

IONIZABLE LIPIDS

FIELD OF THE INVENTION

The present invention generally relates to the field of ionizable (also termed cationic) lipids, and
5 in particular provides a novel type of such lipids as represented by any of the formulae disclosed
herein. The present invention further provides methods for making such lipids as well as uses
thereof, in particular in the preparation of nanoparticle compositions, more in particular
nanoparticle compositions comprising nucleic acids. It further provides vaccine formulations and
10 pharmaceutical formulations comprising nanoparticle compositions based on the ionizable lipids
disclosed herein.

BACKGROUND TO THE INVENTION

Nucleic acid- based drugs are being explored in a growing number of therapeutic areas.
Nonetheless, due to their negative charge, size and instability, the targeted delivery of nucleic
15 acids such as plasmid DNA, messenger RNA, short interfering RNA, single guide RNA and
micro-RNAs to tissues and cells poses a major challenge. A plethora of nanoparticulate carrier
systems has been explored to encapsulate and deliver nucleic acids. These nanoparticles need
to combine efficient and stable encapsulation of the nucleic acid upon storage and in the
extracellular environment, with maximum cellular uptake and efficient release of their payload
20 from endosomes into the cytosol.

Lipid based nanoparticles are clinically used to deliver small interfering RNA and mRNA
vaccines and represent the most advanced class of RNA delivery vehicles. Lipid based
nanoparticles are typically composed of a cationic or ionizable lipid that can be protonated at
acid pH, a helper phospholipid, a PEGylated lipid and a sterol. Each component has specialized
25 functions in LNP stability and activity. The sterol and the PEGylated lipid are vital for LNP
structure and stability, whereas the phospholipid can contribute to stability and endosomal
escape. The cationic or ionizable lipid in turn is considered the main driver of activity and
tolerability by governing mRNA encapsulation, cellular uptake and endosomal escape. Although
effective nucleic acid delivery vehicles, LNPs can induce dose limiting toxicities, such as
30 Complement Activation Related Pseudo-allergy, inflammatory cytokine release and cellular
toxicities by accumulation of non-degradable ionizable lipids into cellular membranes. Further
improvements in cationic or ionizable lipid chemistries are hence needed to improve efficacy
and safety of LNP delivered nucleic acid drugs.

WO2022136641 discloses ionizable lipids and lipid nanoparticles, as well as preparation
35 methods and compositions comprising the same. These lipids differ in their linker moieties, when
compared to the novel ionizable lipids of the present invention.

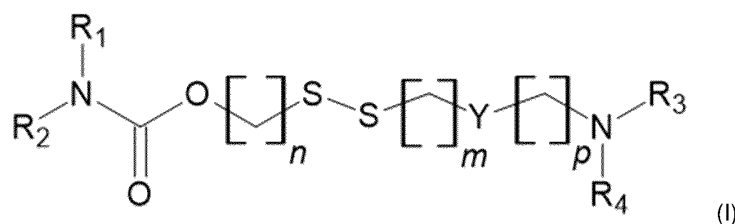
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Accordingly, the present invention relates to a new class of ionizable lipids as defined by the present set of claims, which have improved characteristics over the currently available classes of ionizable lipids.

SUMMARY OF THE INVENTION

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In a first aspect, the present invention provides a lipid, in particular an ionizable lipid represented by formula (I)



wherein

- 10 R_1 and R_2 are each independently selected from -H, - C_{1-20} alkyl, - C_{2-20} alkenyl and - C_{2-20} alkynyl; wherein each of said - C_{1-20} alkyl, - C_{2-20} alkenyl and - C_{2-20} alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl; wherein each
- 15 of said -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl; and wherein the total number of C atoms in R_1 and R_2
- 20 together is at least 8;
- R_3 and R_4 are each independently - C_{1-6} alkyl; wherein each of said - C_{1-6} alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -
- 25 halo, - C_{1-6} alkyl, - C_{3-6} cycloalkyl, OH, -OC $_{1-6}$ alkyl, -SC $_{1-6}$ alkyl, and -NH $_2$; - C_{1-6} alkyl; wherein each of said - C_{1-6} alkyl is optionally and independently substituted with a phenyl substituent;
- or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents
- 30 selected from - C_{1-6} alkyl, and - C_{1-6} alkyl-OH;
- R_5 is -H or - C_{1-6} alkyl; wherein said - C_{1-6} alkyl may optionally be substituted with -NR $_6$ R $_6$ ";
- R_6 and R $_6$ " are each independently selected from -H, and - C_{1-6} alkyl;
- or R $_6$ and R $_6$ " taken together with the N atom to which they are attached form a 5-10 membered

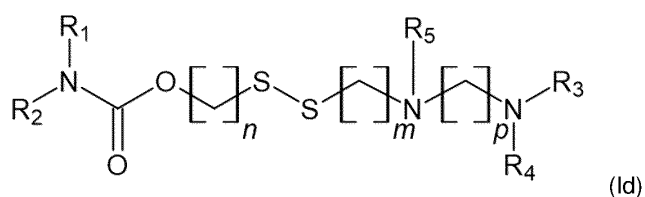
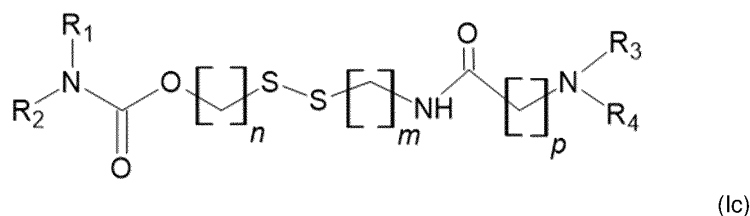
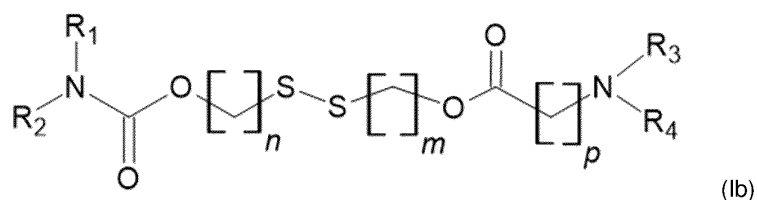
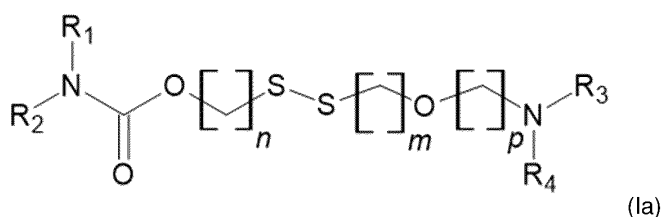
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aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

m, n and p are each independently an integer selected from 1, 2, 3 and 4;

5 Y is selected from the list comprising -O-, -OC(O)-, -C(O)O-, -NR₅-, -NH-C(O)-, -C(O)-NH-.

In a specific embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein and being represented by anyone of formula (Ia), (Ib), (Ic) and (Id)



wherein

R₁ and R₂ are each independently selected from -H, -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl;

15 wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl optionally comprises one or more additional

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heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂ together is at least 8;

- 5 R₃ and R₄ are each independently -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -halo, -C₁₋₆alkyl, -C₃₋₆cycloalkyl, OH, -O-C₁₋₆alkyl, -S-C₁₋₆alkyl, and -NH₂; -C₁₋₆alkyl; wherein
- 10 each of said -C₁₋₆alkyl is optionally and independently substituted with a phenyl substituent; or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- 15 R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆"; R₆ and R₆" are each independently selected from -H, and -C₁₋₆alkyl; or R₆ and R₆" taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents
- 20 selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- m, n and p are each independently an integer selected from 1, 2, 3 and 4.

In yet a further embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein; wherein:

- 25 R₁ and R₂ are each independently selected from -H, -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl; wherein each of said -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C₁₋₂₀alkyl, and -C(O)O-C₂₋₂₀alkenyl; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C₁₋₂₀alkyl,
- 30 and -C(O)O-C₂₋₂₀alkenyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂ together is at least 8;
- 35 R₃ and R₄ are each independently -C₁₋₆alkyl; or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

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R_5 is -H or - C_{1-6} alkyl; wherein said - C_{1-6} alkyl may optionally be substituted with - NR_6R_6'' ;

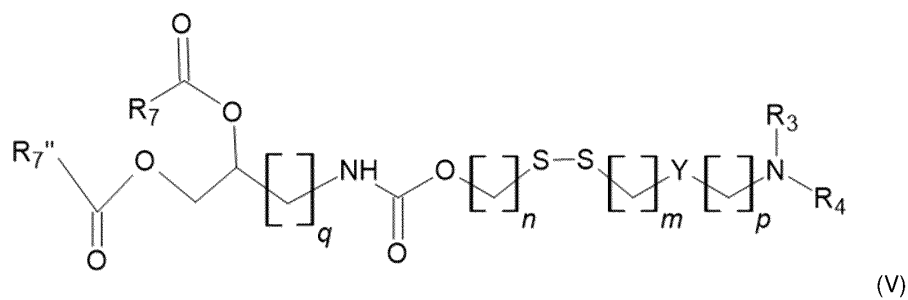
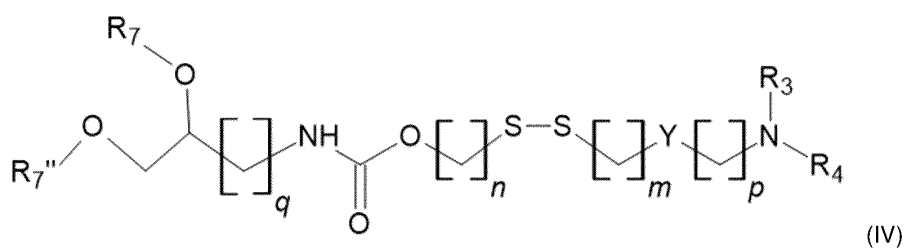
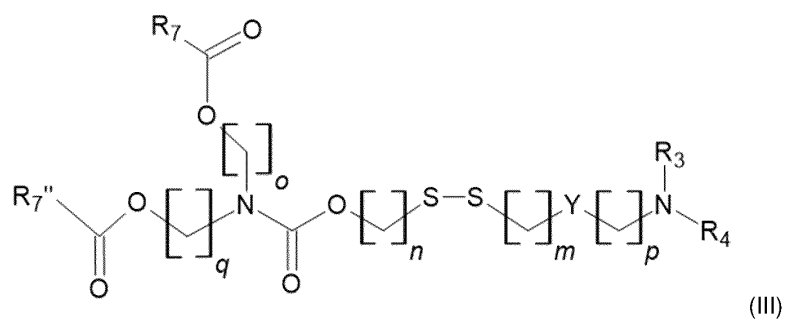
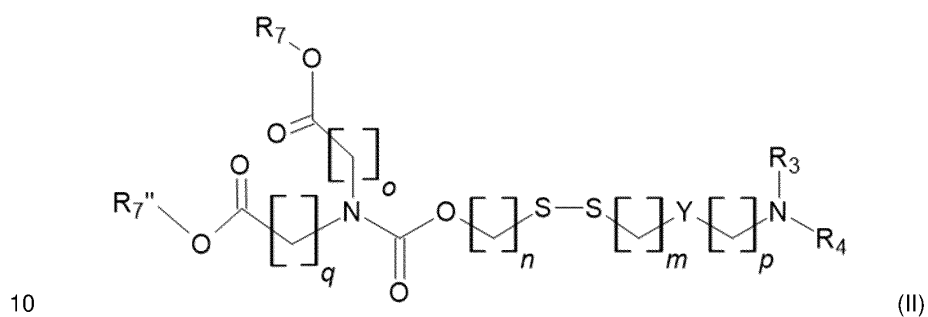
R_6 and R_6'' are each independently selected from -H, and - C_{1-6} alkyl;

or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;

5 m, n and p are each independently an integer selected from 1, 2, 3 and 4;

Y is selected from -O-, -OC(O)-, - NR_5 -, and -NH-C(O)-.

In yet a further embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein and being represented by any one of formula (II), (III), (IV) or (V)



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wherein

- each occurrence of R_7 and R_7'' is independently selected from $-C_{1-20}alkyl$, $-C_{2-20}alkenyl$, $-C_{2-20}alkynyl$; wherein each of said $-C_{1-20}alkyl$, $-C_{2-20}alkenyl$, $-C_{2-20}alkynyl$ may optionally be substituted with from 1-3 substituents selected from $-OH$, $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-OC(O)-C_{2-20}alkynyl$, $-C(O)O-C_{1-20}alkyl$, $-C(O)O-C_{2-20}alkenyl$, and $-C(O)O-C_{2-20}alkynyl$; and the total number of C atoms in R_7 and R_7'' together is at least 6;
- R_3 and R_4 are each independently $-C_{1-6}alkyl$; wherein each of said $-C_{1-6}alkyl$ is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from $-halo$, $-C_{1-6}alkyl$, $-C_{3-6}cycloalkyl$, $-OH$, $-O-C_{1-6}alkyl$, $-S-C_{1-6}alkyl$, and $-NH_2$; $-C_{1-6}alkyl$; wherein each of said $-C_{1-6}alkyl$ is optionally and independently substituted with a phenyl substituent;
- or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}alkyl$, and $-C_{1-6}alkyl-OH$;
- R_5 is $-H$ or $-C_{1-6}alkyl$; wherein said $-C_{1-6}alkyl$ may optionally be substituted with $-NR_6R_6''$;
- R_6 and R_6'' are each independently selected from $-H$, and $-C_{1-6}alkyl$;
- or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}alkyl$, and $-C_{1-6}alkyl-OH$;
- m , n , and p are each independently an integer selected from 1, 2, 3 and 4
- q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10;;
- Y is selected from $-O-$, $-OC(O)-$, $-C(O)O-$, $-NR_5-$, $-NH-C(O)-$, and $-C(O)-NH-$.

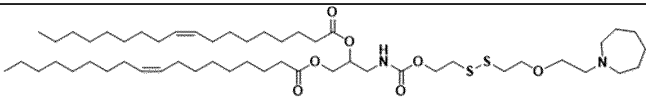
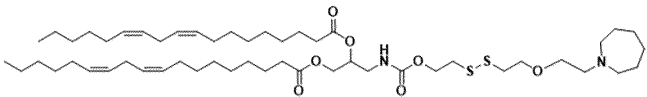
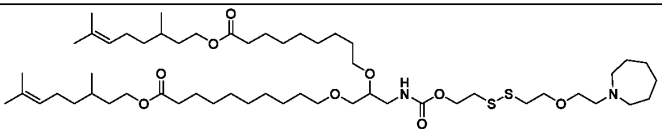
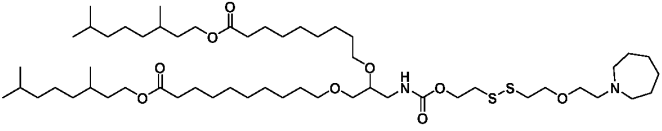
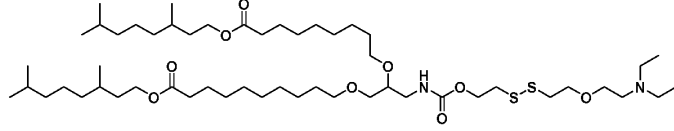
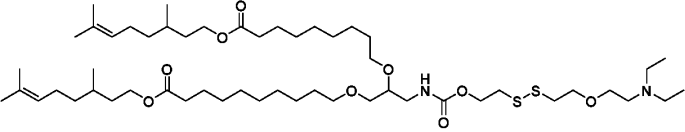
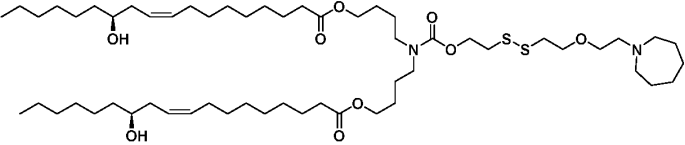
The present invention further provides a lipid, in particular an ionizable lipid as defined herein, wherein:

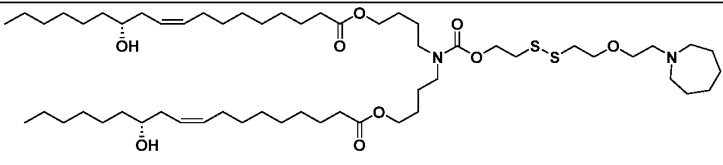
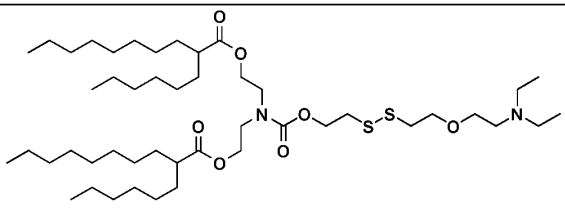
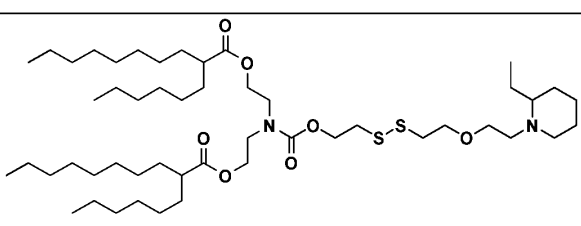
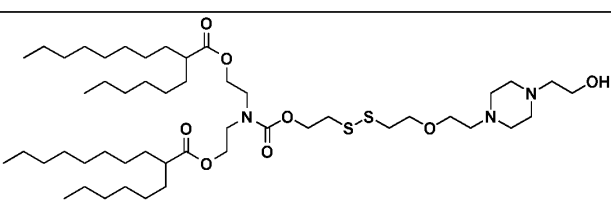
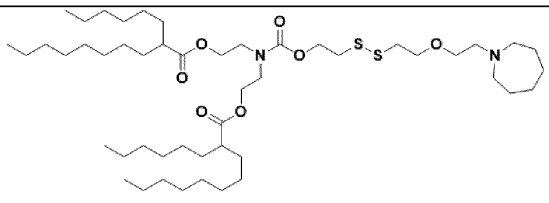
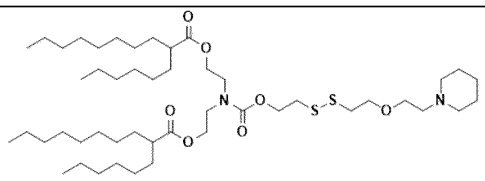
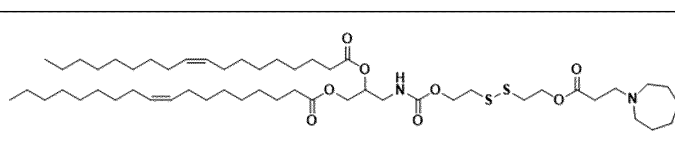
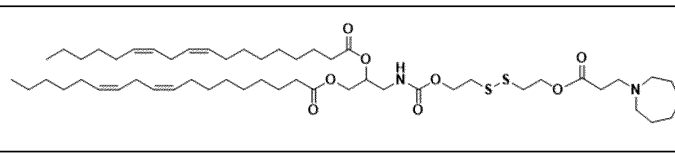
- each occurrence of R_7 and R_7'' is independently selected from $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$;
- wherein each of said $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$ may optionally be substituted with from 1-3 substituents independently selected from $-OH$, $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-C(O)O-C_{1-20}alkyl$, and $-C(O)O-C_{2-20}alkenyl$; and the total number of C atoms in R_7 and R_7'' together is at least 6;
- R_3 and R_4 are each independently $-C_{1-6}alkyl$;
- or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}alkyl$, and $-C_{1-6}alkyl-OH$;
- R_5 is $-H$ or $-C_{1-6}alkyl$; wherein said $-C_{1-6}alkyl$ may optionally be substituted with $-NR_6R_6''$;

- R₆ and R₆^{''} are each independently selected from -H, and -C₁₋₆alkyl;
or R₆ and R₆^{''} taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;
m, n, and p are each independently an integer selected from 1, 2, 3 and 4
5 q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Y is selected from -O-, -OC(O)-, -NR₅-, and -NH-C(O)-.

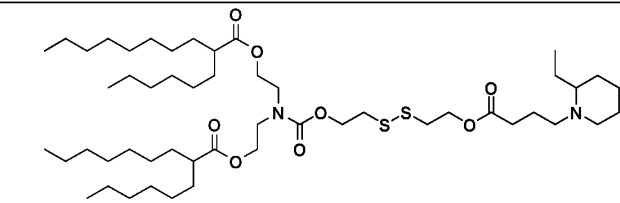
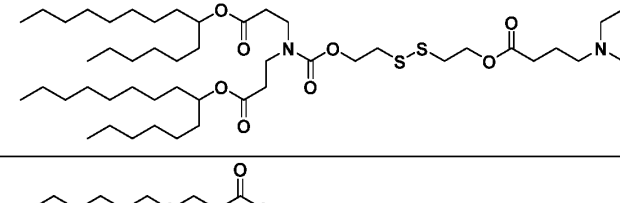
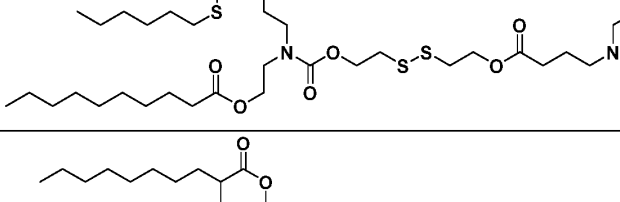
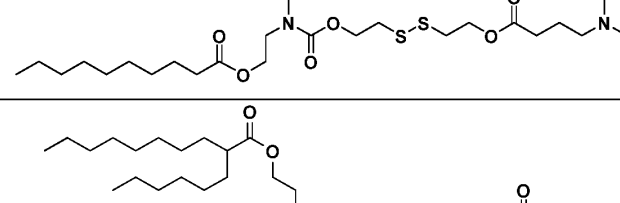
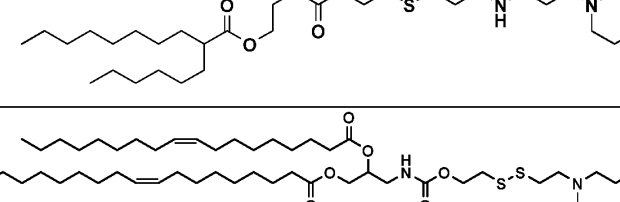
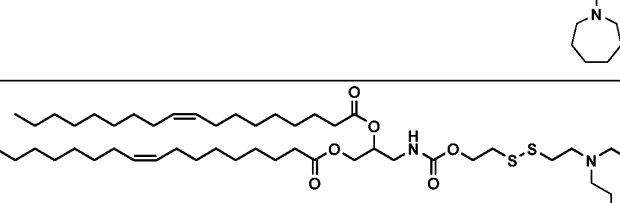
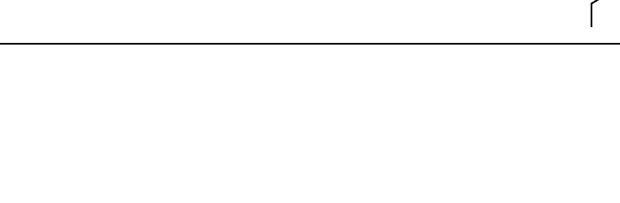
The present invention further provides a lipid, in particular an ionizable lipid as defined herein and being selected from the list comprising:

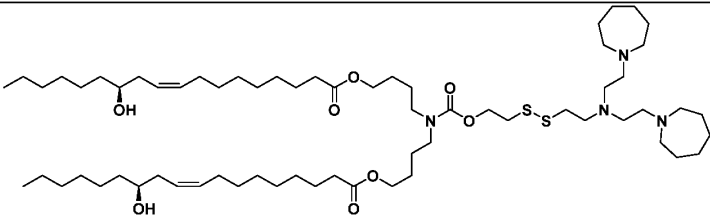
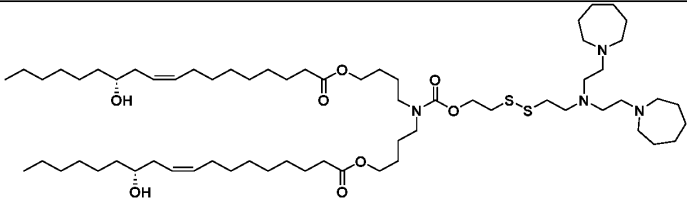
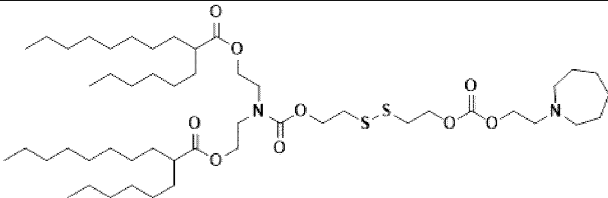
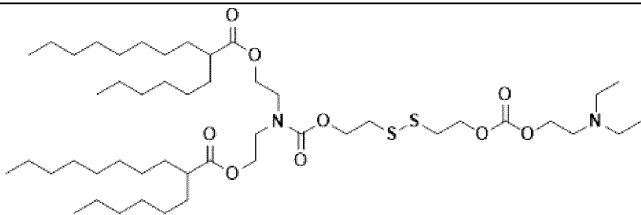
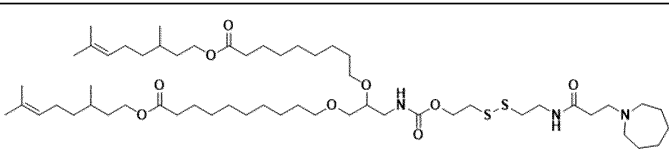
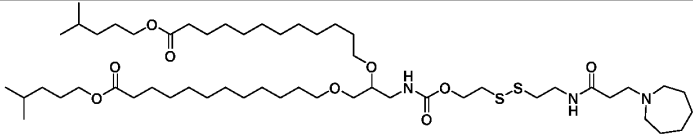
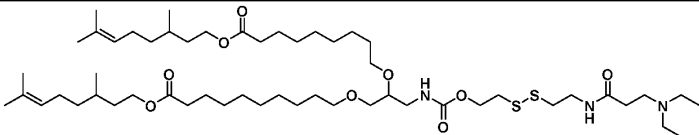
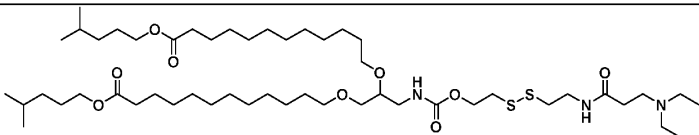
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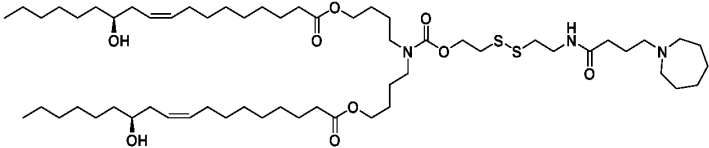
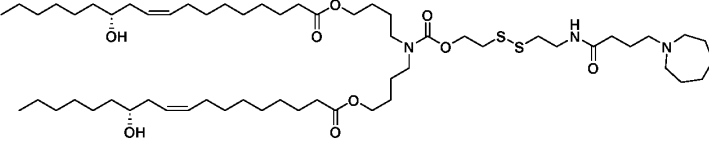
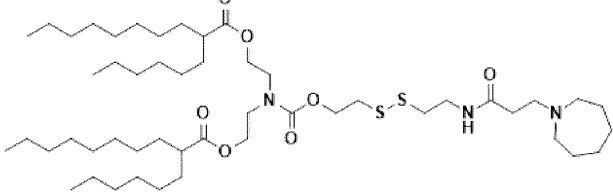
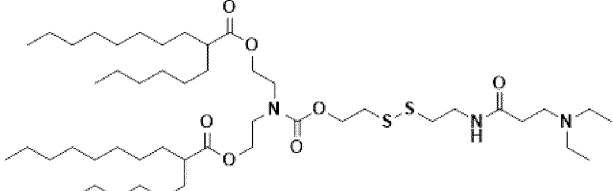
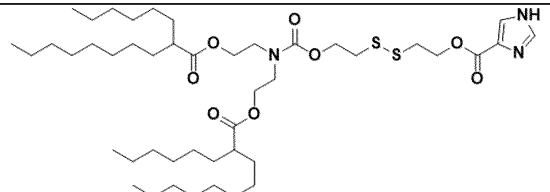
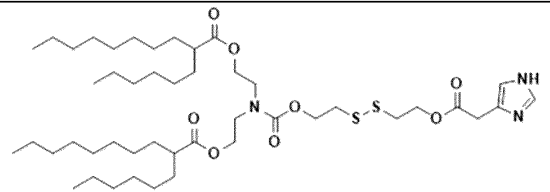
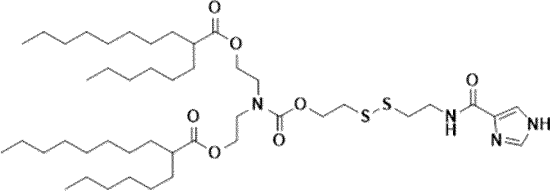
Lipid Name	Chemical Structure
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-13-

IL-46	
IL-47	

In yet a further embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein; wherein the total number of C atoms in R₁ and R₂ together is at least 14.

- 5 The present invention further provides a lipid, in particular an ionizable lipid as defined herein; wherein the total number of C atoms in R₇ and R₇' together is at least 12.

In a further aspect, the present invention provides a lipid nanoparticle or lipid nanoparticle composition comprising a lipid, in particular an ionizable lipid as defined herein. Said
10 nanoparticle composition may further comprise a phospholipid, a sterol and a PEG lipid.

In yet a further embodiment of the present invention, the lipid nanoparticle or lipid nanoparticle composition as defined herein further comprises an active agent, in particular a nucleic acid, preferably mRNA.

- 15 In a further aspect, the present invention provides the use of a lipid, in particular an ionizable lipid as defined herein in the manufacture of a lipid nanoparticle or lipid nanoparticle composition.

- In a final aspect, the present invention provides a pharmaceutical composition comprising a lipid nanoparticle or lipid nanoparticle composition as defined herein and a pharmaceutically acceptable agent.

The invention also provides the pharmaceutical compositions as defined herein for use in human and/or veterinary medicine.

25

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by the following Figures, which show a preferred embodiment of the device according to the invention, and are not intended to limit the scope of the invention in any way, wherein:

- 5
- Figure 1** shows cell viability of DC2.4 cells transfected with LNPs having various ionizable lipids.
- Figure 2** shows cell viability of HEK293T cells transfected with LNPs having various ionizable lipids.
- Figure 3** shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in DC 2.4 cells upon incubation with LNPs having various ionizable lipids, at mRNA concentration of 50 ng and 200 ng/well.
- 10
- Figure 4** shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation with LNPs having various ionizable lipids, at mRNA concentration of 50 ng and 200 ng/well.
- 15
- Figure 5** shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation with LNPs having various ionizable lipids, at mRNA concentration of 50 ng and 200 ng/well.
- 20
- Figure 6** shows cell viability of HEK293T cells transfected with LNPs having various ionizable lipids.
- Figure 7** shows cell viability of HEK293T cells transfected with LNPs having various ionizable lipids.
- Figure 8** shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation with LNPs having various ionizable lipids, at mRNA concentration of 50 ng and 200 ng/well.
- 25
- Figure 9** shows the *in vivo* average radiance (normalized in function of photons/second/cm²/steradian) as captured by the IVIS. The mice are positioned in a manner that the image is taken in the supine view after 24h.
- 30
- Figure 10** reveals the *ex vivo* average radiance (normalized in function of photons/second/cm²/steradian) of both liver and spleen as captured by the IVIS.
- Figure 11** shows the *in vivo* average radiance (normalized in function of photons/second/cm²/steradian) as captured by the IVIS. The mice are positioned in a manner that the image is taken in the supine view after 24h.
- 35
- Figure 12** shows the average hEPO conc in the blood 4h after IV injection with LNPs having various ionizable lipids, expressed as ng/mL.
- Figure 13** shows the IgG1 titers at day 35 as determined by ELISA and expressed as the reciprocal of the dilution of the sera that provides signal that is at least 3 times higher than

-15-

the background $\pm$ 3SD.

