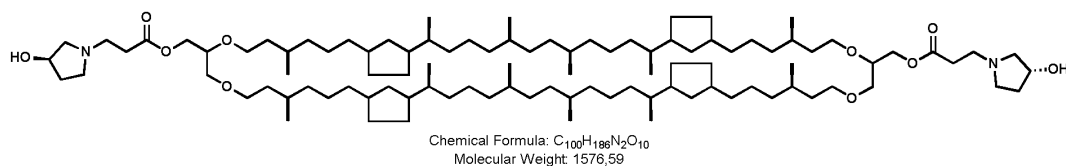
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 51% of bis-(3-pyrrolidin-1-yl) propanoic acid)-GDGT ester as a colorless oil.

bis-3-((R)-(hydroxymethyl)pyrrolidin-1-yl)propanoic acid)-GDGT ester



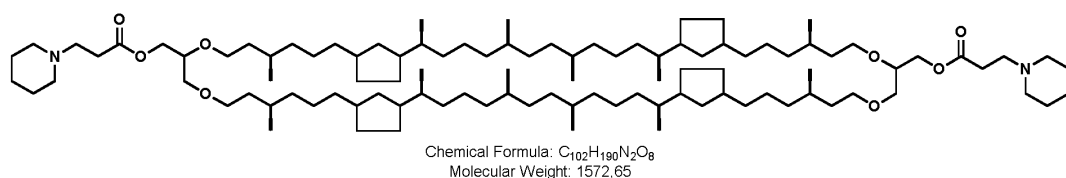
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 67% of bis-3-(2(R)-(hydroxymethyl)pyrrolidin-1-yl) propanoic acid)-GDGT ester as a colorless oil.

bis-3-((S)-(hydroxypyrrolidin-1-yl)propanoic acid)-GDGT ester



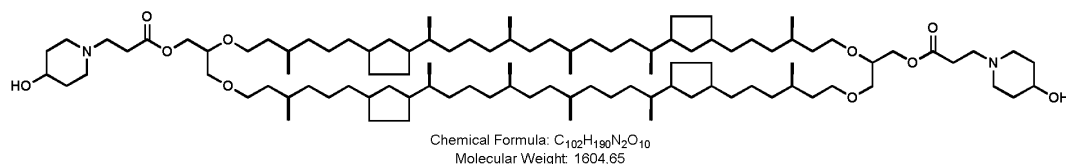
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 62% of bis-3-((S)-(hydroxypyrrolidin-1-yl)propanoic acid)-GDGT ester as a colorless oil.

bis-(3-piperidin-1-yl) propanoic acid)-GDGT ester



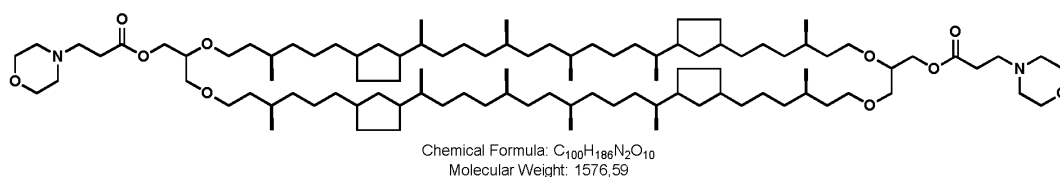
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 64% of bis-(3-piperidin-1-yl) propanoic acid)-GDGT ester as a colorless oil.

bis-(3-(4-hydroxypiperidin-1-yl) propanoic acid)-GDGT ester



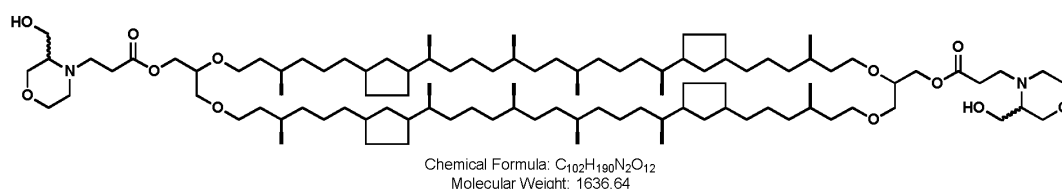
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% of bis-(3-(4-hydroxypiperidin-1-yl) propanoic acid)-GDGT ester as a colorless oil.

bis-(3-morpholino)-propanoic acid)-GDGT ester



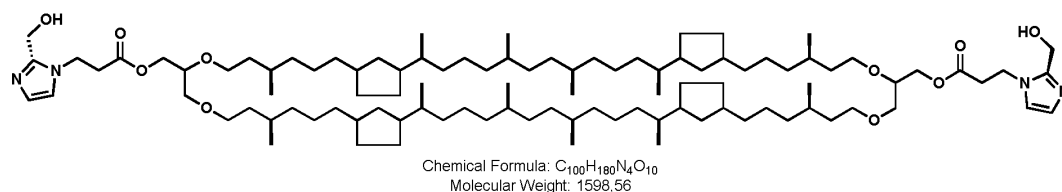
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 54% of bis-(3-morpholino)propanoic acid)-GDGT ester as a colorless oil.

bis-(3-(3-(hydroxymethyl)morpholino)-propanoic acid)-GDGT ester



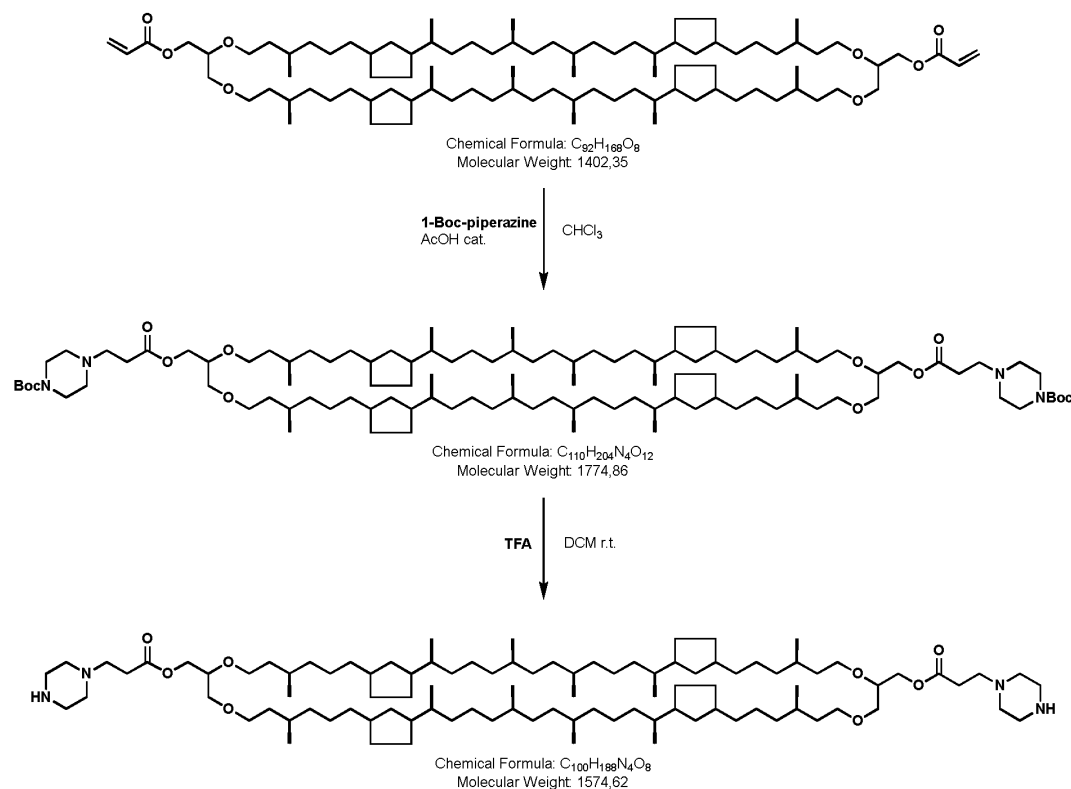
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 47% of a diastereomeric mixture of bis-(3-(3-(hydroxymethyl)morpholino) propanoic acid)-GDGT ester as a colorless oil.

bis-(3-(2-(hydroxymethyl)-1H-imidazolyl) propanoic acid)-GDGT ester



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 75% of bis-(3-(2-(hydroxymethyl)-1H-imidazolyl) propanoic acid)-GDGT ester as a colorless oil.

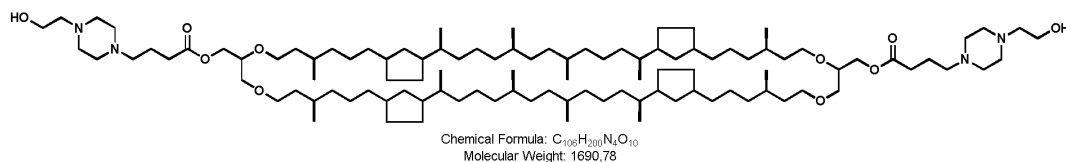
bis-3-(piperazin-1-yl)-propanoic acid) GDGT ester



To bis-acrylic-GDGT ester is added 1-Boc-piperazine in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before dry $CHCl_3$ was added, followed by a catalytic amount of acetic acid (0.1 equiv.). The mixture is heated to $40^\circ C$ for 4 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and saturated aqueous $NaHCO_3$ solution was added. The mixture is extracted with $CHCl_3$ (three times), once washed with brine, dried with $MgSO_4$ filtered and evaporated. The crude material is dissolved in 2 ml dry DCM and 1 ml TFA was added dropwise. The mixture is stirred at room temperature for 2 hours, cooled to $0^\circ C$ and neutralized with sat. aqueous $NaHCO_3$ solution followed by extraction with DCM (3x), drying with $MgSO_4$ and the solvents were evaporated. Purification via flash chromatography (Biotage Sfär Amino column, LP:EE) yields 42 % of

bis-3-(piperazin-1-yl)-propanoic acid)-GDGT ester as a slightly yellow oil.

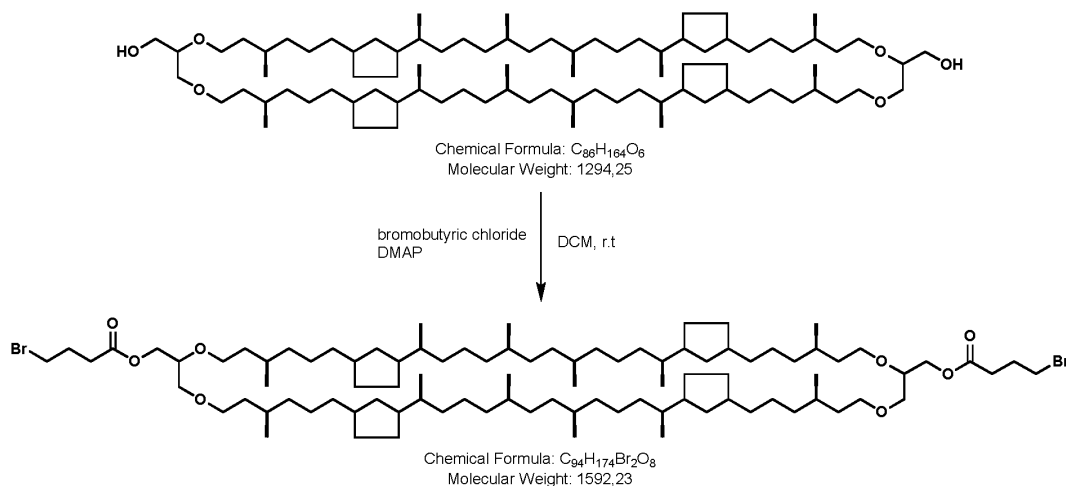
bis-3-(4-(2-hydroxyethyl)piperazin-1-yl)propanoic acid-GDGT ester



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 59% of bis-3-(4-(2-hydroxyethyl)piperazin-1-yl)-propanoic acid)-GDGT ester as a yellow oil.

Example 10 - Synthesis of ionizable GDGTs from bis-(4-bromobutanoic acid)-GDGT ester

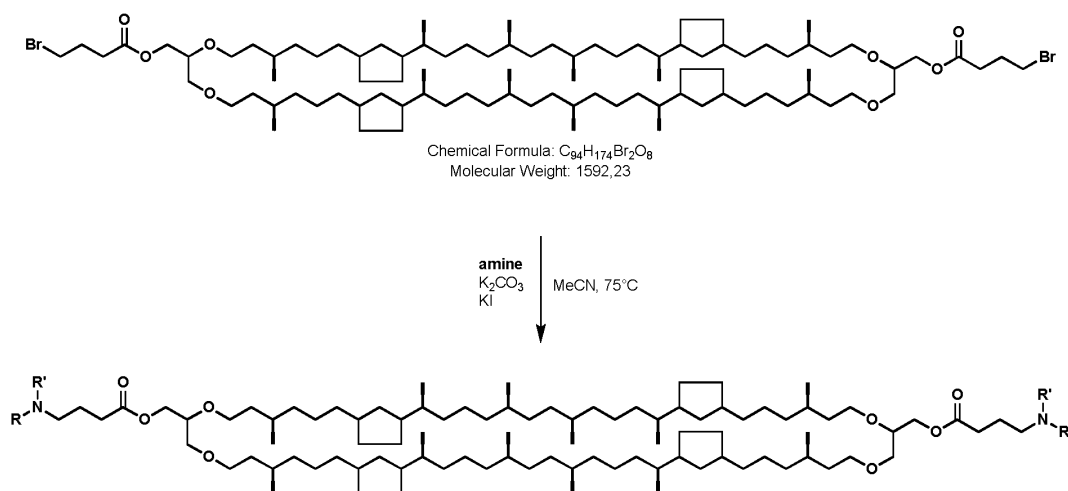
General procedure for the preparation of bis-(4-bromobutanoic acid)-GDGT ester



DMAP (0.4 equiv.) is added to GDGT as preferably obtained according to Example 1 (1 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N₂ (three times) before dry DCM was added via canula. Subsequently the solution is cooled to 0°C by means of an ice/water bath before 4-bromobutyric acid chloride (2.4 equiv.) was added dropwise. The mixture is stirred for 5 minutes at 0°C and then allowed to warm to room temperature and stirred for 18h. After complete consumption of the starting material (checked via TLC), the mixture is quenched with sat. NaHCO₃ and extracted with Et₂O (3x), once washed with brine, dried over Na₂SO₄ and evaporated to dryness. After column chromatography (SiO₂, PE:EE) 72% of bis-(4-bromobutanoic acid)-GDGT ester is isolated as white solid.

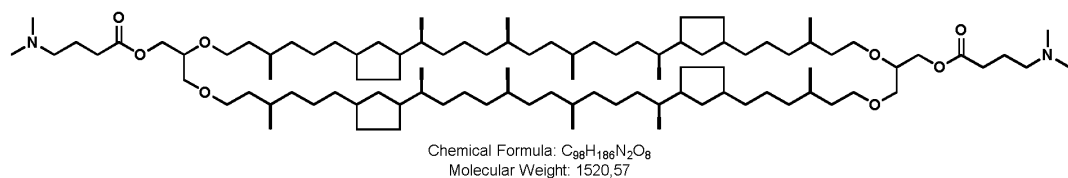
Further steps

To bis-(4-bromobutanoic acid)-GDGT ester (1 equiv.) are added K₂CO₃ (3 equiv.) and KI (0.4 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N₂ (three times) before HPLC-grade MeCN, followed by the appropriate secondary amine (3 equiv.) are added via canula or in one portion as a solid. The turbid mixture is heated to 75°C for 16 to 24 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and the MeCN was evaporated. The solids are taken up in NaHCO₃ and extracted with DCM (three times), once washed with brine, filtered and evaporated. The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain bis-(4-aminobutanoic acid)-GDGT ester as a colorless oil.



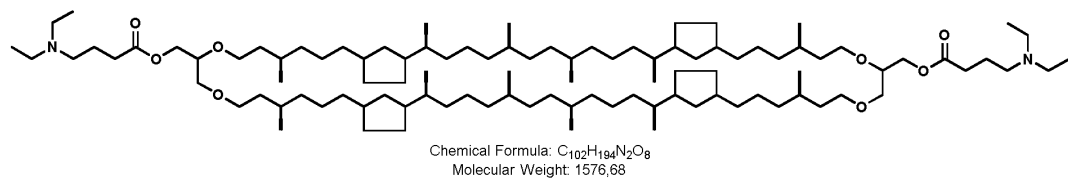
Crude materials obtained depend on the amine used. The products typically are mixtures of several different substituted GDGT scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

bis-(dimethylamino)butanoic acid)-GDGT ester



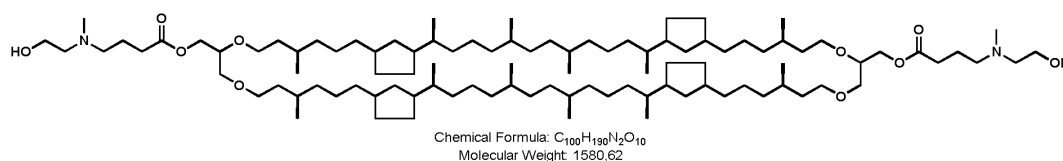
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 48% of bis-(4-(dimethylamino)butanoic acid)-GDGT ester as a colorless oil.

bis-(diethylamino)butanoic acid)-GDGT ester



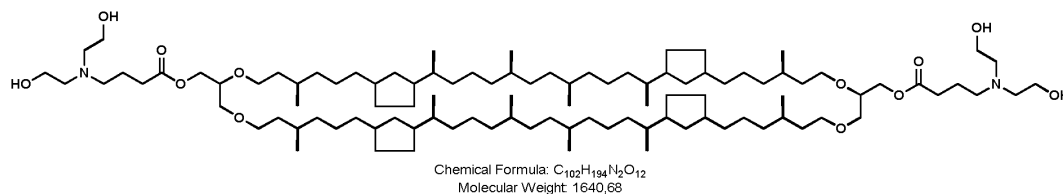
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 63% of bis-(4-(diethylamino)butanoic acid)-GDGT ester as a colorless oil.

bis-(4-(2-hydroxyethyl) (methyl) amino)butanoic acid)GDGT ester



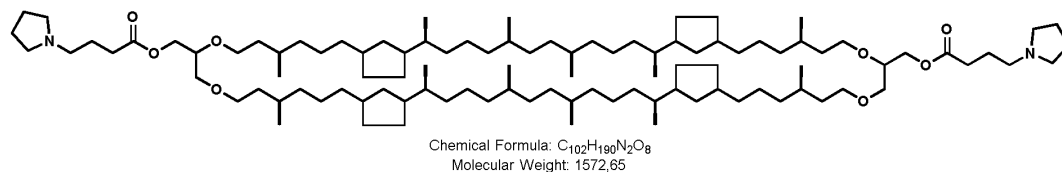
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 68% of bis-(4-(2-hydroxyethyl) (methyl) amino)butanoic acid)GDGT ester as a colorless oil.

bis 4-(bis(2-hydroxyethyl) amino)butanoic acid)-GDGT ester



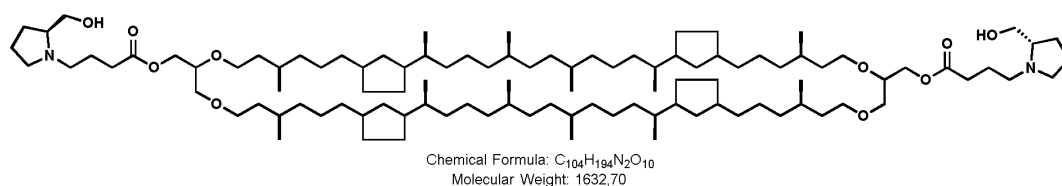
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% 4-(bis(2-hydroxyethyl) amino)butanoic acid)-GDGT ester as a colorless oil.

bis-(4-pyrrolidin-1-yl) butanoic acid)-GDGT ester



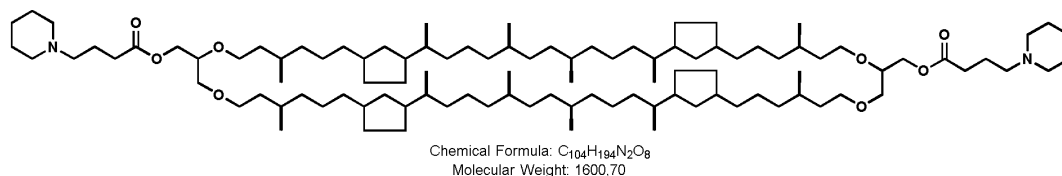
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 53% of bis-(pyrrolidin-1-yl) butanoic acid)-GDGT ester as a colorless oil.