Figure 14 reveals the IgG2a titers at day 35 as determined by ELISA and expressed as the reciprocal of the dilution of the sera that provides signal that is at least 3 times higher than the background $\pm$ 3SD.

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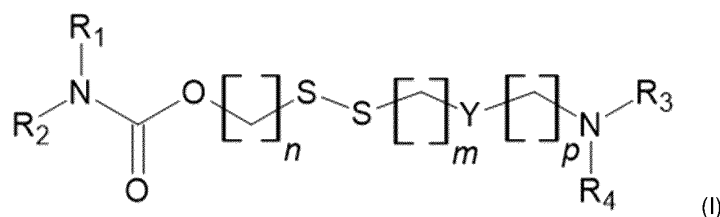
DETAILED DESCRIPTION OF THE INVENTION

The description and drawings merely illustrate the principles of the present invention. It will thus be appreciated that those skilled in the art will be able to devise various arrangements that, although not explicitly described or shown herein, embody the principles of the present invention and are included within its scope. Furthermore, all examples recited herein are principally intended expressly to be only for pedagogical purposes to aid the reader in understanding the principles of the present invention and the concepts contributed by the inventor(s) to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the present invention, as well as specific examples thereof, are intended to encompass equivalents thereof.

In the present invention, expressions such as “comprise”, “include”, “have”, “may comprise”, “may include”, or “may have” indicate existence of corresponding features but do not exclude existence of additional features.

Unless a context dictates otherwise, asterisks are used herein to indicate the point at which a mono- or bivalent radical depicted is connected to the structure to which it relates and of which the radical forms part.

As already mentioned hereinbefore, in a first aspect, the present invention provides a lipid, in particular an ionizable lipid represented by formula (I)



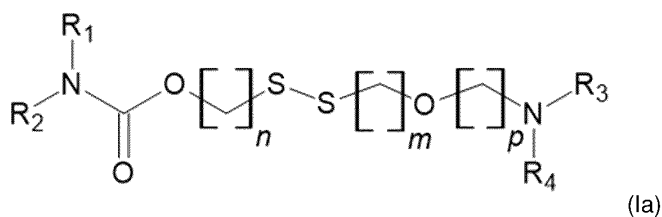
wherein:

R_1 and R_2 are each independently selected from -H, -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl; wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-

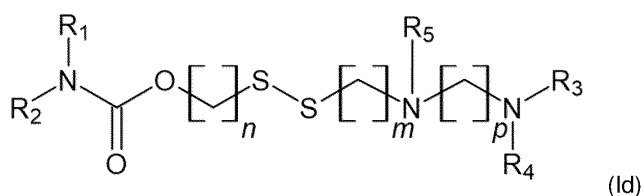
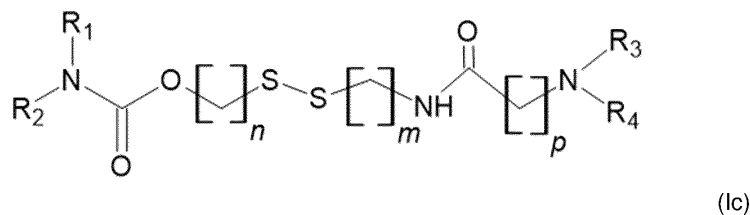
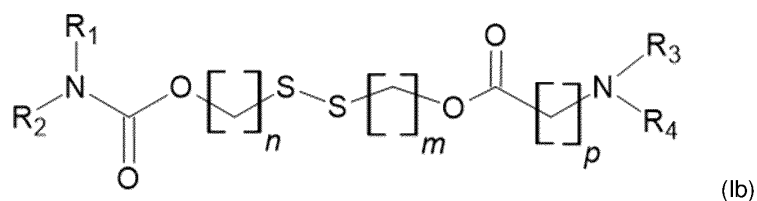
-16-

- C_{2-20} alkynyl, $-C(O)O-C_{1-20}$ alkyl, $-C(O)O-C_{2-20}$ alkenyl, and $-C(O)O-C_{2-20}$ alkynyl; wherein each of said $-OC(O)-C_{1-20}$ alkyl, $-OC(O)-C_{2-20}$ alkenyl, $-OC(O)-C_{2-20}$ alkynyl, $-C(O)O-C_{1-20}$ alkyl, $-C(O)O-C_{2-20}$ alkenyl, and $-C(O)O-C_{2-20}$ alkynyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-OH$, $-OC(O)-C_{1-20}$ alkyl, $-OC(O)-C_{2-20}$ alkenyl, $-OC(O)-C_{2-20}$ alkynyl, $-C(O)O-C_{1-20}$ alkyl, $-C(O)O-C_{2-20}$ alkenyl, and $-C(O)O-C_{2-20}$ alkynyl; and wherein the total number of C atoms in R_1 and R_2 together is at least 8;
- R_3 and R_4 are each independently $-C_{1-6}$ alkyl; wherein each of said $-C_{1-6}$ alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from $-halo$, $-C_{1-6}$ alkyl, $-C_{3-6}$ cycloalkyl, $-OH$, $-OC_{1-6}$ alkyl, $-SC_{1-6}$ alkyl, and $-NH_2$; $-C_{1-6}$ alkyl; wherein each of said $-C_{1-6}$ alkyl is optionally and independently substituted with a phenyl substituent; or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}$ alkyl, and $-C_{1-6}$ alkyl-OH;
- R_5 is $-H$ or $-C_{1-6}$ alkyl; wherein said $-C_{1-6}$ alkyl may optionally be substituted with $-NR_6R_6''$;
- R_6 and R_6'' are each independently selected from $-H$, and $-C_{1-6}$ alkyl; or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}$ alkyl, and $-C_{1-6}$ alkyl-OH;
- m , n and p are each independently an integer selected from 1, 2, 3 and 4;
- Y is selected from $-O-$, $-OC(O)-$, $-C(O)O-$, $-NR_5-$, $-NH-C(O)-$, and $-C(O)-NH-$.

Accordingly, the present invention also provides a lipid, in particular an ionizable lipid as defined herein and being represented by anyone of formula (Ia), (Ib), (Ic) and (Id)



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wherein:

- 5 R_1 and R_2 are each independently selected from -H, - C_{1-20} alkyl, - C_{2-20} alkenyl and - C_{2-20} alkynyl; wherein each of said - C_{1-20} alkyl, - C_{2-20} alkenyl and - C_{2-20} alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl; wherein each
- 10 of said -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)- C_{1-20} alkyl, -OC(O)- C_{2-20} alkenyl, -OC(O)- C_{2-20} alkynyl, -C(O)O- C_{1-20} alkyl, -C(O)O- C_{2-20} alkenyl, and -C(O)O- C_{2-20} alkynyl; and wherein the total number of C atoms in R_1 and R_2
- 15 together is at least 8;
- R_3 and R_4 are each independently - C_{1-6} alkyl; wherein each of said - C_{1-6} alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -
- 20 halo, - C_{1-6} alkyl, - C_{3-6} cycloalkyl, OH, -O- C_{1-6} alkyl, -S- C_{1-6} alkyl, and -NH₂; - C_{1-6} alkyl; wherein each of said - C_{1-6} alkyl is optionally and independently substituted with a phenyl substituent;
- or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected

from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆'';

R₆ and R₆' are each independently selected from -H, and -C₁₋₆alkyl;

or R₆ and R₆' taken together with the N atom to which they are attached form a 5-10 membered

5 aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

m, n and p are each independently an integer selected from 1, 2, 3 and 4.

10 When describing the compounds/lipids of the present invention, the terms used are to be construed in accordance with the following definitions, unless a context dictates otherwise:

The term "alkyl" by itself or as part of another substituent refers to a fully saturated hydrocarbon of Formula C_xH_{2x+1} wherein x is a number greater than or equal to 1. Generally, alkyl groups of
15 this invention comprise from 1 to 20 carbon atoms. Alkyl groups may be linear or branched and may be substituted as indicated herein. When a subscript is used herein following a carbon atom, the subscript refers to the number of carbon atoms that the named group may contain. Thus, for example, C₁₋₄alkyl means an alkyl of one to four carbon atoms. Examples of alkyl groups are methyl, ethyl, n-propyl, i-propyl, butyl, and its isomers (e.g. n-butyl, i-butyl and t-butyl); pentyl and its isomers, hexyl and its isomers, heptyl and its isomers, octyl and its isomers, nonyl and its isomers; decyl and its isomers, undecyl and its isomers, dodecyl and its isomers, tridecyl and its isomers, tetradecyl and its isomers, pentadecyl and its isomers, hexadecyl and its isomers, heptadecyl and its isomers, octadecyl and its isomers, nonadecyl and its isomers, eicosanyl and its isomers.

25 The term "optionally substituted alkyl" refers to an alkyl group optionally substituted with one or more substituents (for example 1 to 4 substituents, for example 1, 2, 3, or 4 substituents) at any available point of attachment. Non-limiting examples of such substituents include esters, carboxylic acids, alkyl moieties, alkene moieties, alkyne moieties, ... and the like.

30 In the context of the present invention, the alkyl, alkenyl and alkynyl moieties as defined herein may also further comprise one or more heteroatoms, such as selected from N, S or O, in that for example a carbon atom in an alkyl, alkene or alkyne chain is replaced by a heteroatom. When two or more C atoms are replaced by heteroatoms, the heteroatoms may be adjacent or separated, as long as it results in a chemically stable compound, i.e. a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into a therapeutic agent. An example of a stable combination of two adjacent heteroatoms is a disulfide (-S-S-) group. Where a carbon atom in an alkyl, alkenyl or alkynyl chain is replaced by an N atom, the N atom may be N or NH depending on the number of bonds

connected to said C atom.

The term "alkenyl" or "alkene", as used herein, unless otherwise indicated, means straight-chain, cyclic, or branched-chain hydrocarbon or internal positions and the like. Generally alkenyl or
5 alkenyl moieties of the present invention comprise from 2 to 20 C atoms. An optionally substituted alkenyl refers to an alkenyl radicals containing at least one carbon-carbon double bond. Examples of alkenyl radicals include ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, pentenyl, hexenyl, hexadienyl, be it in the terminal having optionally one or more substituents (for example 1, 2, 3 or 4), selected from those defined above for substituted alkyl.

10 The term "alkynyl", as used herein, unless otherwise indicated, means straight-chain or branched-chain hydrocarbon radicals containing at least one carbon-carbon triple bond. Examples of alkynyl radicals include ethynyl, propynyl, butynyl, pentynyl, hexynyl, hexadiynyl, be it in the terminal or internal positions, and the like. An optionally substituted alkynyl refers to
15 an alkynyl having optionally one or more substituents (for example 1, 2, 3 or 4), selected from those defined above for substituted alkyl.

The term "cycloalkyl" by itself or as part of another substituent is a cyclic alkyl group, that is to say, a monovalent, saturated, or unsaturated hydrocarbyl group having 1, 2, or 3 cyclic structure.
20 Cycloalkyl includes all saturated or partially saturated (containing 1 or 2 double bonds) hydrocarbon groups containing 1 to 3 rings, including monocyclic, bicyclic, or polycyclic alkyl groups. Cycloalkyl groups may comprise 3 or more carbon atoms in the ring and generally, according to this invention comprise from 3 to 15 atoms. Examples of cycloalkyl groups include but are not limited to cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl,
25 cyclononyl, adamantanyl and cyclodecyl with cyclopropyl being particularly preferred. An "optionally substituted cycloalkyl" refers to a cycloalkyl having optionally one or more substituents (for example 1 to 3 substituents, for example 1, 2, 3 or 4 substituents), selected from those defined above for substituted alkyl.

30 Where alkyl groups as defined are divalent, i.e., with two single bonds for attachment to two other groups, they are termed "alkylene" groups. Non-limiting examples of alkylene groups includes methylene, ethylene, methylenemethylene, trimethylene, propylene, tetramethylene, ethylethylene, 1,2-dimethylethylene, pentamethylene and hexamethylene. Similarly, where alkenyl groups as defined above and alkynyl groups as defined above, respectively, are divalent
35 radicals having single bonds for attachment to two other groups, they are termed "alkenylene" and "alkynylene" respectively.

The term "heterocycle" as used herein by itself or as part of another group refers to non-aromatic, fully saturated or partially unsaturated cyclic groups (for example, 3 to 13 member

monocyclic, 7 to 17 member bicyclic, or 10 to 20 member tricyclic ring systems, or containing a total of 3 to 10 ring atoms) which have at least one heteroatom in at least one carbon atom-containing ring. Each ring of the heterocyclic group containing a heteroatom may have 1, 2, 3 or 4 heteroatoms selected from nitrogen atoms, oxygen atoms and/or sulfur atoms, where the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatoms may optionally be quaternized. The heterocyclic group may be attached at any heteroatom or carbon atom of the ring or ring system, where valence allows. The rings of multi-ring heterocycles may be fused, bridged and/or joined through one or more spiro atoms. An optionally substituted heterocyclic refers to a heterocyclic having optionally one or more substituents (for example 1 to 4 substituents, or for example 1, 2, 3 or 4), selected from those defined above for substituted alkyl. Non-limiting examples of heterocycle comprise: piperidinyl, pyrrolidinyl, azepanyl, morpholinyl,...

The term "aryl" (herein also referred to as aromatic heterocycle) as used herein refers to a polyunsaturated, aromatic hydrocarbyl group having a single ring (i.e. phenyl) or multiple aromatic rings fused together (e.g. naphthalene or anthracene) or linked covalently, typically containing 6 to 10 atoms; wherein at least one ring is aromatic. The aromatic ring may optionally include one to three additional rings (either cycloalkyl, heterocyclyl, or heteroaryl) fused thereto. Aryl is also intended to include the partially hydrogenated derivatives of the carbocyclic systems enumerated herein. Non-limiting examples of aryl comprise phenyl, ...

The aryl ring or heterocycle as defined herein can optionally be substituted by one or more substituents (for example 1 to 5 substituents, for example 1, 2, 3 or 4) at any available point of attachment. Non-limiting examples of such substituents are selected from halogen, hydroxyl, oxo, nitro, amino, hydrazine, aminocarbonyl, azido, cyano, alkyl, cycloalkyl, alkenyl, alkynyl, cycloalkylalkyl, alkylamino, alkoxy, -SO₂-NH₂, aryl, heteroaryl, aralkyl, haloalkyl, haloalkoxy, alkoxycarbonyl, alkylaminocarbonyl, heteroarylalkyl, alkylsulfonamide, heterocyclyl, alkylcarbonylaminoalkyl, aryloxy, alkylcarbonyl, acyl, arylcarbonyl, aminocarbonyl, alkylsulfoxide, -SO₂R^a, alkylthio, carboxyl, and the like, wherein R^a is alkyl or cycloalkyl.

Where a carbon atom in an aryl group is replaced with a heteroatom, the resultant ring is referred to herein as a heteroaryl ring.

The term "heteroaryl" as used herein by itself or as part of another group refers but is not limited to 5 to 12 carbon-atom aromatic rings or ring systems containing 1 to 3 rings which are fused together or linked covalently, typically containing 5 to 8 atoms; at least one of which is aromatic in which one or more carbon atoms in one or more of these rings can be replaced by oxygen, nitrogen or sulfur atoms where the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatoms may optionally be quaternized. Such rings may be fused to an

aryl, cycloalkyl, heteroaryl or heterocyclyl ring. Non-limiting examples of such heteroaryl, include: piridinyl, azepinyl,...

An "optionally substituted heteroaryl" refers to a heteroaryl having optionally one or more substituents (for example 1 to 4 substituents, for example 1, 2, 3 or 4), selected from those defined above for substituted aryl.

The term "oxo" as used herein refers to the group =O.

10 The term "alkoxy" or "alkyloxy" as used herein refers to a radical having the Formula -OR^b wherein R^b is alkyl. Preferably, alkoxy is C₁-C₁₀ alkoxy, C₁-C₆ alkoxy, or C₁-C₄ alkoxy. Non-limiting examples of suitable alkoxy include methoxy, ethoxy, propoxy, isopropoxy, butoxy, isobutoxy, sec-butoxy, tert-butoxy, pentyloxy and hexyloxy. Where the oxygen atom in an alkoxy group is substituted with sulfur, the resultant radical is referred to as thioalkoxy. "Haloalkoxy" is
15 an alkoxy group wherein one or more hydrogen atoms in the alkyl group are substituted with halogen. Non-limiting examples of suitable haloalkoxy include fluoromethoxy, difluoromethoxy, trifluoromethoxy, 2,2,2-trifluoroethoxy, 1,1,2,2-tetrafluoroethoxy, 2-fluoroethoxy, 2-chloroethoxy, 2,2-difluoroethoxy, 2,2,2-trichloroethoxy; trichloromethoxy, 2-bromoethoxy, pentafluoroethyl, 3,3,3-trichloropropoxy, 4,4,4-trichlorobutoxy.

20 The term "carboxy" or "carboxyl" or "hydroxycarbonyl" by itself or as part of another substituent refers to the group -CO₂H. Thus, a carboxyalkyl is an alkyl group as defined above having at least one substituent that is -CO₂H.

25 The term "alkoxycarbonyl" by itself or as part of another substituent refers to a carboxy group linked to an alkyl radical i.e. to form -C(=O)OR^e, wherein R^e is as defined above for alkyl.

The term "alkylcarbonyloxy" by itself or as part of another substituent refers to a -O-C(=O)R^e wherein R^e is as defined above for alkyl.

30 Whenever the term "substituted" is used in the present invention, it is meant to indicate that one or more hydrogens on the atom indicated in the expression using "substituted" is replaced with a selection from the indicated group, provided that the indicated atom's normal valency is not exceeded, and that the substitution results in a chemically stable compound, i.e. a compound
35 that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into a therapeutic agent.

Where groups may be optionally substituted, such groups may be substituted with once or more, and preferably once, twice or thrice. Substituents may be selected from, for example, the group

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comprising halogen, hydroxyl, oxo, nitro, amido, carboxy, amino, cyano haloalkoxy, and haloalkyl.

As used herein the terms such as “alkyl, aryl, or cycloalkyl, each being optionally substituted with” or “alkyl, aryl, or cycloalkyl, optionally substituted with” refers to optionally substituted alkyl, optionally substituted aryl and optionally substituted cycloalkyl.

Furthermore, where groups are divalent, i.e. have two single bonds for attachment to two other groups, each occurrence thereof may be present in either of both directions in the molecule, even if not specifically indicated in the structural formulae or definition of R groups. For example
10 $-(C=N-NH_2)-$ as part of X means that X may for example be represented by $-(C=N-NH_2)-$ or alternatively by the reverse orientation being $-(NH_2-C=N)-$.

In the context of the present invention, the term lipid is meant to be a chemically defined
15 substance that is insoluble in water but soluble in amongst others alcohol, ether and chloroform. Ionizable or cationic lipids are lipids that are typically composed of three sections: an amine head group, a linker moiety and a hydrophobic tail. The term “ionizable” (or alternatively cationic) in the context of a compound or lipid means the presence of any uncharged group in said compound or lipid which is capable of becoming positively charged by receiving an ion (usually
20 an H^+ ion). Alternatively, any uncharged group in said compound or lipid may receive an electron and thus becoming negatively charged.

Accordingly, and in order to obtain their lipid character, the compounds of the present invention comprise a lipid tail being represented by R_1 and R_2 , wherein the total number of C atoms for
25 both groups combined is, at least 8, such as at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19 or at least 20. For example, in the context of the present invention, R_1 may contain 3 C atoms, while R_2 may contain 5 C atoms, thereby the total number of C atoms for both groups combined is at least 8. This also means that each hydrocarbon tail do not need to be identical, while in a specific
30 embodiment, they may be identical to each other.

Accordingly, in a specific embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein, wherein:

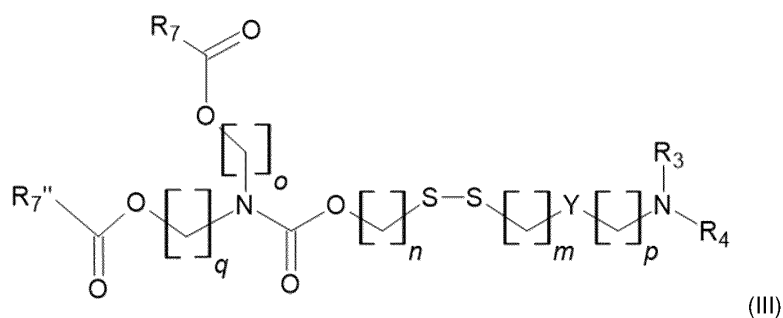
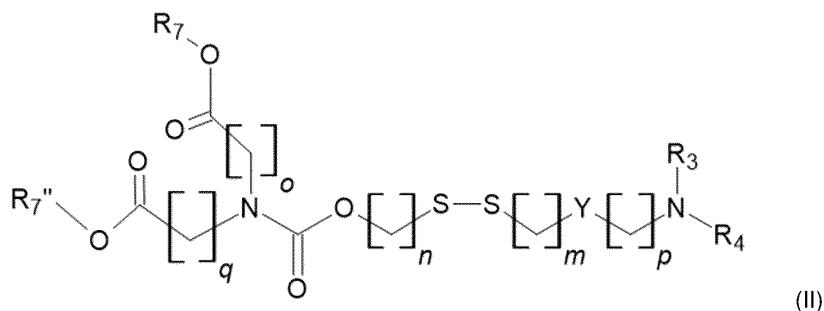
R_1 and R_2 are each independently selected from $-H$, $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$; wherein each
35 of said $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$ optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-C(O)O-C_{1-20}alkyl$, and $-C(O)O-C_{2-20}alkenyl$; wherein each of said $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-C(O)O-C_{1-20}alkyl$, and $-C(O)O-C_{2-20}alkenyl$ optionally comprises one or more additional heteroatoms and/or is

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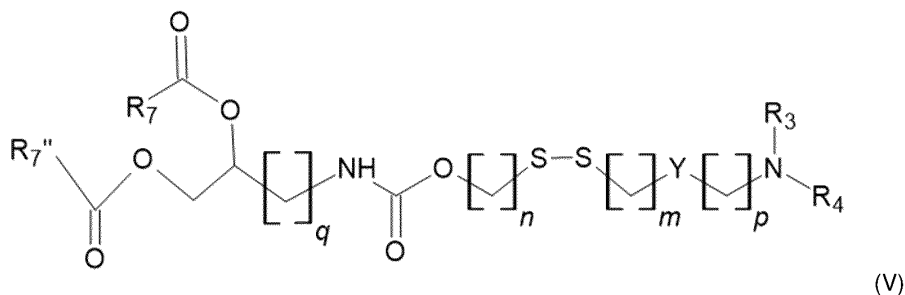
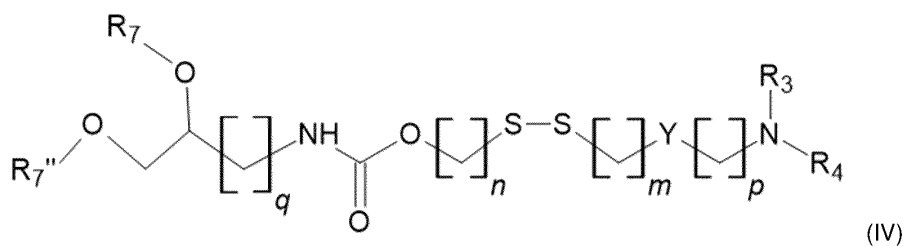
optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂ together is at least 8;

- 5 R₃ and R₄ are each independently -C₁₋₆alkyl;
 or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- 10 R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆";
 R₆ and R₆" are each independently selected from -H, and -C₁₋₆alkyl;
 or R₆ and R₆" taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;
- m, n and p are each independently an integer selected from 1, 2, 3 and 4;
- 15 Y is selected from -O-, -OC(O)-, -NR₅-, and -NH-C(O)-.

In yet a further embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein and being represented by any one of formula (II), (III), (IV) or (V)



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wherein

- each occurrence of R_7 and R_7'' is independently selected from $-\text{C}_{1-20}\text{alkyl}$, $-\text{C}_{2-20}\text{alkenyl}$, $-\text{C}_{2-20}\text{alkynyl}$; wherein each of said $-\text{C}_{1-20}\text{alkyl}$, $-\text{C}_{2-20}\text{alkenyl}$, $-\text{C}_{2-20}\text{alkynyl}$ may optionally be substituted with from 1-3 substituents selected from $-\text{OH}$, $-\text{OC(O)-C}_{1-20}\text{alkyl}$, $-\text{OC(O)-C}_{2-20}\text{alkenyl}$, $-\text{OC(O)-C}_{2-20}\text{alkynyl}$, $-\text{C(O)O-C}_{1-20}\text{alkyl}$, $-\text{C(O)O-C}_{2-20}\text{alkenyl}$, $-\text{C(O)O-C}_{2-20}\text{alkynyl}$; and the total number of C atoms in R_7 and R_7'' together is at least 6;
- R_3 and R_4 are each independently $-\text{C}_{1-6}\text{alkyl}$; wherein each of said $-\text{C}_{1-6}\text{alkyl}$ is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from $-\text{halo}$, $-\text{C}_{1-6}\text{alkyl}$, $-\text{C}_{3-6}\text{cycloalkyl}$, OH , $-\text{O-C}_{1-6}\text{alkyl}$, $-\text{S-C}_{1-6}\text{alkyl}$, and $-\text{NH}_2$; $-\text{C}_{1-6}\text{alkyl}$; wherein each of said $-\text{C}_{1-6}\text{alkyl}$ is optionally and independently substituted with a phenyl substituent;
- or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected from $-\text{C}_{1-6}\text{alkyl}$, and $-\text{C}_{1-6}\text{alkyl-OH}$;
- R_5 is $-\text{H}$ or $-\text{C}_{1-6}\text{alkyl}$; wherein said $-\text{C}_{1-6}\text{alkyl}$ may optionally be substituted with $-\text{NR}_6\text{R}_6''$;
- R_6 and R_6'' are each independently selected from $-\text{H}$, and $-\text{C}_{1-6}\text{alkyl}$;
- or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-\text{C}_{1-6}\text{alkyl}$, and $-\text{C}_{1-6}\text{alkyl-OH}$;
- m , n , and p are each independently an integer selected from 1, 2, 3 and 4
- q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
- Y is selected from $-\text{O}-$, $-\text{OC(O)-}$, $-\text{C(O)O-}$, $-\text{NR}_5-$, $-\text{NH-C(O)-}$, and $-\text{C(O)-NH-}$.

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The present invention further provides a lipid, in particular an ionizable lipid as defined herein, wherein:

each occurrence of R_7 and R_7'' is independently selected from $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$; wherein each of said $-C_{1-20}alkyl$, and $-C_{2-20}alkenyl$ may optionally be substituted with from 1-3 substituents selected from $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-C(O)O-C_{1-20}alkyl$, and $-C(O)O-C_{2-20}alkenyl$; and the total number of C atoms in R_7 and R_7'' together is at least 6;

R_3 and R_4 are each independently $-C_{1-6}alkyl$;

or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from $-C_{1-6}alkyl$, and $-C_{1-6}alkyl-OH$;

R_5 is $-H$ or $-C_{1-6}alkyl$; wherein said $-C_{1-6}alkyl$ may optionally be substituted with $-NR_6R_6''$;

R_6 and R_6'' are each independently selected from $-H$, and $-C_{1-6}alkyl$;

or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;

m , n , and p are each independently an integer selected from 1, 2, 3 and 4

q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

Y is selected from $-O-$, $-OC(O)-$, $-NR_5-$, and $-NH-C(O)-$.

In another specific embodiment, the moiety $-NR_3R_4$ may be selected from the list comprising: $-N-(C_{1-6}alkyl)_2$ or an N-containing non-aromatic heterocycle using said N atom as a point of attachment. In a very specific embodiment, the moiety $-NR_3R_4$ may be selected from the list comprising: $-N-(CH_3)_2$, $-pyrrolidinyl$, $-piperidinyl$, $-morpholinyl$, or $-azepanyl$; in particular $-N-(CH_3)_2$, $-morpholinyl$ or $-azepanyl$.

In another very specific embodiment, the present invention provides a compound according to any of the formulae or variants thereof as disclosed therein, wherein one or more of the following applies:

R_1 and R_2 are each independently selected from $-H$, $-C_{1-20}alkyl$, $-C_{2-20}alkenyl$ and $-C_{2-20}alkynyl$; wherein each of said $-C_{1-20}alkyl$, $-C_{2-20}alkenyl$ and $-C_{2-20}alkynyl$ optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-OC(O)-C_{2-20}alkynyl$, $-C(O)O-C_{1-20}alkyl$, $-C(O)O-C_{2-20}alkenyl$, and $-C(O)O-C_{2-20}alkynyl$; wherein each of said $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-OC(O)-C_{2-20}alkynyl$, $-C(O)O-C_{1-20}alkyl$, $-C(O)O-C_{2-20}alkenyl$, and $-C(O)O-C_{2-20}alkynyl$ optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-OH$, $-OC(O)-C_{1-20}alkyl$, $-OC(O)-C_{2-20}alkenyl$, $-OC(O)-C_{2-20}alkynyl$, $-C(O)O-C_{1-20}alkyl$, $-C(O)O-C_{2-20}alkenyl$, and $-C(O)O-C_{2-20}alkynyl$; and wherein the total number of C atoms in R_1 and R_2 together is at least 8;

In particular: R₁ and R₂ are each independently selected from -H, -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl;
 wherein each of said -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl optionally further comprises one or more
 heteroatoms and/or is optionally and independently substituted with from 1-3 substituents
 independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C₁₋₂₀alkyl, and
 5 -C(O)O-C₂₋₂₀alkenyl; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-
 C₁₋₂₀alkyl, and -C(O)O-C₂₋₂₀alkenyl optionally comprises one or more additional heteroatoms
 and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C<sub>1-
 20</sub>alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl,
 and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂ together is
 10 at least 8;

each occurrence of R₇ and R₇" is independently selected from -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl, -C<sub>2-
 20</sub>alkynyl; wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl, -C₂₋₂₀alkynyl may optionally be
 substituted with from 1-3 substituents selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl,
 -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, -C(O)O-C₂₋₂₀alkynyl; and the
 15 total number of C atoms in R₇ and R₇" together is at least 6;

In particular each occurrence of R₇ and R₇" is independently selected from -C₁₋₂₀alkyl, and -C<sub>2-
 20</sub>alkenyl; wherein each of said -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl may optionally be substituted
 with from 1-3 substituents selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C<sub>1-
 20</sub>alkyl, and -C(O)O-C₂₋₂₀alkenyl; and the total number of C atoms in R₇ and R₇" together is
 20 at least 6;

R₃ and R₄ are each independently -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and
 independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-
 membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S
 and/or optionally and independently substituted with from 1-3 substituents selected from -
 25 halo, -C₁₋₆alkyl, -C₃₋₆cycloalkyl, OH, -OC₁₋₆alkyl, -SC₁₋₆alkyl, and -NH₂; -C₁₋₆alkyl; wherein
 each of said -C₁₋₆alkyl is optionally and independently substituted with a phenyl substituent;

In particular R₃ and R₄ are each independently -C₁₋₆alkyl;

or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered
 aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or
 30 more additional heteroatoms and/or is optionally substituted with from 1-3 substituents
 selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

In particular R₃ and R₄ may be taken together with the N atom to which they are attached form
 a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further
 comprises one or more additional N or O atoms and/or is optionally substituted with from 1-
 35 3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆";

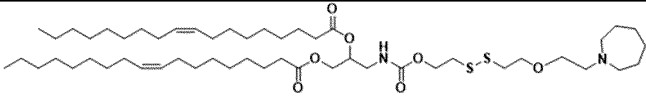
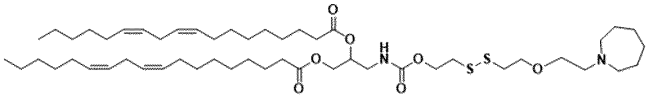
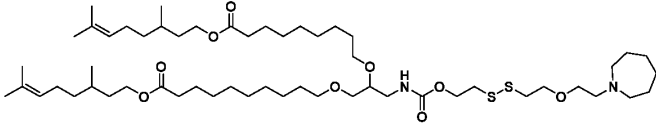
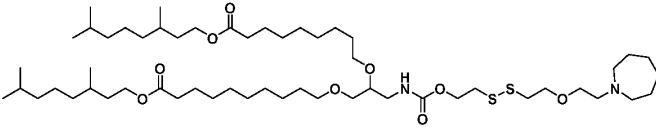
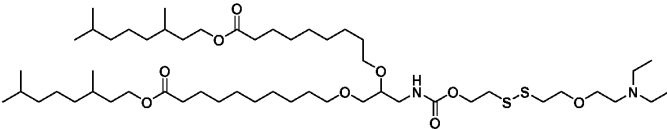
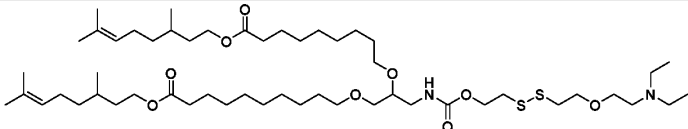
R₆ and R₆" are each independently selected from -H, and -C₁₋₆alkyl;

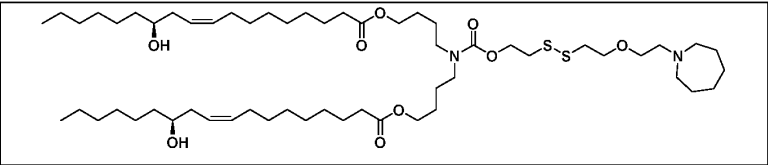
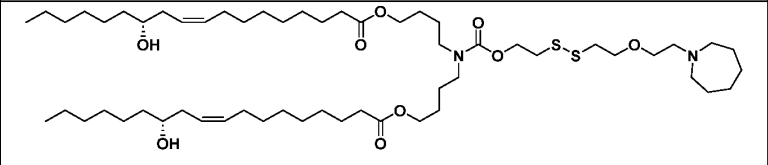
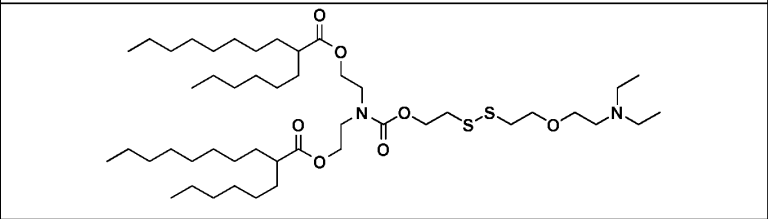
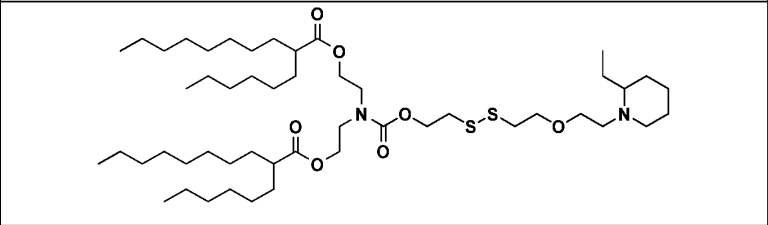
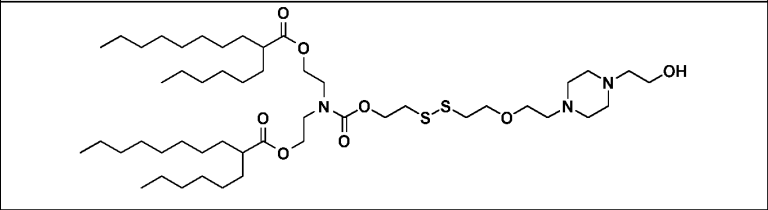
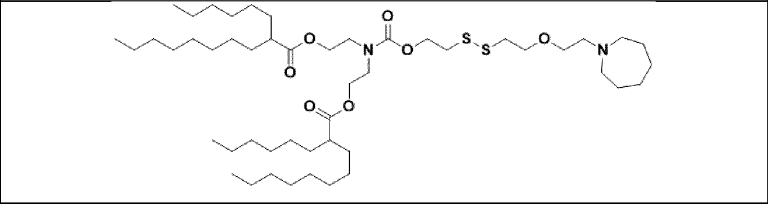
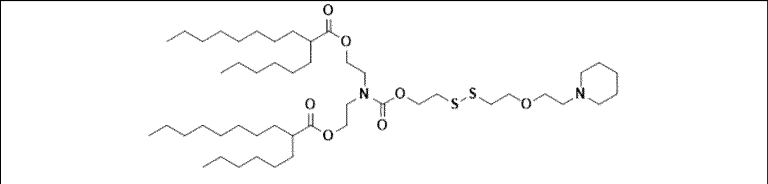
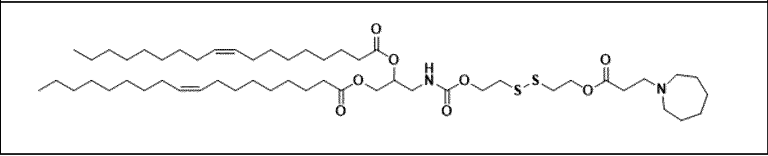
or R₆ and R₆" taken together with the N atom to which they are attached form a 5-10 membered
 aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or

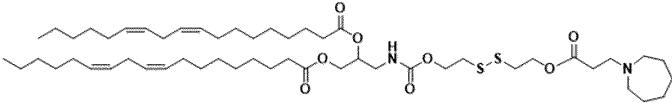
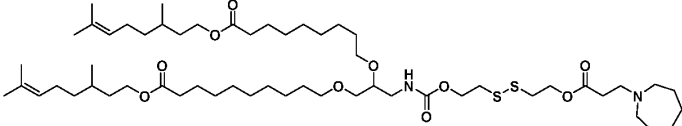
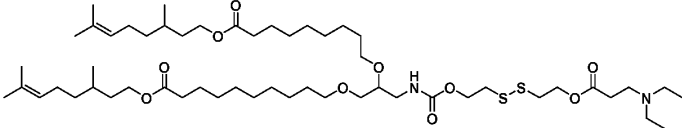
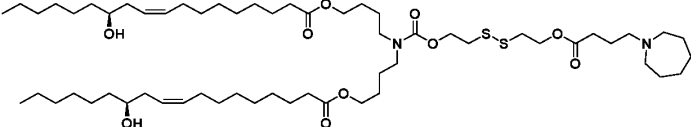
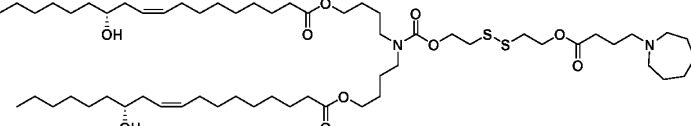
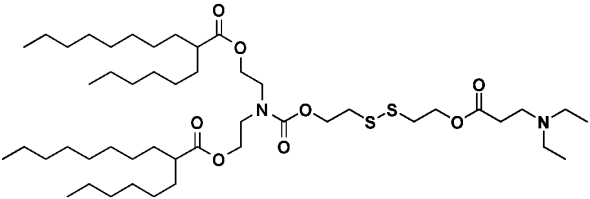
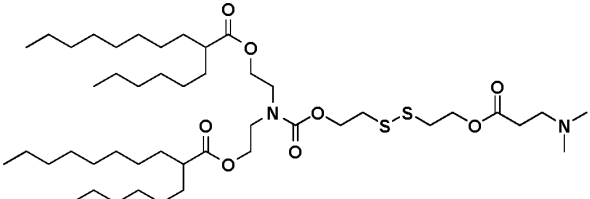
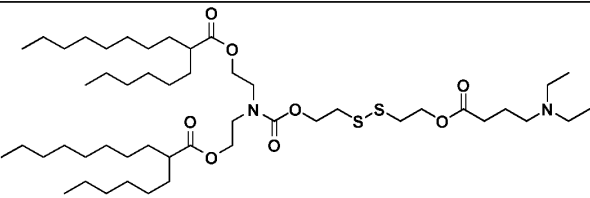
more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

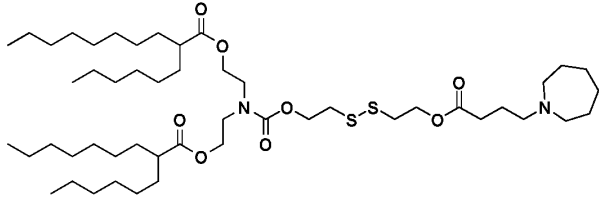
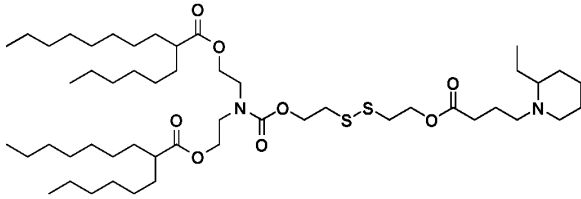
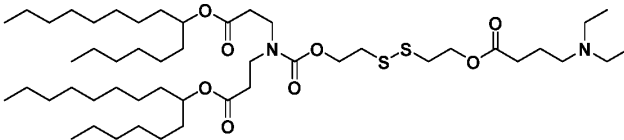
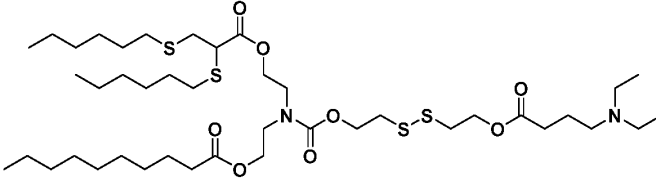
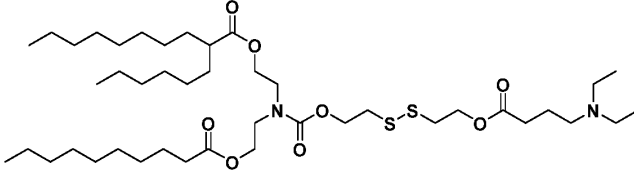
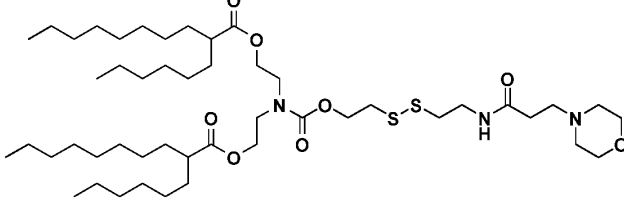
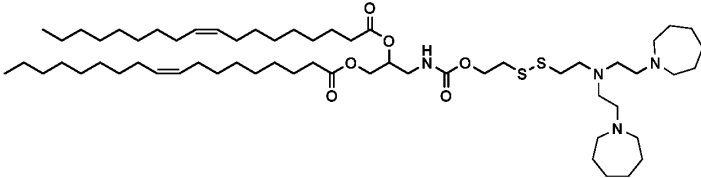
- In particular R₆ and R₆'' taken together with the N atom to which they are attached form a 5-10
5 membered aromatic or non-aromatic heterocycle;
m, n, and p are each independently an integer selected from 1, 2, 3 and 4
q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10;
in particular m, n and p are each 2;
in particular q is 1;
10 in particular o is 2, 3 or 4;
Y is selected from -O-, -OC(O)-, -C(O)O-, -NR₅-, -NH-C(O)-, and -C(O)-NH-;
In particular Y is selected from -O-, -OC(O)-, -NR₅-, and -NH-C(O)-.

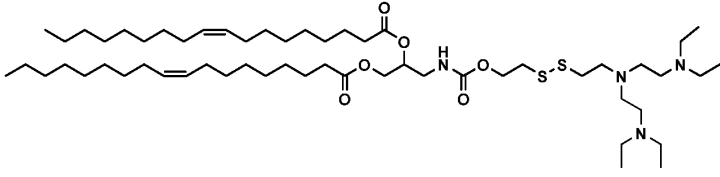
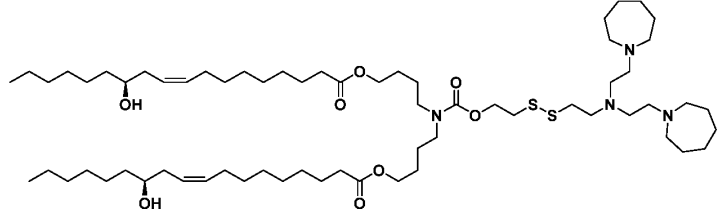
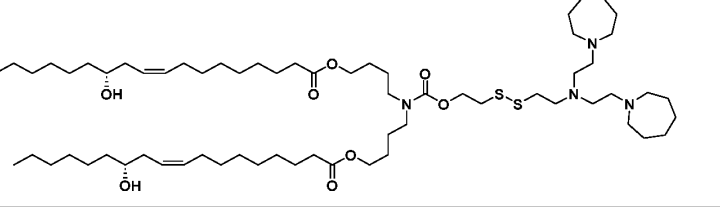
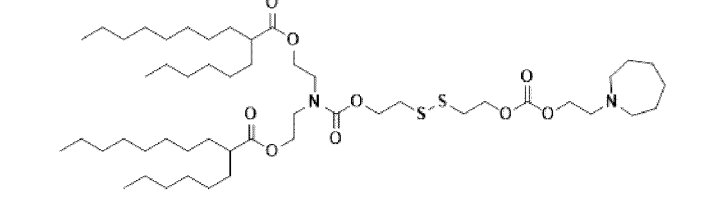
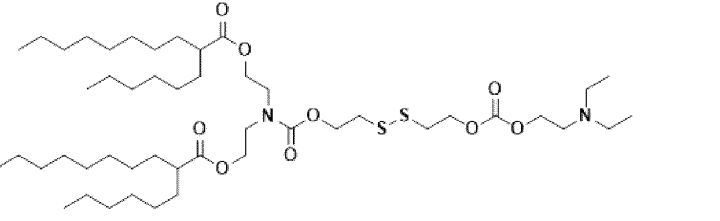
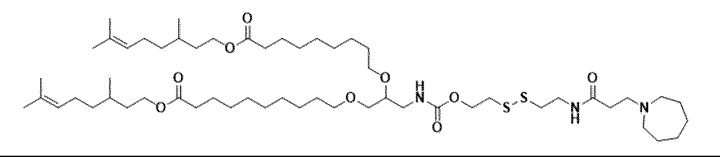
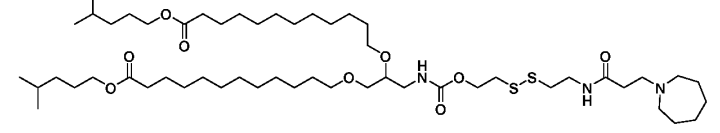
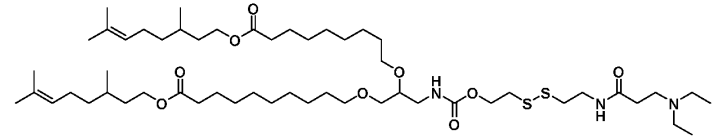
- The present invention further provides a lipid, in particular an ionizable lipid as defined herein
15 and being selected from the list comprising:

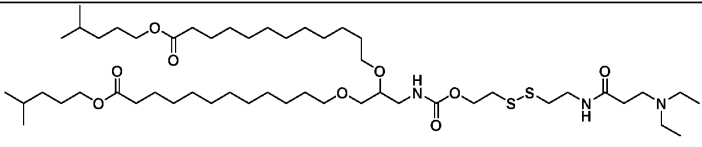
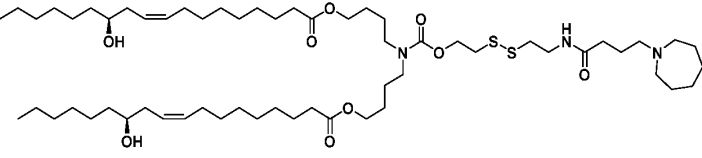
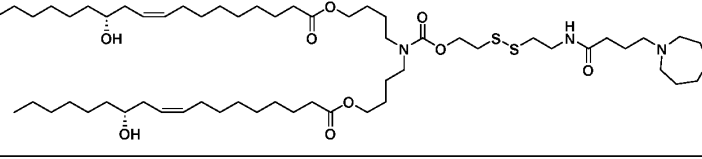
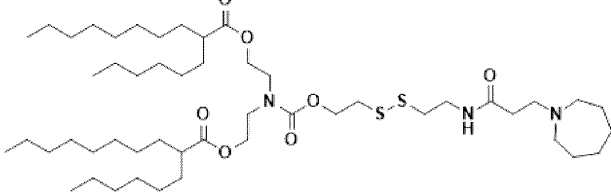
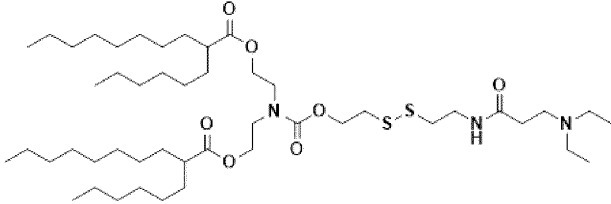
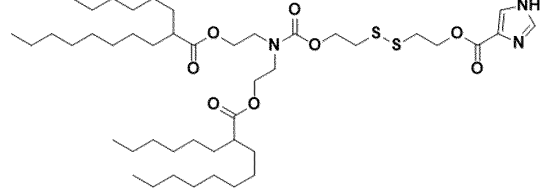
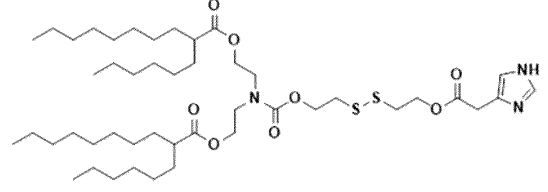
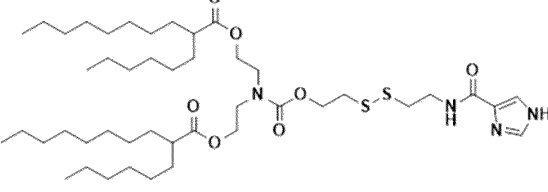
Lipid Name	Chemical Structure
IL-1	
IL-2	
IL-3	
IL-4	
IL-5	
IL-6	

IL-7	
IL-8	
IL-9	
IL-10	
IL-11	
IL-12	
IL-13	
IL-14	

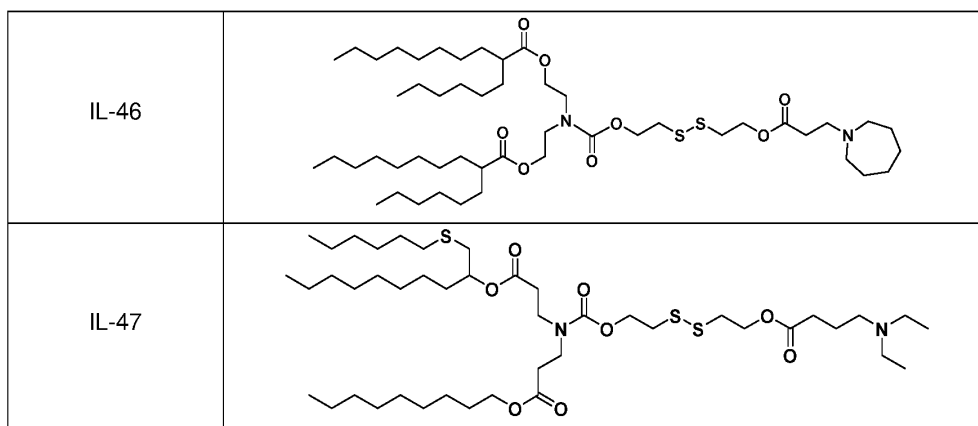
IL-15	
IL-16	
IL-17	
IL-18	
IL-19	
IL-20	
IL-21	
IL-22	

IL-23	
IL-24	
IL-25	
IL-26	
IL-27	
IL-28	
IL-29	

IL-30	
IL-31	
IL-32	
IL-33	
IL-34	
IL-35	
IL-36	
IL-37	

IL-38	
IL-39	
IL-40	
IL-41	
IL-42	
IL-43	
IL-44	
IL-45	

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All of the lipids as defined herein may occur as different isomers/stereoisomers. In particular, the lipids as defined herein may occur in the trans or cis configuration, such as when they contain double bonds. In a preferred embodiment, the lipids as defined herein occur in the cis configuration. In the context of the present invention, the term 'cis' indicates that the functional groups are on the same side of a plane, whereas 'trans' means that they are on opposite sides. As used herein, when a compound has one or more stereocenters, each stereocenter may have the R or S configuration, unless stated otherwise. The compound may therefore be a racemic mixture of enantiomers and/or diastereoisomers, or it may have an excess of one or more of the enantiomers and/or diastereoisomers, such as more than 60 %, more than 70 %, more than 80 %, more than 85 %, more than 90 %, more than 95 %, more than 98 %, more than 99 %.

In yet a further embodiment, the present invention provides a lipid, in particular an ionizable lipid as defined herein; wherein the total number of C atoms in R₁ and R₂ together is at least 14, such as at least 15, at least 17, at least 18, at least 19 or at least 20.

The present invention further provides a lipid, in particular an ionizable lipid as defined herein; wherein the total number of C atoms in R₇ and R₇' together is at least 12.

It was in particular found that by varying different aspects of the lipid structures disclosed herein, finetuning to obtain desired pKa can be achieved. For example, increasing pKa values are typically found between various Y moieties as follows: carbonate < ester < ether < amide. Also linear amides at position -NR₃R₄ were found to result in higher pKa values compared to circular moieties at that position. Furthermore, ester moieties may degrade faster compared to amide moieties which were found to be more stable. Hence, depending on a requirement for fast or slow degradation, these moieties may be varied accordingly.

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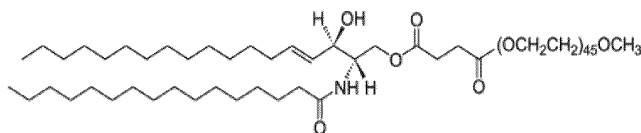
In a further aspect, the present invention provides a lipid nanoparticle or lipid nanoparticle composition comprising a lipid, in particular an ionizable lipid as defined herein.

In the context of the present invention, the term lipid nanoparticle (LNP), also termed solid lipid nanoparticles, is meant to be a nanoparticle comprising lipids. They are often used as a pharmaceutical drug delivery system or pharmaceutical formulation. LNPs as drug delivery vehicle were first approved in 2018, and are currently used in several candidate RNA based vaccines. A lipid nanoparticle is typically spherical with an average diameter between 10 and 1000 nanometers, and possesses a lipid core matrix that can solubilize lipophilic molecules. The term lipid is used here in a broader sense and includes triglycerides, diglycerides, monoglycerides, fatty acids, steroids (e.g. cholesterol) and waxes. Biological membrane lipids such as phospholipids, sphingomyelins, bile acids and sterols are typically used as stabilizers in LNPs.

As used herein, the term "nanoparticle" refers to any particle having a diameter making the particle suitable for systemic, in particular intravenous administration, of, in particular, nucleic acids, typically having a diameter of less than 1000 nanometers (nm), preferably less than 500 nm, even more preferably less than 200 nm, such as for example between 50 and 200 nm; preferably between 60 and 160 nm.

Accordingly, in the context of the present invention, the nanoparticles as disclosed herein further comprise one or more additional lipids either or not acting as stabilizers, such as a phospholipid, a sterol and/or a PEG lipid.

In the context of the present invention, the term "PEG lipid" or alternatively "PEGylated lipid" is meant to be any suitable lipid modified with a PEG (polyethylene glycol) group. Particularly suitable PEG lipids in the context of the present invention are characterized in being C18-PEG lipids, C14-PEG lipids (e.g. DMG-PEG or DMG-PEG2000), C16-PEG lipids, C14-ceramide PEG lipids, C18-ceramide PEG lipids or or C16-ceramide PEG lipids (such as C16 ceramide PEG2000 - N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}).

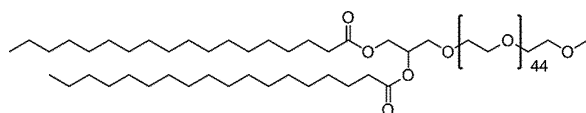


C16-ceramide PEG2000

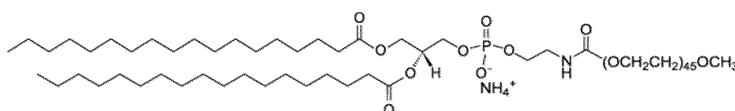
C18-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 18 C-atoms. In a particular embodiment, said C18-PEG2000 lipid is selected from the list comprising: a (distearoyl-based)-PEG2000 lipid such as

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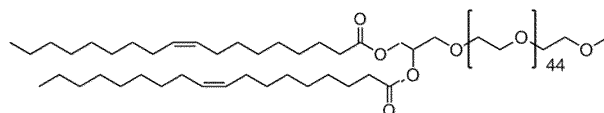
DSG-PEG2000 lipid (2-distearoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000) or DSPE-PEG2000 lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]); or a (dioleoyl-based)-PEG2000 lipid such as DOG-PEG2000 lipid (1,2-Dioleoyl-*rac*-glycerol) or DOPE-PEG2000 lipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]).



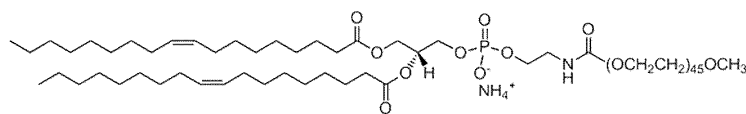
DSG-PEG2000



DSPE-PEG2000

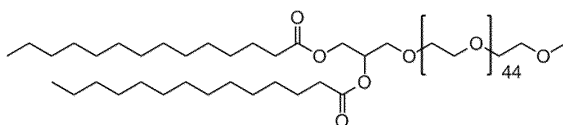


DOG-PEG2000

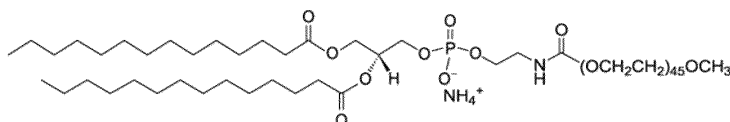


DOPE-PEG2000

C14-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 14 C-atoms. In a particular embodiment, said C14-PEG2000 lipid is based on dimyristoyl, i.e. having 2 C14 tails, such as selected from the list comprising: a (dimyristoyl-based)-PEG2000 lipid such as DMG-PEG2000 lipid (1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000) or 2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine glycol-2000 (DMPE-PEG2000).



DMG-PEG2000



DMPE-PEG2000

In the context of the present invention, the term "phospholipid" is meant to be a lipid molecule consisting of two hydrophobic fatty acid "tails" and a hydrophilic "head" consisting of a phosphate group. The two components are most often joined together by a glycerol molecule, hence, the

phospholipid of the present invention is preferably a glycerol-phospholipid. Furthermore, the phosphate group is often modified with simple organic molecules such as choline (i.e. rendering a phosphocholine) or ethanolamine (i.e. rendering a phosphoethanolamine).

- 5 Suitable phospholipids within the context of the invention can be selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-diundecanoyl-*sn*-glycero-3-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 (OChemPC), 1-hexadecyl-*sn*-glycero-3-phosphocholine (C 16 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, 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate (DOCP), (1,2-dioleoyl-*sn*-glycero-3-phosphate sodium salt (18:1PA) and mixtures thereof.

In a more specific embodiment, said phospholipid is selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and mixtures thereof.

In the context of the present invention, the term "sterol", also known as steroid alcohol, is a subgroup of steroids that occur naturally in plants, animal and fungi, or can be produced by some bacteria. In the context of the present invention, any suitable sterol may be used, such as selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

In a specific embodiment of the present invention one or more of the following applies:

- said LNP comprises about and between 25 mol% and 65 mol% of said ionizable lipid;
 - said LNP comprises about and between 5 mol% and 45 mol% of said phospholipid;
 - said LNP comprises about and between 0.5 mol% and 5.0 mol% of said PEG lipid;
- balanced by the amount of said sterol.

The N:P ratio of the composition refers to the molar ratio of nitrogen atoms in one or more lipids to the number of phosphate groups in an RNA. In general, a lower N:P ratio is preferred. The one or more RNA, lipids, and amounts thereof may be selected to provide an N:P ratio from about 2:1 to about 30:1, such as 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22:1, 24:1, 26:1, 28:1, or 30:1. In certain embodiments, the N:P ratio may be from about 2:1 to about 10:1. In other embodiments, the N:P ratio is from about 4:1 to about 8:1. For example, the N:P ratio may be about 6:1, alternatively the N:P ratio may be about 10:1.

In yet a further embodiment of the present invention, the lipid nanoparticle or lipid nanoparticle composition as defined herein further comprises a cargo molecule such as a pharmaceutically active agent (e.g. small molecule) or a biomolecule, such as a peptide, protein or a nucleic acid. In a particular embodiment, the cargo may be a nucleic acid, such as DNA or RNA; preferably mRNA. In another particular embodiment, the cargo may be a TLR agonist, such as for example the TLR3 agonist polyI:C, or the TLR9 agonist CpG.

Prior to being loaded in the lipid nanoparticles, the cargo molecules may further be modified to induce an overall polyanionic nature to the molecules. This can for example be done by bonding them to a Glu10 moiety as exemplified in the examples part. The Glu10 moiety is a moiety of 10 glutamic acids which increases the polyanionic nature of the molecule to which it is attached.

Accordingly, the lipid nanoparticles and lipid nanoparticle compositions of the present invention are particularly suitable for the intracellular delivery of their cargo molecules. Hence, the present invention provides the use of the lipid nanoparticles and lipid nanoparticle compositions as defined herein for the intracellular delivery of cargo molecules.

In a particular embodiment, the lipid nanoparticle or lipid nanoparticle composition as defined herein further comprises a nucleic acid, preferably mRNA.

A "nucleic acid" in the context of the invention is a deoxyribonucleic acid (DNA) or preferably a ribonucleic acid (RNA), more preferably mRNA. Nucleic acids include according to the invention genomic DNA, cDNA, circRNA, tRNA, mRNA, recombinantly produced and chemically synthesized molecules. A nucleic acid may according to the invention be in the form of a molecule which is single stranded or double stranded and linear or closed covalently to form a circle. A nucleic acid can be employed for introduction into, i.e. transfection of cells, for example, in the form of RNA which can be prepared by in vitro transcription from a DNA template. The RNA can moreover be modified before application by stabilizing sequences, capping and/or polyadenylation.

In the context of the present invention, the term "RNA" relates to a molecule which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. The term includes double stranded RNA, single stranded RNA, isolated
5 RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also
10 comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs. Nucleic acids may be comprised in a vector. The term "vector" as used herein includes any vectors known to the skilled person including plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as adenoviral or baculoviral vectors, or artificial
15 chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial or analogs of naturally-occurring RNA.

According to the present invention, the term "RNA" includes and preferably relates to "mRNA" which means "messenger RNA" and relates to a "transcript" which may be produced using DNA as template and encodes a peptide or protein. mRNA typically comprises a 5' untranslated
20 region (5' -UTR), a protein or peptide coding region and a 3' untranslated region (3'-UTR). mRNA has a limited halftime in cells and in vitro. Preferably, mRNA is produced by in vitro transcription using a DNA template. In one embodiment of the invention, the RNA is obtained by in vitro transcription or chemical synthesis. The in vitro transcription methodology is known to the skilled person. For example, there is a variety of in vitro transcription kits commercially available.