4-(2-(hydroxymethyl)pyrrolidin-1-yl)butanoic acid)-GDGT ester



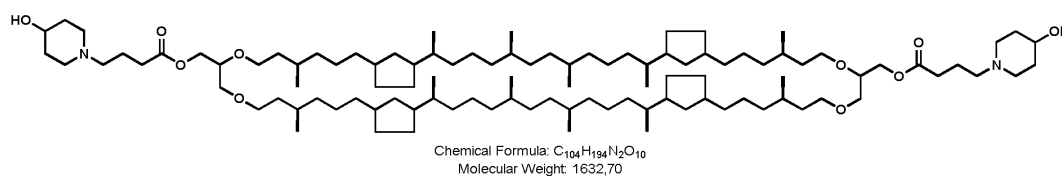
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 49% of bis-(4-(2 (S)-(hydroxymethyl)pyrrolidin-1-yl) butanoic acid)-GDGT ester as a colorless oil.

bis-(4-piperidin-1-yl)) butanoic acid)-GDGT ester



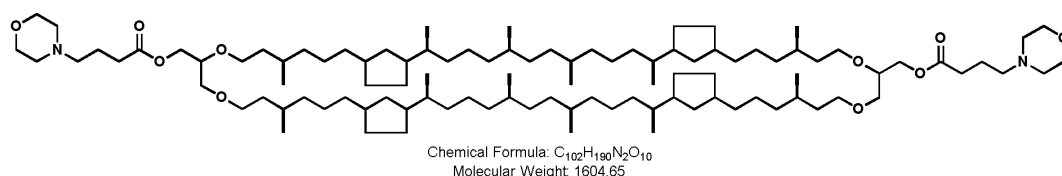
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of bis-(4-piperidin-1-yl)) butanoic acid)-GDGT ester as a colorless oil.

bis-(4-(4-hydroxypiperidin-1-yl)) butanoic acid)-GDGT ester



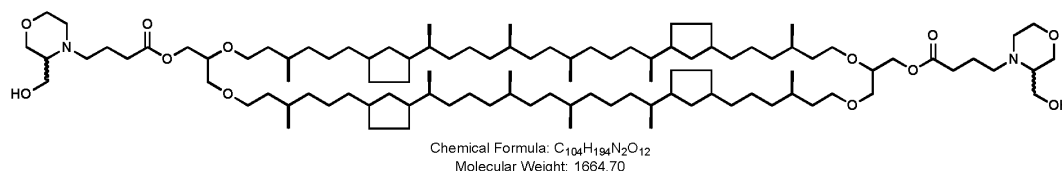
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of bis-(4-(4-hydroxypiperidin-1-yl)) butanoic acid)-GDGT ester as a colorless oil.

bis-(4-morpholino) butanoic acid)-GDGT ester



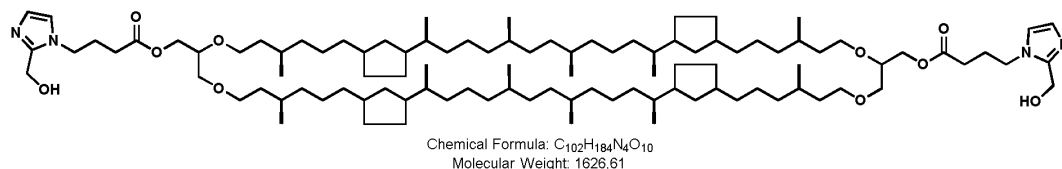
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 60% of bis-(4-morpholino) butanoic acid)-GDGT ester as a colorless oil.

bis-(4-(3-(hydroxymethyl)morpholino) butanoic acid)-GDGT ester



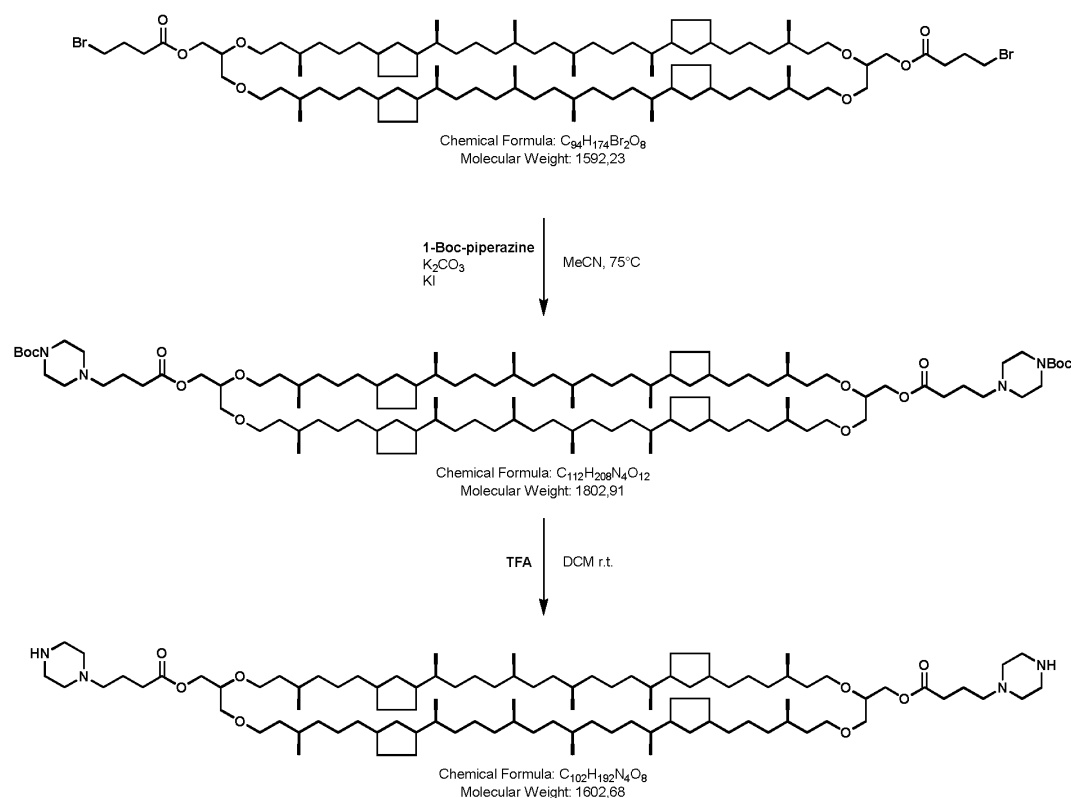
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 41% of bis-(4-(3-(hydroxymethyl)morpholino) butanoic acid)-GDGT ester as a colorless oil.

bis-(4-(2-(hydroxymethyl)-1H-imidazolyl) butanoic acid)-GDGT ester



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 73% of bis-(4-(2-(hydroxymethyl)-1H-imidazolyl) butanoic acid)-GDGT ester as a colorless oil.

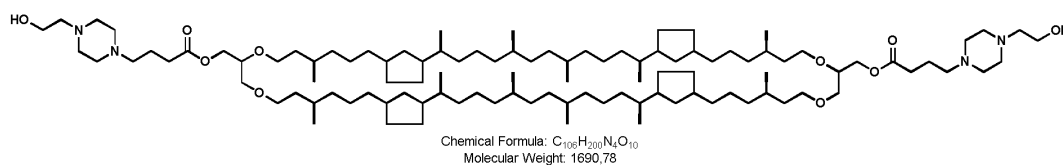
bis-(4-(piperazin-1-yl) butanoic acid)-GDGT ester



To bis-(4-bromobutanoic acid)-GDGT ester (1 equiv.), K_2CO_3 (3 equiv.), KI (0.4 equiv.) and 1-Boc-piperazine are added in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before HPLC-grade MeCN. The turbid mixture is heated to 75°C for 18 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and the MeCN was evaporated. The solids are taken up in $NaHCO_3$ and extracted with DCM (three times), once washed with brine, filtered and evaporated. The crude material

is dissolved in 2 ml dry DCM and 1 ml TFA was added dropwise. The mixture is stirred at room temperature for 2 hours, cooled to 0°C and neutralized with sat. aqueous NaHCO₃ solution. The mixture is 3 times extracted with DCM, dried with MgSO₄ and the solvents evaporated. Purification via flash chromatography (Biotage Sfär Amino column, LP:EE) yields 45 % of bis-(4-(piperazin-1-yl) butanoic acid)-GDGT ester as a slightly yellow oil.

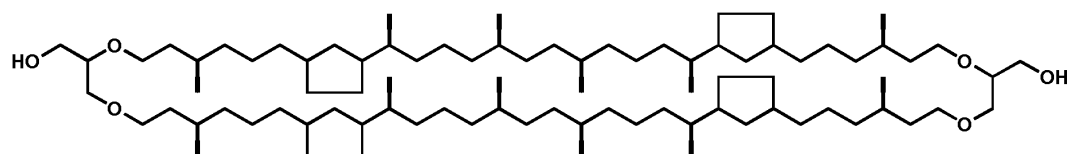
bis-(4-(4-(2-hydroxyethyl)piperazin-1-yl) butanoic acid)-GDGT ester



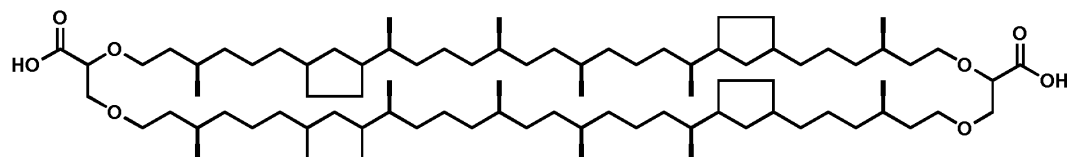
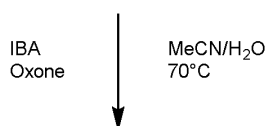
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 54% of bis-4-(4-(2-hydroxyethyl)piperazin-1-yl)-GDGT ester as a yellow oil.

Example 11 - Synthesis of ionizable GDGTs from GDGT acid

Preparation of bis-GDGT-acid



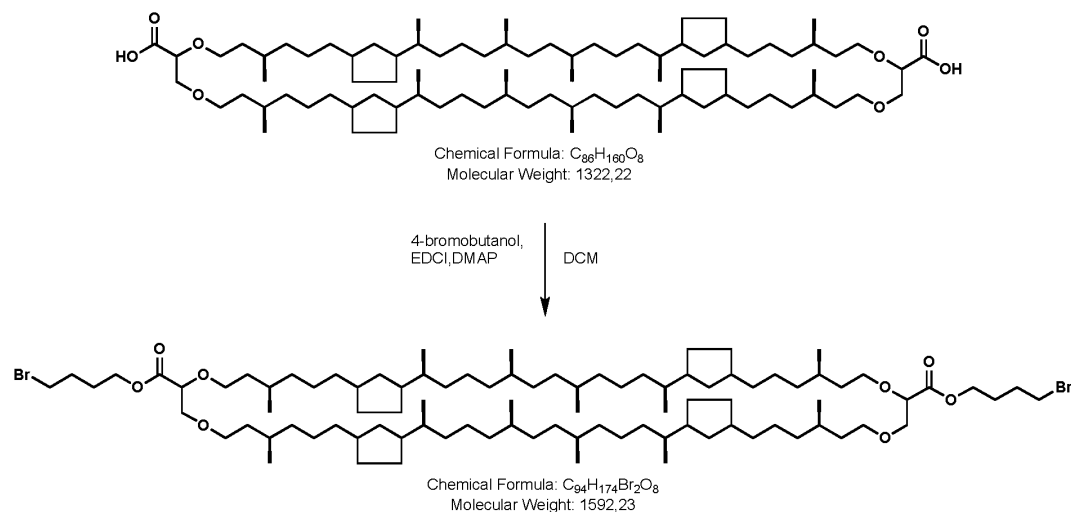
Chemical Formula: $C_{86}H_{164}O_6$
Molecular Weight: 1294,25



Chemical Formula: $C_{86}H_{160}O_8$
Molecular Weight: 1322,22

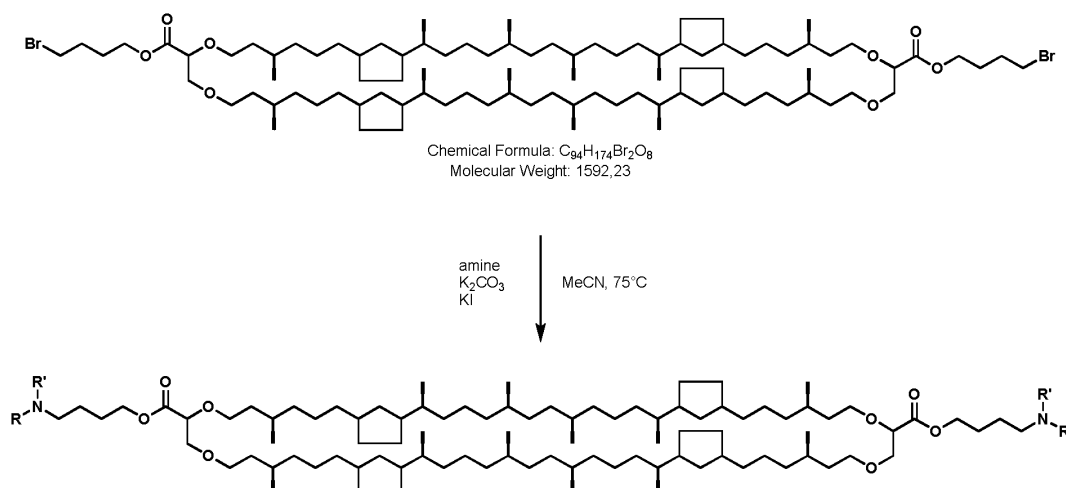
GDGT as preferably obtained according to Example 1 (1 equiv.), 2-iodobenzoic acid (0.4 eq.) and Oxone (2.8 eq.) are dissolved in MeCN/H₂O=2:1 in an 8 mL vial, equipped with a septum and a magnetic stirring bar. The mixture is heated to 70°C for 6 hours and then cooled to room temperature. The hypervalent iodine precipitant is removed via filtration and washed with water and DCM. The solvent mixture is extracted with DCM (3x) and dried over MgSO₄. Evaporation of the solvent yields 97% of crude bis-GDGT-acid which is used without further purification.

General procedure for the preparation of bis-(4-aminobutyl)-GDGT esters



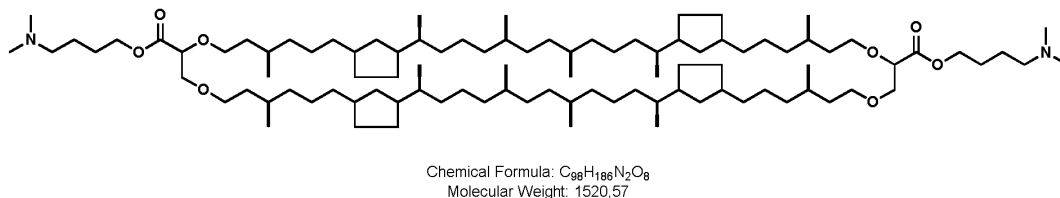
To bis-GDGT acid (1 equiv.) are added EDCI (2.2 equiv.) and DMAP (0.4 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The mixture is dissolved in DCM before 4-bromobutanol (2.8 equiv.) is added and stirred at room temperature for 18 hours. After quenching with saturated aqueous $NaHCO_3$ solution the mixture is extracted with DCM (3x), washed with brine, dried over $MgSO_4$ and evaporated. The crude material is purified via flash chromatography (SiO_2 , LP:EE) to obtain bis-(4-bromobutane)-GDGT-ester as a colorless oil.

To bis-(4-bromobutyl)-GDGT ester (1 equiv.) were added K_2CO_3 (3 equiv.) and KI (0.4 equiv.) in an 8 mL vial, equipped with a septum and a stirring bar. The vial was evacuated and backfilled with N_2 (three times) before HPLC-grade MeCN, followed by the appropriate secondary amine (3 equiv.) were added via canula or in one portion as a solid. The turbid mixture was heated to $75^\circ C$ for 16 to 24 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and the MeCN was evaporated. The solids were taken up in $NaHCO_3$ and extracted with DCM (three times), once washed with brine, filtered and evaporated. The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain bis-(4-aminobutyl)-GDGT ester as a colorless oil.



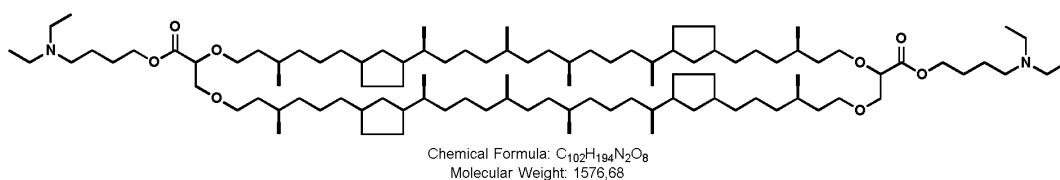
Crude materials obtained depend on the amine used. The products typically are mixtures of several different substituted GDGT scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

bis-(dimethylamino)butyl)-GDGT ester



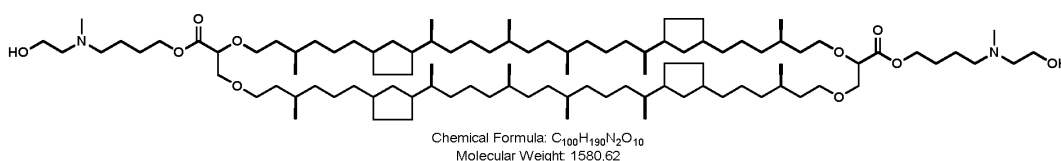
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 65% of bis-(4-(dimethylamino)butyl)-GDGT ester as a colorless oil.

bis-(diethylamino)butyl)-GDGT ester



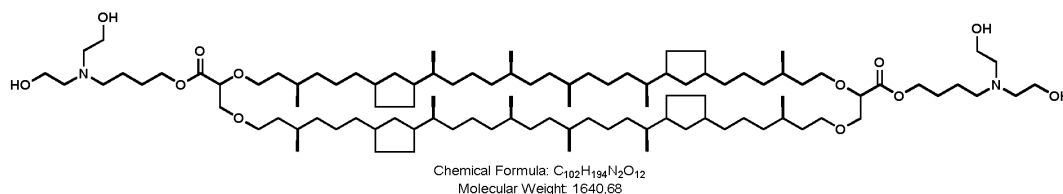
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 70% of bis-(4-(diethylamino)butyl)-GDGT ester as a colorless oil.

bis-(4-(2-hydroxyethyl)(methyl)aminobutyl)-GDGT ester



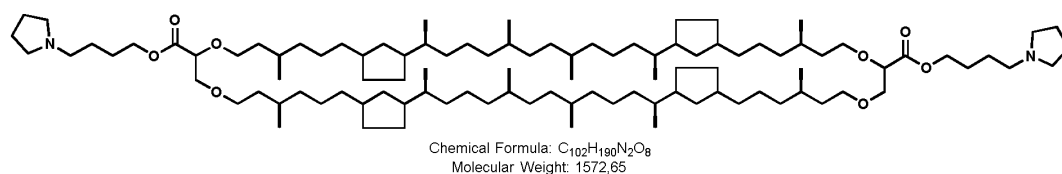
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 68% of bis-(4-(2-hydroxyethyl)(methyl)aminobutyl)-GDGT ester as a colorless oil.

bis 4-(bis-(2-hydroxyethyl)aminobutyl)-GDGT ester



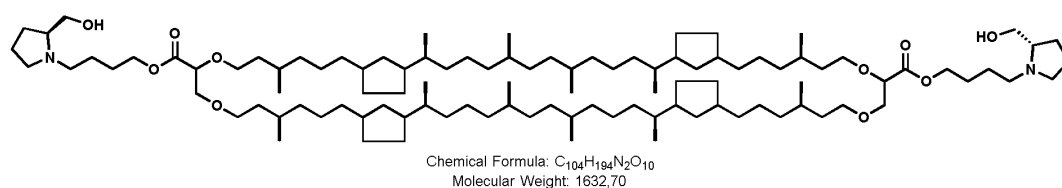
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 58% bis-4-(bis-(2-hydroxyethyl)aminobutyl)-GDGT ester as a colorless oil.

bis-(4-pyrrolidin-1-yl)butyl)-GDGT ester



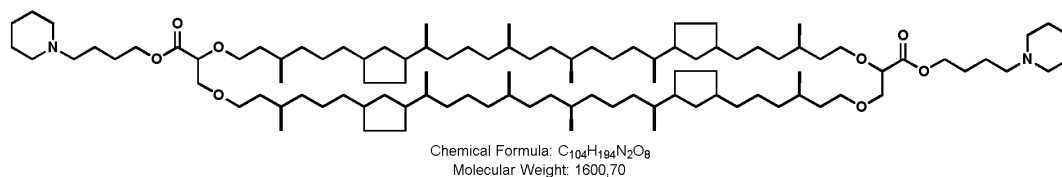
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 56% of bis-pyrrolidin-1-yl)butyl)-GDGT ester as a colorless oil.

4-(2(*S*)-(hydroxymethyl)pyrrolidin-1-yl)butyl)-GDGT ester



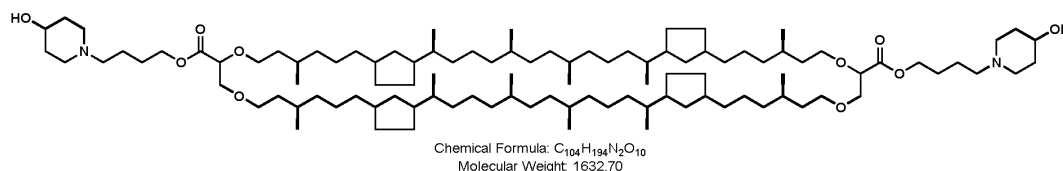
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% of bis-(4-(2(*S*)-(hydroxymethyl)pyrrolidin-1-yl)butyl)-GDGT ester as a colorless oil.

bis-(4-piperidin-1-yl) butyl)-GDGT ester



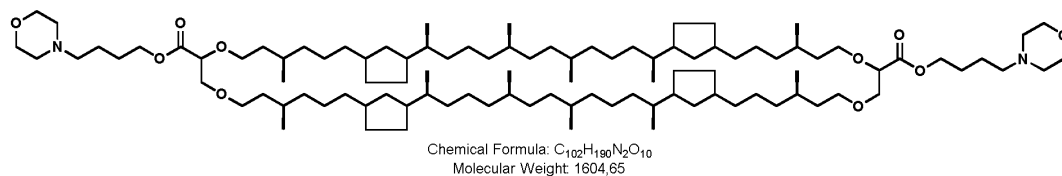
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 65% of bis-(4-piperidin-1-yl) butyl)-GDGT ester as a colorless oil.

bis-(4-(4-hydroxypiperidin-1-yl) butyl)-GDGT ester



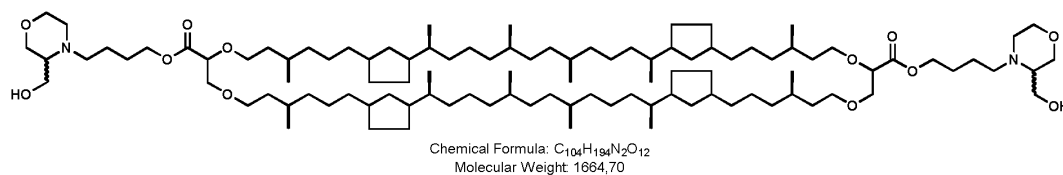
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 64% of bis-(4-(4-hydroxypiperidin-1-yl) butyl)-GDGT ester as a colorless oil.

bis-(4-morpholino) butyl)-GDGT ester



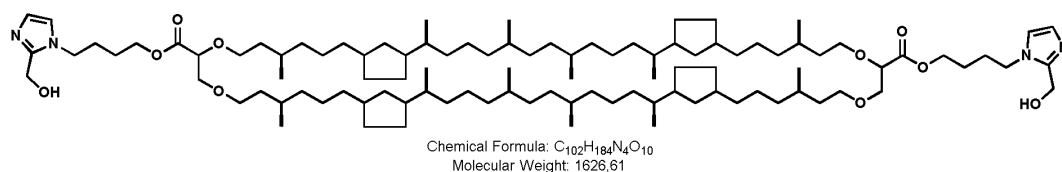
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 66% of bis-(4-morpholino) butyl)-GDGT ester as a colorless oil.

bis-(4-(3-(hydroxymethyl)morpholino) butyl)-GDGT ester



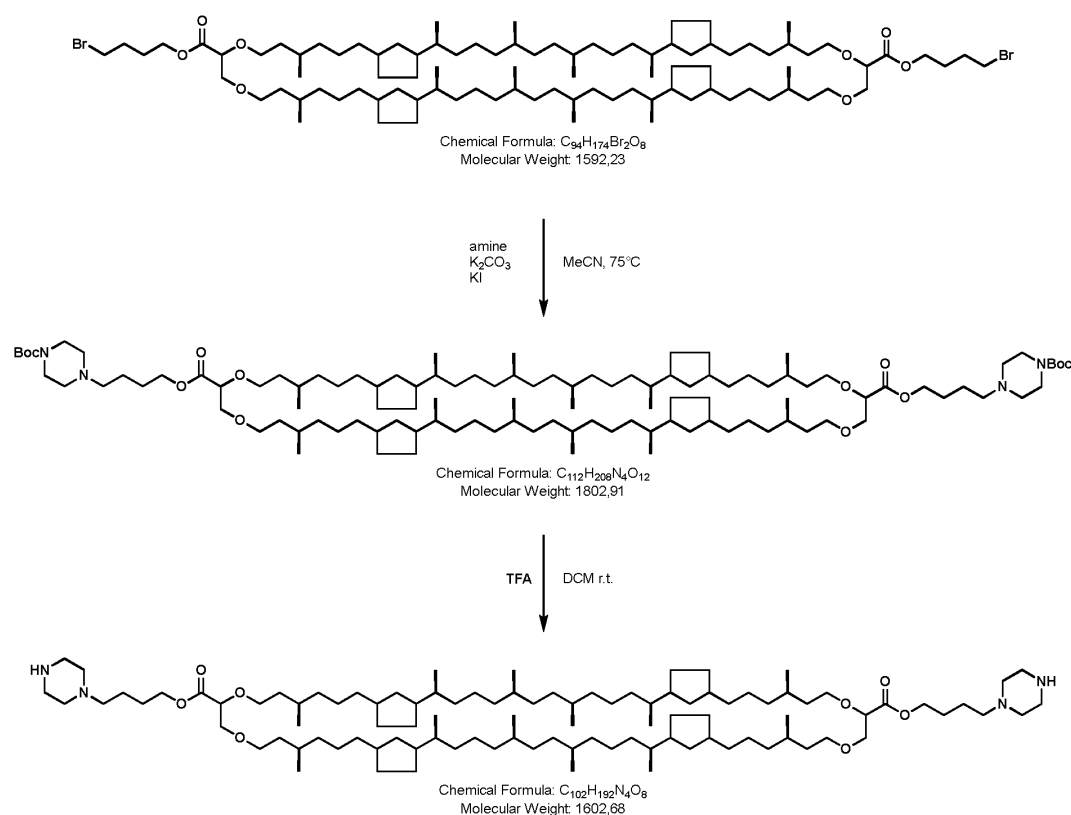
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 48% as a mixture of diastereomers of bis-(4-(3-(hydroxymethyl)morpholino) butyl)-GDGT ester as a colorless oil.

bis-(4-(2-(hydroxymethyl)-1H-imidazolyl) butyl)-GDGT ester



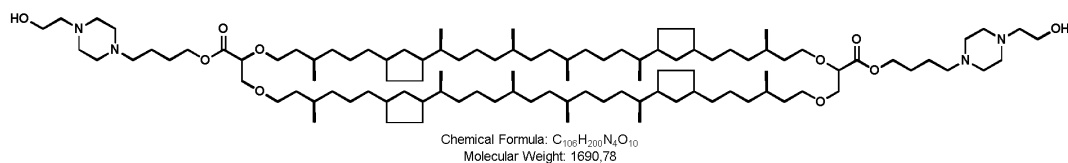
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 63% of bis-(4-(2-(hydroxymethyl)-1H-imidazolyl) butyl)-GDGT ester as a colorless oil.

bis-4-(piperazin-1-yl) butyl-GDGT ester



To bis-(4-bromobutyl)-GDGT ester (1 equiv.), K_2CO_3 (3 equiv.), KI (0.4 equiv.) and 1-Boc-piperazine is added in an 8 mL vial, equipped with a septum and a stirring bar. The vial is evacuated and backfilled with N_2 (three times) before HPLC-grade MeCN. The turbid mixture is heated to 75°C for 18 hours (full consumption of the starting material, checked via TLC), cooled to room temperature and the MeCN was evaporated. The solids are taken up in $NaHCO_3$ and extracted with DCM (three times), once washed with brine, filtered and evaporated. The crude material is dissolved in 2 ml dry DCM and 1 ml TFA was added dropwise. The mixture is stirred at room temperature for 2 hours, cooled to 0°C and neutralized with sat. aqueous $NaHCO_3$ solution. The mixture is 3 times extracted with DCM, dried with $MgSO_4$ and the solvents evaporated. Purification via flash chromatography (Biotage Sfär Amino column, LP:EE) yields 48 % of bis-4-(piperazin-1-yl)butyl-GDGT ester as a slightly yellow oil.

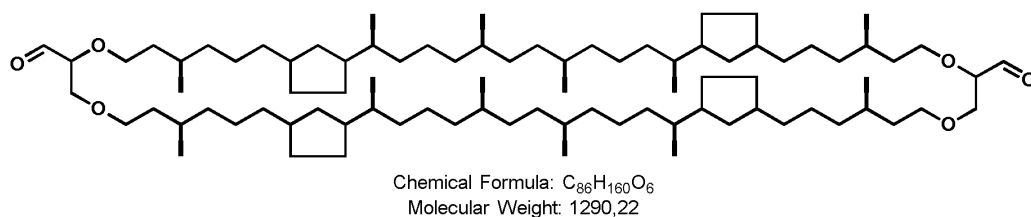
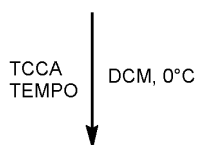
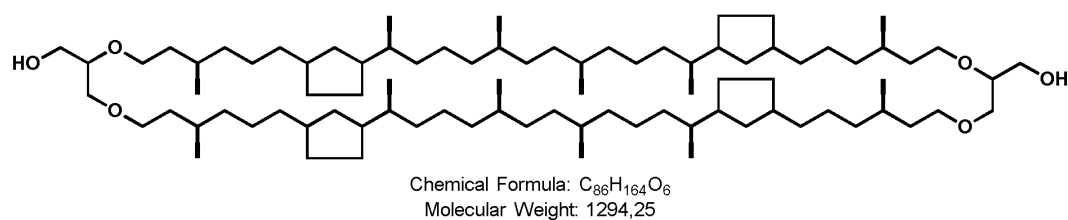
bis-4-(4-(2-hydroxyethyl)piperazin-1-yl)butyl-GDGT ester



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 65% of bis-4-(4-(2-hydroxyethyl)piperazin-1-yl)butyl-GDGT ester as a yellow oil.

Example 12 - Synthesis of further ionizable GDGTs from GDGT aldehyde

Preparation of bis-GDGT-aldehyde

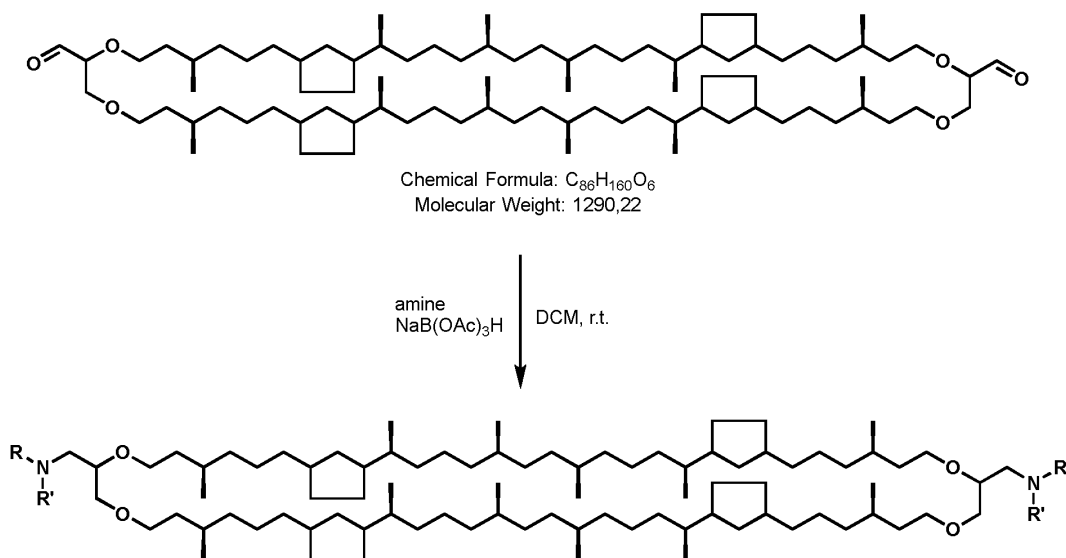


GDGT as preferably obtained according to Example 1 (1 equiv.) and TCCA (2.4 equiv.) are dissolved in dry DCM in an 8 mL vial equipped with a septum and a magnetic stirring bar. The mixture is cooled to 0°C and TEMPO (0.2 eq.) is added and stirred for 10

minutes. Then the yellow solution is poured into LP, filtered via celite, washed with LP and the solvents are evaporated to yield 99% of crude bis-GDGT-aldehyde which is used without further purification.

General procedure for the preparation of bis-GDGT amines

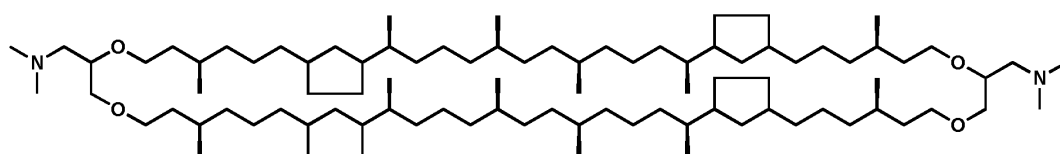
Bis-GDGT-aldehyde (1 equiv.) is dissolved in dry DCM and the appropriate amine (4 equiv.) is added and the mixture is stirred for 10 minutes at room temperature. Then sodium triacetoxyhydroborate (6 equiv.) is added in one portion and the heterogenous mixture was stirred for 2 hours, before being carefully quenched with 2 N NaOH. After extraction with ether (3x), washing with brine, drying over MgSO₄ and filtration the solvents were evaporated. Column Chromatography (Biotage Sfär Amino, LP:EE) yields GDGT amines as colorless to yellow oils.



Crude materials obtained depend on the amine used. The products typically are mixtures of several different substituted GDGT

scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

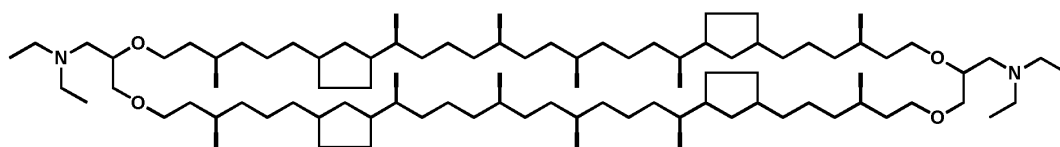
bis-(dimethylamino)-GDGT



Chemical Formula: $C_{90}H_{174}N_2O_4$
Molecular Weight: 1348,39

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 51% of bis-(dimethylamino)-GDGT as a colorless oil.

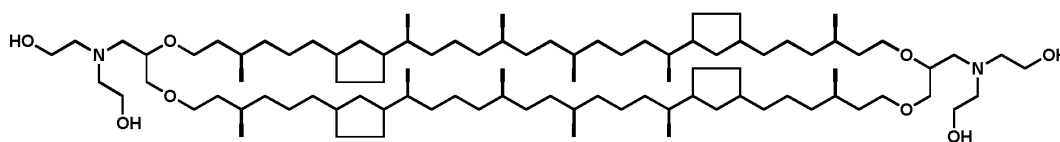
bis-(diethylamino)-GDGT



Chemical Formula: $C_{94}H_{182}N_2O_4$
Molecular Weight: 1404,50

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 62% of bis-(diethylamino)-GDGT as a colorless oil.

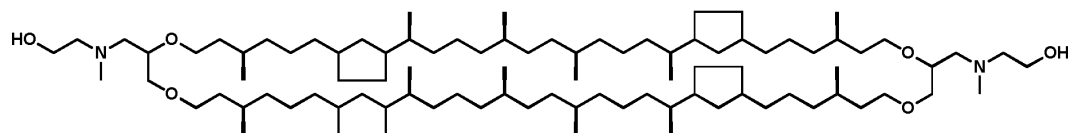
bis-(bis-(hydroxyethyl)amino)-GDGT



Chemical Formula: $C_{94}H_{182}N_2O_8$
Molecular Weight: 1468,50

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 65% of bis-(bis(hydroxyethyl)amino)-GDGT as a colorless oil.

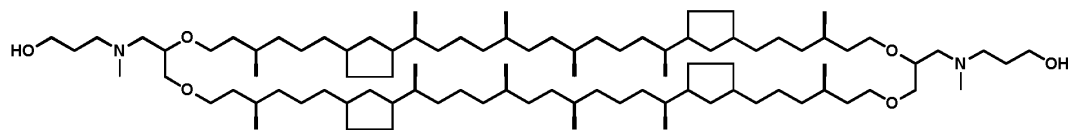
bis-(methyl(hydroxyethyl)amino)-GDGT



Chemical Formula: $C_{92}H_{178}N_2O_6$
Molecular Weight: 1408,44

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 64% of bis-(methyl(hydroxyethyl)amino)-GDGT as a colorless oil.

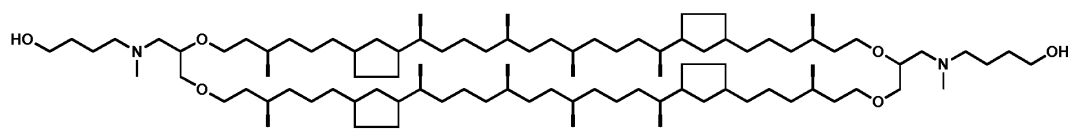
bis-(methyl(hydroxypropyl)amino)-GDGT



Chemical Formula: $C_{94}H_{182}N_2O_6$
Molecular Weight: 1436,50

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 48% of bis-(methyl(hydroxyethyl)amino)-GDGT as a colorless oil.