25 In a further aspect, the present invention provides a pharmaceutical composition comprising one or more LNP's as defined herein and a pharmaceutically acceptable agent, such as a carrier, excipient,.... Such pharmaceutical compositions are particularly suitable as a vaccine. Thus, the invention also provides a vaccine comprising one or more LNP's according to the present invention.

30 In the context of the present invention, the term "vaccine" as used herein is meant to be any preparation intended to provide adaptive immunity (antibodies and/or T cell responses) against a disease. To that end, a vaccine as meant herein contains at least one nucleic acid molecule, e.g. mRNA molecule encoding an antigen to which an adaptive immune response is mounted.
35 This antigen can be present in the format of a weakened or killed form of a microbe, a protein or peptide, or an antigen encoding a nucleic acid. An antigen in the context of this invention is meant to be a protein or peptide recognized by the immune system of a host as being foreign,

thereby stimulating the production of antibodies against is, with the purpose of combating such antigens. Vaccines can be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (example, to actively treat or reduce the symptoms of an ongoing disease). The administration of vaccines is called vaccination.

- 5 The vaccine of the invention may be used for inducing an immune response, in particular an immune response against a disease-associated antigen or cells expressing a disease-associated antigen, such as an immune response against cancer. Accordingly, the vaccine may be used for prophylactic and/or therapeutic treatment of a disease involving a disease-associated antigen or cells expressing a disease-associated antigen, such as cancer.
- 10 Preferably said immune response is a T cell response. In one embodiment, the disease-associated antigen is a tumor antigen. The antigen encoded by the RNA comprised in the nanoparticles described herein preferably is a disease-associated antigen or elicits an immune response against a disease-associated antigen or cells expressing a disease-associated antigen.
- 15 The present invention also provides the LNP's, pharmaceutical compositions and vaccines according to this invention for use in human or veterinary medicine. The use of the LNP's, pharmaceutical compositions and vaccines according to this invention for human or veterinary medicine is also intended. Finally, the invention provides a method for the prophylaxis and treatment of human and veterinary disorders, by administering the LNP's, pharmaceutical compositions and vaccines according to this invention to a subject in need thereof. Such pharmaceutical compositions are particularly suitable in various fields such as prophylactic vaccines, therapeutic vaccines, protein replacement therapies, gene editing, gene silencing, small molecule delivery, etc.
- 20
- 25 The present invention further provides the use of an LNP, a pharmaceutical composition or a vaccine according to the present invention for the immunogenic delivery of said one or more nucleic acid molecules. As such the LNP's, pharmaceutical compositions and vaccine of the present invention are highly useful in the treatment several human and veterinary disorders. Thus, the present invention provides the LNP's, pharmaceutical compositions and vaccines of
- 30 the present invention for use in the treatment of cancer or infectious diseases.

The lipid nanoparticles of the present invention may be prepared in accordance with the protocols as specified in the Examples part. More generally, the LNP's may be prepared using a method comprising:

- 35
- preparing a first alcoholic composition comprising said ionizable lipid, said phospholipid, said sterol, said PEG lipid, and a suitable alcoholic solvent;
 - preparing a second aqueous composition comprising said one or more nucleic acids and

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an aqueous solvent;

- mixing said first and second composition in a microfluidic mixing device.

5 In further detail, the lipid components are combined in suitable concentrations in an alcoholic vehicle such as ethanol. Thereto, an aqueous composition comprising the nucleic acid is added, and subsequently loaded in a microfluidic mixing device.

10 The aim of microfluidic mixing is to achieve thorough and rapid mixing of multiple samples (i.e. lipid phase and nucleic acid phase) in a microscale device. Such sample mixing is typically achieved by enhancing the diffusion effect between the different species flows. Thereto several microfluidic mixing devices can be used, such as for example reviewed in Lee et al., 2011. A particularly suitable microfluidic mixing device according to the present invention is the NanoAssemblr from Precision Nanosystems.

15 Other technologies suitable for preparing the LNP's of the present invention include dispersing the components in a suitable dispersing medium, for example, aqueous solvent and alcoholic solvent, and applying one or more of the following methods: ethanol dilution method, a simple hydration method, sonication, heating, vortex, an ether injecting method, a French press method, a cholic acid method, a Ca^{2+} fusion method, a freeze-thaw method, a reversed-phase
20 evaporation method, T-junction mixing, Microfluidic Hydrodynamic Focusing, Staggered Herringbone Mixing, and the like.

25 The ionizable lipids of the present invention can be prepared according to the reaction schemes provided in the examples hereinafter, but those skilled in the art will appreciate that these are only illustrative for the invention and that the compounds of this invention can be prepared by any of several standard synthetic processes commonly used by those skilled in the art of organic chemistry.

EXAMPLESPREPARATION OF THE LIPIDS5 **1. General information**

Unless otherwise stated, all glassware was oven dried before use and all reactions were carried out under an argon atmosphere using standard Schlenk-techniques. Dry solvents were purchased from Acros Organics or Sigma-Aldrich and used without further purification. All reagents were purchased from commercial sources and were used without further purification unless otherwise stated. Reaction progress was monitored by thin layer chromatography (TLC) performed on aluminum plates coated with Kieselgel F254 with 0.2 mm thickness. Visualization was achieved by ultraviolet light (254 nm) or by staining with either ninhydrine, cerium molybdate or potassium permanganate. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck and co.). Mass spectra were obtained using a Finnigan MAT 8200 (70 eV), an Agilent 5973 (70 eV), using electrospray ionization (ESI) or electron impact ionization (EI). All ¹H NMR, ¹³C NMR were recorded on a Bruker AV-400 in Chloroform-d₁ or DMSO-d₆. Chemical shifts are given in parts per million (ppm), referenced to tetramethylsilane using the solvent peak as internal standard (CDCl₃: ¹H = 7.26 ppm, ¹³C = 77.16 ppm; CD₃SOCD₃: ¹H = 2.50 ppm, ¹³C = 39.52 ppm). Coupling constants were quoted in Hz. ¹H NMR splitting patterns were designated as singlet (s), broad (brd), doublet (d), triplet (t), quartet (q), quintet (qu), pentet (p), sextet (se), septet (sep), octet (o) or combinations thereof. Splitting patterns that could not be interpreted were designated as multiplet (m).

GC-MS were measured on an Agilent System with the following conditions:

25 Column: HP-5MS 30 m, 0.25 mm ID, 0.25 μm
Flow: 2.0 mL/min. He
Injection: 1 μl @ 100 μl/s; 250 °C; Split Flow: 20 mL/min. Split Ratio: 10
Detection: Agilent 5977E Source: EI; FID (300 °C; H₂:
30 mL/min.; Air: 400 mL/min. Makeup: 25 mL/min. He)
30 Gradient: 0 min. 50 °C; 0.5 min. 50 °C; 35 °C/min. 300 °C; 2 min.

UHPLC were measured on Agilent System with the following conditions:

Column: ACQUITY UHPLC BEH C18 (1.7 μm) 2.1 mm x 50 mm
Temperature: 40 °C
35 Detection: DAD + ELSD + 6120 Quadrupole
Flow: 0.6 mL/min.
Solvent A: Water + 0.1% formic acid
Solvent B: ACN + 0.1% formic acid

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Gradient: 0 min. 2% B; 0.5 min. 2% B; 4.0 min. 98% B; 4.8 min.
98% B; 5.0 min. 2% B

5 Column: ACQUITY UHPLC BEH C18 (1.7 μ m) 2.1 mm x 50 mm
Temperature: 40 °C
Detection: DAD + ELSD + 6120 Quadrupole
Flow: 0.8 mL/min.
Solvent A: Water + 0.1% formic acid
Solvent B: ACN + 0.1% formic acid
10 Gradient: 0 min. 75% B; 0.2 min. 75% B; 2 min. 100% B; 8 min.
100% B; 8.1 min. 75% B

Column: ACQUITY UHPLC BEH C18 (1.7 μ m) 2.1 mm x 50 mm
Temperature: 40 °C
15 Detection: DAD + ELSD + 6120 Quadrupole
Flow: 0.7 mL/min.
Solvent A: Water + 0.05% formic acid
Solvent B: ACN + 0.05% formic acid
Gradient: 0 min. 70% B; 0.5 min. 70% B; 3.0 min. 98% B; 5.0 min.
20 98% B; 5.1 min. 70% B; 6.0 min. 70% B

Column: Nucleodur Phenyl Hexyl (1.8 μ m) 2.0 mm x 100 mm
Temperature: 40 °C
Detection: DAD + ELSD + 6120 Quadrupole
25 Flow: 0.5 mL/min.
Solvent A: Water + 0.1% TFA
Solvent B: ACN + 0.1% TFA
Gradient: 0 min. 10% B; 0.5 min. 10% B; 10.0 min. 98% B; 15.0 min.
98% B; 15.1 min. 10% B

30 Column: Waters XBridge Phenyl (4.6 x150 mm; 3.5 μ m)
Solvent A: 10 mM NH₄OAc (Water/Methanol/Acetonitrile 900/60/40)
Solvent B: 10 mM NH₄OAc (Water/Methanol/Acetonitrile 100/540/360)
Gradient: Solvent A/Solvent B: 20/80 (0 min) --> (4.0 min) --> 0/100 (41.0 min)

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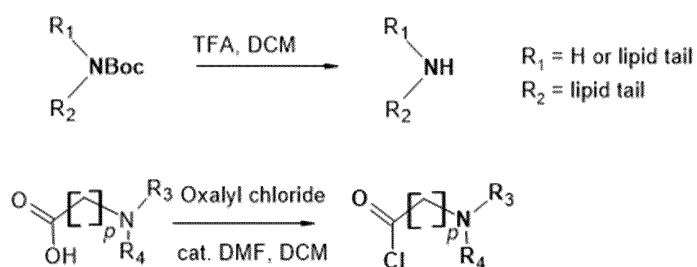
All compounds are named using autonomenclature.

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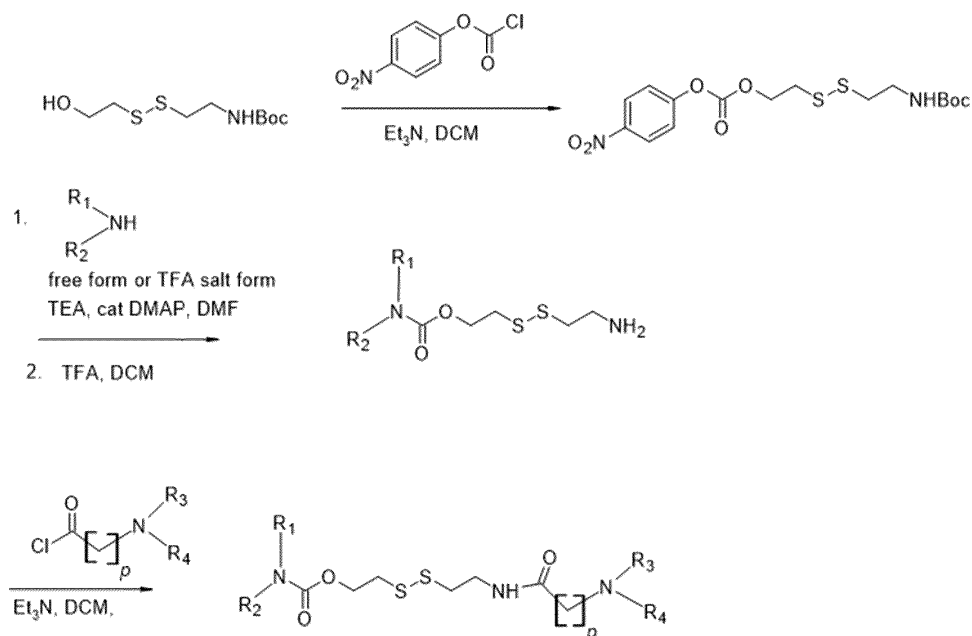
2. Synthesis of lipids

Note: in the below synthesis schemes n and m are each 2, both can be easily varied to other values where applicable.

5 PREPARATION OF PARTICULAR REAGENTS USED IN SOME OF THE FOLLOWING REACTION SCHEMES

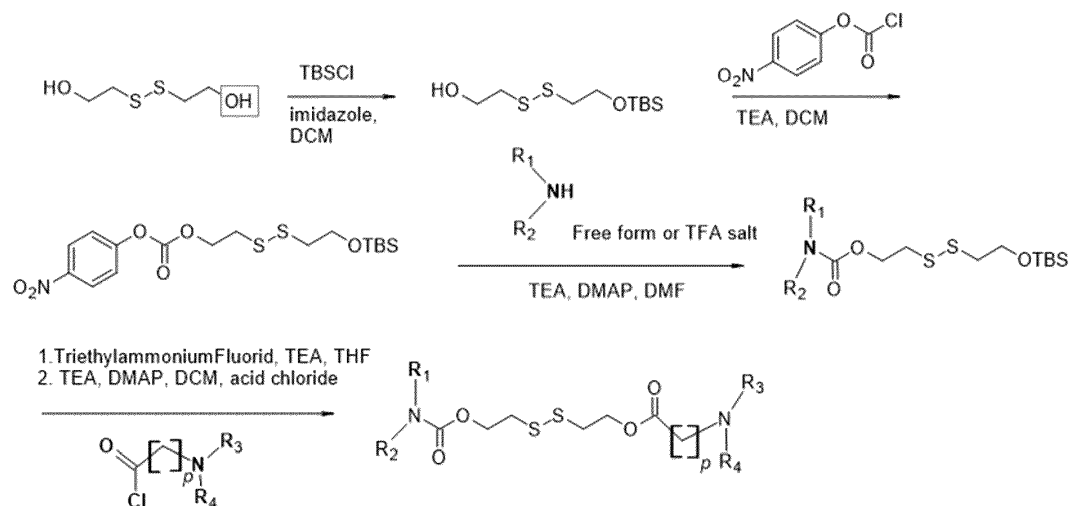


GENERAL SYNTHESIS SCHEME FOR COMPOUNDS IN WHICH Y IS -NH-C(O)-



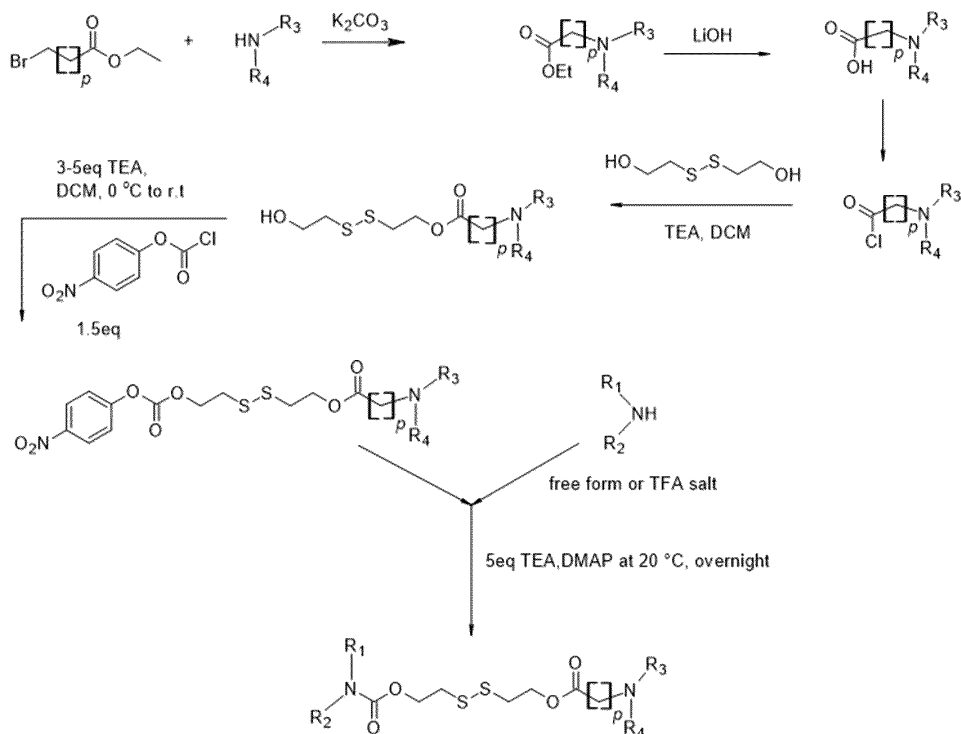
GENERAL SYNTHESIS SCHEME FOR COMPOUNDS IN WHICH Y IS -OC(O)-

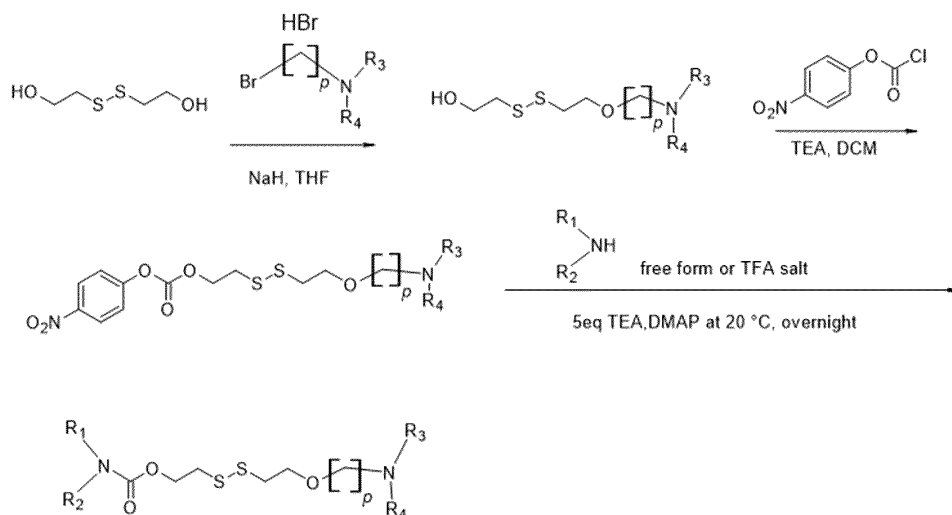
OPTION 1



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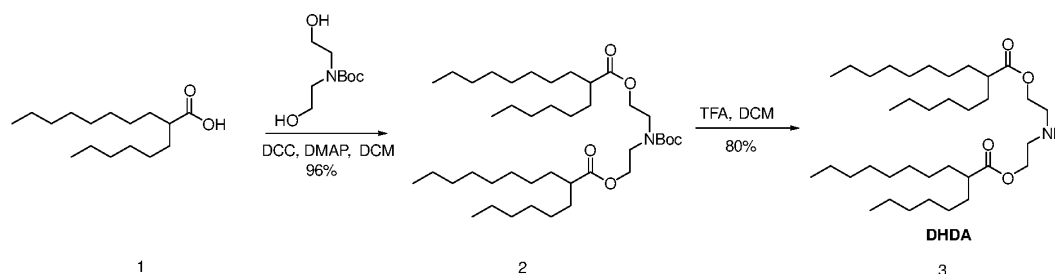
OPTION 2



GENERAL SYNTHESIS SCHEME FOR COMPOUNDS IN WHICH Y IS -O-EXAMPLARY IONIZABLE LIPIDS OF THE INVENTION

5

Herein below are provided detailed synthesis routes for ionizable lipids and intermediates used in the synthesis of exemplary ionizable lipids of the invention.

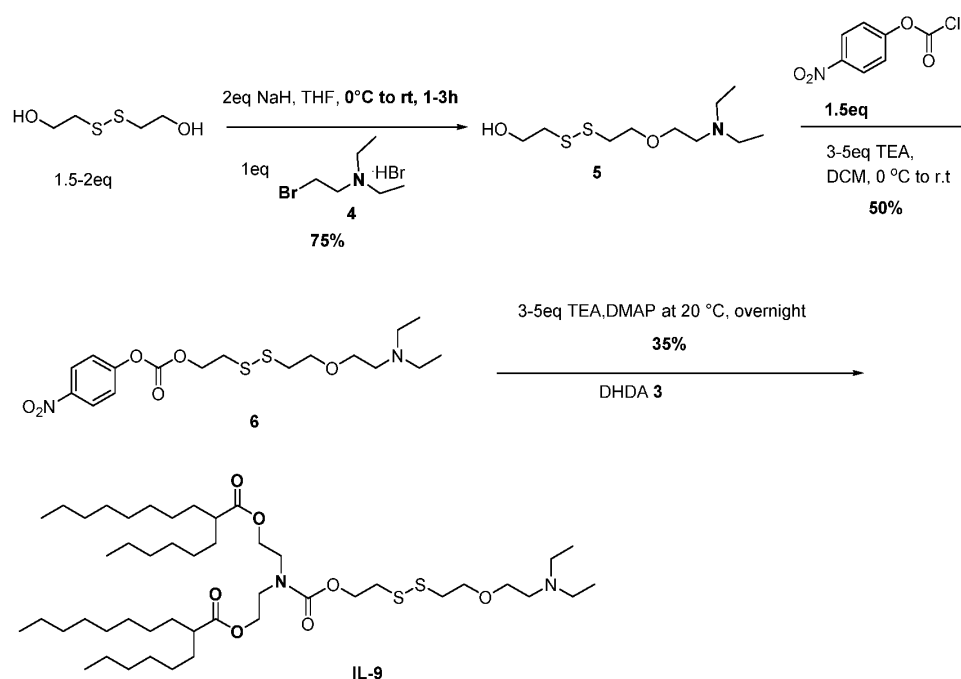


- 10 **Intermediate 2**; *tert*-butoxycarbonyl)azanediy)bis(ethane-2,1-diyl) bis(2-hexyldecanoate
- DCC (7.30g, 2.2eq, 35.4mmol) was added to the ice-cooled DCM (160 mL) solution of 2-hexyldecanoic acid (9.07g, 2.2eq, 35.4mmol). The RM was allowed to come to rt. *tert*-butyl bis(2-hydroxyethyl)carbamate (3.30g, 1eq, 16.1mmol) and DMAP (196mg, 0.1eq, 1.61mmol) were added to the RM, and stirred at rt for 18h. The reaction mixture (RM) was filtered over a P3 glass
- 15 filter, the solid was washed with 100 mL DCM and the filtrate was evaporated. The crude residue was dissolved in 250 mL TBME and stirred for 15 min at an ice-cooled temperature. The content was filtered off and the filtrate was evaporated. The crude product was purified by column chromatography (heptane/EtOAc gradient). Intermediate 2 (10.50g, 15.39mmol, 96%) was isolated as a colorless oil.

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Intermediate 3: Azanediybis(ethane-2,1-diyl) bis(2-hexyldecanoate) (DHDA)

Intermediate **2** (10.5 g, 1 Eq, 15.4 mmol) was dissolved in DCM (50 mL) and TFA (37 g, 25 mL, 21 eq, 0.32 mol) was added, and the mixture was stirred at room temperature (rt) for 2 hours, after which the mixture was evaporated thoroughly. The product TFA salt was dissolved in 150 mL DCM and washed with 2x100 mL NaHCO₃ (sat. aq.), dried over sodium sulfate and evaporated. Intermediate **3** (DHDA, 7.15 g, 12.3 mmol, 80 %) was isolated as a slightly yellow oil.

**Intermediate 5; 2-((2-(2-(diethylamino)ethoxy)ethyl)disulfaneyl)ethan-1-ol:**

2-bromo-*N,N*-diethylethylamine HBr (516mg, 96% wt, 1eq, 1.90mmol) and 2,2'-dithiodiethanol (760mg, 90% wt, 2.34eq, 4.43mmol) were dissolved in THF (15.8mL) and the mixture was cooled to 0 °C using an ice bath. Sodium hydride (159mg, 0.13 mL, 60% wt, 2.1 eq, 3.99mmol) was added slowly and when bubbling ceased the ice bath was removed and the mixture was stirred at room temperature for 4h. The RM was quenched with water, diluted with 20 mL DCM, and washed with 1x20 mL Na₂CO₃ (sat. aq.), 20 mL water, dried over sodium sulfate and evaporated. Purification was performed by column chromatography (DCM/MeOH gradient). Intermediate **5** (360mg, 1.42mmol, 74.8%) was obtained as a pale-yellow oil.

Intermediate 6; 2-((2-(2-(diethylamino)ethoxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate
Intermediate **5** (360 mg, 1 Eq, 1.42 mmol) and triethylamine (359mg, 495μL, 2.5eq, 3.55mmol) were dissolved in DCM (1.9mL) and the solution was cooled to 0 °C using an ice-water bath. A solution of 4-nitrophenyl carbonochloridate (344mg, 1.2eq, 1.70mmol) in DCM (1.9mL) was

-47-

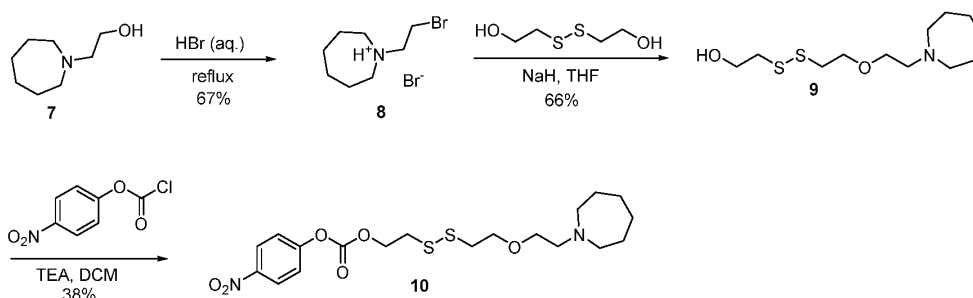
added dropwise at 0°C over 15 minutes. The RM was diluted with 50 mL DCM and washed with 2x50 mL 0.5M HCl, 3x50 mL Na₂CO₃ (sat. aq.), 50 mL water, dried over sodium sulfate and evaporated. Purification was performed by column chromatography (DCM/MeOH gradient). Intermediate **6** (300mg, 717µmol, 50.5%) was obtained as a yellow oil.

5

IL-9; 15-ethyl-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4-oxo-5,12-dioxo-8,9-dithia-3,15-diazaheptadecyl 2-hexyldecanoate

Intermediate **6** (114mg, 1.0eq, 273µmol) was dissolved in 1mL DMF and triethylamine (82.9mg, 114µL, 3eq, 819µmol) was added, followed by DMAP (3.34mg, 0.1eq, 27.3µmol). DHDA **3** (380mg, 2.0eq, 546µmol) in 1 mL DMF was added and the mixture was stirred at room temperature over 3 days. The reaction mixture was evaporated, and the residue was re-dissolved in 50 mL TBME and washed with 2x50mL Na₂CO₃ (sat. aq.), 2x50mL 3% LiCl (aq.), dried over sodium sulfate and evaporated. Purification was performed by column chromatography (DCM/MeOH gradient). **IL-9** (83 mg, 96 µmol, 35 %) was obtained as a pale-yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.28 (t, J = 6.5Hz, 2H), 4.12 (q, J = 5.18Hz, 4H), 3.70-3.60 (m, 4H), 3.58-3.41 (m, 4H), 2.91-2.67 (m, 10H), 2.33-2.20 (m, 2H), 1.58-1.42 (m, 4H), 1.42-1.30 (m, 4H), 1.28-1.05 (m, 46H), 0.89-0.71 (m, 12H); ¹³C NMR (75 MHz, Chloroform-*d*): 176.2, 155.5, 69.3, 63.4, 62.3, 61.9, 51.7, 47.6, 47.5, 46.9, 45.7, 38.6, 37.5, 32.3, 31.8, 31.7, 29.7, 29.6, 29.4, 29.3, 29.2, 27.5, 27.4, 22.7, 22.6, 14.1, 14.0, 10.4. MS: 861.60 m/z [M+H]

20



Intermediate 8; 1-(2-Bromoethyl)azepan-1-ium bromide

2-(Azepan-1-yl)ethan-1-ol, **7** (10.00g, 1eq, 69.82mmol) was dissolved in aqueous HBr (35.31g, 23.86mL, 48% wt, 3eq, 209.5mmol) and the RM was heated to 175 °C (ext. temp.). The flask was fitted with a distillation head with a collecting flask. The distillation head was insulated with aluminum foil and distillates were collected for 6 hours until no more distillate was observed. The RM was allowed to cool to room temperature, after which a large lump of brown solid remained in the flask. The solid was broken into pieces and triturated with 250 mL acetone and agitated by sonication until a homogeneous suspension of grey solid in brown filtrate was obtained. The resulting grey solid was collected by filtration, washed with acetone and dried under vacuo. Intermediate **8** (13.52g, 47.10mmol, 67%) was isolated as a grey solid.

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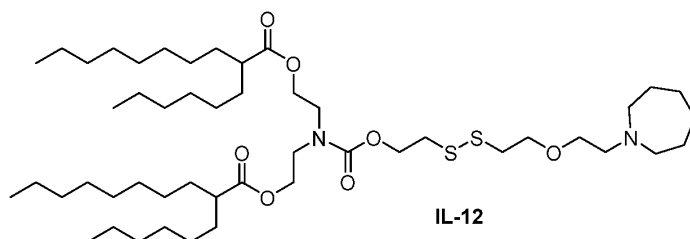
-48-

Intermediate 9; 2-((2-(2-(Azepan-1-yl)ethoxy)ethyl)disulfaneyl)ethan-1-ol

Intermediate 9 was prepared from intermediate 8 in an analogy to intermediate 5. The crude was purified by column chromatography (DCM/MeOH gradient, later again with DCM/MeOH containing 2M ammonia to obtain intermediate 9 (161mg, 576μmol, 66.1%) as a slightly yellow oil.

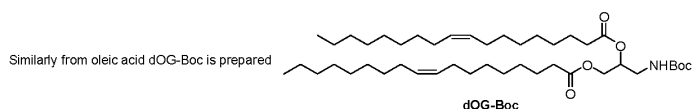
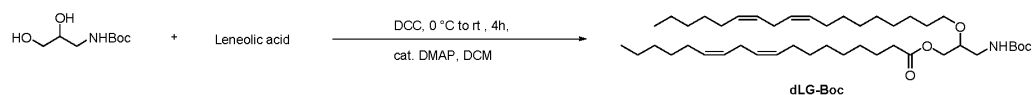
Intermediate 10; 2-((2-(2-(Azepan-1-yl)ethoxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate

Intermediate 9 (161 mg, 1 Eq, 576 μmol) was dissolved in DCM (5.0 mL) and the mixture was cooled to 0 °C using an ice bath. 4-nitrophenyl carbonochloridate (139mg, 1.2eq, 691 μmol) was added at 0 °C. After 5 minutes the ice bath was removed, and the mixture was stirred at room temperature for 3 hours. The mixture was diluted with 25 mL DCM and washed with 2x25 mL half-saturated Na₂CO₃ (aq.), 25 mL water. DCM layer was dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient 0-10% MeOH). 2-((2-(2-(Azepan-1-yl)ethoxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate (10, 86 mg, 0.19 mmol, 34 %) was obtained as a yellow oil.

IL-12; 14-(Azepan-1-yl)-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4-oxo-5,12-dioxa-8,9-dithia-3-azatetradecyl 2-hexyl decanoate

IL-12 was prepared from the intermediate 3 and intermediate 10 using the method employed IL-

9. The crude product was purified by column chromatography (DCM/MeOH gradient). IL-12 (40mg, 45μmol, 31%) was obtained as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.28 (t, J = 6.6Hz, 2H), 4.24-4.13 (m, 4H), 3.86 (bs, 2H), 3.71 (t, J = 6.12Hz, 2H), 3.60-3.49 (m, 4H), 3.21-3.09 (bs, 6H), 2.91 (t, J = 6.6Hz, 2H), 2.86 (t, J = 6.1Hz, 2H), 2.36-2.25 (m, 2H), 1.96-1.80 (bs, 4H), 1.76-1.63 (bs, 4H), 1.62-1.48 (m, 4H), 1.48-1.35 (m, 4H), 1.35-1.15 (m, 40H), 0.84 (t, 4x3H = 12H). MS: 887.40 m/z [M+H].