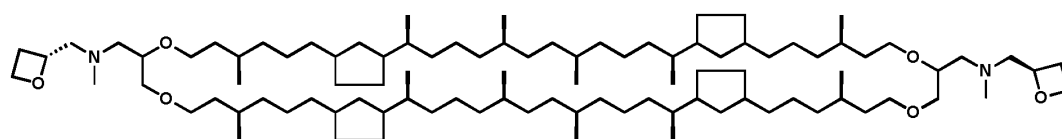
bis-(methyl(hydroxybutyl)amino)-GDGT



Chemical Formula: $C_{96}H_{186}N_2O_6$
Molecular Weight: 1464,55

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 59% of bis-(methyl(hydroxybutyl)amino)-GDGT as a colorless oil.

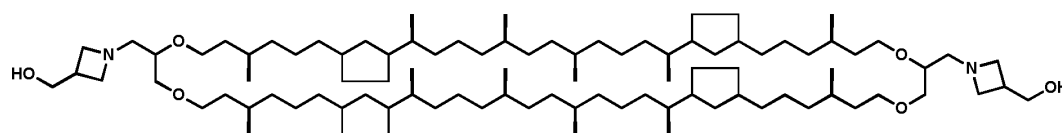
bis-((S)-oxetane-2-methane-N-methyl-amine)-GDGT



Chemical Formula: $C_{96}H_{182}N_2O_6$
Molecular Weight: 1460,52

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% of bis-((S)-oxetane-2-methane-N-methyl-amine)-GDGT as a colorless oil.

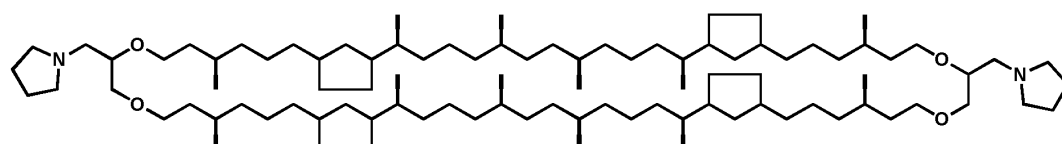
bis-(azetidine-3-methanoyl)-GDGT



Chemical Formula: $C_{94}H_{178}N_2O_6$
Exact Mass: 1431,37

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 48% of bis-(azetidine-3-methanoyl)-GDGT as a colorless oil.

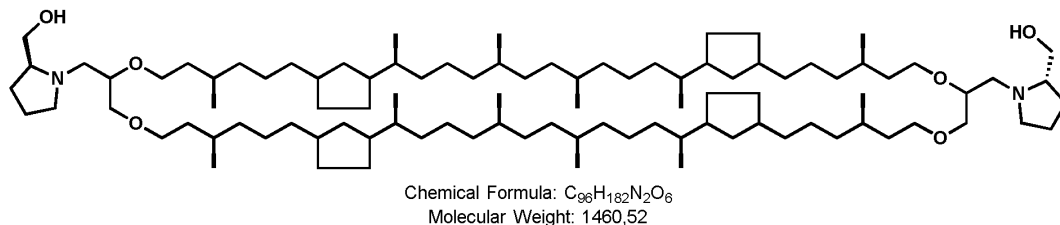
bis-(pyrrolidine)-GDGT



Chemical Formula: $C_{94}H_{178}N_2O_4$
Exact Mass: 1399,38

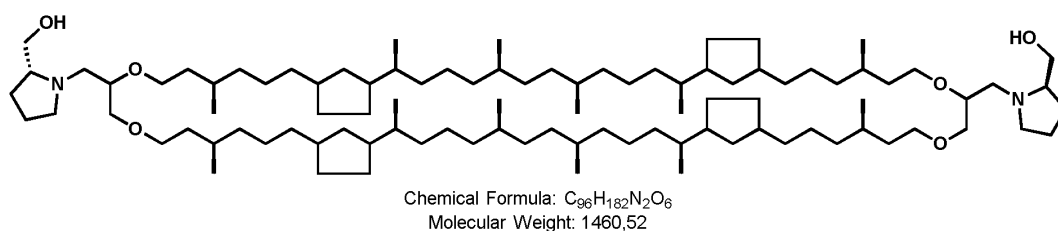
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 57% of bis-(pyrrolidine)-GDGT as a colorless oil.

bis-(*L*-prolinol)-GDGT



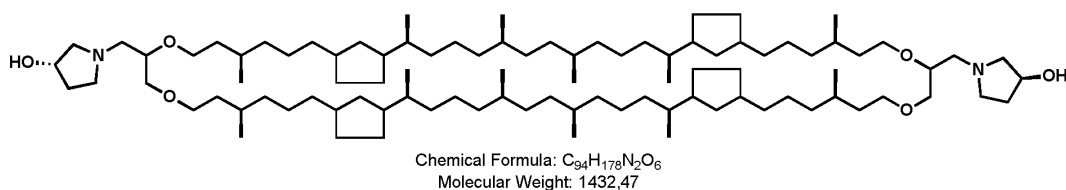
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 55% of bis-(*L*-prolinol)-GDGT as a colorless oil.

bis-(*D*-prolinol)-GDGT



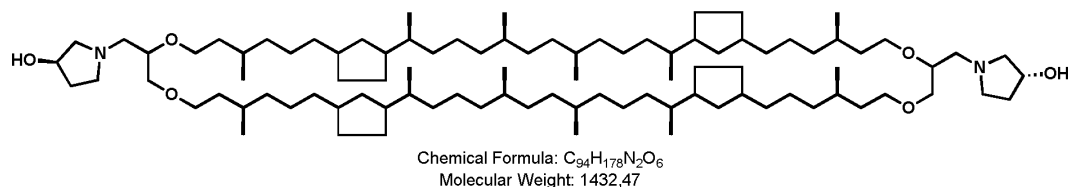
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 55% of bis-(*D*-prolinol)-GDGT as a colorless oil.

bis-(*S*-pyrrolidine-3-ol)-GDGT



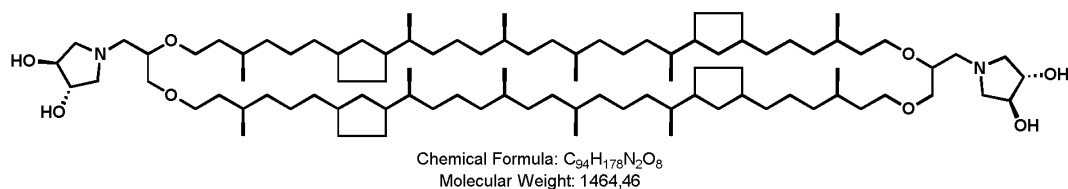
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 60% of bis-(*S*-pyrrolidine-3-ol)-GDGT as a colorless oil.

bis-(*R*-pyrrolidine-3-ol)-GDGT



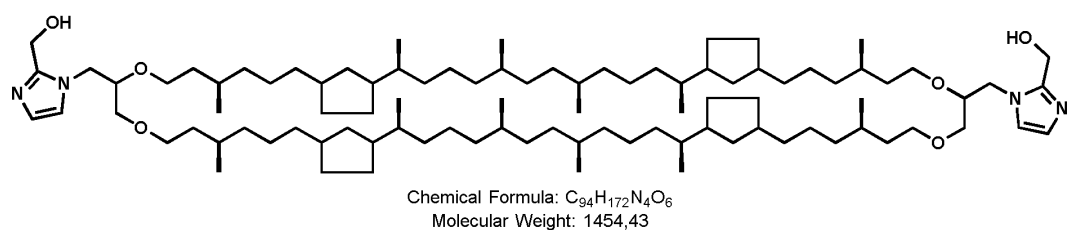
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 62% of bis-(*R*-pyrrolidine-3-ol)-GDGT as a colorless oil.

Bis-((3*S*, 4*S*)-pyrrolidine-3,4-diol)-GDGT



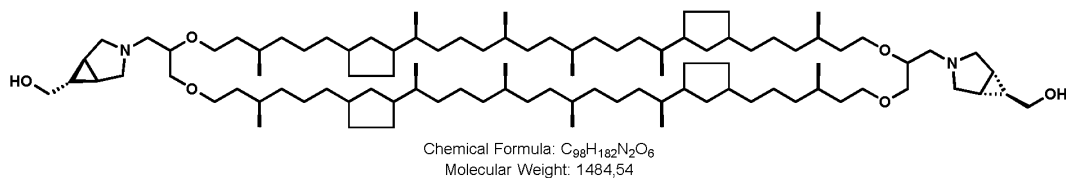
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 49% of bis-((3*S*, 4*S*)-pyrrolidine-3,4-diol)-GDGT as a colorless oil.

Bis-(2-(hydroxymethyl)imidazol)-GDGT



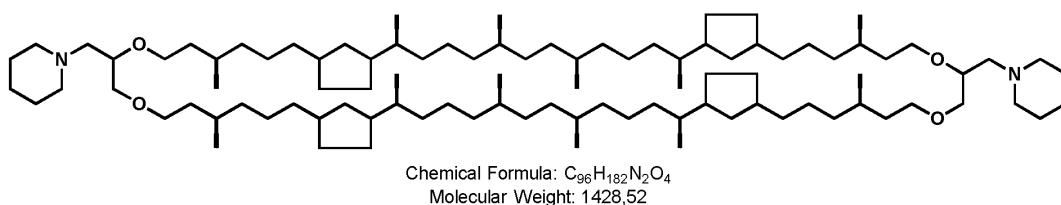
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 60% of bis-(2-(hydroxymethyl)imidazol)-GDGT as a colorless oil.

Bis-((1R,5S,6r)-3-azabicyclo[3.1.0]hexan-6-yl)methanol)-GDGT



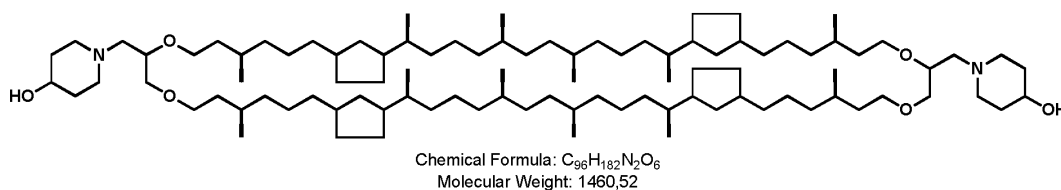
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% of bis-((1R,5S,6r)-3-azabicyclo[3.1.0]hexan-6-yl)methanol)-GDGT as a colorless oil.

Bis-(piperidine)-GDGT



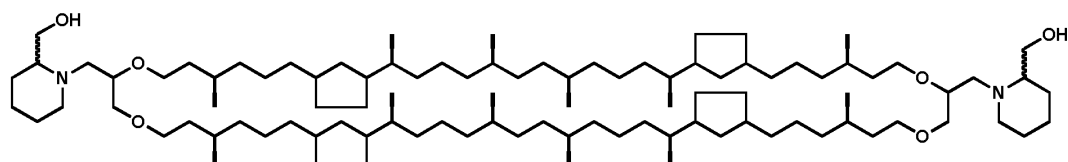
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 70% of bis-(piperidine)-GDGT as a colorless oil.

Bis-(4-hydroxypiperidine)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 70% of bis-(4-hydroxypiperidine)-GDGT as a colorless oil.

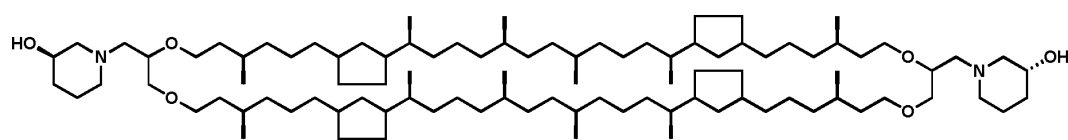
Bis-(2-hydroxymethylpiperidine)-GDGT



Chemical Formula: $C_{98}H_{186}N_2O_6$
Molecular Weight: 1488,57

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 49% as a diastereomeric mixture of bis-(2-hydroxymethylpiperidine)-GDGT as a colorless oil.

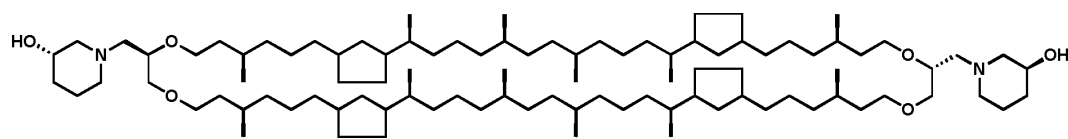
Bis-((*R*)-piperidine-3-ol)-GDGT



Chemical Formula: $C_{98}H_{182}N_2O_6$
Exact Mass: 1459,40

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 58% of bis-((*R*)-piperidine-3-ol)-GDGT as a colorless oil.

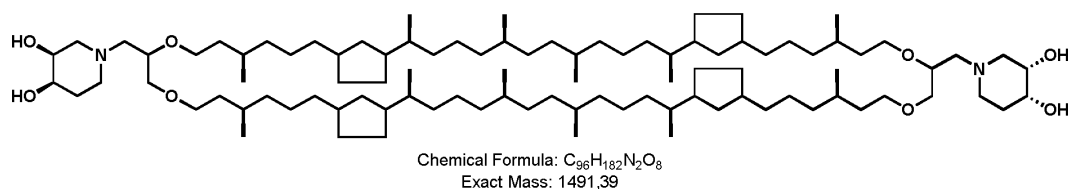
Bis-((*S*)-piperidine-3-ol)-GDGT



Chemical Formula: $C_{98}H_{182}N_2O_6$
Exact Mass: 1459,40

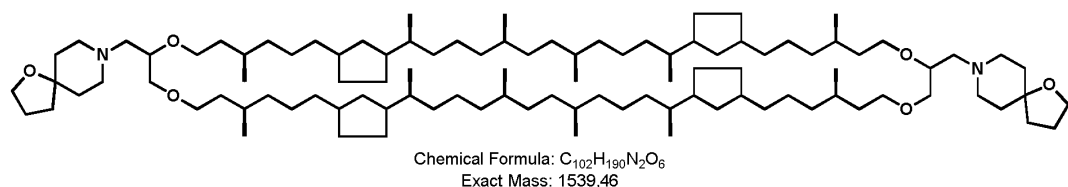
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% as of bis-((*S*)-piperidine-3-ol)-GDGT as a colorless oil.

Bis-((3R,4S)-piperidine-3,4-diol)-GDGT



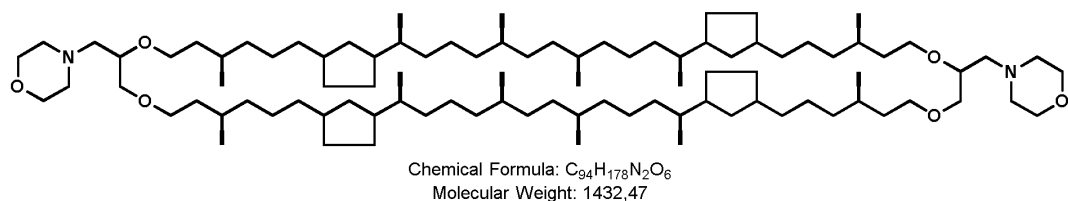
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% of bis-((3R,4S)-piperidine-3,4-diol)-GDGT as a colorless oil.

Bis-(2-oxa-8-azaspiro[4.5]decane)-GDGT



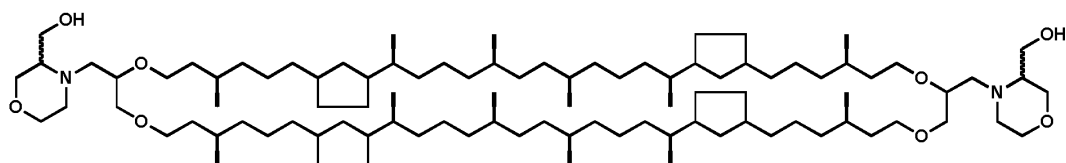
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of bis-(2-oxa-8-azaspiro[4.5]decane)-GDGT as a colorless oil.

Bis-(morpholine)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 70% of bis-(morpholine)-GDGT as a colorless oil.

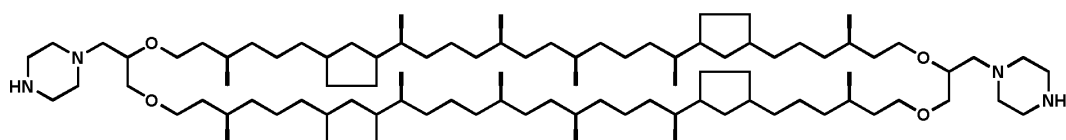
Bis-(morpholine-3-yl-methanol)-GDGT



Chemical Formula: $C_{96}H_{182}N_2O_8$
Molecular Weight: 1492,52

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 52% as a diastereomeric mixture of bis-(morpholine-3-yl-methanol)-GDGT as a colorless oil.

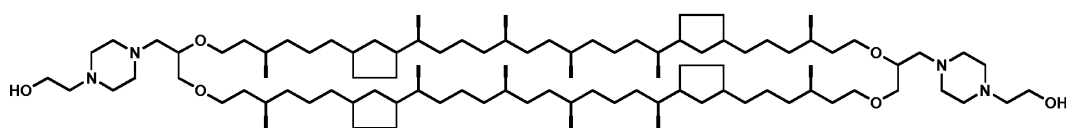
Bis-(piperazine)-GDGT



Chemical Formula: $C_{94}H_{180}N_4O_4$
Molecular Weight: 1430,50

Bis-(piperazine)-GDGT is obtained via reductive amination with N-Boc-piperidine and subsequent Boc-cleavage with TFA. The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 50% of bis-(piperazine)-GDGT as a colorless oil.

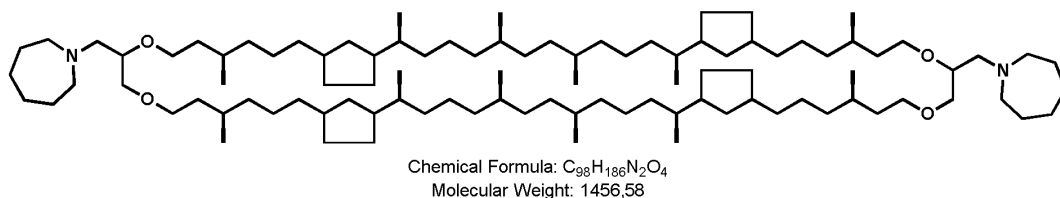
Bis-(4-(hydroxyethyl)piperazine)-GDGT



Chemical Formula: $C_{98}H_{188}N_4O_6$
Molecular Weight: 1518,60

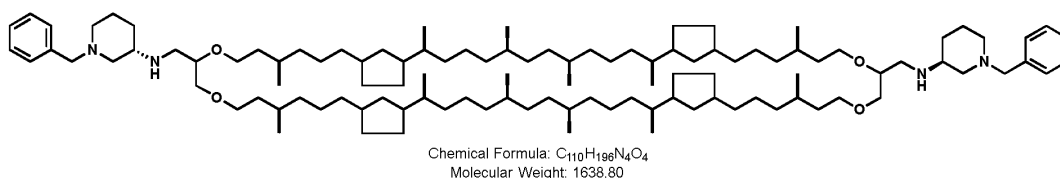
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 50% of bis-(4-(hydroxyethyl)piperazine)-GDGT as a colorless oil.

Bis-(azocane)-GDGT



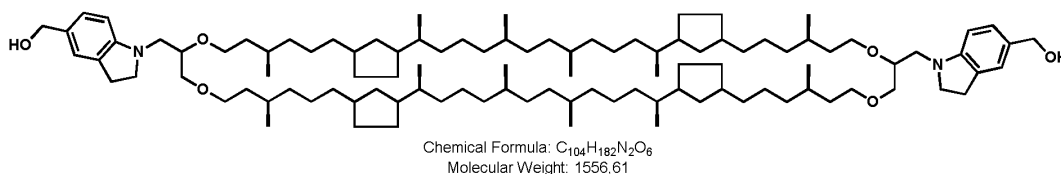
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of bis-(azocane)-GDGT as a colorless oil.

Bis-((R)-3-amino-1-benzylpiperidine)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 58% of bis-((R)-3-amino-1-benzylpiperidine)-GDGT as a colorless oil.

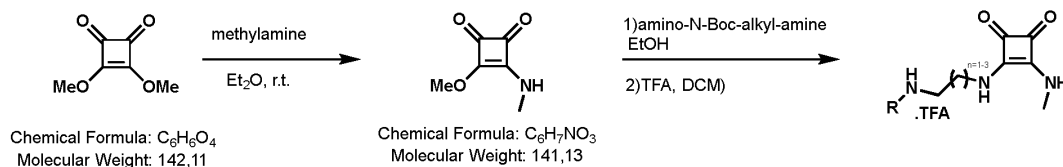
Bis-(indolin-5-yl-methanol)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of bis-(indolin-5-yl-methanol)-GDGT as a colorless oil.

Example 13 - Synthesis of ionizable squareamide amino GDGTs from GDGT aldehyde

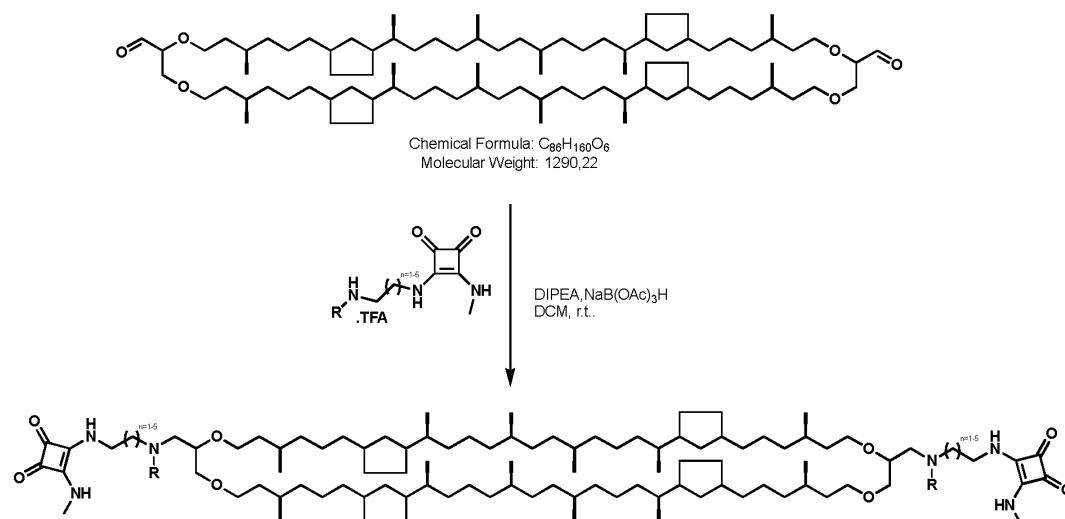
Preparation of 3-methoxy-4-(methylamino)cyclobut-3-ene-1,2-dione



3,4-dimethoxycyclobut-3-ene-1,2-dione (1 equiv.) is dissolved in Et_2O before methylamine (2M in THF, 1.1 eq.) is added dropwise. The mixture is stirred for 24h whereupon a white precipitate formed. The solid is removed via filtration and washed with ether. Pure 3-methoxy-4-(methylamino)cyclobut-3-ene-1,2-dione is obtained via recrystallisation in ethyl acetate in 70% yield.

Pure 3-methoxy-4-(methylamino)cyclobut-3-ene-1,2-dione (1 equiv.) is dissolved in EtOH and amino-NBoc-alkylamine is added (1.2 equiv.) and stirred for 22 hours. Column Chromatography (Biotage Sfär Amino, LP:EE) yields pure N-Boc-amino-squareamides as white solids. Boc deprotection is performed by treating N-Boc-amino-squareamides dissolved in DCM with TFA(15 equiv.) for 2 hours. Evaporation of the solvent yields amino-squareamide TFA-salts, which are used without further purification.

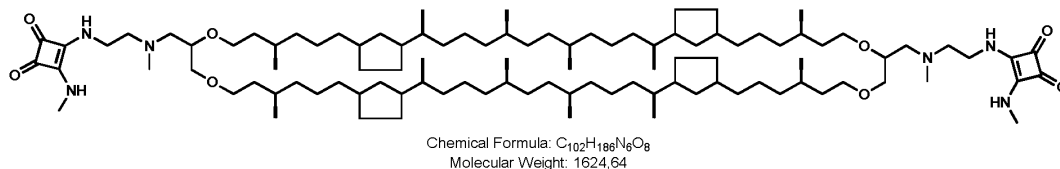
General procedure for the preparation of bis-(squareamide amino)-GDGT



Bis-GDGT-aldehyde as preferably obtained according to Example 7 or 12 (1 equiv.) and amino-squareamide TFA-salt (2.2 equiv.) were dissolved in dry DCM before N,N-Diisopropylethylamine (4.4 equiv.) is added via syringe and the mixture is stirred for 10 minutes at room temperature. Then sodium triacetoxyhydroborate (6 equiv.) is added in one portion and the heterogenous mixture was stirred for 2 hours, before being carefully quenched with 2 N NaOH. After extraction with ether (3x), washing with brine, drying over $MgSO_4$ and filtration the solvents are evaporated. Column Chromatography (Biotage Sfär Amino, LP:EE) yields bis-(squareamide amino)-GDGT as white solids.

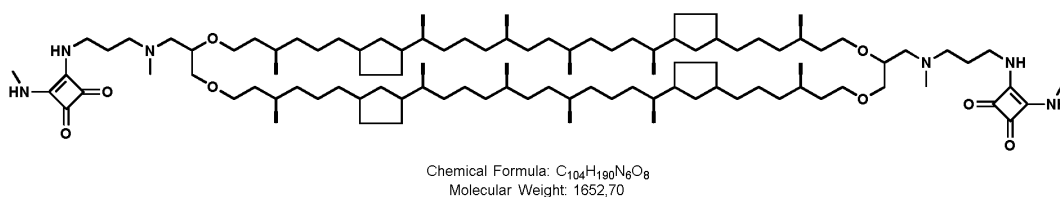
Materials obtained depend on the squareamide amine used. The products typically are mixtures of several different substituted GDGT scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

bis-((3-(methylamino)-4-((3-(methylamino)ethyl) amino) cyclobut-3-ene-1,2-dione)-GDGT



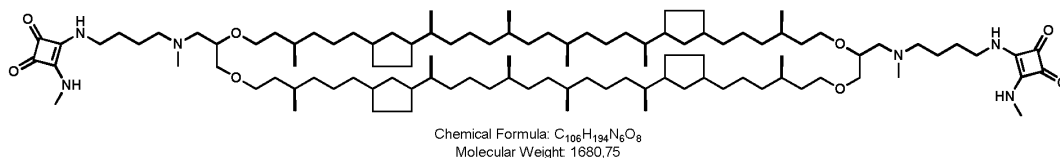
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 40% of bis-((3-(methylamino)-4-((3-(methylamino)ethyl) amino) cyclobut-3-ene-1,2-dione)-GDGT as a white solid.

bis-((3-(methylamino)-4-((3-(methylamino)propyl) amino) cyclobut-3-ene-1,2-dione)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 41% of bis-((3-(methylamino)-4-((3-(methylamino)propyl) amino) cyclobut-3-ene-1,2-dione)-GDGT as a white solid.

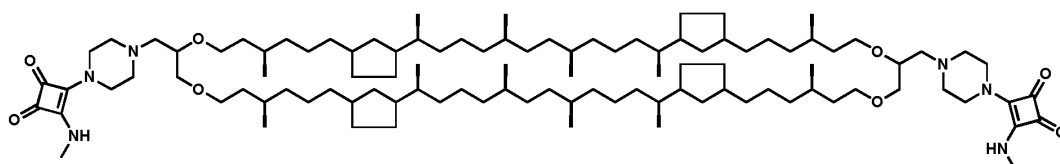
bis-((3-(methylamino)-4-((3-(methylamino)butyl) amino) cyclobut-3-ene-1,2-dione)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 38% of bis-((3-

(methylamino)-4-((3-(methylamino)butyl)amino)cyclobut-3-ene-1,2-dione)-GDGT as a white solid.

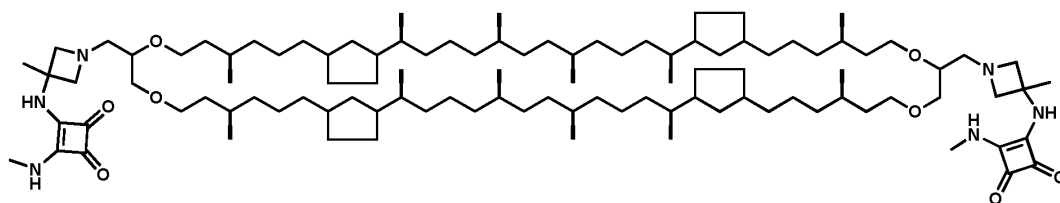
bis-((3-(3-methylazetidin-3-yl)amino)-4-(methylamino)cyclobut-3-ene-1,2-dione))-GDGT



Chemical Formula: $C_{104}H_{186}N_6O_8$
Molecular Weight: 1648,67

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 45% of bis-((3-(3-methylazetidin-3-yl)amino)-4-(methylamino)cyclobut-3-ene-1,2-dione))-GDGT as a white solid.

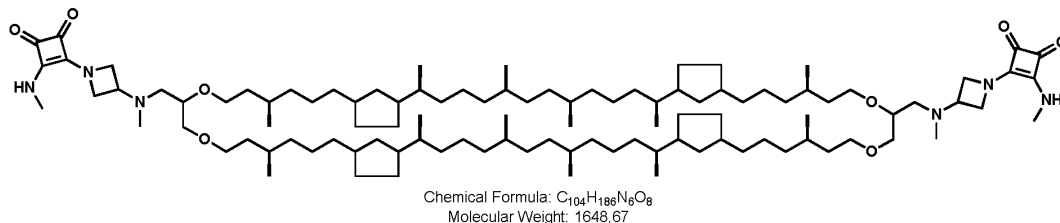
bis-((3-(3-methylazetidin-3-yl)amino)-4-(methylamino)cyclobut-3-ene-1,2-dione))-GDGT



Chemical Formula: $C_{104}H_{186}N_6O_8$
Molecular Weight: 1648,67

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 32% of bis-((3-(3-methylazetidin-3-yl)amino)-4-(methylamino)cyclobut-3-ene-1,2-dione))-GDGT as a white solid.

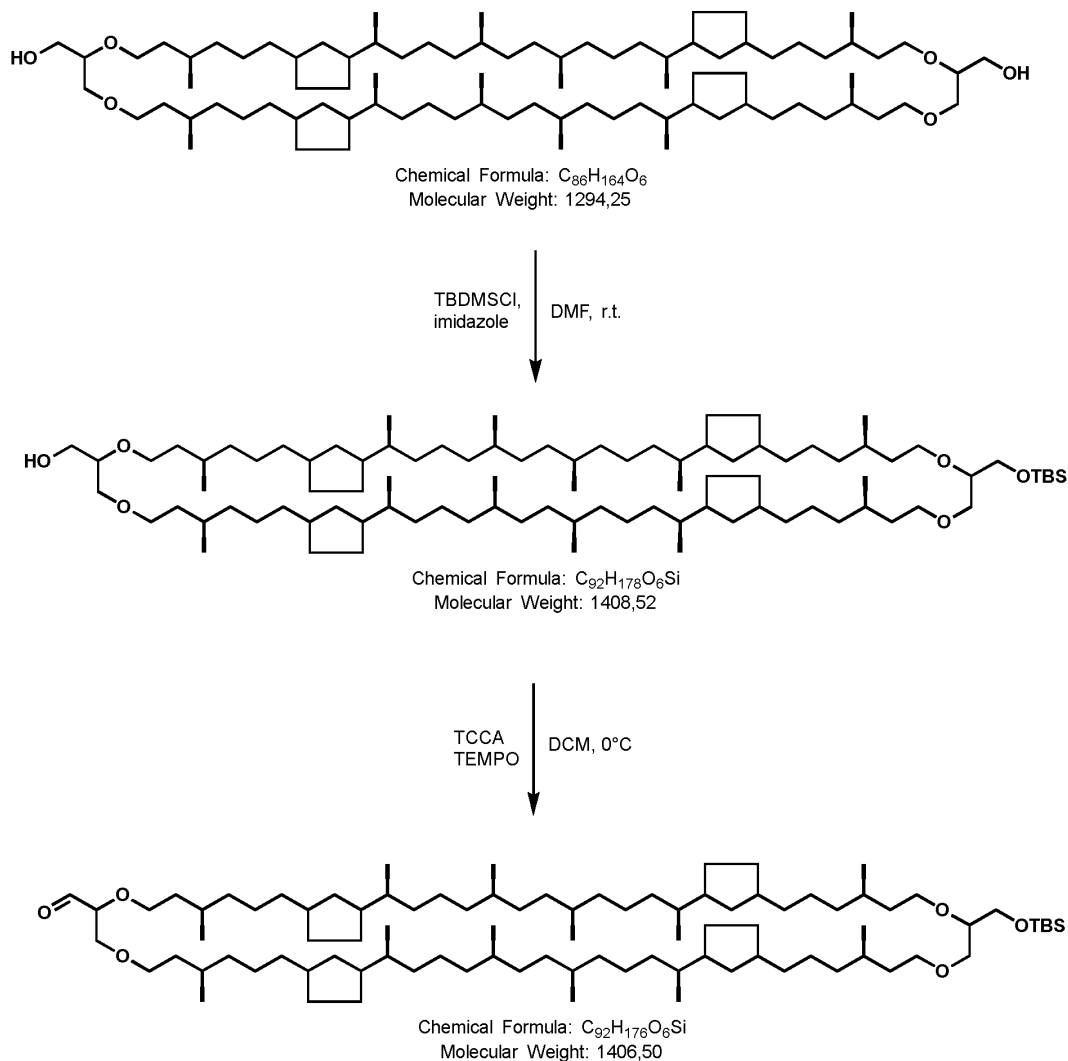
bis-((3-(3-(methyl) amino) azetidin-1-yl)-4-(methylamino) cyclobut-3-ene-1,2-dione)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 28% of bis-((3-(3-methylazetidin-3-yl) amino)-4-(methylamino) cyclobut-3-ene-1,2-dione))-GDGT as a white solid.

Example 14 - Synthesis of ionizable monosubstituted GDGTs

Preparation of mono-TBSO-GDGT-aldehyde

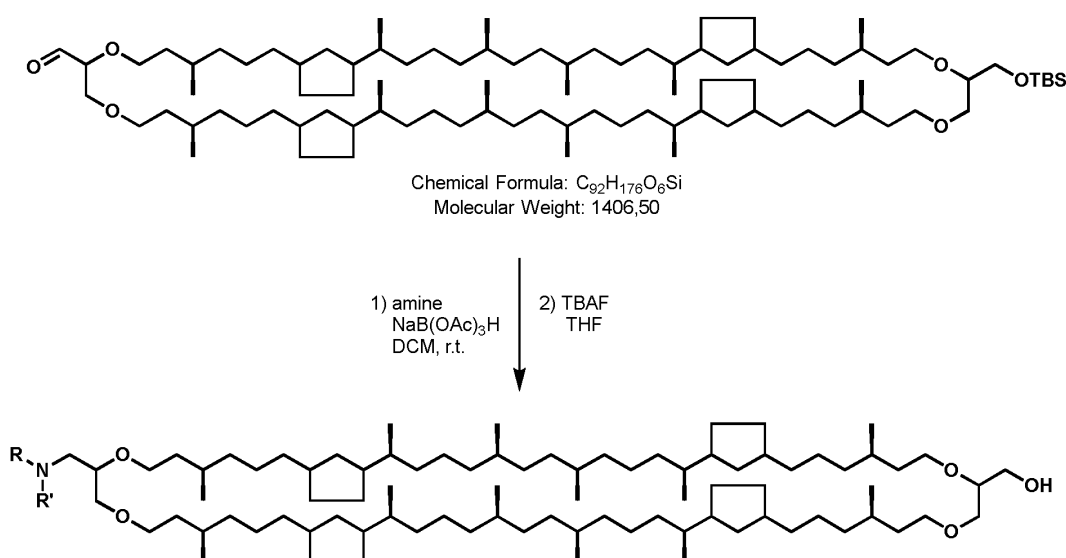


GDGT as preferably obtained according to Example 1 (1 equiv.) and imidazole (2.2 equiv.) is dissolved in DMF dry and *tert*-Butyldimethylsilyl chloride (50%w/w in toluene, 1.2 equiv.) is added dropwise. The suspension is stirred for 20 h, taken up in diethyl ether and washed with 1 M aqueous LiCl solution (3x), dried over MgSO₄, filtered and evaporated. The crude material is purified via column chromatography (SiO₂, LP:EE) to yield 42% of mono-TBSO-GDGT as colorless oil.

Mono-TBSO-GDGT (1 equiv.) and TCCA (1.2 equiv.) are dissolved in dry DCM in an 8 mL vial equipped with a septum and a magnetic stirring bar. The mixture is cooled to 0°C and TEMPO (0.1 eq.) is added and stirred for 10 minutes. Then the yellow solution is poured into LP, filtered via celite, washed with LP and the solvents were evaporated to yield 99% of crude mono-TBSO-GDGT-aldehyde which was used without further purification.

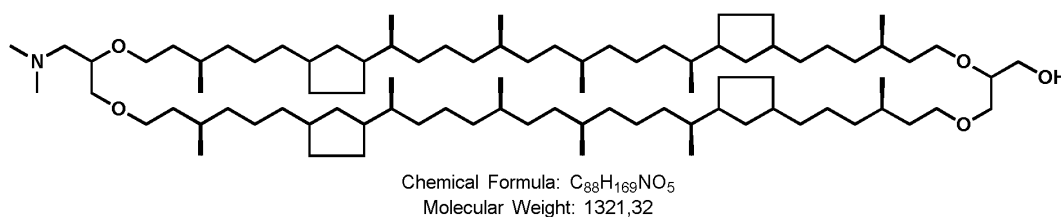
General procedure for the preparation of mono-GDGT amines

Mono-TBSO-GDGT-aldehyde (1 equiv.) is dissolved in dry DCM and the appropriate amine (2 equiv.) is added and the mixture was stirred for 10 minutes at room temperature. Then STAB (3 equiv.) is added in one portion and the heterogenous mixture is stirred for 2 hours, before being carefully quenched with 2 N NaOH. After extraction with ether (3x), washing with brine, drying over MgSO₄ and filtration the solvents are evaporated. The crude material was dissolved in dry THF and Tetra-*n*-butylammonium fluoride (1 M in THF, 1.5 equiv.) is added and stirred for 20 hours. The mixture is quenched via the addition of 1 M aqueous CaCO₃ solution and extracted with ether (3x), washed with brine and dried over MgSO₄. After filtration of the dessicant the solvents are removed under vacuum. Column Chromatography (Biotage Sfär Amino, LP:EE) yields pure mono-GDGT amines as colorless to yellow oils.