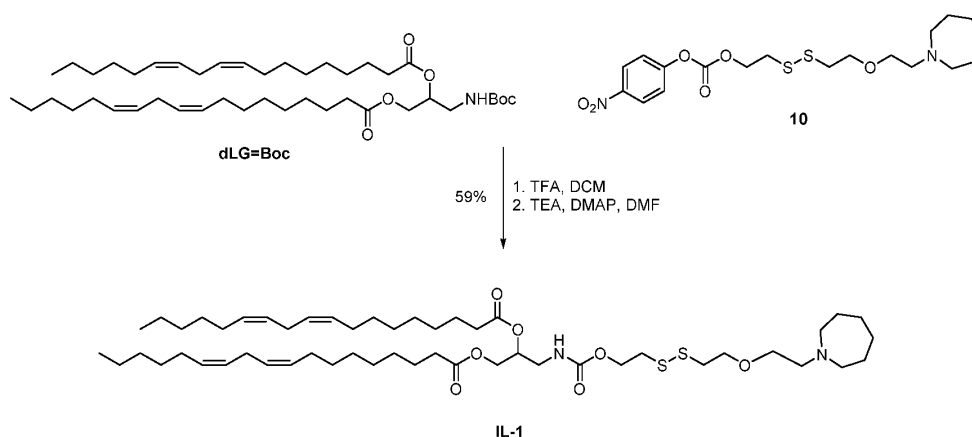
-49-

dLG-Boc; 3-((tert-butoxycarbonyl)amino)propane-1,2-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate)

Linoleic acid (8.2g, 2.2eq, 29mmol) was dissolved in DCM (100mL) under nitrogen atmosphere and the mixture was cooled to 0 °C using an ice-water bath. DCC (5.9g, 2.2eq, 29mmol) was added and the mixture was stirred at 0°C for 5min, the ice-water bath was removed and the mixture was stirred at rt for 10 min. *tert*-Butyl (2,3-dihydroxypropyl)carbamate (2.5g, 1eq, 13mmol) and DMAP (0.17 g, 0.11 Eq, 1.4 mmol) were added and the mixture was stirred at rt for 4 h. Heptane (100 mL) was added and the resulting suspension was filtered and washed with heptane. The filtrate was concentrated *in vacuo* and the crude product was purified by column chromatography (0-15% TBME/heptane gradient). The titled compound dLG-Boc (8.36g, 11.7mmol, 89%) was obtained as a colorless oil.

General procedure for the coupling of dOG and dLG lipids to intermediate 10 (Lipid-3 and Lipid-4).

3-((tert-butoxycarbonyl)amino)propane-1,2-diyl dioleate/dilinoleate (**dOG-Boc** or **dLG-Boc**, 900 mg, 1 Eq, 1.25 mmol) was dissolved in DCM (2.5mL) and TFA (3.7 g, 2.5 mL, 26 Eq, 32 mmol) was added. The RM was stirred at rt for 2 hours, after which volatiles were removed and dried under high vacuo. Meanwhile, intermediate **10** (722mg, 1.3eq, 1.62mmol) and triethylamine (632mg, 871μL, 5eq, 6.25mmol) were dissolved in DMF (5.0mL) and DMAP (15.3mg, 0.1eq, 125μmol) was added. The Boc deprotected lipid in DMF (5.0 mL) was added and RM was stirred at room temperature for 16h. The volatiles were removed, and the residue was dissolved in 50 mL TBME and washed with 4x50mL half-saturated Na₂CO₃ (aq.), 2x50mL 5% LiCl (aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient).



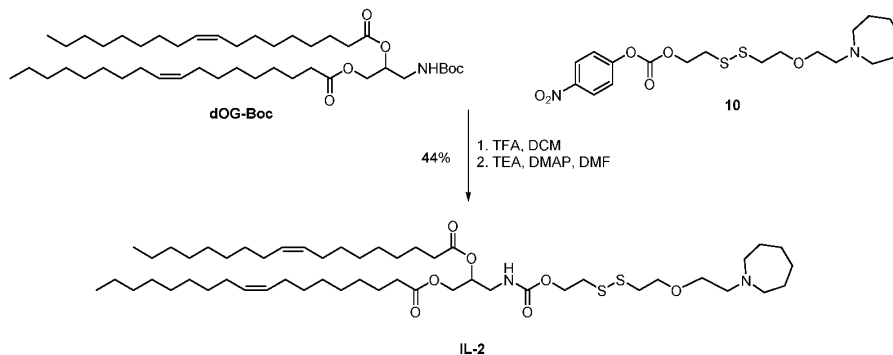
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IL-1; 1-(Azepan-1-yl)-11-oxo-3,10-dioxa-6,7-dithia-12-azapentadecane-14,15-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate)

IL-1 (678 mg, 736 μmol, 59 %) was isolated as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*):

-50-

5.42-5.30 (m, 8H), 5.15-5.30 (m, 2H), 4.30 (t, J = 6.4Hz, 2H), 4.26 (dd, J = 4.4, 11.7Hz, 1H), 4.12 (dd, J = 4.4, 11.7Hz, 1H), 3.69 (t, J = 6.2Hz, 2H), 3.62 (bs, 2H), 3.49-3.30 (m, 2H), 2.96-2.64 (m, 12H), 2.30 (app. dt, J = 1.7, 7.7Jz, 4H), 2.08-1.98 (m, 8H), 1.79-1.53 (m, 12H), 1.39-1.20 (m, 28H), 0.89 (t, 2x3H = 6H); MS: 921.60 m/z [M⁺].

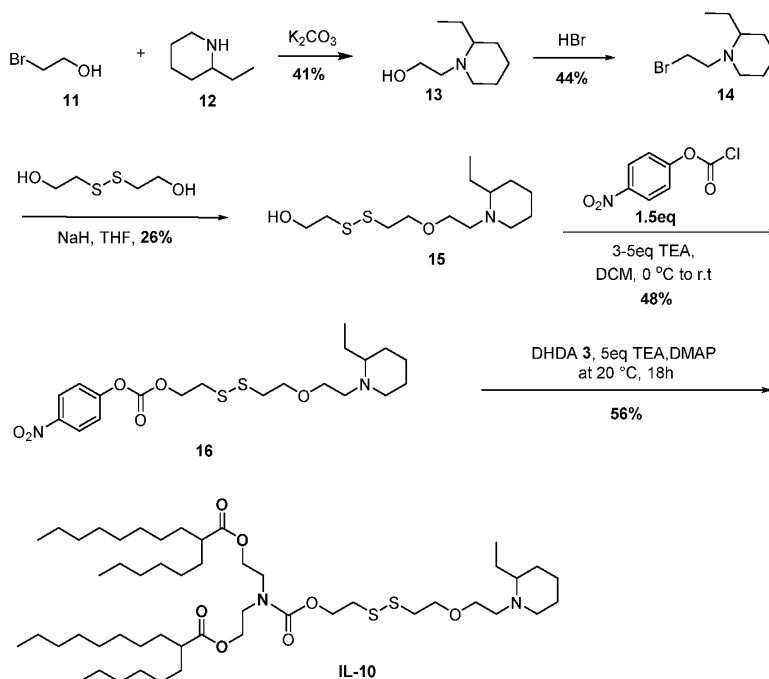


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IL-2; 1-(Azepan-1-yl)-11-oxo-3,10-dioxo-6,7-dithia-12-azapentadecane-14,15-diyl dioleate

IL-2 (514 mg, 555 μ mol, 44 %) was isolated as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 5.42-5.30 (m, 4H), 5.18-5.07 (m, 2H), 4.33 (t, J = 6.5Hz, 2H), 4.30 (dd, J = 5.6, 12.2Hz, 1H), 4.12 (dd, J = 5.6, 12.2Hz, 1H), 3.73 (t, J = 6.6Hz, 2H), 3.62 (bs, 2H), 3.53-3.34 (m, 2H), 3.08-2.96 (m, 10H), 2.30 (app. dt, J = 1.9, 7.3Hz, 4H), 2.08-1.98 (m, 8H), 1.79-1.53 (m, 12H), 1.41-1.20 (m, 40H), 0.90 (t, 2x3H = 6H); MS: 925.60 m/z [M⁺].

10



Intermediate 13; 2-(2-ethylpiperidin-1-yl)ethan-1-ol

To 2-ethyl piperidine **12** (3.0g, 3.5mL, 1eq, 27mmol) in acetonitrile (50 mL) was added potassium carbonate (11g, 4.7mL, 3eq, 80mmol) and ethylene bromohydrin **11** (3.6g, 2.1mL, 1.1eq, 29mmol). The reaction mixture was stirred at 85°C for 6h and then cooled and partitioned
5 between ethyl acetate (100 mL) and water (100 mL). The organic layer was washed with brine (100 mL), dried and evaporated to dryness to afford 2-(2-ethylpiperidin-1-yl)ethan-1-ol **13** (1.7 g, 11 mmol, 41 %), which was used in the next step without further purification.

Intermediate 14; 1-(2-bromoethyl)-2-ethylpiperidin-1-ium bromide

2-(2-ethylpiperidin-1-yl)ethan-1-ol **13** (1.7 g, 1eq, 11 mmol) was dissolved in aqueous HBr (5.5g, 3.7mL, 48% wt, 3eq, 32mmol) and the flask was fitted with a distillation head and a collection
10 flask. The mixture was heated to 175 °C (ext. temp.) and the distillation head was insulated with aluminium foil. During 2h of heating 3.2 mL of distillate was collected. The reaction mixture was allowed to cool to room temperature. The mixture was added to 50 mL acetone and agitated by sonication. The resulting grey solid was collected by filtration, washed with acetone, and dried
15 *in vacuo* to afford **14** (1.43 g, 4.75 mmol, 44%) as a grey solid.

Intermediate 15; 2-((2-(2-(2-ethylpiperidin-1-yl)ethoxy)ethyl)disulfaneyl)ethan-1-ol

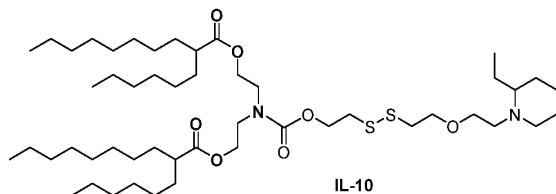
Suspension of 2,2'-dithiodiethanol (789.0mg, 2.2eq, 5.11mmol) and intermediate **14** (700.0mg, 1eq, 2.325mmol) in THF (15.5mL) was cooled to 0°C using an ice bath. NaH (279.0mg, 60% wt, 3eq, 6.97mmol) was added slowly. The RM was allowed to come to rt and stirred for another 2h
20 at rt. The RM was ice cooled and quenched with a few drops of water, filtered, and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient 0 to 35%). Fractions of the desired material were collected to obtain titled intermediate **15** (177mg, 603µmol, 25.9%) as a pale yellow oil.

Intermediate 16; 2-((2-(2-(2-ethylpiperidin-1-yl)ethoxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate

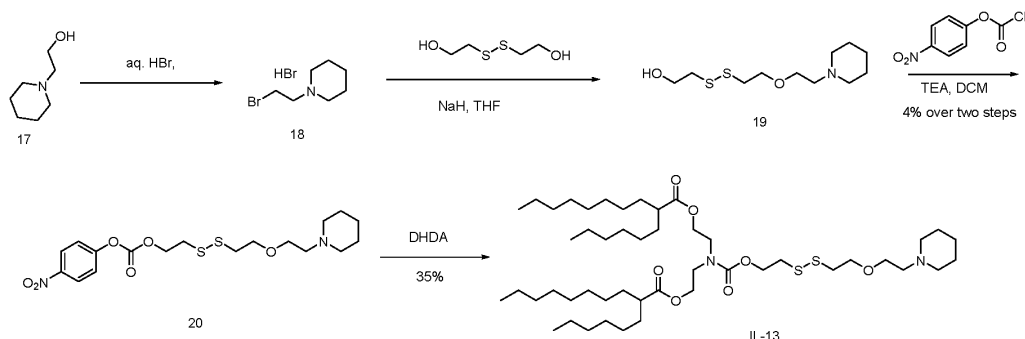
Intermediate **15** (216.0mg, 1eq, 736.0µmol) and triethylamine (186.2mg, 256µL, 2.5eq, 1.84mmol) were dissolved in DCM (1.5mL) and the solution was cooled to 0 °C using an ice-water bath. Solution of 4-nitrophenyl carbonochloridate **7** (222.5mg, 1.5eq, 1.10mmol) in DCM (1.5mL) was added dropwise at 0°C over 10min. After 1 hour of stirring at room temperature the
30 reaction mixture was diluted with DCM (100 mL), washed with Na₂CO₃ (sat. aq.) (2 x 100 mL), brine (2 x 100 mL), dried over sodium sulfate, and evaporated. The crude material was purified by column chromatography (DCM/MeOH gradient 0 to 10%) to obtain **16** (163.8mg, 357.2µmol, 48%) as a yellow oil.

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IL-10; 14-(2-ethylpiperidin-1-yl)-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4-oxo-5,12-dioxa-8,9-dithia-3-azatetradecyl 2-hexyldecanoate



Intermediate **16** (163.8mg, 1eq, 357.2μmol) was dissolved in DMF (0.9mL) and triethylamine (79.5mg, 110μL, 2.2eq, 785.8μmol) was added, followed by DMAP (4.36mg, 0.1eq, 35.7μmol). A solution of DHDA **3** (228.7mg, 1.1eq, 392.9μmol) in DMF (0.9 mL) was added and RM was stirred at room temperature over 3 days. The mixture was evaporated, and the residue was re-dissolved in 100 mL TBME and washed with 2x100 mL Na₂CO₃ (sat. aq.), 2x100 mL 3 wt% LiCl (aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient 0 to 10%). Pure fractions were collected to obtain **IL-10** (180 mg, 200 μmol, 55.9 %) as a pale yellow viscous oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.36 (t, J = 6.6Hz, 2H), 4.24-4.13 (m, 4H), 3.69 (t, J = 6.4Hz, 2H), 3.62-3.49 (m, 6H), 2.96-2.83 (m, 6H), 2.63-2.52 (m, 1H), 2.40-2.27 (m, 3H), 2.22 (bs, 1H), 1.73-1.50 (m, 9H), 1.50-1.36 (m, 5H), 1.35-1.15 (m, 42H), 0.93-0.81 (m, 15H). ¹³C NMR (100 MHz, Chloroform-*d*); 176.2, 155.5, 69.3, 68.9, 63.5, 62.3, 62.0, 61.9, 53.1, 52.3, 47.5, 46.9, 45.7, 38.6, 37.6, 32.3, 31.9, 29.6, 29.3, 29.2, 27.5, 27.4, 25.2, 24.3, 23.6, 22.7, 22.6, 14.11, 14.07, 10.0; MS: 901.60 m/z [M+H].



Intermediate 18; 1-(2-Bromoethyl)piperidine hydrobromide

To 2-(piperidin-1-yl)ethan-1-ol (9.7g, 10mL, 1eq, 75mmol) was added aq. hydrogen bromide (38g, 26mL, 48% wt, 3eq, 0.23mol), the flask was fitted with a distillation head with collection flask and reaction mixture was stirred for 2h at 180°C (ext. temp.). Water and volatiles were removed, the reaction mixture was allowed to cool down to room temperature, and acetone was added. The solid was triturated by adding 200ml of acetone and sonicated. The solid was filtrated off, filtrate was discarded, solid was dried *under vacuo* to obtain 1-(2-bromoethyl)piperidine **18** (7.2 g, 37 mmol, 50 %) as a grey solid.

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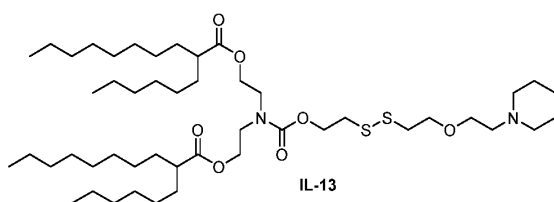
Intermediate 19; 2-((2-(2-(Piperidin-1-yl)ethoxy)ethyl)disulfaneyl)ethan-1-ol

2,2'-dithiodiethanol (5.76 g, 2 Eq, 37.4 mmol) and **18** were reacted to obtain **19** using conditions used to obtain **15**.

Intermediate 20; 4-Nitrophenyl (2-((2-(2-(piperidin-1-yl)ethoxy)ethyl)disulfaneyl)ethyl) carbonate

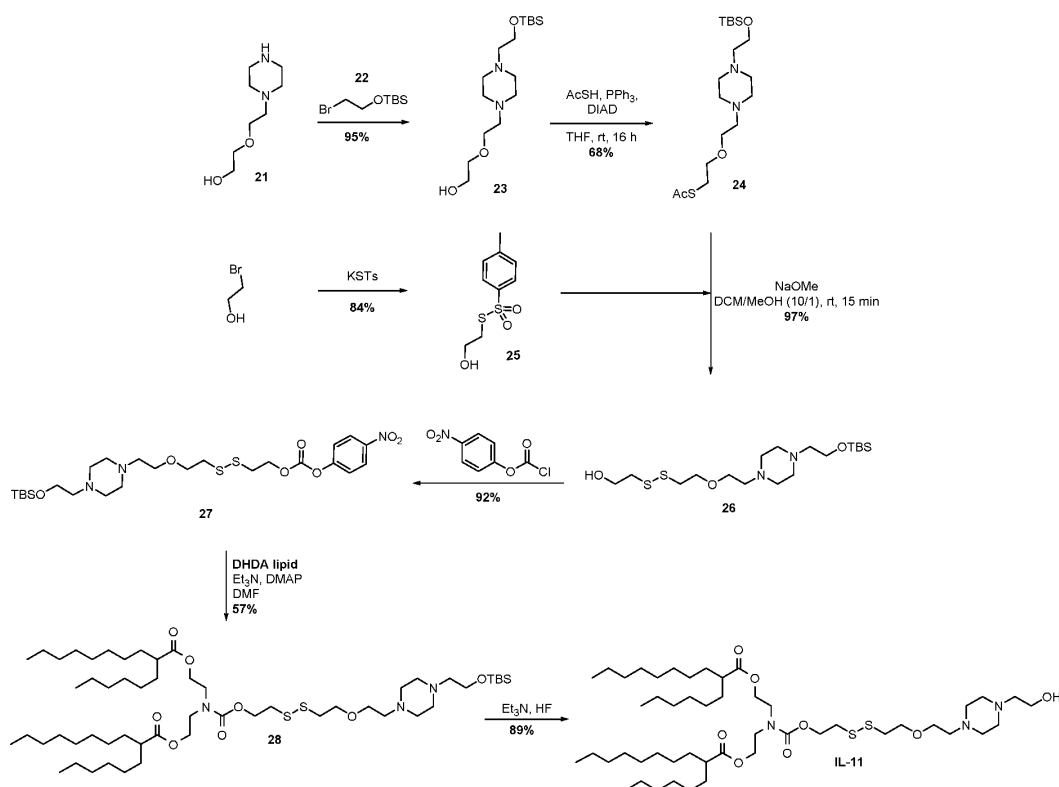
Intermediate **19** and 4-nitrophenyl carbonochloridate were reacted to obtain **20** under similar conditions which were used to obtain intermediate **16**.

IL-13; 3-(2-((2-hexyldecanoyl)oxy)ethyl)-4-oxo-14-(piperidin-1-yl)-5,12-dioxa-8,9-dithia-3-azatetradecyl 2-hexyldecanoate



Azanediybis(ethane-2,1-diyl) bis(2-hexyldecanoate), DHDA (**3**, 324 mg, 1 Eq, 557 μ mol) and **19** (312mg) were reacted to obtain **IL-13** (173 mg, 198 μ mol, 35.6 %) as a yellow oil using protocol similar to that was used for IL-10. ^1H NMR (400 MHz, Chloroform-*d*): 4.36 (t, J = 6.5Hz, 2H), 4.24-4.13 (m, 4H), 3.70 (t, J = 6.5Hz, 2H), 3.64 (t, J = 5.8Hz, 2H), 3.57 (t, J = 5.8Hz, 2H), 3.53 (t, J = 5.8Hz, 2H), 2.93 (t, J = 6.5Hz, 2H), 2.89 (t, J = 6.5Hz, 2H), 2.67-2.38 (m, 6H), 2.37-2.27 (m, 2H), 1.73-1.50 (m, 8H), 1.50-1.36 (m, 6H), 1.35-1.15 (m, 40H), 0.88 (t, J = 6.6Hz, 4x3H=12H). ^{13}C NMR (100 MHz, Chloroform-*d*): 176.3, 155.6, 77.2, 69.3, 68.7, 63.5, 62.3, 61.9, 58.4, 55.0, 53.4, 47.5, 46.9, 45.7, 38.6, 37.6, 32.3, 31.9, 31.7, 29.7, 29.6, 29.5, 29.32, 29.27, 27.5, 27.4, 25.7, 24.1, 22.7, 22.6, 14.13, 14.10; MS: 873.60 m/z [M^+].

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Intermediate 23; 2-(2-(4-(2-((tert-butyldimethylsilyl)oxy)ethyl)piperazin-1-yl)ethoxy)ethan-1-ol
 2-(2-(piperazin-1-yl)ethoxy)ethan-1-ol **21** (1.19g, 1.18eq, 6.83mmol), KI (920mg, 0.961eq,
 5.54mmol) and K_2CO_3 (1.20g, 1.51eq, 8.68mmol) were suspended in acetonitrile (23mL). (2-
 5 bromoethoxy)(tert-butyl)dimethylsilane **22** (1.38g, 1.20mL, 1eq, 5.77mmol) was added and the
 RM was refluxed for 16h. Volatiles were removed and aqueous work up was performed with 3/1
 sat. Na_2CO_3 /water (50mL), extract with DCM (3x 50mL), dried over Na_2SO_4 , filter, and
 concentrate *in vacuo* to obtain 1.83g of compound **23** as a colorless oil was obtained (95% yield).

Intermediate 24; S-(2-(2-(4-(2-((tert-butyldimethylsilyl)oxy)ethyl)piperazin-1-yl)ethoxy)ethyl)
 10 ethanethioate

PPh_3 (1.73g, 1.2eq, 6.60mmol) was dissolved in THF (22.0mL), cool to 0 °C and add DIAD
 (1.34g, 1.28mL, 1.2eq, 6.60mmol) was added dropwise. A solution of intermediate **23** (1.83g,
 1eq, 5.50mmol) and thioacetic acid (419mg, 395 μ L, 1eq, 5.50mmol) in 10 ml THF was prepared
 in a separate flask. After 15min the mixture of alcohol and AcSH was added to the DIAD and
 15 PPh_3 mixture. After completion of the addition, the cooling bath was removed and the resultant
 yellow solution was stirred at rt for 16h. The crude was purified by silica column, EtOAc/heptane
 (0-90% gradient) then 0-3% gradient of 7M NH_3 in MeOH/DCM. Intermediate **24** (1.46 g, 68%)
 was obtained as a yellow oil.

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Intermediate 26; 2-((2-(4-(2-((tert-butyldimethylsilyl)oxy)ethyl)piperazin-1-yl)ethoxy)ethyl)disulfaneyl)ethan-1-ol

To a mixture of **24** (775mg, 1eq, 1.98mmol), intermediate **25** (700 mg, 1.52 eq, 3.01 mmol) in DCM (19 mL) and MeOH (2.0 mL) was added sodium methoxide in methanol (161mg, 551μL, 5.4M, 1.5eq, 2.98mmol) at rt. Upon addition a white suspension formed (sodium sulfinate) and the mixture was stirred at rt for 15 min. Sat. NaHCO₃ (25 ml) was added, and the layers were partitioned. The aqueous layer was extracted with DCM (20 mL) and the combined organic layers were dried over Na₂SO₄, filtered, and concentrated in vacuo. The crude (1.0 g) was purified by column chromatography (24 g silica, 0-3% gradient of 7M NH₃ in MeOH/DCM). The intermediate **26** (814 mg, 97%) was obtained as a light-yellow oil.

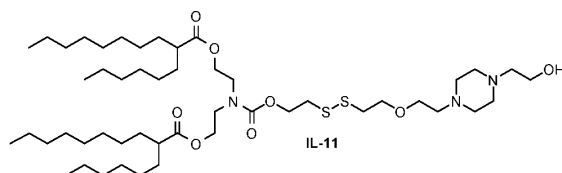
Intermediate 27; 2-((2-(4-(2-((tert-butyldimethylsilyl)oxy)ethyl)piperazin-1-yl)ethoxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate.

Intermediate **27** (814mg, 1eq, 1.92mmol) was dissolved in DCM (9.58mL) followed by addition of Et₃N (388mg, 534μL, 2eq, 3.83mmol). The mixture was cooled to 0 °C and 4-nitrophenyl carbonochloridate (579mg, 1.5eq, 2.87mmol) in DCM (3mL) was added dropwise over 5min. After stirring for 15min at 0 °C the mixture was allowed to warm to rt and stirred for 2h at rt. 10mL of DCM was added and washed with half sat. Na₂CO₃ (25 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo* to obtain 1.04 g of intermediate **27** as a yellow oil (92% yield).

Intermediate 28; 14-(4-(2-((tert-butyldimethylsilyl)oxy)ethyl)piperazin-1-yl)-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4-oxo-5,12-dioxo-8,9-dithia-3-azatetradecyl 2-hexyldecanoate

DHDA (**3**, 330mg, 1eq, 567μmol) was dissolved in DMF (1.0mL) followed by addition of Et₃N (115mg, 0.3mL, 4eq, 2.3mmol). A solution of intermediate **27** (398mg, 1.19eq, 675μmol) in DMF (1mL) was added followed by the addition of DMAP (4.0mg, 0.058eq, 33μmol). The RM was stirred at rt for 2h and warmed to 50 °C 18h. The aqueous workup was performed by the addition of 2 ml of water and 1ml sat. Na₂CO₃, extract with heptane (5mL), wash the organic layer with a mixture of water, sat. Na₂CO₃ and MeOH (3mL, 1/1/1). Organics were dried over Na₂SO₄, filtered, and concentrated in vacuo to obtain 580 mg of crude as orange oil. Purification by column (eluent A: 1% 7M NH₃ in DCM, eluent B: MeOH) gradient of 0-3% eluent B in A to obtain intermediate **28** (336 mg, 57%) as an orange oil.

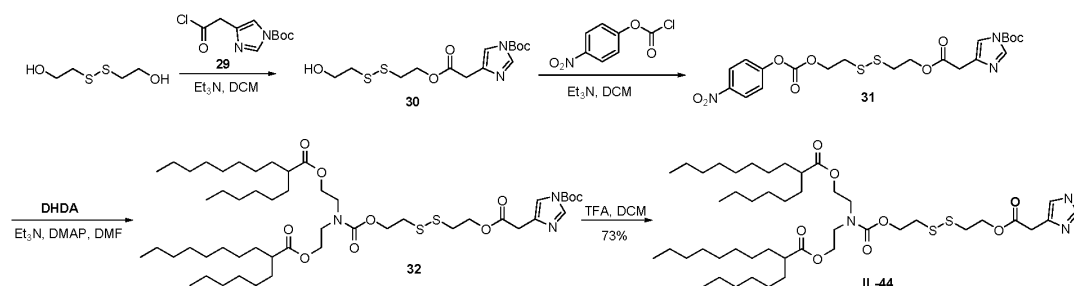
IL-11; 3-(2-((2-hexyldecanoyl)oxy)ethyl)-14-(4-(2-hydroxyethyl)piperazin-1-yl)-4-oxo-5,12-dioxo-8,9-dithia-3-azatetradecyl 2-hexyldecanoate



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To a mixture of intermediate **27** (335mg, 1eq, 324 μ mol) and Et₃N (223mg, 181 μ L, 4eq, 1.30mmol) in THF (1.08mL) was added TBAF (92mg, 0.35mL, 1.0M, 1.1eq, 0.35mmol). The RM was stirred at rt for 18h. The aqueous workup was performed with 20 ml of sat. Na₂SO₄ and extraction with 3x20 ml heptane, combined organics were dried over Na₂SO₄, filtered, and concentrated *in vacuo* to obtain 300 mg crude oil. Purification by silica column (1-5% gradient of 7M NH₃ in MeOH/DCM) to obtain 265mg of IL-11 as a slightly orange oil (89% yield). ¹H NMR (400 MHz, Chloroform-*d*): 4.32 (t, J = 6.5Hz, 2H), 4.24-4.11 (m, 4H), 3.67 (t, J = 6.5Hz, 2H), 3.58 (t, J = 5.6Hz, 4H), 3.54 (t, J = 5.7Hz, 2H), 3.50 (t, J = 5.7Hz, 2H), 2.90 (t, J = 6.5Hz, 2H), 2.86 (t, J = 6.5Hz, 2H), 2.62-2.40 (m, 10H), 2.34-2.51 (m, 2H), 1.63-1.48 (m, 4H), 1.48-1.33 (m, 4H), 1.32-1.10 (m, 40H), 0.85 (t, J = 6.6Hz, 4x3H=12H). ¹³C NMR (100 MHz, Chloroform-*d*); 176.3, 155.5, 69.3, 68.8, 63.5, 62.3, 61.9, 59.2, 57.72, 57.68, 53.7, 52.7, 47.5, 46.9, 45.7, 38.6, 37.6, 32.3, 31.9, 31.7, 29.6, 29.5, 29.3, 29.2, 27.5, 27.4, 22.6, 14.12, 14.07; MS: 918.60 m/z [M⁺].



15

Intermediate 29; tert-Butyl 4-(2-chloro-2-oxoethyl)-1H-imidazole-1-carboxylate

Triethylamine (1.31g, 1.80mL, 3eq, 12.9mmol) and di-tert-butyl dicarbonate (1.03g, 1.1eq, 4.74mmol) in DMF (5.2mL) were added to the suspension of (3H-imidazol-4-yl)-acetic acid HCl (700mg, 1eq, 4.31mmol) in DMF (5.2mL). After 4 h at room temperature, the solution was concentrated. The residue was taken up in EtOAc and washed with H₂O. After separation, the organic phase was dried over Na₂SO₄ and concentrated *in vacuo*. The residue was suspended in DCM (15 mL), and oxalyl chloride (0.26 mL, 3.07 mmol, 2.4 eq) followed by DMF (15.7 mg, 16.8 μ L, 0.05 Eq, 215 μ mol) were added. The solution was stirred for 2h at rt and concentrated *in vacuo* to provide the crude tert-butyl 4-(2-chloro-2-oxoethyl)-1H-imidazole-1-carboxylate (480mg, 1.96mmol, 45%) as a yellow oil.

25

Intermediate 30; tert-Butyl 4-(2-((2-hydroxyethyl)disulfaneyl)ethoxy)-2-oxoethyl)-1H-imidazole-1-carboxylate

Intermediate **29** (480.0mg, 1eq, 1.962mmol) was added over 30 min at 0 °C to a DCM (4.0 mL) solution of 2,2'-dithiodiethanol (907.7mg, 3eq, 5.885mmol) and triethylamine (992.5mg, 1.37mL, 5eq, 9.809mmol). The reaction mixture was stirred at 0°C 1h and 30min at rt. The reaction content was diluted with DCM and washed sequentially with NaHCO₃ (100mL) and then brine

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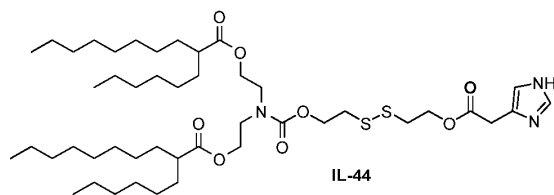
(100mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude was purified by column chromatography to obtain the intermediate **30** (198mg, 0.55mmol, 27%) as a pale yellow oil.

5 **Intermediate 31**; *tert*-Butyl 4-(2-(2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfaneyl)ethoxy)-2-oxoethyl)-1H-imidazole-1-carboxylate
Intermediate **30** (198mg, 1eq, 546μmol) and triethylamine (138mg, 190μL, 2.5eq, 1.37mmol) were dissolved in DCM (1.0 mL) and the solution was cooled to 0 °C using an ice-water bath. 4-nitrophenyl carbonochloridate (143mg, 1.3eq, 710μmol) in DCM (1.0 mL) was added dropwise
10 at 0°C over 15min. The RM was diluted with 50 mL DCM and washed with 2x50mL 0.5M HCl, 3x50mL Na₂CO₃ (sat. aq.), 50mL water, dried over sodium sulfate and evaporated. The crude was purified by column chromatography to obtain the intermediate **31** (210mg, 0.40mmol, 72.9%) as a yellow oil.

Intermediate 32; *tert*-butyl 4-(2-(2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfaneyl)ethoxy)-2-oxoethyl)-1H-imidazole-1-carboxylate
15 Intermediate **31** (210mg, 1eq, 398μmol) was dissolved in DMF (1.0mL) and the solution was placed under nitrogen atmosphere. To the carbonate solution was added triethylamine (101mg, 139μL, 2.5eq, 995 μmol), followed by DMAP (4.86mg, 0.1eq, 39.8μmol). DHDA (**3**, 301mg, 1.3eq, 517μmol) was dissolved in DMF (1.0mL) and the solution was added dropwise to the
20 reaction mixture over 5min and allowed to stir at room temperature for 48h. The DMF was evaporated and the residue was dissolved in DCM 100mL and washed with 2x 50 mL 0.5MHCl (aq.), 3x50 mL half sat. Na₂CO₃ (aq.), 2x 3% LiCl solution, dried over sodium sulfate and evaporated. The crude was purified by column chromatography to obtain intermediate **32** (105mg, 0.11mmol, 27%) as a pale yellow oil.

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IL-44; 3-(2-((2-Hexyldecanoyl)oxy)ethyl)-14-(1H-imidazol-4-yl)-4,13-dioxo-5,12-dioxa-8,9-dithia-3-azatetradecyl 2-hexyldecanoate



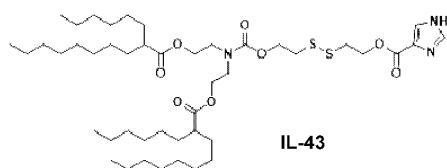
Intermediate **32** (335mg, 1eq, 345μmol) was dissolved in DCM (2.0mL) and TFA (3.0g, 2.0mL, 75eq, 26mmol) was added and the mixture was stirred at room temperature for 2 hours. Volatiles were removed under vacuum and the crude product was purified by column chromatography (DCM/MeOH gradient). **IL-44** (218mg, 0.250mmol, 73%) was isolated as yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 8.49 (s, 1H), 7.25 (s, 1H), 4.38 (t, J = 6.5Hz, 2H), 4.33 (t, J = 6.5Hz, 2H), 4.25-4.12 (m, 4H), 3.8 (s, 4H), 3.59-3.45 (m, 4H), 2.90 (t, J = 6.5Hz, 4H), 2.36-2.24 (m, 2H),
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1.63-1.48 (m, 4H), 1.48-1.33 (m, 4H), 1.32-1.11 (m, 40H), 0.85 (t, J = 6.6Hz, 4x3H=12H). ¹³C NMR (100 MHz, Chloroform-d): 176.43, 176.39, 168.3, 155.6, 133.6, 126.4, 117.7, 63.4, 62.3, 61.9, 47.6, 47.1, 45.7, 37.3, 36.8, 32.3, 31.9, 31.7, 30.1, 29.6, 29.4, 29.3, 29.2, 27.7, 27.4, 22.7, 22.6, 14.12, 14.07; MS: 870.4 m/z [M⁺].

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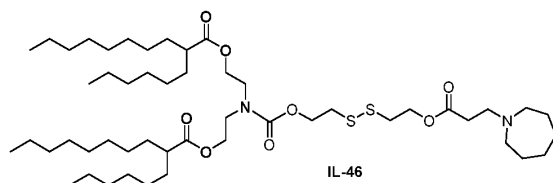
IL-43; 11-(2-((2-Hexyldecanoyl)oxy)ethyl)-1-(1H-imidazol-4-yl)-1,10-dioxo-9-oxa-5,6-dithia-2,11-diazatridecan-13-yl 2-hexyldecanoate:



Starting from 1H-imidazole-4-carboxylic acid, **IL-43** was obtained by analogous protocols as used for Boc protected **IL-44**. ¹H NMR (400 MHz, Chloroform-d): 7.84 (s, 1H), 7.80 (s, 1H), 4.53 (t, J = 6.5Hz, 2H), 4.34 (t, J = 6.5Hz, 2H), 4.25-4.12 (m, 4H), 3.59-3.47 (m, 4H), 3.00 (t, J = 6.5Hz, 2H), 2.94 (t, J = 6.5Hz, 2H), 2.38-2.23 (m, 2H), 1.65-1.48 (m, 4H), 1.48-1.33 (m, 4H), 1.32-1.11 (m, 40H), 0.85 (t, J = 6.6Hz, 4x3H=12H); MS: 856.4 m/z [M⁺].

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IL-46; 2-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl) 3-(azepan-1-yl)propanoate

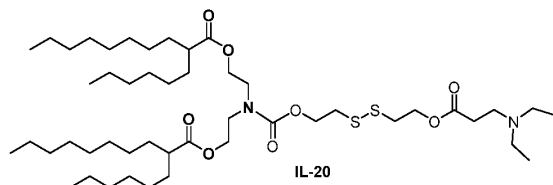


3-Azepan-1-yl-propanoic acid was converted into its acid chloride and used to prepare **IL-46** as mentioned in the example to prepare Boc-protected **IL-44**.

¹H NMR (400 MHz, Chloroform-d): 4.31 (q, J = 6.3Hz, 4H), 4.16 (q, J = 7.9Hz, 4H), 3.59-3.45 (m, 4H), 2.97-2.80 (m, 6H), 2.66 (appt t, J = 5.3Hz, 4H), 2.53 (t, J = 7.3Hz, 2H), 2.38-2.23 (m, 2H), 1.68-1.48 (m, 10H), 1.48-1.33 (m, 4H), 1.32-1.11 (m, 40H), 0.85 (t, J = 6.9Hz, 4x3H=12H); MS: 915.4 m/z [M⁺].

20

IL-20; 2-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl) 3-(diethylamino)propanoate

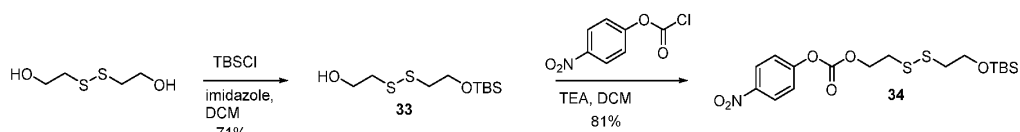


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3-(diethylamino)propanoic acid hydrochloride (1.0g, 1eq, 5.5mmol) was converted to its acid chloride and used to prepare **IL-20** as a pale-yellow oil in an analogy to the Boc-protected **IL-44**.

¹H NMR (400 MHz, Chloroform-*d*): 4.30 (q, *J* = 6.5Hz, 4H), 4.16 (q, *J* = 7.6Hz, 4H), 3.59-3.45 (m, 4H), 2.95-2.82 (q, *J* = 7.0Hz, 4H), 2.77 (t, *J* = 7.1Hz, 2H), 2.50 (q, *J* = 7.1Hz, 4H), 2.44 (t, *J* = 7.1Hz, 2H), 2.35-2.22 (m, 2H), 1.62-1.47 (m, 4H), 1.47-1.32 (m, 4H), 1.32-1.08 (m, 40H), 0.99 (t, *J* = 7.1Hz, 2x3H=6H), 0.84 (t, *J* = 6.4Hz, 2x3H=6H); MS: 889.40 *m/z* [*M*⁺].



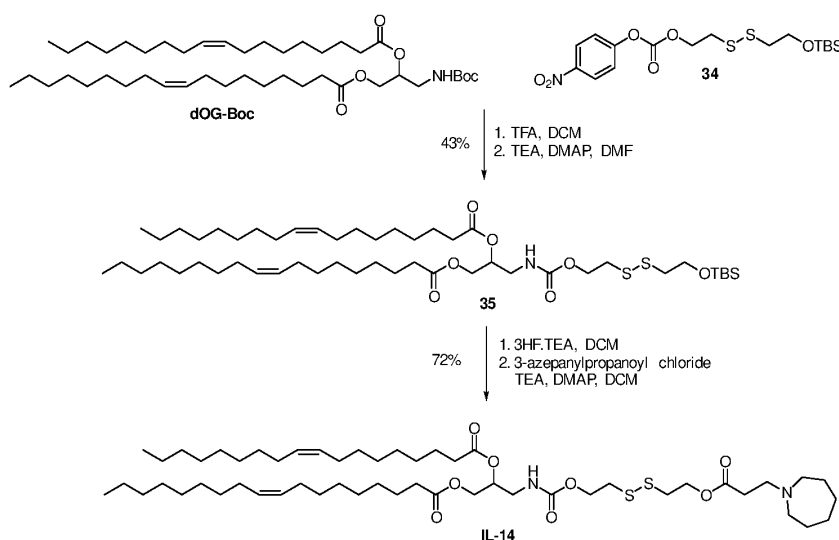
10 Intermediate 33; 2-((2-((Tert-butyldimethylsilyl)oxy)ethyl)disulfaneyl)ethan-1-ol

2,2'-Dithiodiethanol (2.50g, 1.5eq, 16.2mmol) and imidazole (883mg, 1.2eq, 13.0mmol) were dissolved in DCM (100mL) and cooled to 0°C. *tert*-butyldimethylchlorosilane (1.63g, 1.80mL, 1eq, 10.8mmol) was added and the mixture was stirred at 0°C for 30min and at room temperature for another 30min. The mixture was transferred to a separatory funnel, washed with 100mL water and 100mL brine, dried over sodium sulfate, and evaporated. The product was purified by column chromatography (heptane/EtOAc) to obtain the intermediate **33** (2.05g, 7.63mmol, 71%) was isolated as a colorless oil.

Intermediate 34; 2-((2-((Tert-butyldimethylsilyl)oxy)ethyl)disulfaneyl)ethyl (4-nitrophenyl) carbonate

20 Intermediate **33** (2.05g, 1eq, 7.63mmol) and triethylamine (1.55g, 2.13mL, 2eq, 15.3mmol) were dissolved in DCM (50mL) and the solution was cooled to 0°C using an ice-water bath. 4-nitrophenyl carbonochloridate (2.31g, 1.5eq, 11.5mmol) was added at 0°C and the mixture was stirred at room temperature for 16h. The reaction mixture was diluted with 100mL DCM and washed with 4x100mL half-saturated Na₂CO₃ (aq.), 50mL water, dried over sodium sulfate and 25 evaporated. The crude product was purified by column chromatography (heptane/EtOAc gradient). Intermediate **34** (2.68g, 6.18mmol, 81%) was isolated as a colorless oil.

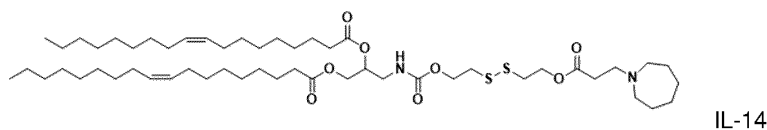
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Intermediate 35; General procedure for the coupling of dOG or dLG lipids to intermediate 34

3-((tert-butoxycarbonyl)amino)propane-1,2-diyl diolate/dilinoleate (**dOG** or **dLG**, 1.00g, 1eq, 1.4mmol) was dissolved in DCM (2.5mL) and TFA (3.7g, 2.5mL, 26eq, 32mmol) was added and the mixture was stirred at room temperature for 2 hours, after which volatiles were removed and the residue was coevaporated with 50mL toluene. Meanwhile, the intermediate **34** (787mg, 1.3eq, 1.82mmol) and triethylamine (632mg, 871μL, 5eq, 6.25mmol) were dissolved in DMF (5.0mL) and DMAP (15.3mg, 0.1eq, 125μmol) was added. The deprotected lipid in DMF (5.0mL) was added and the mixture was stirred at rt for 16h. The mixture was evaporated thoroughly, and the residue was dissolved in 50mL TBME and washed with 4x50mL half-saturated Na₂CO₃ (aq.), 2x50mL 5% LiCl (aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient).

IL-14; 16-(azepan-1-yl)-5,14-dioxo-6,13-dioxo-9,10-dithia-4-azahexadecane-1,2-diyl diolate



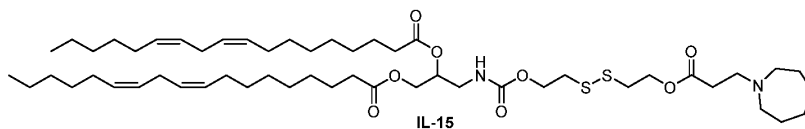
Compound **34** (910 mg, 1 eq, 1.00 mmol) was dissolved in THF (5.0 mL) and triethylamine (506 mg, 697 μL, 5 Eq, 5.00 mmol) was added, followed by triethylammonium fluoride (242 mg, 0.24 mL, 1.5 Eq, 1.50 mmol) and the mixture was stirred at room temperature for 4 hours. The mixture was diluted with 50 mL TBME and washed with 2x50 mL water, dried over sodium sulfate and evaporated. Meanwhile, 3-(azepan-1-yl)propanoic acid (342 mg, 2 Eq, 2.00 mmol) was dissolved in THF (5.0 mL) and oxalyl chloride (381 mg, 262 μL, 3 Eq, 3.00 mmol) was added and the mixture was stirred at room temperature for 1 hour. The volatiles were removed under vacuume and the material was handled under Ar atm. The desilylated lipid was dissolved in DCM

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(5.0 mL) and triethylamine (506 mg, 697 μ L, 5 Eq, 5.00 mmol) and DMAP (12.2 mg, 0.1 Eq, 99.9 μ mol) were added and the mixture was cooled to 0 °C using an ice bath. The 1.5 ml DCM solution of acyl chloride was added reaction mixture at 0 °C, the ice bath was removed, and the mixture was stirred at rt for 1 hour. The mixture was diluted with 50 mL TBME and washed with 2x50 mL NaHCO₃ (sat. aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient) to obtain **IL-14** (260.8 mg, 273.5 μ mol, 71.5 %) as a pale-yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 5.37-5.15 (m, 5H), 5.13-5.02 (m, 1H), 4.40-4.20 (m, 5H) 4.10 (dd, *J* = 12.0, 5.6Hz, 1H), 3.50-3.27 (m, 2H), 2.96-2.82 (m, 6H), 2.67 (appt. t *J* = 5.5Hz, 2H), 2.52 (t, *J* = 7.2Hz, 2H), 2.28 (appt dt, *J* = 7.3, 1.9Hz, 4H), 2.04-1.90 (m, 8H), 1.69-1.50 (m, 12H), 1.36-1.13 (m, 40H), 0.85 (t, *J* = 6.8Hz, 2x3H=6H). ¹³C NMR (100 MHz, Chloroform-*d*); 173.4, 173.1, 172.4, 156.1, 130.03, 130.01, 129.70, 129.68, 70.2, 62.7, 62.5, 62.4, 55.1, 53.3, 41.3, 37.8, 37.0, 34.2, 34.0, 32.6, 31.9, 29.8, 29.7, 29.5, 29.3, 29.21, 29.19, 29.13, 29.10, 29.09, 27.6, 27.6, 27.2, 27.2, 27.0, 24.9, 24.8, 22.7, 14.1; MS: 953.60 *m/z* [*M*⁺].

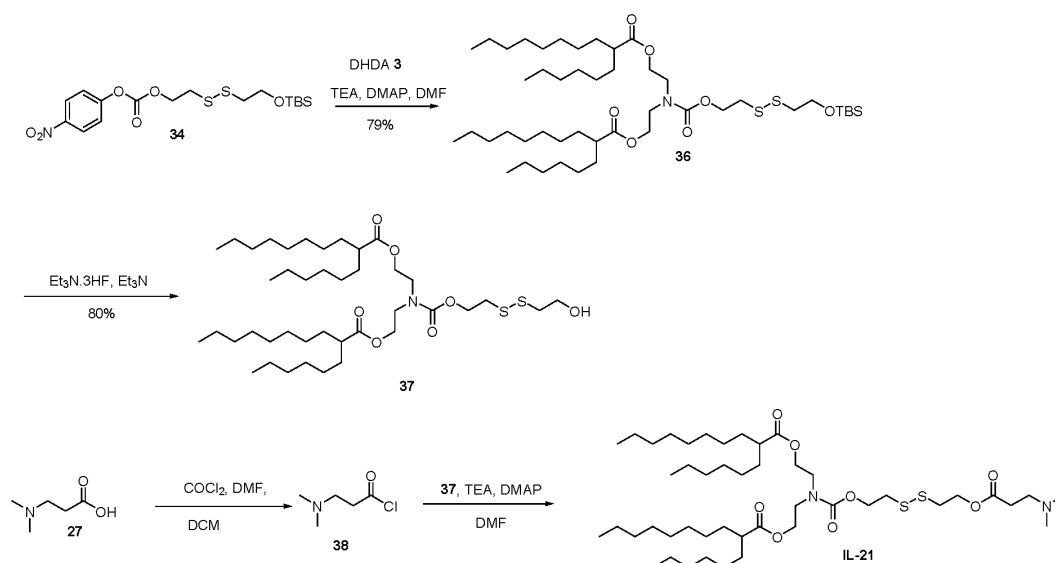
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IL-15; 16-(azepan-1-yl)-5,14-dioxo-6,13-dioxo-9,10-dithia-4-azahexadecane-1,2-diyl (9*Z*,9'*Z*,12*Z*,12'*Z*)-bis(octadeca-9,12-dienoate)



20 Starting from dLG-Boc and intermediate **34**, **IL-15** (620 mg, 653 μ mol, 65 %) was isolated as a colorless oil. ¹H NMR (400 MHz, Chloroform-*d*): 5.42-5.25 (m, 6H), 5.21-5.04 (m, 2H), 4.42-4.20 (m, 4H), 4.11 (dd, *J* = 5.7, 11.8Hz, 1H), 3.51-3.27 (m, 2H), 2.97-2.79 (m, 5H), 2.75 (appt t, *J* = 6.1Hz), 2.64 (bs, 3H), 2.55-2.45 (m, 2H), 2.28 (appt dt, *J* = 7.3, 1.6Hz, 4H), 2.08-1.94 (m, 8H), 1.69-1.53 (m, 10H), 1.39-1.20 (m, 28H), 0.91-0.81 (t, 2x3H = 6H); MS: 949.60 *m/z* [*M*⁺].

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Intermediate 36; 13-(2-((2-hexyldecanoyl)oxy)ethyl)-2,2,3,3-tetramethyl-12-oxo-4,11-dioxo-7,8-dithia-13-aza-3-silapentadecan-15-yl 2-hexyldecanoate

- 5 Triethylamine (197mg, 272μL, 2.2eq, 1.95mmol) and DMAP (10.8mg, 0.1eq, 88.7μmol) were added to the DMF (5.0mL) solution of the intermediate **34** (500mg, 1.3eq, 1.15mmol). DHDA **3** (516mg, 1eq, 887μmol) as a solution in 1mL of DMF was added and the reaction mixture was stirred at rt for 16h. The volatiles were removed, and the residue was dissolved in 50 mL TBME and washed with 4x50 mL half-saturated Na₂CO₃ (aq.), 2x50 mL 5% LiCl (aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient). Intermediate **36** (610mg, 696μmol, 79%) was isolated as a colorless oil.

Intermediate 37; (((2-((2-Hydroxyethyl)disulfaneyl)ethoxy)carbonyl)azanediyl)bis(ethane-2,1-diyl) bis(2-hexyldecanoate)

- Intermediate **36** (610.0mg, 1 eq, 696.0μmol) was dissolved in THF (5.0mL) and triethylamine (352mg, 485μL, 5eq, 3.48mmol) was added, followed by triethylammoniumfluoride (336mg, 0.34mL, 3eq, 2.08mmol) and the RM was stirred at room temperature for 18h. The reaction mixture was diluted with 50 mL TBME and washed with 2x50mL water, dried over sodium sulfate and evaporated to afford intermediate **37** (422mg, 554μmol, 79%) as a colorless oil.

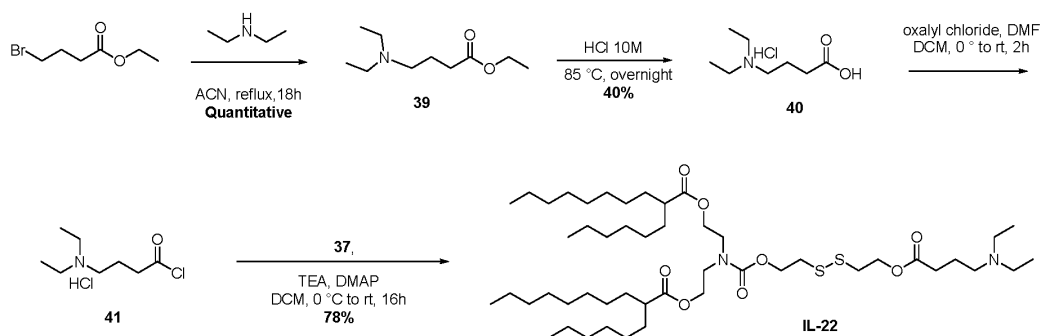
Intermediate 38; 3-(dimethylamino)propanoyl chloride

- 20 To a suspension of 3-(dimethylamino)propanoic acid (1.0g, 1eq, 8.5mmol) in dry DCM (37mL) at 0°C under an nitrogen atmosphere, oxalyl chloride (2.2g, 1.5mL, 2.0eq, 17mmol) was added followed by one drop of dry DMF. The reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was dried under a high vacuum to obtain 3-(dimethylamino)propanoyl chloride (1.2g, 8.9mmol, 100%) as an off-white solid, which was used immediately in the next step.

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IL-21: 1-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxo-3,4-dithia-9-azadocosyl) 3-(dimethylamine-yl)propanoate

Intermediate acyl chloride **38** (62.97 mg, 3 eq, 0.47 mmol) was added as a 2ml solution in dry DCM to the ice-cold DCM (3.5 mL) solution of the intermediate **37** (118.0 mg, 1 eq, 154.8 μ mol) containing triethylamine (78.33 mg, 108 μ L, 5 eq, 774.1 μ mol) and DMAP (5.67 mg, 0.3 eq, 46.44 μ mol). The ice bath was removed and the reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was diluted with 50mL TBME and washed with 2x50 mL NaHCO₃ (sat. aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient). After purification, **IL-21** (85 mg, 64%) was obtained as a pale-yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.23 (dt, *J* = 6.5, 2.1Hz, 4H), 4.20-4.11 (m, 4H), 3.58-3.44 (m, 4H), 2.89 (q, *J* = 6.5Hz, 4H), 2.62-2.42 (m, 4H), 2.35-2.25 (m, 2H), 2.21 (s, 6H), 1.62-1.47 (m, 4H), 1.47-1.32 (m, 4H), 1.32-1.12 (m, 40H), 0.84 (t, 4x3H = 12H); ¹³C NMR (100 MHz, Chloroform-*d*): 176.2, 172.2, 155.5, 63.4, 62.2, 61.9, 54.7, 47.4, 46.9, 45.7, 45.3, 34.5, 37.2, 36.8, 32.8, 32.3, 31.9, 31.7, 29.7, 29.6, 29.5, 29.3, 29.2, 27.5, 27.4, 22.7, 22.6, 14.10, 14,.06; MS: 861.30 m/z [M⁺].



Intermediate 39; Ethyl 4-(diethylamino)butanoate

A solution of ethyl 4-bromobutanoate **10** (20 g, 1 Eq, 0.10 mol) and diethylamine (37 g, 53 mL, 5 eq, 0.51 mol) in ACN (200 mL) was refluxed for 18h. The solvent was removed in vacuo and NaOH 1M (150 mL) was added. The solution was extracted with heptanes (3 x 200 mL). The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The desired product **11** (19 g, quantitative) was recovered as an orange oil.

Intermediate 40; 4-(Diethylamino)butanoic acid hydrochloride

39 (19 g, 1 Eq, 0.10 mol) was taken into aq HCl (37 g, 0.10 L, 10 molar, 10 eq, 1.0 mol) and the resulting mixture was maintained at 85 °C for 16h. The solvent was evaporated under reduced pressure and the resulting dark oil was suspended in water and evaporated again. This was performed three times. The crude dark solid was dissolved in hot acetic acid (50 mL) and precipitated with addition of diethyl ether (250 mL). The resulting white solid was filtered and washed with diethyl ether to provide 4-(Diethylamino)butanoic acid hydrochloride (8 g, 40%) as a white powder.

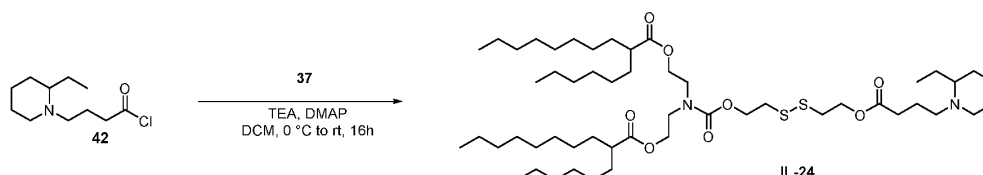
-64-

Intermediate 41; 4-(Diethylamino)butanoyl chloride hydrochloride

To a cooled (0°C) suspension of acid 12 (2.4g, 1eq, 12mmol) in DCM (50mL) was added oxalyl chloride (3.1g, 2.1mL, 2eq, 25mmol) followed by DMF (90mg, 95μL, 0.1eq, 1.2mmol). The resulting brown suspension cleared up after 10-15 min. The solution was stirred for 2 hours. The solvent was then evaporated to give intermediate **41** as a brown solid which was used in the next step without any treatment.

IL-22; 1-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl)-4-(diethylamine-yl)butanoate

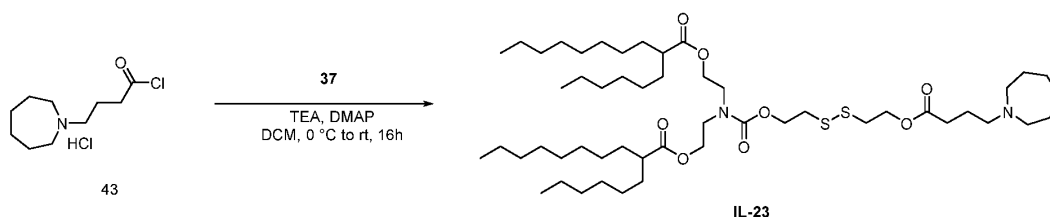
To a solution of alcohol **37** (7.5g, 1eq, 9.8mmol) in DCM (75 mL) was added DMAP (0.60 g, 0.5 eq, 4.9 mmol) and Et₃N (3.0g, 4.1mL, 3eq, 30mmol). The resulting mixture was cooled down to 0°C and a solution of acyl chloride **41** (2.6g, 1.5eq, 15mmol) in DCM (25mL) was added. The reaction mixture was stirred at room temperature 16h. The reaction mixture was diluted with DCM (200mL) and washed with NaHCO₃ (2 x 200mL). The organic layer was dried over sodium sulfate, filtered and evaporated. Purification of the crude residue (DCM/MeOH gradient from 100/0 to 88/12 and then 85/15) gave **IL-22** (6.9 g, 78%) as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.32 (q, J = 6.5Hz, 4H), 4.21-4.08 (m, 4H), 3.55-3.42 (m, 4H), 2.86 (q, J = 6.6Hz, 4H), 2.71-2.53 (m, 6H), 2.33 (t, J = 7.33Hz, 2H), 2.30-2.20 (m, 2H), 1.89-1.76 (m, 2H), 1.58-1.44 (m, 4H), 1.44-1.29 (m, 4H), 1.29-1.11 (m, 40H), 1.07 (t, J = 7.3Hz, 6H), 0.80 (t, J = 7.0Hz, 4x3H = 12H); ¹³C NMR (100 MHz, Chloroform-*d*): 175.2, 171.9, 154.4, 62.3, 62.1, 61.27, 61.24, 61.0, 60.9, 50.4, 46.5, 46.0, 45.7, 44.7, 36.5, 36.2, 35.8, 31.3, 30.9, 30.7, 30.6, 30.5, 28.6, 28.4, 28.3, 28.2, 26.5, 26.4, 21.7, 21.6, 20.2, 13.1, 13.07, 9.7; MS: 903.60 m/z [M⁺].

**IL-24**; 1-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl) 4-(2-ethylpiperidin-1-yl)butanoate

Intermediate **42** [(4-(2-Ethylpiperidin-1-yl)butanoyl chloride] was obtained from 2-ethyl piperidine in analogy to the conversion of diethyl amine to intermediate **41**.

37 (114.0mg, 1eq, 0.15mmol) and intermediate **42** (97.7mg, 3eq, 0.45mmol) were subjected to analogous conditions used for **IL-22** to obtain **IL-24** (100 mg, 71%) as a pale-yellow viscous oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.32 (q, J = 6.5Hz, 4H), 4.21-4.10 (m, 4H), 3.57-3.45 (m, 4H), 2.99-2.23 (m, 13H), 1.87(bs, 2H), 1.80-1.10 (m, 56H), 0.93-0.76 (m, 5x3H = 15H); MS: 943.60 m/z [M⁺].

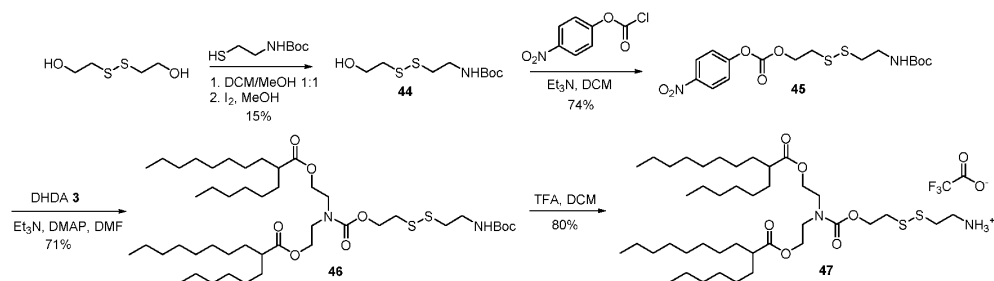
-65-



IL-23; 1-(14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl)-4-(azepan-1-yl)butanoate

4-(azepan-1-yl)butanoic acid hydrochloride was converted to its acid chloride intermediate **43** in analogy to the conversion of **40** to **41**.

Intermediate 37 (297mg, 1eq, 0.39mmol) and intermediate **43** (2eq) were subjected to analogous conditions used for **IL-22** to obtain **IL-23** (144 mg, 155 μ mol, 39.8 %) as a yellow oil. ^1H NMR (400 MHz, Chloroform-*d*): 4.32 (q, $J = 6.3\text{Hz}$, 4H), 4.21-4.12 (m, 4H), 3.59-3.45 (m, 4H), 2.97-2.74 (m, 8H), 2.40 (t, $J = 7.1\text{Hz}$, 2H), 2.35-2.25(m, 2H), 2.06-1.92 (m, 2H), 1.87-1.74 (bs, 4H), 1.69-1.61 (bs, 4H), 1.61-1.48 (m, 4H), 1.48-1.33 (m, 4H), 1.33-1.13 (m, 40H), 0.85 (t, $J = 6.7\text{Hz}$, $4 \times 3\text{H} = 12\text{H}$); ^{13}C NMR (100 MHz, Chloroform-*d*); 176.4, 172.6, 155.5, 135.3, 128.5, 126.9, 62.4, 62.33, 62.29, 61.9, 56.7, 54.8, 47.5, 47.0, 45.7, 41.9, 37.5, 37.1, 36.7, 32.3, 31.9, 31.9, 31.7, 31.3, 29.7, 29.7, 29.6, 29.5, 29.3, 29.2, 27.5, 27.4, 26.9, 25.4, 22.7, 22.6, 20.8, 14.11, 14.08; MS: 929.60 m/z [M^+].