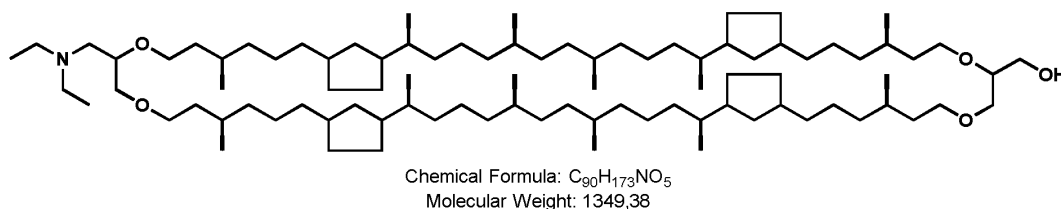
Crude materials obtained depend on the amine used. The products typically are mixtures of several different substituted GDGT scaffolds. Representative structures (with a GDGT-4 scaffold) are shown in the following.

mono-(dimethylamino)-GDGT



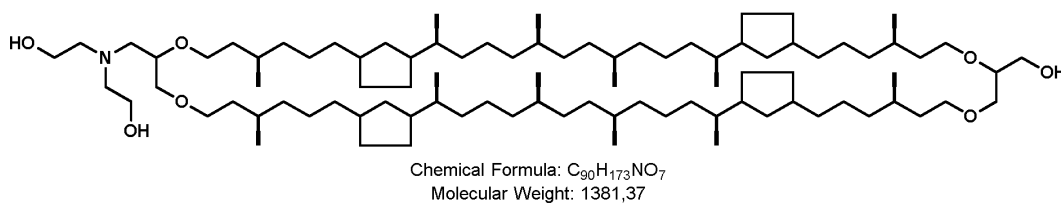
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% of mono-(dimethylamino)-GDGT as a colorless oil.

mono-(diethylamino)-GDGT



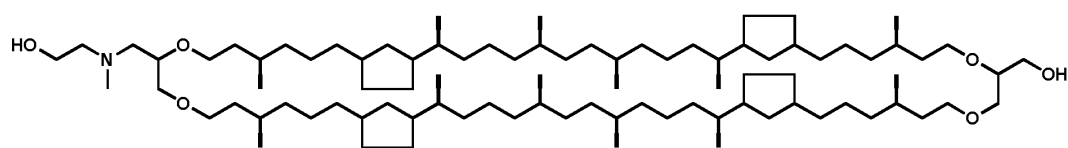
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 55% of mono-(diethylamino)-GDGT as a colorless oil.

mono-(bis-(hydroxyethyl) amino)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 54% of mono-(bis(hydroxyethyl)amino)-GDGT as a colorless oil.

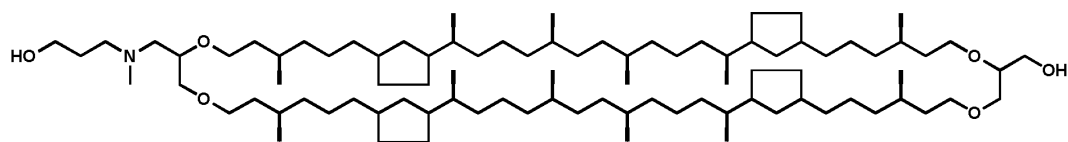
mono-(methyl(hydroxyethyl)amino)-GDGT



Chemical Formula: $C_{89}H_{171}NO_6$
Molecular Weight: 1351,35

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 60% of mono-(methyl(hydroxyethyl)amino)-GDGT as a colorless oil.

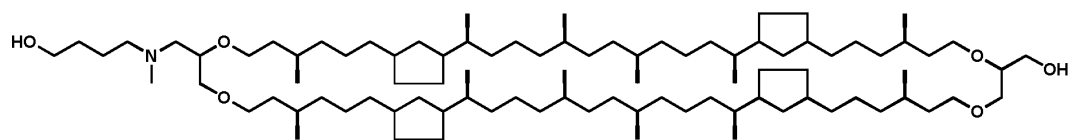
mono-(methyl(hydroxypropyl)amino)-GDGT



Chemical Formula: $C_{90}H_{173}NO_6$
Molecular Weight: 1365,38

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% of mono-(methyl(hydroxyethyl)amino)-GDGT as a colorless oil.

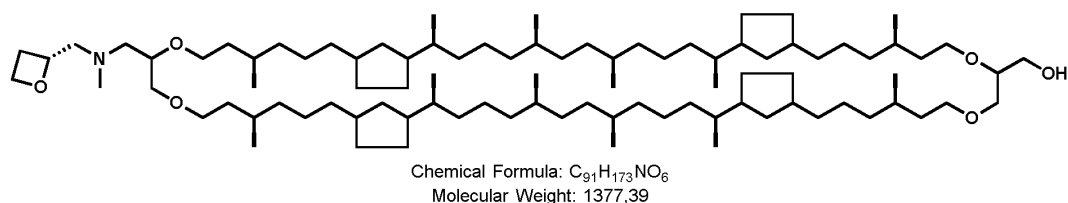
mono-(methyl(hydroxybutyl)amino)-GDGT



Chemical Formula: $C_{91}H_{175}NO_6$
Molecular Weight: 1379,40

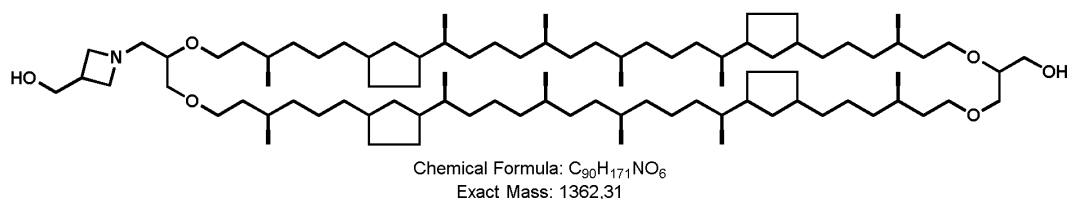
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 64% of mono-(methyl(hydroxybutyl)amino)-GDGT as a colorless oil.

Mono-((S)-oxetane-2-methane-N-methyl-amine)-GDGT



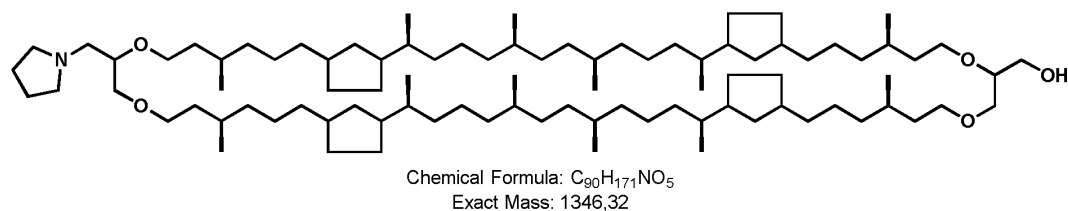
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 41% of mono-((S)-oxetane-2methane-N-methyl-amine)-GDGT as a colorless oil.

mono-(azetidine-3-methanoyl)-GDGT



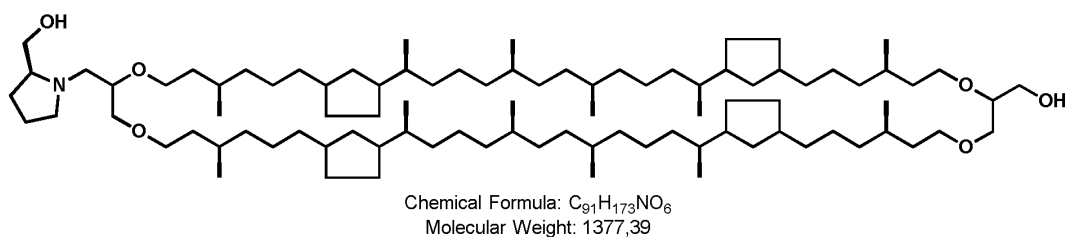
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 46% of mono-(azetidine-3-methanoyl)-GDGT as a colorless oil.

mono-(pyrrolidine)-GDGT



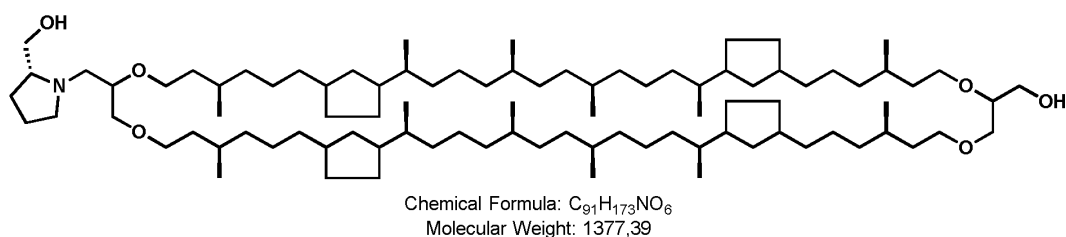
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of mono-(pyrrolidine)-GDGT as a colorless oil.

mono-(*L*-prolinol)-GDGT



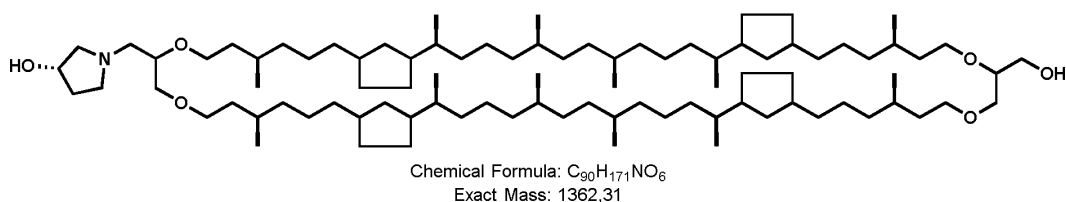
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 52% of mono-(*L*-prolinol)-GDGT as a colorless oil.

mono-(*D*-prolinol)-GDGT



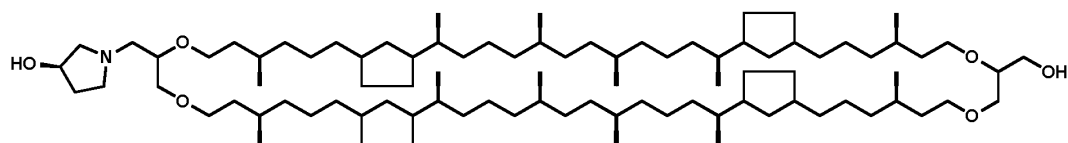
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% of mono-(*D*-prolinol)-GDGT as a colorless oil.

mono-(*S*-pyrrolidine-3-ol)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 49% of mono-(*S*-pyrrolidine-3-ol)-GDGT as a colorless oil.

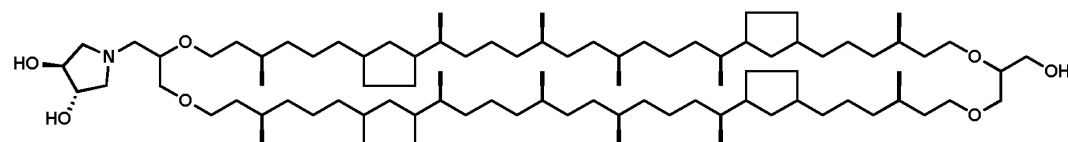
mono-(*R*-pyrrolidine-3-ol)-GDGT



Chemical Formula: $C_{90}H_{171}NO_8$
Exact Mass: 1362,31

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 69% of mono-(*R*-pyrrolidine-3-ol)-GDGT as a colorless oil.

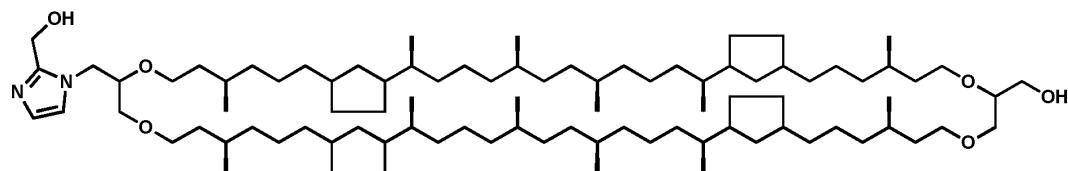
mono-((3*S*,4*S*)-pyrrolidine-3,4-diol)-GDGT



Chemical Formula: $C_{90}H_{171}NO_7$
Exact Mass: 1378,31

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 59% of mono-((3*S*,4*S*)-pyrrolidine-3,4-diol)-GDGT as a colorless oil.

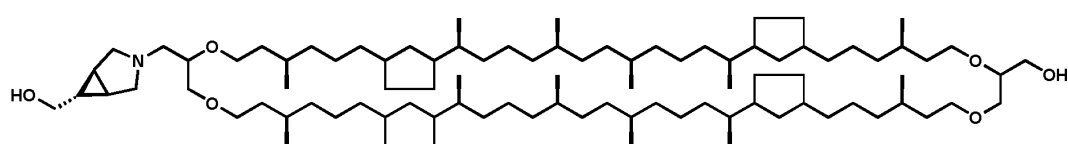
mono-(2-(hydroxymethyl)imidazol)-GDGT



Chemical Formula: $C_{90}H_{168}N_2O_6$
Molecular Weight: 1374,34

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 64% of mono-(2-(hydroxymethyl)imidazol)-GDGT as a colorless oil.

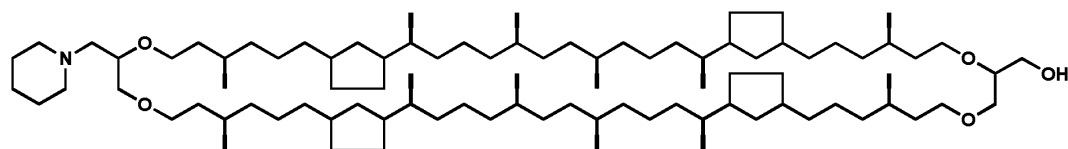
mono-((1R, 5S, 6r)-3-azabicyclo[3.1.0]hexan-6-yl)methanol)-GDGT



Chemical Formula: C₉₂H₁₇₃NO₆
Molecular Weight: 1389,40

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 55% of mono-((1R, 5S, 6r)-3-azabicyclo[3.1.0]hexan-6-yl)methanol)-GDGT as a colorless oil.

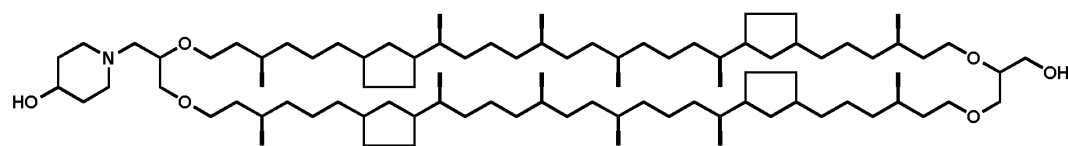
mono-(piperidine)-GDGT



Chemical Formula: C₉₁H₁₇₃NO₅
Molecular Weight: 1361,39

The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 66% of mono-(piperidine)-GDGT as a colorless oil.

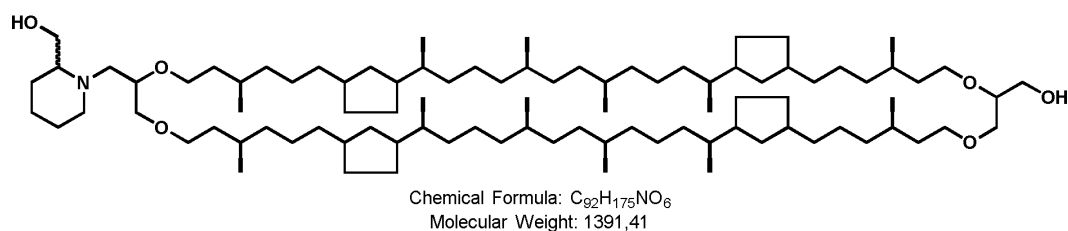
mono-(4-hydroxypiperidine)-GDGT



Chemical Formula: C₉₁H₁₇₃NO₆
Molecular Weight: 1377,39

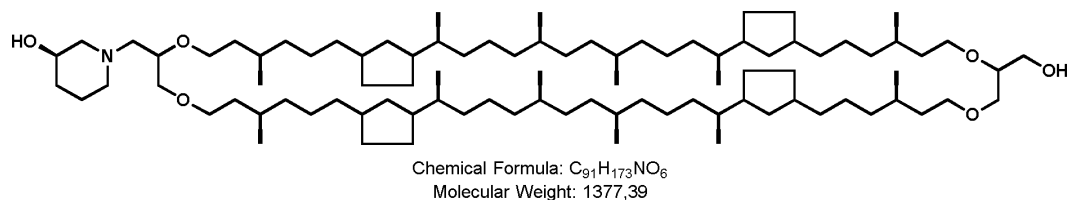
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 61% of mono-(4-hydroxypiperidine)-GDGT as a colorless oil.

mono-(2-hydroxymethylpiperidine)-GDGT



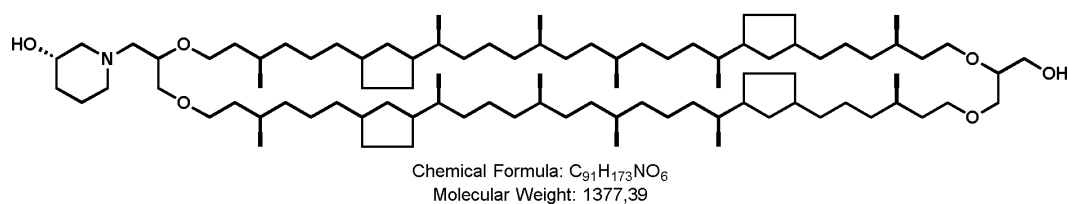
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% as a diastereomeric mixture of mono-(2-hydroxymethylpiperidine)-GDGT as a colorless oil.

mono-((R)-piperidine-3-ol)-GDGT



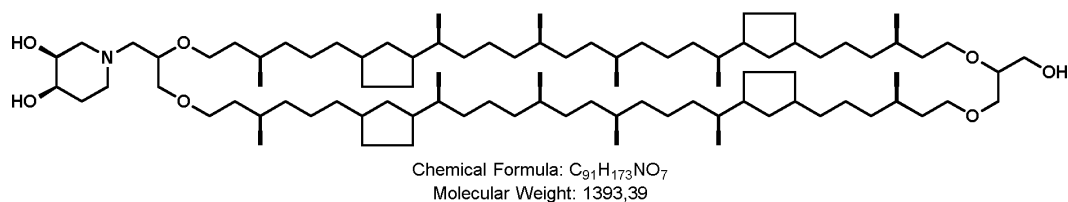
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 48% of mono-((R)-piperidine-3-ol)-GDGT as a colorless oil.

mono-((*S*)-piperidine-3-ol)-GDGT



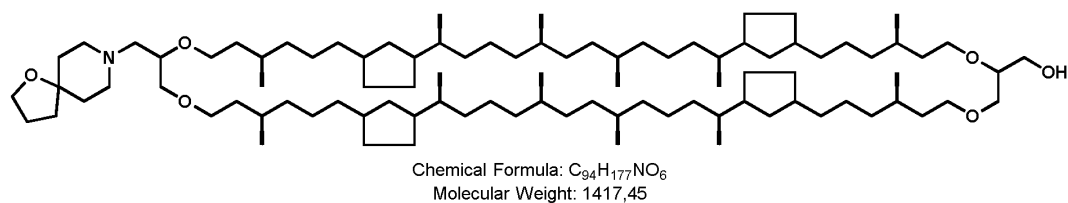
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 54% as of mono-((*S*)-piperidine-3-ol)-GDGT as a colorless oil.