Intermediate 44; *tert*-Butyl (2-((2-hydroxyethyl)disulfaneyl)ethyl)carbamate

2,2'-Dithiodiethanol (2.74g, 3eq, 17.8mmol) was dissolved in DCM/MeOH 1:1 (75mL) and *tert*-butyl (2-mercaptoethyl)carbamate (1.05g, 1.00mL, 1eq, 5.92mmol) was added in 5 portions over 3 hours. The RM was stirred at room temperature for 18h. A solution of iodine (752mg, 0.5eq, 2.96mmol) in MeOH (15mL) was added until the solution retained the yellow color. The solvent was evaporated, the residue was dissolved in 150mL EtOAc and washed with 3x100mL NaHCO_3 (sat. aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (heptane/EtOAc gradient). The intermediate **44** (230mg, 0.91mmol, 15%) was isolated as a thick, light yellow oil.

Intermediate 45; *tert*-Butyl (2-((2-((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfaneyl)ethyl)carbamate

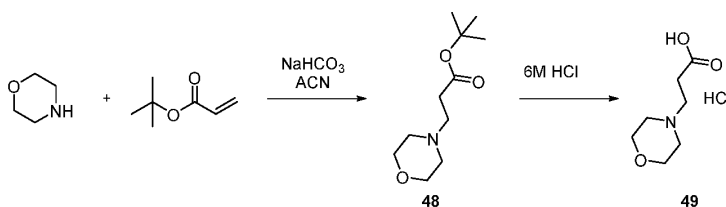
Intermediate **44** (150mg, 1eq, 592μmol) was dissolved in DCM (3.5mL) and the solution was cooled to 0 °C using an ice bath. Triethylamine (150mg, 206μL, 2.5eq, 1.48mmol) was added, followed by 4-nitrophenyl carbonochloridate (143mg, 1.2eq, 0.71mmol) and the mixture was stirred at 0°C for 5 min. The ice bath was removed, allowed to warm to room temperature and stirred at room temperature for 18h. The reaction mixture was diluted with 50mL DCM, washed with 2x50mL half-saturated Na₂CO₃ (aq.), 50mL water, dried over sodium sulfate and evaporated. The crude was purified by column chromatography (DCM/MeOH gradient). Intermediate **45** (184mg, 0.44mmol, 74.3%) was obtained as a white crystalline solid.

Intermediate 46; 3-(2-((2-Hexyldecanoyl)oxy)ethyl)-15,15-dimethyl-4,13-dioxo-5,14-dioxo-8,9-dithia-3,12-diazahexadecyl 2-hexyl decanoate

Intermediate **45** (184mg, 1.2eq, 0.44mmol) was dissolved in 2mL DMF and triethylamine (111mg, 153μL, 3eq, 1.10mmol) was added, followed by DMAP (4.47mg, 0.1eq, 36.6μmol). DHDA **3** lipid (213mg, 1eq, 0.37mmol) in 1.5mL DMF was added to the stirring solution and stirred at room temperature for 5h. A few drops of DCM were added and then the mixture became a clear yellow solution. Volatiles were removed and the residue was dissolved in 50 mL TBME and washed with 2x50 mL 0.5M HCl (aq.), 2x50mL half-saturated Na₂CO₃ (aq.), 2x50mL 5% LiCl (aq.), dried over sodium sulfate and evaporated. The crude product was purified by column chromatography (DCM/MeOH gradient). Intermediate **46** (223mg, 0.26mmol, 71%) was obtained as yellow oil.

Intermediate 47; 14-Hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxo-3,4-dithia-9-azadocosan-1-aminium 2,2,2-trifluoroacetate

Intermediate **46** (223mg, 1eq, 0.26mmol) was dissolved in DCM (1.0mL) and TFA (1.5g, 1.0mL, 50eq, 13mmol) was added to the reaction mixture and stirred at room temperature for 1h. Volatiles were removed, and the crude product was purified by column chromatography (DCM/MeOH gradient). Intermediate **47** (181mg, 0.21mmol, 80%) was isolated as a slightly yellow oil.



Intermediate 49: 3-morpholinopropanoic acid hydrochloride

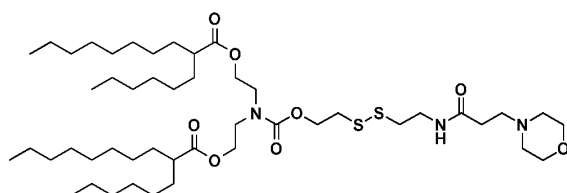
Morpholine (4.0g, 4.0mL, 1eq, 46mmol) and *tert*-butyl acrylate (6.5g, 7.3mL, 1.1eq, 51mmol) were dissolved in ACN (130mL), sodium bicarbonate (3.9g, 1eq, 46mmol) was added and the mixture was heated to reflux 18h. The solid was filtered off and the ACN was evaporated. The

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residue was dissolved in 150 mL DCM, washed with 100 mL brine, dried over sodium sulfate and evaporated. The crude was dissolved in THF (20 mL) and HCl (20g, 92mL, 6.0M, 12eq, 0.55mol) was added and the mixture was heated to reflux (150 degrees ext.) for 2h. The mixture was allowed to cool down and the aqueous HCl evaporated. The solid was re-dissolved twice in distilled water and concentrated again. The residue was suspended in 200 mL acetone, stirred on ice for 20 minutes and the solid was filtered off, washed with 100 mL fresh acetone and dried *in vacuo* to afford 3-morpholinopropanoic acid hydrochloride (4.2 g, 9.2 mmol, 20 %, 43% purity). The purity was determined by Q-NMR.

10 **General procedure for IL with amide moiety:**

Carboxylic acid (2 eq, 315 μ mol) was dissolved in DCM (1.0 mL) and oxalyl chloride (2.5eq, 394 μ mol) was added, followed by one drop of DMF and the mixture was stirred at room temperature for 30min, after which volatiles were removed under vacuo. Meanwhile, intermediate **47** (120mg, 1eq, 0.16mmol) was dissolved in DCM (1.0mL) and triethylamine (63.8mg, 87.9 μ L, 4eq, 0.63mmol) was added. The intermediate acyl chloride in DCM (1.0 mL) was added slowly at 0°C. The ice bath was removed, and the reaction mixture was stirred at room temperature for 30 minutes. The mixture was diluted with TBME and washed with 2x50 mL NaHCO₃ (sat. aq.), dried over Na₂SO₃ and evaporated. The crude material was purified by automated column chromatography (DCM/MeOH gradient) afforded amide ILs



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In an analogy intermediate **47** upon treatment with aminoacyl chloride gave IL-45 IL-41, and IL-42. The aminoacyl chlorides that were used are 1H-imidazole-4-carboxylic acid, 3-Azepan-1-yl-propanoic acid and 3-(diethylamino)propanoic acid respectively.

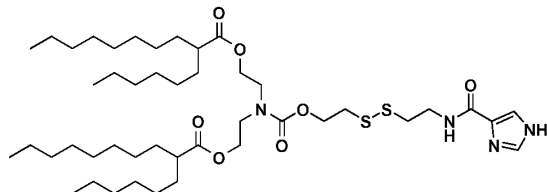
25 **IL-28;** 3-(2-((2-hexyldecanoyl)oxy)ethyl)-15-morpholino-4,13-dioxo-5-oxa-8,9-dithia-3,12-diazapentadecyl 2-hexyldecanoate

Starting from 3-morpholinopropanoic acid hydrochloride (466mg, 43%wt, 2eq, 1.02mmol), IL-28 (109mg, 121 μ mol, 23.6%) was obtained as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.32 (t, J = 6.7Hz, 2H), 4.22-4.12 (m, 4H), 3.71 (J = 4.6Hz, 4H), 3.59-3.45 (m, 6H), 2.88 (t, J = 6.7Hz, 2H), 2.78 (t, J = 6.0Hz, 2H), 2.60 (t, J = 6.0Hz, 2H), 2.48 (bs, 2H), 2.36 (t, J = 6.4Hz, 2H), 2.33-2.23 (m, 2H), 1.66-1.47 (m, 4H), 1.47-1.33 (m, 4H), 1.31-1.12 (m, 40H), 0.84 (t, J = 6.8Hz, 4x3H=12H); ¹³C NMR (100 MHz, Chloroform-*d*): 176.2, 172.4, 155.5, 66.9, 63.4, 62.3, 61.9, 54.4, 53.0, 47.5, 46.9, 45.7, 38.1, 37.0, 36.9, 32.3, .31.8, 31.8, 31.7, 29.7, 29.6, 29.5, 29.3, 29.2, 27.5, 27.4, 22.7, 22.6, 14.10, 14.07; MS: 902.60 m/z [M⁺].

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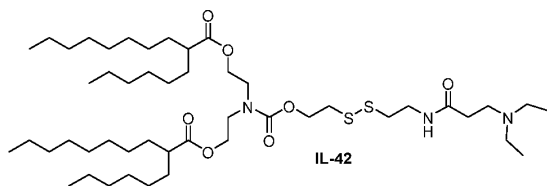
-68-

IL-45; *tert*-butyl 4-((14-hexyl-9-(2-((2-hexyldecanoyl)oxy)ethyl)-8,13-dioxo-7,12-dioxa-3,4-dithia-9-azadocosyl) carbamoyl)-1H-imidazole-1-carboxylate



- 5 Starting from *tert*-butyl 4-(chlorocarbonyl)-1H-imidazole-1-carboxylate (95.4mg, 2eq, 0.41mmol), boc protected **IL-45** was obtained which upon standard TFA mediated Boc-deprotection condition gave **IL-45** (137mg, 0.16mmol, 77%) as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 8.53 (bs, 1H), 8.44 (bs, 1H), 8.04 (s, 1H), 4.35 (t, J = 6.6Hz, 2H), 4.25-4.13 (m, 4H), 3.76-3.63 (m, 2H), 3.60-3.48 (m, 4H), 2.98-2.82 (m, 4H), 2.38-2.24 (m, 2H), 1.63-1.48 (m, 4H), 1.48-1.35 (m, 4H), 1.35-1.11 (m, 40H), 0.91-0.79 (m, 4x3H=12H); MS: 855.40 m/z [M⁺].
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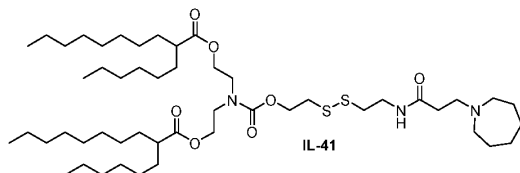
IL-42; 16-Ethyl-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4,13-dioxo-5-oxa-8,9-dithia-3,12,16-triazaoctadecyl 2-hexyldecanoate



- 15 Starting from 3-(diethylamino)propanoic acid, **IL-42** (121mg, 136μmol, 86%) was isolated as a yellow oil starting. ¹H NMR (400 MHz, Chloroform-*d*): 4.32 (t, J = 6.6Hz, 2H), 4.24-4.09 (m, 4H), 3.61-3.44 (m, 6H), 2.88 (t, J = 6.6Hz, 2H), 2.77 (q, J = 6.4Hz, 4H), 2.64 (q, J = 7.1Hz, 4H), 2.45 (appt t, J = 6.0Hz, 2H), 2.35-2.23 (m, 2H), 1.66-1.47 (m, 4H), 1.47-1.33 (m, 4H), 1.31-1.12 (m, 40H), 1.08 (t, J = 7.3Hz, 6H), 0.84 (t, J = 7.3Hz, 4x3H=12H); ¹³C NMR (100 MHz, Chloroform-*d*): 176.3, 172.4, 155.5, 63.5, 62.3, 61.9, 48.8, 47.5, 46.9, 46.1, 45.7, 38.2, 37.2, 32.3, 32.26, 31.8, 31.7, 29.6, 29.4, 29.3, 29.2, 27.5, 27.4, 22.7, 22.6, 14.10, 14.06, 10.8; MS: 888.40 m/z [M⁺].
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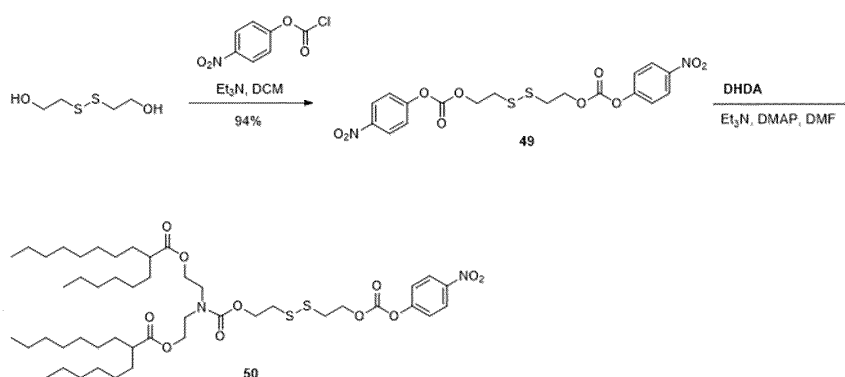
IL-41; 15-(Azepan-1-yl)-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4,13-dioxo-5-oxa-8,9-dithia-3,12-diazapentadecyl 2-hexyl decanoate

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Starting from 3-Azepan-1-yl-propanoic acid, **IL-41** (122mg, 133 μ mol, 85%) was isolated as a yellow oil. ^1H NMR (400 MHz, Chloroform- d): 4.32 (t, J = 6.7Hz, 2H), 4.22-4.11 (m, 4H), 3.59-3.44 (m, 6H) 2.88 (t, J = 6.7Hz, 2H), 2.79 (t, J = 6.2Hz, 2H), 2.76, 2.69 (m, 2H), 2.47-2.37 (m, 2H), 2.34-2.24 (m, 2H), 1.77-1.47 (m, 12), 1.47-1.33 (m, 4H), 1.31-1.11 (m, 40H), 0.84 (t, J = 7.1Hz, 4x3H=12H); ^{13}C NMR (100 MHz, Chloroform- d): 176.3, 172.6, 155.5, 63.5, 62.3, 61.9, 55.8, 53.8, 47.5, 47.0, 45.7, 38.2, 37.5, 37.1, 32.6, 32.3, 31.9, 31.7, 29.6, 29.5, 29.3, 29.2, 27.5, 27.4, 27.3, 26.9, 22.7, 22.6, 14.11, 14.08; MS: 914.10 m/z [M^+].



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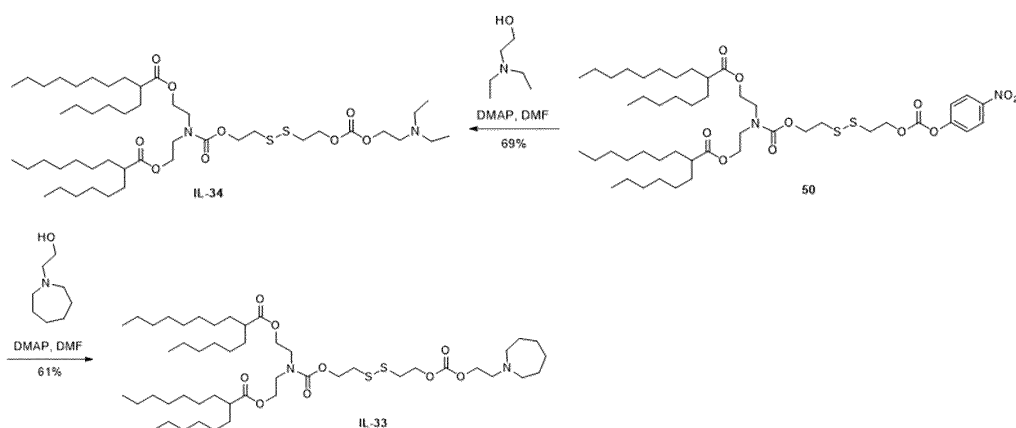
Intermediate 49; Disulfanediylbis(ethane-2,1-diyl) bis(4-nitrophenoxy) bis(carbonate)

A DCM (20 mL) solution of 2,2'-dithiodiethanol (2.00g, 1eq, 13.0mmol) and triethylamine (4.59g, 6.33mL, 3.5eq, 45.4mmol) was cooled to 0°C using an ice-water bath. 4-nitrophenyl carbonochloridate (6.53g, 2.5eq, 32.4mmol) in DCM (20mL) was added dropwise at 0°C over 30min. The ice bath was removed, and the reaction mixture was stirred at room temperature for 20h. The RM was diluted with 50mL DCM and washed with 2x50mL 0.5M HCl, 3x50 mL Na₂CO₃ (sat. aq.), 50 mL water, dried over sodium sulfate and evaporated. Intermediate **49** (5.93g, 12.2mmol, 94%) was isolated as a thick slightly yellow oil and used as it for next step.

Intermediate 50; 11-(2-((2-Hexylnonanoyl)oxy)ethyl)-1-(4-nitrophenoxy)-1,10-dioxo-2,9-dioxo-5,6-dithia-11-azatridecan-13-yl 2-hexyldecanoate

Intermediate **49** (2.56g, 3eq, 5.28mmol) was dissolved in DMF (5.0mL) and triethylamine (356mg, 491 μ L, 2eq, 3.52mmol) was added, followed by DMAP (21.5mg, 0.1eq, 0.18mmol). DHDA, **3** (1.00g, 1eq, 1.76mmol) in DMF (5.0mL) was added dropwise over 10min and the mixture was stirred at room temperature overnight. Volatiles were removed and crude residue was suspended in 25 mL heptane and the suspension was kept at ice-cool conditions for 5 minutes. The heptane layer was carefully decanted. This step was repeated a couple of times. The combined organics were filtered over Celite and the clear filtrate was evaporated. Intermediate **50** (1.50g, 1.6mmol, 93%) was isolated as a yellow oil.

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General procedure for IL's with carbonate moiety (**IL-33** and **IL-34**) with amino alcohols.

- 5 Intermediate **49** (250mg, 1eq, 270 μ mol) was dissolved in DMF (1.0mL) and DMAP (65.9mg, 2eq, 539 μ mol) was added, followed by the amino alcohol (2eq, 539 μ mol) and the mixture was stirred at room temperature for 2h. Volatiles were removed and the residue was dissolved in 25 mL DCM and washed with 2x25mL half-saturated Na₂CO₃ (aq.), 25mL water, and 25mL 5% LiCl (aq.), dried over sodium sulfate and evaporated. The crude was purified by column chromatography (DCM/MeOH gradient).
- 10

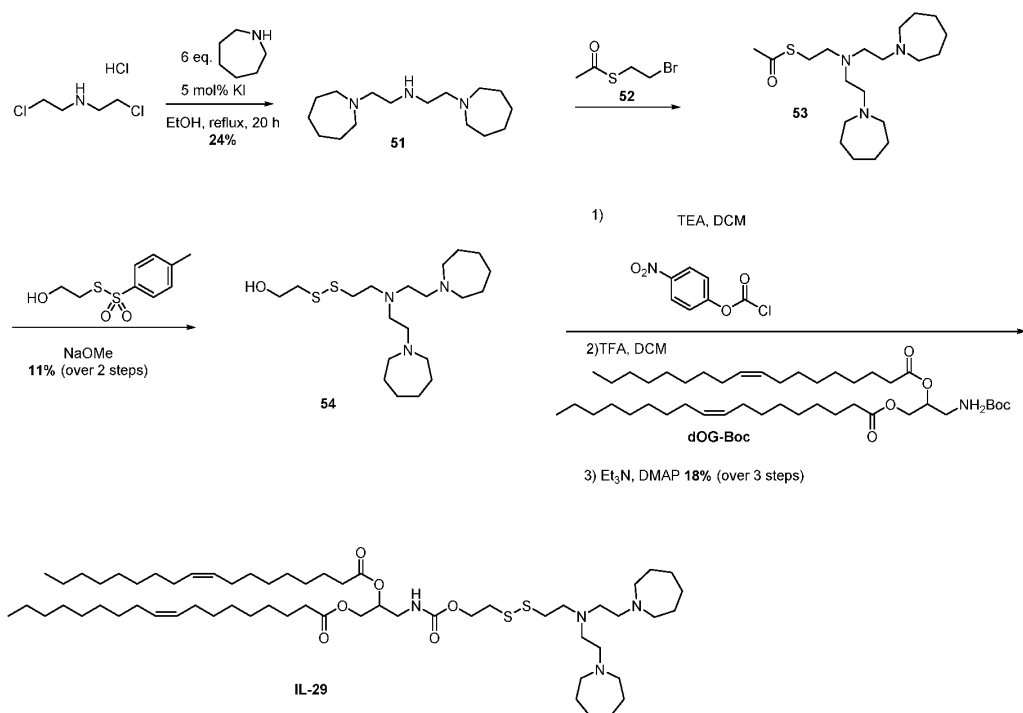
IL-33; 16-(azepan-1-yl)-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4,13-dioxo-5,12,14-trioxa-8,9-dithia-3-azahexadecyl 2-hexyl decanoate

- Starting from 2-(azepan-1-yl)ethanol and intermediate **50**, **IL-33** (153 mg, 164 μ mol, 61 %) was obtained as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.42 (m, 4H), 4.25-4.13 (m, 6H), 3.59-3.46 (m, 4H) 2.98-2.89 (m, 4H), 2.80 (t, *J* = 6.0Hz, 2H), 2.69 (appt t, *J* = 5.2Hz), 2.35-2.25 (m, 2H), 1.66-1.48 (m, 10H), 1.48-1.33 (m, 4H), 1.33-1.14 (m, 40H), 0.86 (t, *J* = 6.9Hz, 4x3H=12H); ¹³C NMR (100 MHz, Chloroform-*d*): 176.3, 155.5, 155.0, 66.2, 65.5, 63.6, 62.3, 61.9, 55.8, 55.6, 47.5, 46.9, 45.7, 37.5, 37.0, 32.3, 31.9, 31.7, 29.6, 29.5, 29.3, 29.2, 28.1, 27.5, 27.4, 27.0, 22.67, 22.6, 14.11, 14.08; MS: 914.10 m/z [*M*⁺].
- 15

- 20 **IL-34**; 17-ethyl-3-(2-((2-hexyldecanoyl)oxy)ethyl)-4,13-dioxo-5,12,14-trioxa-8,9-dithia-3,17-diazanonadecyl 2-hexyl decanoate

- Starting from 2-(diethylamino)ethanol, **IL-34** (169 mg, 187 μ mol, 69 %) was isolated as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.33 (q, *J* = 7.5Hz, 4H), 4.22-4.11 (m, 4H), 3.59-3.46 (m, 4H) 3.58-3.48 (m, 4H), 2.98-2.87 (m, 4H), 2.69 (t, *J* = 6.3Hz, 2H), 2.50 (q, *J* = 7.1Hz, 4H), 2.35-2.25 (m, 2H), 1.62-1.48 (m, 4H), 1.48-1.33 (m, 4H), 1.33-1.12 (m, 40H), 0.99 (t, *J* = 7.1Hz, 6H), 0.84 (t, *J* = 6.8Hz, 4x3H=12H); ¹³C NMR (100 MHz, Chloroform-*d*): 176.2, 155.5, 155.0, 66.3, 65.8, 65.5, 63.3, 62.2, 61.9, 51.0, 47.7, 47.5, 46.9, 45.7, 37.5, 36.9, 36.9, 36.4, 32.3, 31.8, 31.8, 31.7, 29.6, 29.4, 29.3, 29.2, 27.5, 27.4, 22.7, 22.6, 14.10, 14.06, 11.9; MS: 905.40 m/z [*M*⁺].
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**Intermediate 52; S-(2-bromoethyl) ethanethioate**

The THF (60 mL) solution of potassium ethanethioate (3.26g, 1eq, 28.5mmol) and 1,2-dibromoethane (10.7g, 4.94mL, 2eq, 57.1mmol) was refluxed for 20h. The RM was cooled to rt and ~100 ml TBME was added. The suspension was filtered over a paper filter, washed with TBME and the filtrate was concentrate *in vacuo* to obtain 5.9 g of crude brown oil. Purification by column chromatography (0-15% EtOAc/heptane gradient) afforded intermediate **52** (1.9g, 36% yield).

Intermediate 51; Bis(2-(azepan-1-yl)ethyl)amine

- 10 To a EtOH (9.97mL) solution of bis(2-chloroethyl)amine hydrochloride (2.49g, 1eq, 14.0mmol) in a microwave tube, sodium iodide (105 mg, 0.05 eq, 698 μ mol) and azepane (8.99g, 10mL, 6.5eq, 90.7mmol) were added and stirred at reflux for 22h. 2M NaOH (40ml) was added to the RM and extract with DCM (3x40ml), dry, filter, conc in vacuo and obtained 2.26g brown oil. Purification by silica column chromatography (0-8% gradient of MeOH/DCM with 1% 7M NH₃ in MeOH) gave intermediate **51** (920mg, 24%) as an orange oil

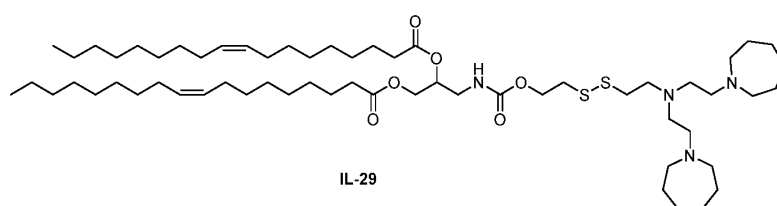
Intermediate 53; S-(2-(bis(2-(azepan-1-yl)ethyl)amino)ethyl) ethanethioate

- Intermediate **52** (366 mg, 1.12 eq, 2.00 mmol) and then KI (295 mg, 1 eq, 1.78 mmol) were added to the mixture of intermediate **51** (476mg, 1eq, 1.78 mmol) and K₂CO₃ (246 mg, 1 eq, 1.78 mmol) in acetonitrile (2.0mL) and DMF (2.0mL). The mixture was stirred at 50°C for 18h. 1ml sat. Na₂CO₃ and 20ml water were added to the RM and extracted with DCM (2x20mL), combined organic layers dried over Na₂SO₄, filtered, and conc in vacuo to obtain intermediate **53** (760mg) as a brown oil.

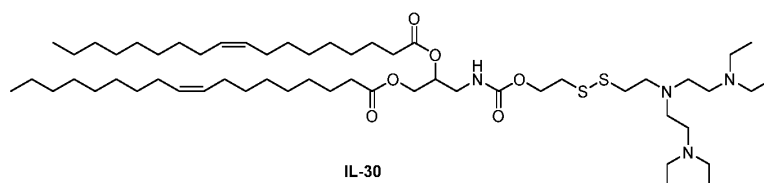
-72-

Intermediate 54; 2-((2-(bis(2-(azepan-1-yl)ethyl)amino)ethyl)disulfaneyl)ethan-1-ol

Sodium methoxide in methanol (220mg, 753 μ L, 5.4M, 2.3eq, 4.07mmol) was added to the mixture of crude intermediate **53** (760mg, 86% wt, 1eq, 1.77mmol), S-(2-hydroxyethyl) 4-methylbenzenesulfonothioate (724mg, 1.76eq, 3.12mmol) in DCM (15.9mL) and MeOH (1.77mL) at rt for 30 min. Sat. NaHCO₃ (25 ml) was added to the RM and the layers were partitioned. The aqueous layer was extracted with DCM (2x20 mL) and the combined organic layers were dried over Na₂SO₄, filtered, and concentrated in vacuo. The crude (800 mg) was purified twice by column chromatography (1-6% gradient of 7M NH₃ in MeOH/DCM). The intermediate **55** (80mg, 11%) was obtained as an orange oil.

**IL-29** 1-(azepan-1-yl)-3-(2-(azepan-1-yl)ethyl)-11-oxo-10-oxa-6,7-dithia-3,12-diazapentadecane-14,15-diyl dioleate

Et₃N (0.13g, 0.18mL, 6.5eq, 1.3mmol) and 4-nitrophenyl carbonochloridate (54mg, 1.4eq, 0.27mmol) were added to the DCM (0.4mL) solution of intermediate **54** (80mg, 1eq, 0.20mmol) at 0°C. The RM was stirred at rt for 30min. DMAP (5.0 mg, 0.21 Eq, 41 μ mol) and 3-aminopropane-1,2-diyl dioleate (dOG, 0.44 g, 3 eq, 0.59 mmol) in DMF (0.4 mL) were added. The mixture was stirred at rt for 18 h. Volatiles were removed under vacuo and residue was suspended in 7 ml heptane. The mixture was washed twice with sat. Na₂CO₃/water/methanol (1/1/1, 2x 7mL). Later the heptane layer was washed with methanol/water (1/1, 1x7 mL) and then with methanol/water (9/1, 1x7mL). The resulting heptane layer was concentrated in vacuo and 325 mg of orange oil was obtained. The crude was purified by silica column (eluent A: DCM, eluent B: 2M NH₃ in MeOH) to obtain **IL-29** as a light yellow oil (34 mg, 18% yield). ¹H NMR (400 MHz, Chloroform-*d*): 5.55-5.24 (m, 4H), 5.14-5.04 (m, 1H), 4.41-4.20 (m, 4H), 4.11 (dd, J = 5.2, 11.8Hz, 1H), 3.70-3.26 (m, 4H), 2.93-2.53 (m, 18H), 2.36-2.15 (m, 6H), 2.60-1.76 (m, 12H), 1.72-1.49 (m, 18H), 1.38-1.14 (m, 40H), 0.86 (t, J = 6.9Hz, 2x3H = 6H); MS: 1049.8 m/z [M⁺].

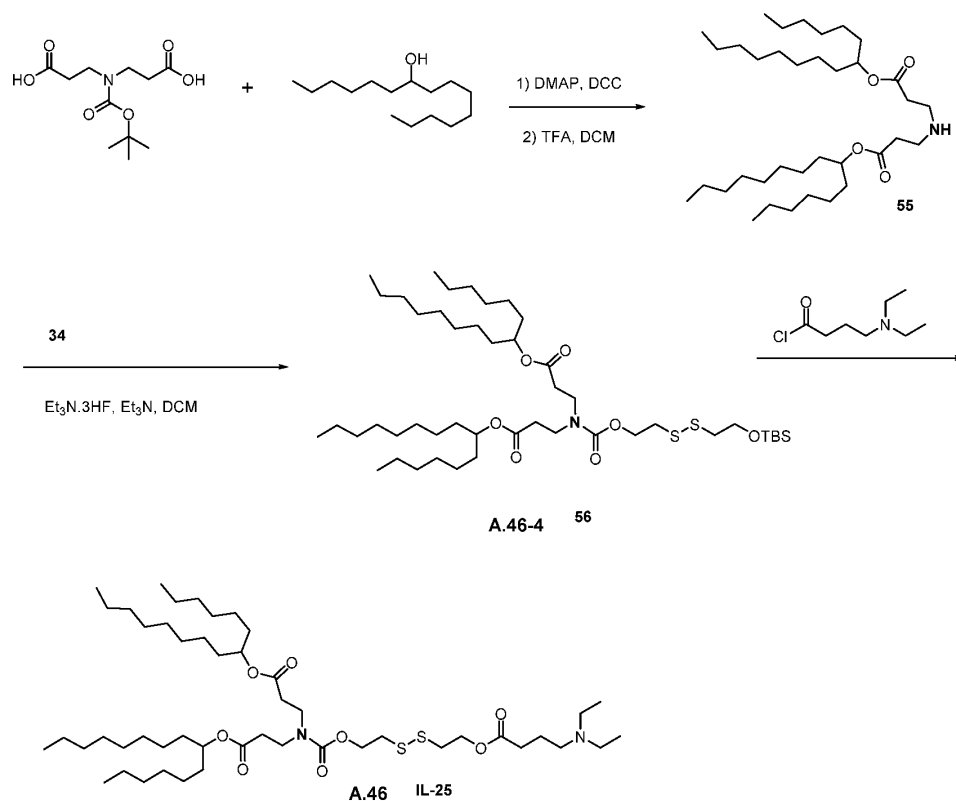
**IL-30**; 6-(2-(diethylamino)ethyl)-3-ethyl-14-oxo-13-oxa-9,10-dithia-3,6,15-triazaoctadecane-17,18-diyl dioleate

In an analogy to IL-29, starting from *N,N,N',N'*-tetraethyldiethylenetriamine, **IL-30** was obtained

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as a light yellow oil (87mg, 17% yield). ¹H NMR (400 MHz, Chloroform-*d*): 5.55-5.28 (m, 4H), 5.14-5.04 (m, 1H), 4.41-4.22 (m, 3H), 4.11 (dd, *J* = 5.2, 11.8Hz, 1H), 3.49-3.26 (m, 2H), 2.93-2.73 (m, 6H), 2.64-2.46 (m, 14H), 2.35-2.25 (m, 4H), 2.06-1.90 (m, 8H), 1.66-1.52 (m, 6H), 1.38-1.18 (m, 40H), 1.01 (t, *J* = 6.9Hz, 12H), 0.87 (t, *J* = 6.9Hz, 2x3H = 6H); MS: 997.80 *m/z* [*M*⁺].