mono-((3*R*,4*S*)-piperidine-3,4-diol)-GDGT



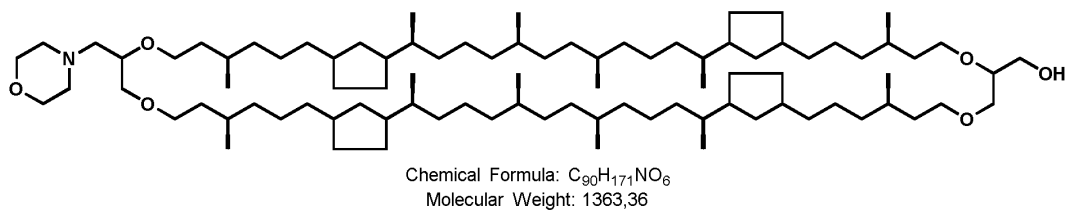
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 51% of mono-((3*R*,4*S*)-piperidine-3,4-diol)-GDGT as a colorless oil.

mono-(2-oxa-8-azaspiro[4.5]decane)-GDGT



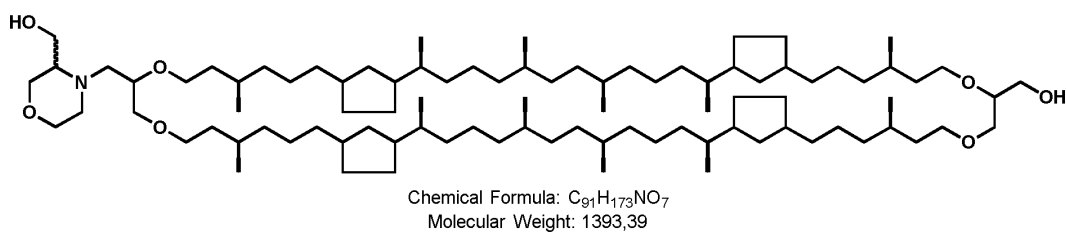
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 66% of mono-(2-oxa-8-azaspiro[4.5]decane)-GDGT as a colorless oil.

mono-(morpholine)-GDGT



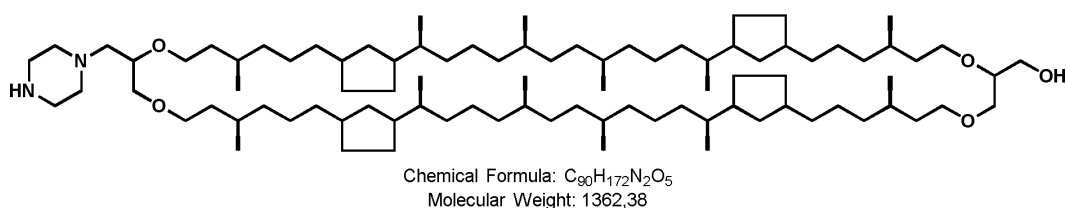
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 59% of mono-(morpholine)-GDGT as a colorless oil.

mono-(morpholine-3-yl-methanol)-GDGT



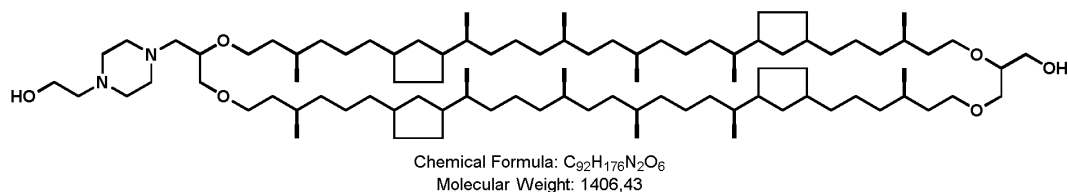
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 43% as a diastereomeric mixture of mono-(morpholine-3-yl-methanol)-GDGT as a colorless oil.

mono-(piperazine)-GDGT



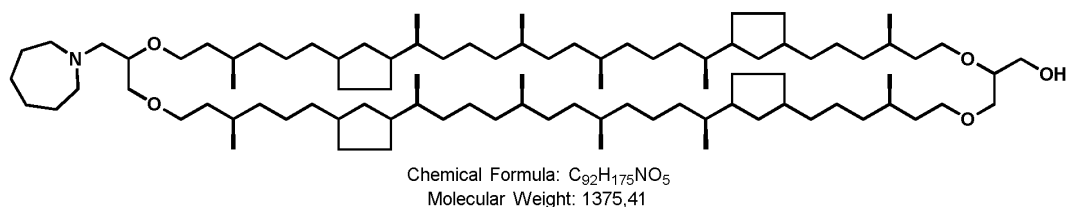
Bis-(piperazine)-GDGT is obtained via reductive amination with N-Boc-piperidine and subsequent Boc-cleavage with TFA. The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 51% of mono-(piperazine)-GDGT as a colorless oil.

mono-(4-(hydroxyethyl)piperazine)-GDGT



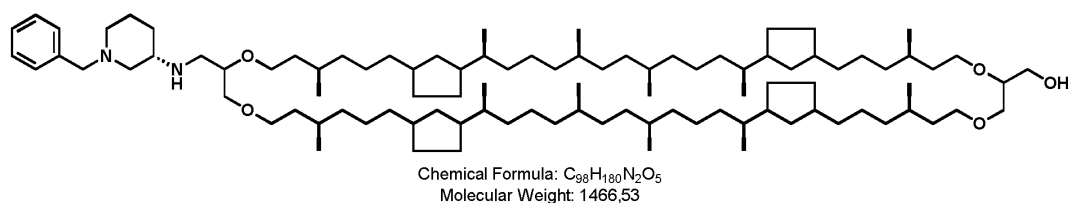
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 50% of mono-(4-(hydroxyethyl)piperazine)-GDGT as a colorless oil.

mono-(azocane)-GDGT



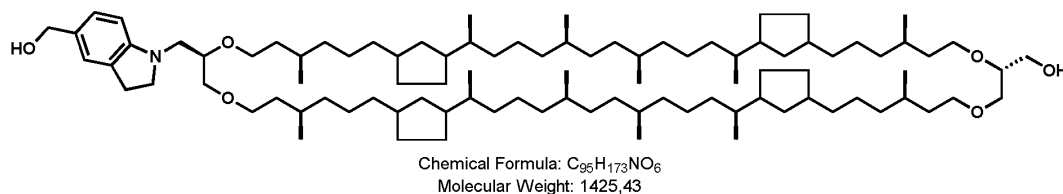
The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 65% of mono-(azocane)-GDGT as a colorless oil.

mono-((R)-3-amino-1-benzylpiperidine)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 71% of mono-((R)-3-amino-1-benzylpiperidine)-GDGT as a colorless oil.

mono-(indolin-5-yl-methanol)-GDGT



The crude material is purified via flash chromatography (Biotage Sfär Amino column, LP:EE) to obtain 38% of bis-(indolin-5-yl-methanol)-GDGT as a colorless oil.

Example 15 - Formulation of LNPs

Lipid stock solutions (archaeal membrane lipids obtained from *Sulfolobus acidocaldarius* including GDGTs (see Table 1 below; see also WO 2020/187526 A1), DSPC, ALC-0159, cholesterol, and ionizable GDGTs as obtained according to any one of examples 3, 4, 5, 6, and 8 (or any one of the ionizable GDGTs of examples 9, 10, 11, 12, 13 and 14) are filtered through a 0.2 µm polytetrafluoroethylene (PTFE) filter and mixed according to the desired molar ratios. A mixture of dimethyl sulfoxide (DMSO) and 2-propanol (2:3) is used as the organic solvent. The aqueous phase is prepared by dissolving the desired mRNA (e.g. the mRNA which codes for an antigen, such as SARS-CoV-2 spike protein) in a 10 mM citrate buffer (pH=4.0). LNPs are formulated using a NanoAssembler Ignite (Precision Nanosystems) at a total flow rate of 12 mL/min and a flow rate ratio of 3:1 (aqueous phase:organic phase). Immediately after formulation, LNPs are diluted 1:2 in a 10 mM PBS (pH=7.4). Removal of organic solvent is achieved via dialysis (SpectraPor, 6-8 kDa) against 10 mM phosphate-buffered saline (PBS) buffer (pH=7.4). Physicochemical characterization of the LNPs may be performed via the Ribogreen assay (mRNA content) and Zetasizer analysis (size, polydispersity index (PDI), Zeta potential). Zetasizer is available from Malvern Panalytical Ltd, UK.

A typical lipid composition for the GDGT-containing archaeal membrane lipid stock solution is given in Table 1:

Lipid component	Relative abundance within the ether lipid fraction (mol%)
archaeols	5-25%
unsubstituted caldarchaeols	15-50%
Hex-caldarchaeols	8-14%
PI-caldarchaeols	3-10%
2Hex-caldarchaeols	10-22%
2Hex-PI-caldarchaeols	3-10%
sulfono-3Hex-PI-caldarchaeols	0-5%

After formulation, LNPs are stored in 2 mL Eppendorf tubes at 25°C (room temperature), respectively. Physiochemical characterization via Ribogreen assay and ZetaSizer analysis may be conducted in certain time intervals over a period of 6 weeks.

The LNPs may be used to formulate mRNA vaccines.

Example 16 - Formulation and characterization of further LNPs

The primary objective was to characterize a range of ionizable GDGTs utilized LNPs. By modifying the lipid ratios of ionizable GDGTs, we several key parameters were evaluated, including size, PDI (polydispersity index) and encapsulation efficiency. Moreover, zeta potential and pKa value determinations were conducted with the objective of acquiring further insights into the physicochemical properties of the ionizable GDGTs. Finally, an *in vivo* study in rats was performed.

Buffers

The buffer for the formulation of LNPs was a 10 mM citric acid buffer at pH 3.0. The buffer for dilution and dialysis was a 10 mM PBS at pH of 7.4

LNP formulations

Conventional lipids for LNPs were dissolved in ethanol to achieve a concentration of between 10 and 20 g/L, while the ionizable GDGTs were dissolved in isopropanol to reach a concentration of 30 g/L. The total lipid concentration was 10-20 mM, with 20 mM being used to determine the pKa and zeta potential value.

Furthermore, an investigation was conducted into the effect of varying the N/P ratio (4 - 6 - 8 - 12). (For LNPs, the N/P ratio corresponds to the ratio between the amine groups (N) of the ionizable lipid and the phosphate group (P) of the nucleic acid cargo (1 per base for RNA, 2 for DNA). The N/P ratio of 6 yielded the best results and was chosen for further experiments.

Poly(A) was used as cargo (dissolved in citric acid buffer) and encapsulated into lipid nanoparticles via microfluidic techniques, using a flow rate ratio of 3:1 (aqueous to organic phase) and a total flow rate of 12 mL/min. Furthermore, a 2:1 dilution with PBS buffer was performed to reduce the organic solvent content, followed by a dialysis step.

The Zetasizer ZSP (Malvern) was used to determine the size, PDI and zeta potential of the particles, while a RiboGreen assay was employed to ascertain the encapsulation efficiency.

The results shown in the following tables were obtained for the ionizable GDGT used (a mixture of several different GDGT scaffolds was used; a GDGT-4 scaffold is shown as a representative structure, respectively). The lipid ratio of the ionizable GDGTs among the conventional LNP lipid composition is given in Mol%.

Table 2

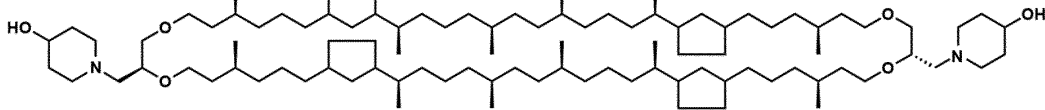
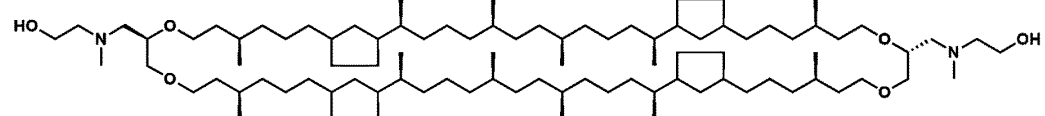
			
OHPIPD-GDGT			
Molecular mass [g/mol]		1460.52	
Chemical formula		C ₉₆ H ₁₈₂ N ₂ O ₆	
pKa value		6.59 +/- 0.08	
Zeta potential [mV]		5.24 +/- 0.24	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	82.24	0.08	78.34
34.7	72.17	0.08	87.91
46.3	73.93	0.15	91.39
53.15	86.05	0.12	89.67
60	103.06	0.10	93.86

Table 3



NMEA-GDGT			
Molecular mass [g/mol]		1408.44	
Chemical formula		C ₉₂ H ₁₇₈ N ₂ O ₆	
pKa value		6.61 +/- 0.09	
Zeta potential [mV]		6.79 +/- 1.22	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	84.38	0.01	89.38
34.7	82.68	0.08	100.15
46.3	81.35	0.15	95.37
53.15	94.78	0.11	97.50
60	109.72	0.10	95.15

Table 4

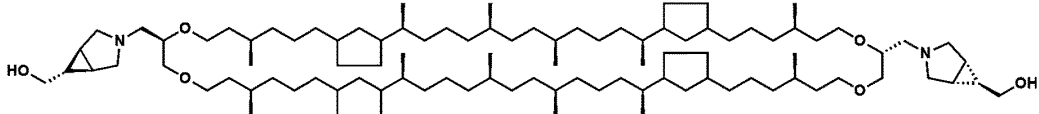
			
EABM-GDGT			
Molecular mass [g/mol]		1484.54	
Chemical formula		C ₉₈ H ₁₈₂ N ₂ O ₆	
pKa value		6.47 +/- 0.07	
Zeta potential [mV]		2.99 +/- 0.30	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	78.05	0.06	73.93
34.7	79.90	0.05	85.38
46.3	94.71	0.12	86.95
53.15	95.20	0.09	94.76
60	128.29	0.12	93.20

Table 5

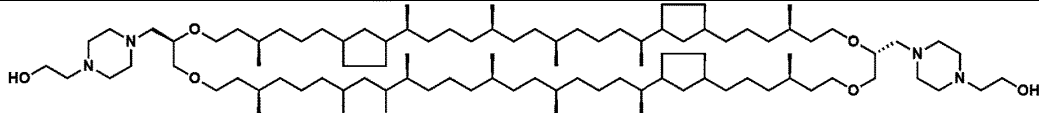
			
HEPIA-GDGT			
Molecular mass [g/mol]		1518.60	
Chemical formula		C ₉₈ H ₁₈₈ N ₄ O ₆	
pKa value		6.07 +/- 0.1	
Zeta potential [mV]		3.25 +/- 0.65	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	81.24	0.14	86.62
34.7	82.78	0.15	91.60
46.3	89.46	0.16	93.11
53.15	129.00	0.17	99.39
60	141.45	0.14	99.24

Table 6

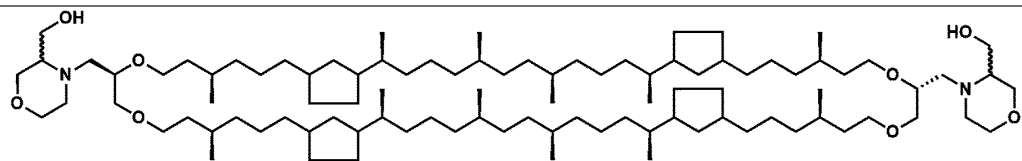
			
MORPHO-GDGT			
Molecular mass [g/mol]		1492.52	
Chemical formula		C ₉₆ H ₁₈₂ N ₂ O ₈	
pKa value		4.85 +/- 0.10	
Zeta potential [mV]		-7.35 +/- 0.23	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	75.19	0.05	71.28
34.7	63.29	0.05	72.53
46.3	71.76	0.07	89.90
53.15	79.68	0.07	78.81
60	97.19	0.07	83.73

Table 7

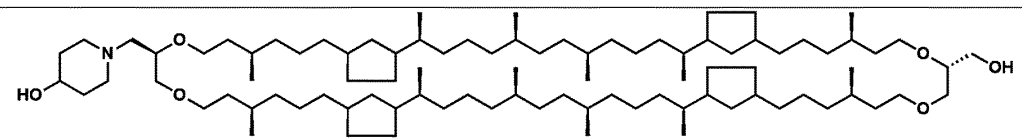
			
Mono-OHPIPD-GDGT			
Molecular mass [g/mol]		1377.39	
Chemical formula		C ₉₁ H ₁₇₃ NO ₆	
pKa value		6.33 +/- 0.10	
Zeta potential [mV]		2.04 +/- 0.55	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	67.33	0.06	58.77
34.7	69.97	0.09	73.44
46.3	77.10	0.09	82.78
53.15	84.20	0.13	78.15
60	85.23	0.11	81.73

Table 8

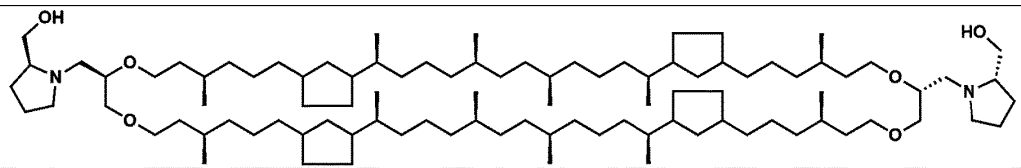
			
L-PRO-GDGT			
Molecular mass [g/mol]		1460.52	
Chemical formula		C ₉₆ H ₁₈₂ N ₂ O ₆	
pKa value		6.67 +/- 0.11	
Zeta potential [mV]		-2.40 +/- 1.07	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	88.0	0.22	83.36
34.7	83.72	0.16	88.72
46.3	89.74	0.01	88.39
53.15	103.73	0.15	94.83
60	130.40	0.27	92.16

Table 9

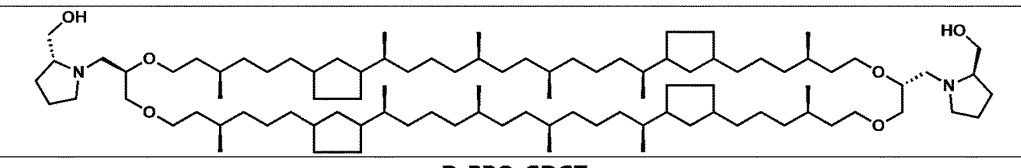
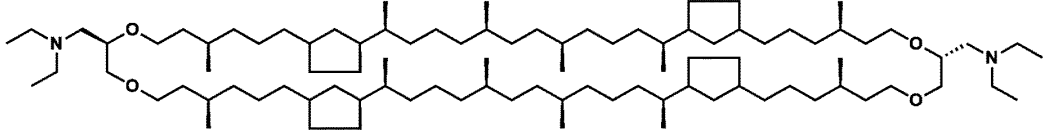
			
D-PRO-GDGT			
Molecular mass [g/mol]		1460.52	
Chemical formula		C ₉₆ H ₁₈₂ N ₂ O ₆	
pKa value		6.84 +/- 0.05	
Zeta potential [mV]		-2.99 +/- 0.0	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	79.45	0.14	72.67
34.7	97.72	0.09	76.34
46.3	100.93	0.16	92.87
53.15	113.90	0.13	76.34
60	110.83	0.21	72.67

Table 10

			
DEA-GDGT			
Molecular mass [g/mol]		1404.50	
Chemical formula		C ₉₄ H ₁₈₂ N ₂ O ₄	
pKa value		6.16 +/- 0.10	
Zeta potential [mV]		-1.74	
Characterization of LNPs			
Mol-%	Size [nm]	PDI	Encapsulation Efficiency [%]
23.15	82.21	0.11	73.22
34.7	87.66	0.09	80.30
46.3	98.82	0.09	96.67
53.15	123.07	0.11	85.89
60	132.17	0.11	87.32

In vivo study

The in vivo study was conducted with some of the LNPs characterized in Tables 2 to 10 above. Formulations containing 46.3 mol-% (lipid ratio) of the respective ionizable GDGT (N/P ratio 6 or 12) and encapsulating erythropoietin (EPO) mRNA were administered intramuscularly to Wistar rats. The objective of the in vivo study was to evaluate the properties of the different ionizable GDGTs in LNP formulations and their efficiency in transporting the EPO mRNA into muscle cells. Following the administration of the LNPs, the plasma concentration of EPO (AUC levels) was monitored and quantified through the use of an ELISA assay to determine the extent of induced EPO protein.

The results are shown in Table 11 below:

EPO concentration [mIU/mL]			
OHPIPD – 46mol% / N/P ratio 6	345.8 +/- 578.4	OHPIPD – 46/ NP 12	418.5 +/- 103.1
NMEA – 46/ NP 6	323.7 +/- 59.5	NMEA – 46/ NP 12	373.0 +/- 25.8
EABM – 46/ NP 6	842.4 +/- 104.6	EABM – 46/ NP 12	607.0 +/- 19.6
MORPHO – 46/ NP 6	678.6 +/- 178.2	MORPHO – 46/ NP 12	837.9 +/- 235.0
Mono-OHPIPD – 46/ NP 6	2191.9 +/- 496.6	Mono-OHPIPD – 46/ NP 12	1726.6 +/- 169.2

In general, the EPO protein concentrations achieved were higher compared to a control without mRNA. For several LNPs (such as e.g., mono-OHPIPD based LNPs), the protein concentrations achieved were much higher.

Taken together, these results show that ionizable GDGTs are very suitable for LNPs encapsulating mRNA cargo.

Non-patent references

Aldosari, Basmah N., et al. "Lipid nanoparticles as delivery systems for RNA-based vaccines." *Pharmaceutics* 13.2 (2021): 206.

Benvegna, Thierry, Loïc Lemiègre, and Sandrine Cammas-Marion. "New generation of liposomes called archaeosomes based on natural or synthetic archaeal lipids as innovative formulations for drug delivery." *Recent patents on drug delivery & formulation* 3.3 (2009): 206-220.

Cornebise, Mark, et al. "Discovery of a novel amino lipid that improves lipid nanoparticle performance through specific interactions with mRNA." *Advanced Functional Materials* 32.8 (2022): 2106727.

Daswani, Varsha P., et al. "The Polar Lipid Fraction E from *Sulfolobus acidocaldarius* Can Be Used as Liposomal Drug Stabilizing Agents to Reduce the Leakage of the Antivascular Drug Combretastatin A4 Disodium Phosphate from Tetraether/Diester Hybrid Archaeosomes." *Biophysica* 1.4 (2021): 474-486.

Engelhardt, Konrad H., et al. "Transfection studies with colloidal systems containing highly purified bipolar tetraether lipids from *Sulfolobus acidocaldarius*." *Archaea* 2017 (2017).

Eygeris, Yulia, et al. "Chemistry of lipid nanoparticles for RNA delivery." *Accounts of chemical research* 55.1 (2021): 2-12.

Hou, Xucheng, et al. "Lipid nanoparticles for mRNA delivery." *Nature Reviews Materials* 6.12 (2021): 1078-1094.

Jain, Samta, et al. "Biosynthesis of archaeal membrane ether lipids." *Frontiers in microbiology* 5 (2014): 641.

Kaur, Gurmeet, et al. "Archaeosomes: an excellent carrier for drug and cell delivery." *Drug delivery* 23.7 (2016): 2497-2512.

Markova, Natalia, et al. "Biophysical Characterization of Viral and Lipid-Based Vectors for Vaccines and Therapeutics with Light Scattering and Calorimetric Techniques." *Vaccines* 10.1 (2021): 49.

Midoux, Patrick, et al. "Lipid-based mRNA vaccine delivery systems." *Expert review of vaccines* 14.2 (2015): 221-234.

Morii, Hiroyuki, and Yosuke Koga. "Asymmetrical topology of diether-and tetraether-type polar lipids in membranes of *Methanobacterium thermoautotrophicum* cells." *Journal of Biological Chemistry* 269.14 (1994): 10492-10497.

Patel, Girishchandra B., et al. "Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems." *Critical reviews in biotechnology* 19.4 (1999): 317-357.

Paunovska, Kalina, et al. "Drug delivery systems for RNA therapeutics." *Nature Reviews Genetics* 23.5 (2022): 265-280.

Sateesh, Ananthu, et al. "Surface modification of medical-grade polyurethane by cyanurichloride-activated tetraether lipid (a new approach for bacterial antiadhesion)." *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials* 84.3 (2008): 672-681.

Schouten, Stefan, et al. "The organic geochemistry of glycerol dialkyl glycerol tetraether lipids: A review." *Organic geochemistry* 54 (2013): 19-61.

Schuster, Bernhard, Dietmar Pum, and Uwe B. Sleytr. "Voltage clamp studies on S-layer-supported tetraether lipid membranes." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1369.1 (1998): 51-60.

Schwarzmann, Günter, Bernadette Breiden, and Konrad Sandhoff. "Membrane-spanning lipids for an uncompromised monitoring of membrane fusion and intermembrane lipid transfer." *Journal of Lipid Research* 56.10 (2015): 1861-1879.

Siliakus, Melvin F., et al. "Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure." *Extremophiles* 21.4 (2017): 651-670.

Szoka Jr, Frank, et al. "Comparative properties and methods of preparation of lipid vesicles (liposomes)." *Annual review of biophysics and bioengineering* 9.1 (1980): 467-508.

van Hoogevest, Peter. "Review-an update on the use of oral phospholipid excipients." *European journal of pharmaceutical sciences* 108 (2017): 1-12.

Vaz, W. L. C., et al. "A comparison of the translational diffusion of a normal and a membrane-spanning lipid in L α phase 1-palmitoyl-2-oleoylphosphatidylcholine bilayers." *European Biophysics Journal* 12 (1985): 19-24.

Vishwakarma, Nikhar, et al. "Lipid-based nanocarriers for lymphatic transportation." *AAPS PharmSciTech* 20.2 (2019): 1-13.

Weijers, J. W. H., et al. "Carbon isotopic composition of branched tetraether membrane lipids in soils suggest a rapid turnover and a heterotrophic life style of their source organism (s)." *Biogeosciences* 7.9 (2010): 2959-2973.

Zhang, Xiaoping, Varun Goel, and Gabriel J. Robbie. "Pharmacokinetics of Patisiran, the first approved RNA interference therapy in patients with hereditary transthyretin-mediated amyloidosis." *The Journal of Clinical Pharmacology* 60.5 (2020): 573-585.

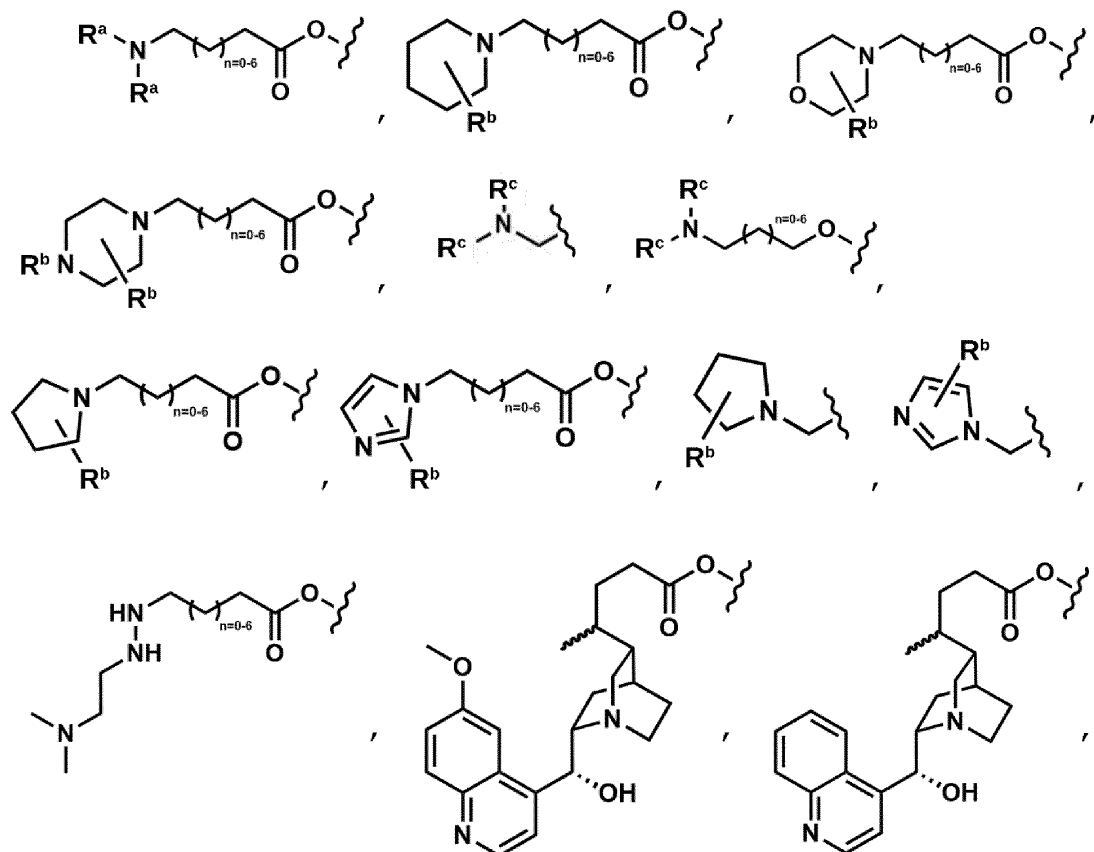
Claims

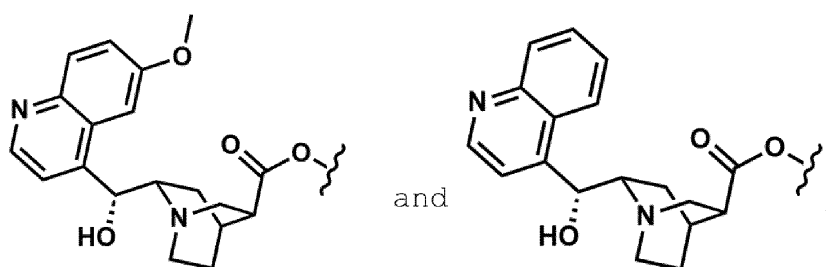
1. A lipid nanoparticle (LNP) encapsulating a nucleic acid cargo, wherein the LNP comprises at least

- an ionizable lipid fraction and
- a stabilizer fraction;

wherein the ionizable lipid fraction comprises at least one ionizable glycerol dialkyl glycerol tetraether (GDGT) lipid;

wherein the at least one ionizable GDGT lipid comprises at least one ionizable head group S; preferably wherein S is selected from the group consisting of:





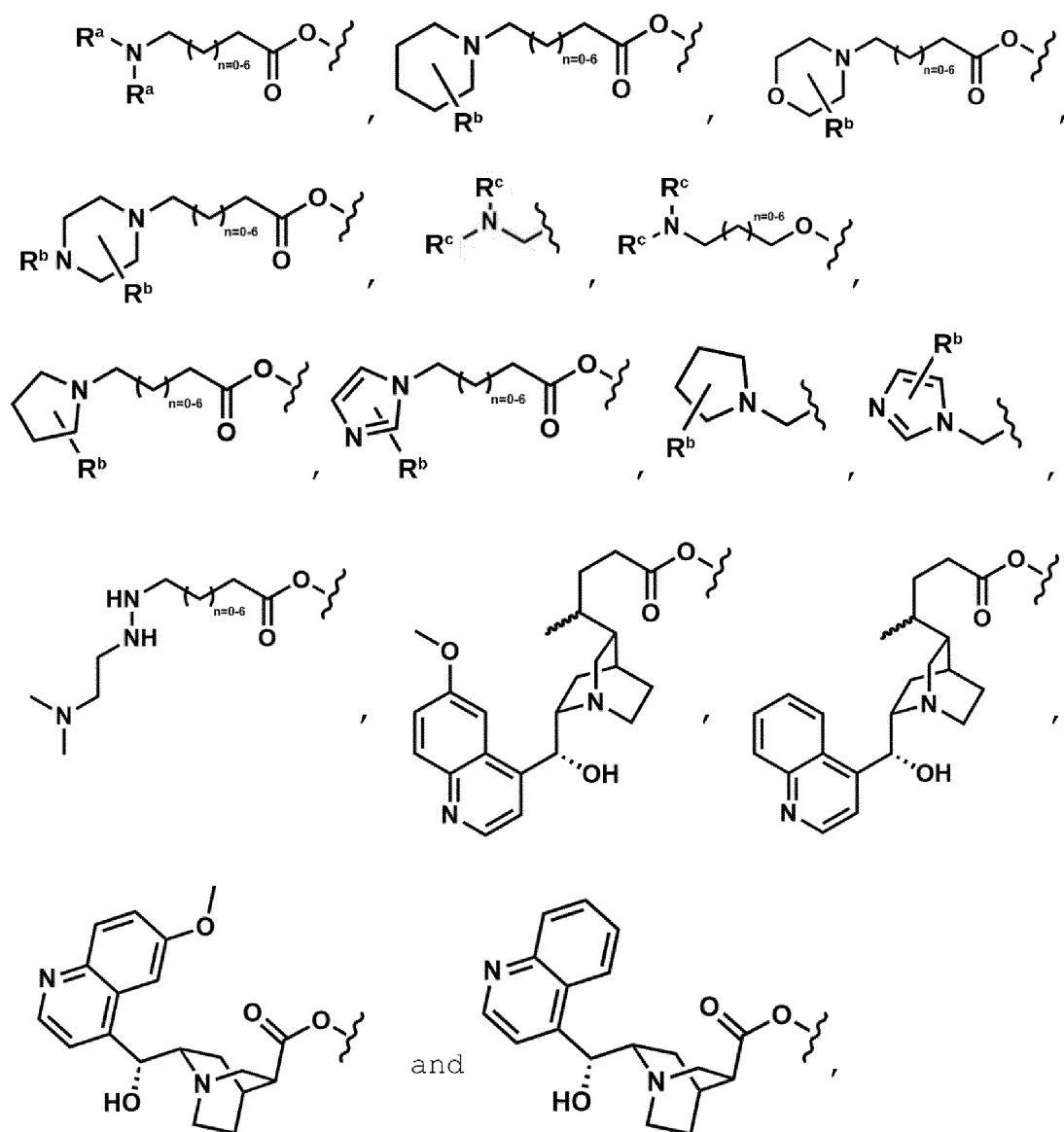
wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine, each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide and alkyl thiol, preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol, and each occurrence of R^c is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide.

2. The LNP of claim 1, wherein the nucleic acid cargo comprises a messenger ribonucleic acid (mRNA).

3. The LNP of claim 1 or 2, wherein the stabilizer fraction comprises at least one polyethylene glycol (PEG) lipid.

4. The LNP of any one of claims 1 to 3, wherein the LNP further comprises a sterol lipid fraction, preferably comprising cholesterol.

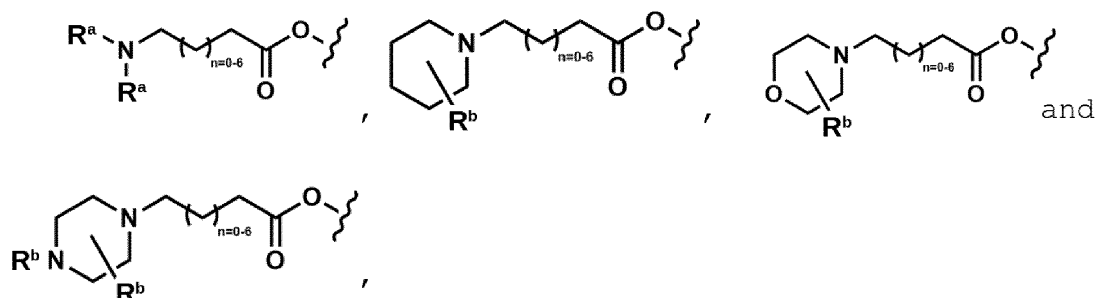
5. A GDGT lipid suitable for use as an ionizable lipid in an LNP, wherein the GDGT lipid comprises at least one ionizable head group S, wherein S is selected from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkylamine, alkyl ether, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate,

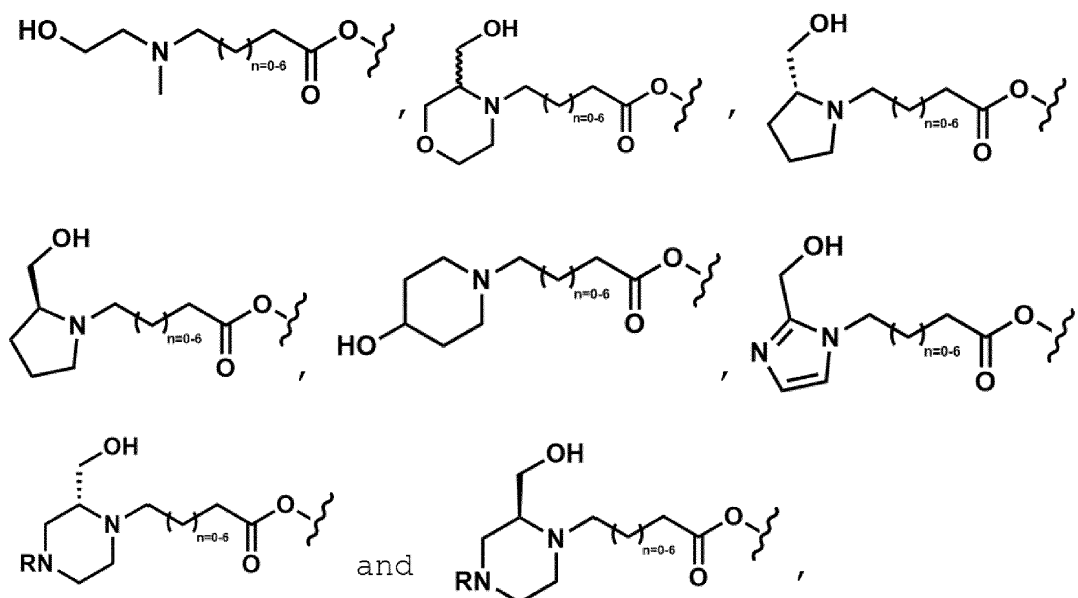
alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine, each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate, alkyl sulfonamide and alkyl thiol, preferably from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol, and each occurrence of R^c is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide, preferably from alkyl, cycloalkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine, alkyl thiol, alkyl ester, alkyl amide, alkyl carbamate, alkyl sulfonate and alkyl sulfonamide.

6. The GDGT lipid of claim 5, wherein S is selected from the group consisting of:



wherein each occurrence of R^a is independently selected from H, alkyl, cycloalkyl, alkenyl, hydroxyalkyl and alkylamine, and each occurrence of R^b is independently selected from H, alkyl, alkenyl, hydroxyalkyl, alkyl ether, alkylamine and alkyl thiol.

7. The GDGT lipid of claim 5 or 6, wherein the head group S is selected from:



wherein each occurrence of R is independently selected from H and alkyl; preferably wherein the alkyl is selected from methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, pentyl, iso-pentyl, hexyl, iso-hexyl, heptyl, and iso-heptyl.

8. An ether lipid fraction comprising the GDGT lipid of any one of claims 5 to 7; preferably obtainable by extraction from an archaeal culture, preferably a *Sulfolobus* culture, more preferably a *Sulfolobus acidocaldarius* culture, followed by substitution with the ionizable head group S.

9. A pharmaceutical composition comprising the LNP of any one of claims 1 to 4 and preferably at least one excipient.

10. A method for producing the GDGT lipid of any one of claims 5 to 7, comprising the steps of:

- obtaining a lipid fraction comprising one or more precursor GDGT lipids from an archaeal culture, preferably a

Sulfolobus culture, more preferably a *Sulfolobus acidocaldarius* culture;

- purifying the one or more precursor GDGT lipids; and
- contacting the one or more precursor GDGT lipids with one or more reagents to produce the GDGT lipid with the at least one ionizable head group S.