5



Intermediate 55; di(pentadecan-7-yl) 3,3'-((tert-butoxycarbonyl)azanediyl)dipropionate (A37-3) DCC (908mg, 2.3eq, 4.4mmol) was added to the DCM (8.70 mL) solution of *N*-(2-Carboxyethyl)-*N*-[(1,1-dimethylethoxy)carbonyl]-β-alanine (500mg, 1eq, 1.91mmol) in at 0°C. The RM was stirred at 0°C for 15min before adding 7-pentadecanol (962mg, 2.2eq, 4.21mmol) followed by DMAP (23.4 mg, 0.1eq, 191μmol). The RM was stirred at room temperature for 18h. The solids were filtered off and washed with DCM and the filtrate evaporated. The crude was purified by silica column purification (heptanes/EtOAc gradient from 100/0 to 60/40). Boc-protected intermediate **55** (842mg, 1.23mmol, 64%) was obtained as pale yellow oil.

TFA (14.8g, 10.0mL, 105eq, 130mmol) was added to a solution of Boc-protected intermediate **55** (842mg, 1eq, 1.23mmol) in DCM (20 mL). The RM stirred for 3h at rt. The RM was concentrated to dryness and stripped with DCM (3x). Intermediate **55** (875 mg, 1.26 mmol, 102 %) was isolated as a brown oil and was used for the next step.

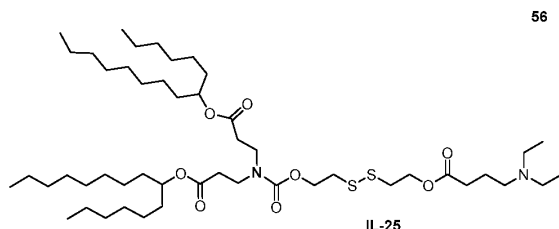
-74-

Intermediate 56: Pentadecan-7-yl 2,2,3,3-tetramethyl-12-oxo-13-(3-oxo-3-(pentadecan-7-yloxy)propyl)-4,11-dioxo-7,8-dithia-13-aza-3-silahexadecan-16-oate (A46-3)

To a solution of intermediate **55** (520 mg, 1.2 Eq, 747 μ mol) in DMF (2.0 mL), triethylamine (252 mg, 347 μ L, 4eq, 2.49mmol) and DMAP (7.61mg, 0.1eq, 62.3 μ mol) were successively added to a solution of intermediate **34** (270mg, 1eq, 623 μ mol) in DMF (3.0mL). The homogeneous yellow RM was stirred for 18h at rt. The RM was partitioned between TBME and water. The aqueous layer was further extracted with TBME (2x). The combined organic layers were washed with water (2x), dried over sodium sulfate, filtered and concentrated. The crude oil was purified by silica gel chromatography (2% to 10% MeOH in DCM) to obtain TBS-protected intermediate **56** (321mg, 366 μ mol, 58%) was isolated as a yellow oil.

Triethylamine trihydrofluoride (177.13 mg, 0.18 mL, 3 Eq, 1.0987 mmol) was added to a mixture of TBS-protected intermediate **56** (321.00mg, 1eq, 366.24 μ mol) and triethylamine (111.18mg, 153 μ L, 3eq, 1.1mmol) in THF (5.0mL). The reaction mixture was stirred overnight at room temperature. An aliquot of was worked-up (TBME/aq NaHCO₃) and analyzed by NMR, showing ~ full conversion. Another portion of triethylammonium fluoride (59.0mg, 60 μ L, 1eq, 366.2 μ mol) was added and the reaction mixture was further 3h. The reaction mixture was carefully poured in aqueous saturated NaHCO₃ and the product was extracted with TBME (3x). The organic layer was washed with aq NaHCO₃ (2x), brine (1x), dried over sodium sulfate, filtered and concentrated to yield intermediate **56** (279mg, 366 μ mol, 99%) as a yellow oil.

IL-25; Di(pentadecan-7-yl) 3,3'-((14-ethyl-10-oxo-2,9-dioxo-5,6-dithia-14-azahexadecanoyl)azanediyl)dipropionate

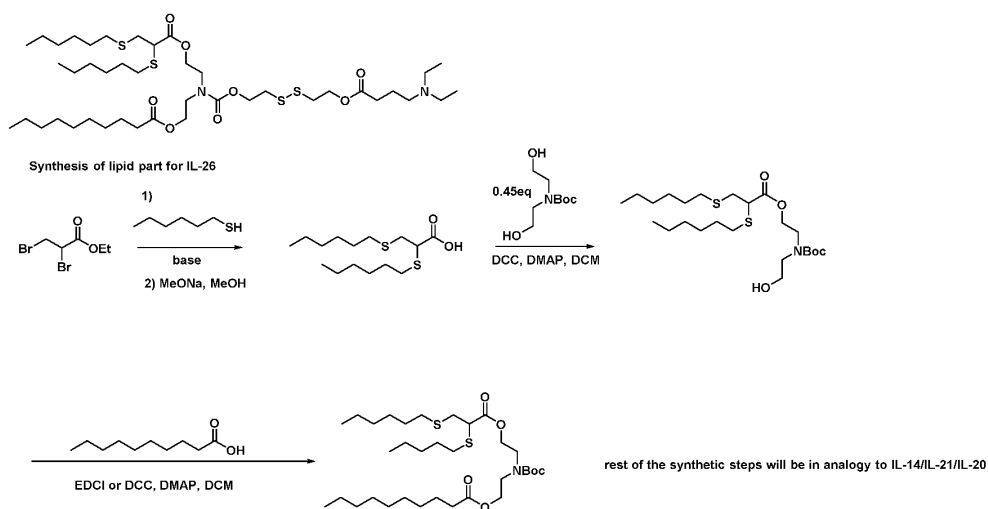


A solution of intermediate **41** (77.8mg, 1.2eq, 438 μ mol) in DCM (2.5 mL) was added to a mixture of intermediate **56** (278mg, 1eq, 36 μ mol), DIPEA (118mg, 159 μ L, 2.5eq, 912 μ mol) and DMAP (4.46 mg, 0.1eq, 36.5 μ mol) in DCM (5.0 mL). The resulting brown reaction mixture was stirred for 3 days at room temperature. The reaction mixture was partitioned between TBME and sat NaHCO₃. The aqueous layer was further extracted with TBME (2x). The organic layers were washed with water (1x), dried over sodium sulfate, filtered and concentrated to yield yellow oil. The crude was dissolved in DCM and purified by silica gel chromatography (2% to 10% MeOH in DCM) to obtain **IL-25** (200mg, 221 μ mol, 60%) as a yellow oil. ¹H NMR (400 MHz, Chloroform-*d*): 4.83 (p, J = 6.2Hz, 2H), 4.32 (t, J = 6.5Hz, 4H), 3.52 (bs, 4H), 2.93-2.74 (m, 8H), 2.63-2.49 (m, 4H), 2.42 (t, J = 6.8Hz, 2H), 2.03-1.91 (m, 2H), 1.56-1.42 (m, 8H), 1.13-1.15 (m, 46H), 0.84

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(t, J = 6.8 Hz, 4x3H = 12H); ^{13}C NMR (100 MHz, Chloroform-d); 172.5, 171.4, 171.2, 155.4, 74.9, 74.8, 63.1, 62.4, 51.0, 46.7, 44.4, 37.5, 37.1, 34.2, 34.0, 31.9, 31.7, 31.3, 29.7, 29.6, 29.5, 29.3, 29.2, 25.3, 23.27, 22.7, 22.6, 20.1, 14.1, 14.08, 9.84; MS: 903.60 m/z [M^+].

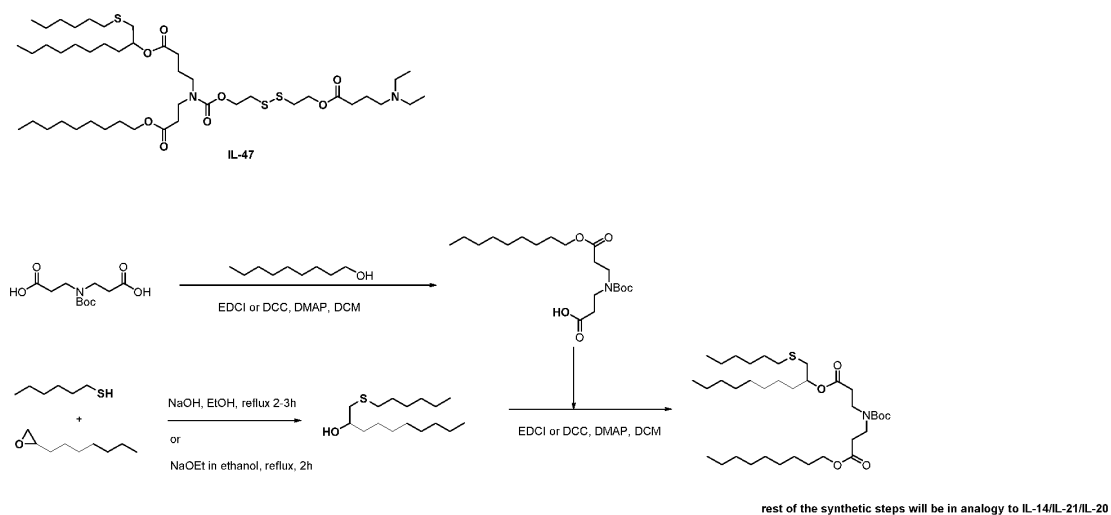
5 IL-26 synthesis route:



IL-27: the lipid tail part synthesis route is analogous to IL-26, using 2-hexyldecanoic acid.

IL-47 synthesis route:

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EXAMPLE: LNP SYNTHESIS**mRNA synthesis:**

All mRNA's were prepared in vitro by T7-mediated transcription from linearized DNA templates (peTheRNA vector), which incorporates 5' and 3' UTRs and a polyA tail. The final mRNA utilizes
5 Cap1 and 100% replacement of uridine with N1-methyl-pseudo-uridine. Ionizable lipids of the invention were prepared according to the reaction schemes defined herein above.

LNP synthesis:

Lipid based nanoparticles were produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100mM, pH 4) and lipid solution in a 2:1 or 3:1 volume ratio at a speed of from 9
10 - 16 mL/min using the NanoAssemblr Benchtop (Precision Nanosystems). LNPs were produced at a standard molar ratio ionizable lipid/DSPC (Avanti Polar Lipids) /cholesterol (Sigma) /DMG-PEG2000 (Avanti Polar Lipids) of about 50/10/38.5/1.5 eGFP mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/3 – 1/20. LNPs were dialyzed
15 against TBS (10000 times more TBS volume than LNP volume) using slide-a-lyzer dialysis cassettes (20K MWCO, 3mL, ThermoFisher). Size, polydispersity and zeta potential were measured with a Zetasizer Nano (Malvern) or DynaPro Plate reader (Wyatt). mRNA encapsulation was measured by standard Ribogreen RNA assay (Invitrogen).

EXAMPLE 2: IN VITRO EXPERIMENTS**20 mRNA synthesis**

mRNA encoding for eGFP was prepared *in vitro* by T7-mediated transcription from linearized DNA templates (peTheRNAvs3 vector), which incorporates 5' and 3' UTRs and a polyA tail. The final mRNA utilizes Cap1 and 100% replacement of uridine with N1-methyl-pseudo-uridine.

LNP synthesis

25 Lipid based nanoparticles are produced by microfluidic or T-junction mixing of an mRNA solution in a sodium acetate buffer (100mM, pH4) and lipid solution in a 3:1 volume ratio at a speed of 12mL/min, 16 ml/min or 20 mL/min using the NanoAssemblr Benchtop (Precision Nanosystems) or alternative T-junction device. The lipid solution contained a mixture of the ionizable lipid of interest, DSPC or DOPE (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti). LNPs were
30 dialyzed against TBS (10000 times more TBS volume than LNP volume) using slide-a-lyzer dialysis cassettes (20K MWCO, 3mL, ThermoFisher). Size, polydispersity and zeta potential were measured with a Zetasizer Nano (Malvern). mRNA encapsulation was measured by standard Ribogreen RNA assay (Invitrogen).

SM-102 and MC3 are ionizable lipids considered to be known in the state-of-the art and were used as control.

- 5 The apparent pKa of formulated LNPs was determined via TNS binding assay as described by Sabnis et al. (Molecular Therapy, Vol. 26, No 6, 2018).

Cell lines

The most optimal culturing conditions per cell type including growth medium, sub cultivation ratio, and medium renewal recommendations are summarized below. To harvest adherent cells, used-up growth medium was discarded and cells were rinsed twice with phosphate buffered saline (PBS) (Sigma) before addition of trypsin-EDTA (0.05%) (Gibco, Thermo Fisher Scientific) to loosen the cells. Medium renewal needs to occur every 2 to 3 days, whenever cells reached confluency of approximately 70%. Cell viability was determined using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter).

Cell type	Description	Source	Growth medium	Subcultivation ratio
HEK-293T	Adherent human embryonic kidney cells	ATCC	DMEM – high glucose with 10% FBS, 4mM glutamin, 2 mM P/S	1:2 to 1:10
DC2.4	Immortalized murine dendritic cells	Merck	RPMI-1640 with 10% FBS, 2mM glutamin, 1 mM sodium pyruvate, 2 mM P/S	1:2 to 1:10

Abbreviations: DMEM: Dulbecco’s Modified Eagle Medium; P/S: Penicillin/Streptomycin; FBS: Foetal Bovine Serum

Transfection

- 20 Cells were plated in a 96-well plate at a density of 2.0-3.0 x10e4 cells/100µl complete growth medium (specific per cell type). Transfection was performed when cells reached 70-90% confluency. The positive control Lipofectamine (MessengerMAX, Invitrogen) was diluted in OptiMEM (serum reduced, Gibco) and incubated for 10 minutes. In the meantime, eGFP mRNA and LNPs encapsulating eGFP mRNA were diluted in OptiMEM to get to a concentration of the mRNA content of 200 and 50ng/well. mRNA : lipid complexes were incubated in a 1 : 1 ratio for 5 minutes and were added to each condition in quadruplicate. Cells were incubated for 24 hours at 37°C 5% CO2. Afterwards cells were harvested using 1 x TrypLE select enzyme (Gibco) and stained with a live/dead marker SYTOX blue (Life Technologies) in FACS buffer (PBS
- 25

supplemented with 1% bovin serum albumin (BSA) and 0.09% azide (all from Sigma)). Cells were immediately acquired after addition of the live dead marker using the Attune Nxt Flow Cytometer (ThermoFisher Scientific).

5 eGFP expression

For assessment of eGFP expression, cells were stained with SYTOX blue. Within the gate of SYTOX blue negative cells, expression levels of eGFP were determined. The relative mean fluorescence intensity (rel MFI) was calculated as the MFI value of the expression marker divided by that of untransfected cells.

10

Data was acquired on an Attune Nxt cytometer and analyzed with Flow Jo Software. Flow cytometric data were analyzed using the Flowjo version 10 software.

Example 2a

15 LNPs were produced at a standard molar ratio ionizable lipid/DSPC/cholesterol/DMG-PEG2000 of about 50/10/38.5/1.5. eGFP mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10, except for SM-102 bearing LNP where mRNA/ionizable lipid molar ratio of 1/6 was used.

A list of relevant physico-chemical properties for different LNP compositions, based on IL-9, IL-10, IL-12, IL-13, IL-21, IL-22, IL-23, IL-24 and SM-102 is shown below in Table 1.

20

Table 1. Physico-chemical characteristics of LNPs

Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	EE%
IL-9	IL-9 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	73.7	0.139	100
IL-10	IL-10 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	76.6	0.034	99
IL-12	IL-12 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	81.8	0.128	99
IL-13	IL-13 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	73.5	0.057	98
IL-21	IL-21 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	55.4	0.157	96
IL-22	IL-22 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	66.8	0.087	100
IL-23	IL-23 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	63.2	0.160	97
IL-24	IL-24 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	63.4	0.091	98
SM-102	SM-102 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	79.2	0.075	98

Figure 1 shows that LNPs containing ionizable lipids according to the invention have no significant impact on the viability of the transfected DC2.4 cells.

Figure 2 shows that LNPs containing ionizable lipids according to the invention have no significant impact on the viability of the transfected HEK293T cells.

5 Figure 3 shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in DC 2.4 cells upon incubation with the indicated LNPs at mRNA concentration of 50 ng and 200 ng/well.

Figure 4 shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation
10 with the indicated LNPs at mRNA concentration of 50 ng and 200 ng/well.

Example 2b

LNPs were produced at a standard molar ratio ionizable lipid/DSPC/cholesterol/DMG-PEG2000 of about 50/10/38.5/1.5. eGFP mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10.

15 A list of relevant physico-chemical properties for different LNP compositions, based on IL-20, IL-33, IL-34, IL-41, IL-42 and IL-46 is shown below in Table 2.

Table 2. Physico-chemical characteristics of LNPs

Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	EE%
IL-20	IL-20 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	64.4	0.193	95
IL-33	IL-33 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	66.7	0.211	95
IL-34	IL-34 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	82.3	0.191	91
IL-41	IL-41 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	77.1	0.158	97
IL-42	IL-42 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	67.1	0.179	98
IL-46	IL-46 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	70.1	0.214	95

20 Figure 5 shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation with the indicated LNPs at mRNA concentration of 50 ng and 200 ng/well.

Figure 6 shows that LNPs containing ionizable lipids according to the invention have no significant impact on the viability of the transfected HEK293T cells.

Example 2c

LNPs were produced at a standard molar ratio ionizable lipid/DSPC/cholesterol/DMG-PEG2000 of about 50/10/38.5/1.5. eGFP mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10.

- 5 A list of relevant physico-chemical properties for different LNP compositions, based on IL-11, IL-13, IL-21, IL-24, IL-28 and MC3 is shown below in Table 3.

Table 3. Physico-chemical characteristics of LNPs

Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	EE%
IL-11	IL-11 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	96.2	0.069	97
IL-13	IL-13 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	74.6	0.099	96
IL-21	IL-21 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	71.2	0.244	94
IL-24	IL-24 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	73.2	0.163	95
IL-28	IL-28 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	72.2	0.117	90
MC3	MC3 / DSPC/ Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	63.3	0.106	98

- 10 Figure 7 shows that LNPs containing ionizable lipids according to the invention may show to have no significant impact on the viability of the transfected HEK293T cells.
- Figure 8 shows the Relative Mean Fluorescence Intensity (measured as the fold-increase in eGFP MFI compared to untreated cells) of eGFP expression in HEK-293T cells upon incubation with the indicated LNPs at mRNA concentration of 50 ng and 200 ng/well.

15

EXAMPLE 3: IN VIVO EXPERIMENTS

Example 3a: Bioluminescence imaging after IV administration of Fluc mRNA containing LNPs

20 **Materials and methods**

Animals

- Mice were housed in IVC under specific pathogen-free conditions. All animal experiments were performed with approval from the Ethical Committee and animal care was according to established guidelines. Female Balb/C 6-weeks old (20-23g) were obtained from Charles River International Laboratories, Inc. (France) and housed (max 5 per cage) with free access to water and standard laboratory animal chow.
- 25

Intravenous injection

For intravenous injection, mice are either placed in a warming chamber for vein dilation for no more than 10 min or the cage was placed under a red lamp for 10-20 min. When appropriately warm, mice were restrained individually. The tail was swabbed with gauze dampened in 70% ethanol. The needle of a BD microfine syringe (20-25G) with LNP solution was carefully inserted into one of the side tail veins and applying slow pressure to the plunger a maximum volume of 200µL (for mice of 20-25g) was injected. The needle was removed from the vein and the local bleeding stopped by applying slight pressure to the puncture site with dry gauze. Animals were subsequently observed for at least 10 minutes in their cage. Each injection was equivalent to 10 µg Fluc mRNA.

Luminescence imaging

Imaging was performed 24h after IV injection of LNP formulations. Each mouse was injected i.p. with 100 µl D-luciferin (30 mg/mL). Mice were then placed in an anesthesia induction chamber with oxygen supply (0.4-0.8 L/min) and isoflurane (5%) until they undergo narcosis. The flow of isoflurane was then reduced (3%) to maintain narcosis until mice are ready to be imaged. Mice are placed in a maximum of 3 per group in a stage inside the IVIS Lumina II (PerkinElmer) using the same anesthesia flow (3-4% Isoflurane). Imaging is performed with the parameters set to Luminescence, auto exposure with background overlay and medium binning (4), using field of view D. Imaging of the animals was performed no longer than 15 min after luciferin injection (peak of the signal). Once in vivo images were taken, the mice were sacrificed by means of cervical dislocation, dissected and imaging of respectively the liver, spleen and inguinal lymph node under similar imaging conditions.

mRNA synthesis

FireFly luciferase (Fluc) mRNA was produced from a linearized peTheRNA vector using eTheRNA-optimized in vitro transcription (IVT) reaction conditions and purified via cellulose. Uridine was fully substituted by N1-Methylpseudouridine (N1ψ) to generate N1ψ-modified mRNA.

LNP production

Lipid based nanoparticles are produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100mM, pH4) and lipid solution in a 3:1 volume ratio at a speed of 12mL/min, 16 mL/min or 20 mL/min using a microfluidic mixing device with T-junction. The lipid solution contained a mixture of the ionizable lipid of interest, DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti). MC3 and SM-102 were taken along as benchmarks.

LNPs were produced at a standard molar ratio ionizable lipid/DSPC/cholesterol/DMG-PEG2000 of about 50/10/38.5/1.5. eGFP mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/6.

A list of relevant physico-chemical properties for different LNP compositions, based on IL-9, IL-10, IL-12, IL-22, MC3 and SM-102 is shown below in Table 4.

Table 4. Physico-chemical characteristics of LNPs

LNP	Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	EE%
1	IL-9	IL-9 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	73.5	0.058	97
2	IL-10	IL-10 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	79.4	0.041	97
3	IL-12	IL-12 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	77.6	0.077	99
4	IL-22	IL-22 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	77.1	0.135	97
5	MC3	MC3 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	56.9	0.107	98
6	SM-102	SM-102 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	70.8	0.053	98

Figure 9 reveals the *in vivo* average radiance (normalized in function of photons/second/cm²/steradian) as captured by the IVIS. The mice are positioned in a manner that the image is taken in the supine view after 24h.

Figure 10 reveals the *ex vivo* average radiance (normalized in function of photons/second/cm²/steradian) of both liver and spleen as captured by the IVIS.

Example 3b: combination of Bioluminescence imaging after IV administration of Fluc mRNA containing LNPs and hEPO expression of hEPO mRNA containing LNPs

Materials and methods:

Animals

Mice were housed in IVC under specific pathogen-free conditions. All animal experiments were performed with approval from the Ethical Committee for Animal Experiments and animal care was according to established guidelines. Female Balb/C 6-weeks old (20-23g) were obtained from Charles River International Laboratories, Inc. (France) and housed (max 5 per cage) with free access to water and standard laboratory animal chow.

Intravenous injection

For intravenous injection, mice are either placed in a warming chamber for vein dilation for no more than 10 min or the cage was placed under a red lamp for 10-20 min. When appropriately warm, mice were restrained individually. The tail was swabbed with gauze dampened in 70% ethanol. The needle of a BD microfine syringe (20-25G) with LNP solution was carefully inserted into one of the side tail veins and applying slow pressure to the plunger a maximum volume of 100µL (for mice of 20-25g) was injected. The needle was removed from the vein and the local bleeding stopped by applying slight pressure to the puncture site with dry gauze. Animals were subsequently observed for at least 10 minutes in their cage. Each injection was equivalent to 5 µg Fluc mRNA and each mouse was injected 3 times in one week intervals using the batch of LNP formulation. LNP formulations were kept at 4°C and monitored for their physico-chemical characteristics prior to their usage (Table 6). 24h post injection, blood samples were taken and hEPO protein content was determined using a commercially available hEPO-ELISA kit according to the manufacturers protocol.

mRNA synthesis

- FireFly luciferase (Fluc) mRNA was produced from a linearized peTheRNA vector using eTheRNA-optimized in vitro transcription (IVT) reaction conditions and purified via cellulose. Uridine was fully substituted by N1-Methylpseudouridine (N1ψ) to generate N1ψ-modified mRNA.
- Human erythropoietin (hEPO) mRNA was produced from a linearized peTheRNA vector using eTheRNA-optimized in vitro transcription (IVT) reaction conditions and purified via cellulose. Uridine was fully substituted by N1-Methylpseudouridine (N1ψ) to generate N1ψ-modified mRNA.

LNP production

- Lipid based nanoparticles are produced by T-mixing of an mRNA solution in sodium acetate buffer (100mM, pH4) and lipid solution in a 3:1 volume ratio at a speed of 12 mL/min or 16mL/min using the NanoAssemblr Benchtop (Precision Nano). The lipid solution contained a mixture of the ionizable lipid of interest, DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti).

- LNPs were dialyzed against TBS (10000 times more TBS volume than LNP volume) using slide-a-lyzer dialysis cassettes (20K MWCO, 3mL, ThermoFisher). Size, polydispersity and zeta potential were measured with a Zetasizer Nano (Malvern). mRNA encapsulation was measured by standard Ribogreen RNA assay (Invitrogen).

- LNPs were produced at a standard molar ratio ionizable lipid/DOPE/cholesterol/DMG-PEG2000 of about 50/10/38.5/1.5. Fluc and hEPO mRNA was encapsulated in all LNPs, at a mRNA/ionizable lipid molar ratio of 1/6. Each LNP contains both mRNA's.

A list of relevant physico-chemical properties for the LNP composition based on IL1 at different timepoints is shown below in Table 5.

Table 5. Physico-chemical characteristics of LNPs

LNP	Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	EE%
1	IL-9	IL-9 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	74.8	0.192	98
2	IL-10	IL-10 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	77.4	0.096	97
3	IL-12	IL-12 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	76.3	0.183	98
4	IL-21	IL-21 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	75.0	0.249	97
5	IL-22	IL-22 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	69.6	0.145	98
6	IL-23	IL-23 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	70.4	0.181	98
7	IL-24	IL-24 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	71.6	0.143	97
8	MC3	MC3 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	72.7	0.158	96

5 Figure 11 reveals the *in vivo* average radiance (normalized in function of photons/second/cm²/steradian) as captured by the IVIS. The mice are positioned in a manner that the image is taken in the supine view after 24h.

10 Figure 12 reveals the average hEPO conc in the blood 4h after IV injection, expressed as ng/mL.

EXAMPLE 4: IN VIVO EXPERIMENTS

15 **Example 4: induction of anti-HA (hemagglutinin) immune responses upon intramuscular mRNA vaccination**

Materials and methods

Animals

20 Mice were housed in IVC under specific pathogen-free conditions. All animal experiments were performed with approval from the Ethical Committee for Animal Experiments and animal care was according to established guidelines. Female Balb/C 6-weeks old (20-23g) were obtained from Charles River International Laboratories, Inc. (France) and housed (max 5 per cage) with free access to water and standard laboratory animal chow.

Intramuscular injections and muscle thickness assessment:

All mice were housed under specific pathogen-free conditions, and animal studies were conducted under protocols and guidelines approved by the Ghent University animal care and use committee (ECD22-05). Mice were injected in quadriceps muscle with mRNA LNPs in TBS (50 µl volume, 0.5 µg of mRNA) on 2 timepoints (day 0 prime and day 21 boost). The thickness of the muscle at the injection site was measured with an electronic external measuring gauge (K220T, Kroeplin) at day 1 up to day 4 after injection.

Assessment of mouse endpoint Immunoglobulin titers

10 100 µl of mouse whole blood was collected on day 21 and day 35 in serum gel tubes (SarsTedt). Serum was separated from the blood clot by centrifugation at 10 000 g for 10 min at 4°C. Black flat bottom maxisorp 96 well plates (437111, Life Technologies) were coated overnight at 4 °C with 100 µl 1µg/ml of recombinant H1N1 (A/Puerto Rico/8/1934) HA protein (Sino Biological, 11684-V08H) in carbonate/bicarbonate buffer (0.1 M, pH 9.6). Plates were subsequently blocked with 100 µl of 3%BSA (05479-250g, Sigma) in PBS (w/v) for 2h. Subsequently, plates were washed 3 times with PBS/0.1%Tween (10113103, Fisher Scientific). A serial dilution of serum samples was added to the plates (initial 100X dilution of serum for d21 and initial 5000x serum dilution for d35; 5X dilution steps). After 2h incubation at RT plates were washed 5 times and solutions of rabbit anti-mouse IgG1 conjugated with HRP (1:15 000, Biorad, OBT1508P) or goat anti-mouse IgG2a conjugated with HRP (1:8000, STAR133P Biorad) were added for another 1h. After a final wash, the fluorescent Amplex UltraRed Reagent (A36006, Invitrogen) was used to develop plates according to the manufacturers' instructions. Plates were read on a Tecan Infinite 200 Pro with $\lambda_{ex}=540nm$, $\lambda_{em}=590nm$. The dilutions of serum of TBS treated mice served for cut-off determination, being the average fluorescence measured in the TBS samples plus 3 standard deviations. All points beyond cut-off were considered to be below quantification limit. The curves were fitted with a 5PL algorithm in (Prism) to the dilution data then the endpoint titer was calculated at cross point of the modeled curve with the cut-off.

LNP production:

30 LNP formulations were prepared as indicated above. All formulations were prepared in a sterile manner with a N/P ratio of 10, except for SM-102 bearing LNPs where an N/P ratio of 6 was used. All formulations were characterized for size and PDI using Dynamic Light Scattering Zetasizer Nano-ZS (Malvern Pananalytical Ltd., Malvern, UK) and stored afterwards at 4°C. The physico-chemical characteristics of each formulation can be found in Table 6.

Table 6: List of relevant physico-chemical properties of different LNP formulations. These include the type of ionizable lipid, the lipid composition and the mol fraction of each component, the size and PDI (as determined by dynamic light scattering) and the encapsulation efficiency. Encapsulation efficiency has been measured by Ribogreen assay.

5

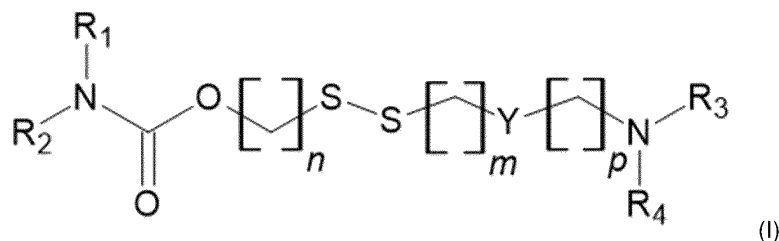
Ionizable Lipid	Composition	Ratio [mol %]	Size [nm]	PDI	E. E. [%]
IL-9	IL-9 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	71.8	0.168	99
IL-10	IL-10 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	76.1	0.074	98
IL-12	IL-12 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	79.6	0.176	98
IL-21	IL-21 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	66.4	0.219	98
IL-22	IL-22 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	65.7	0.134	99
IL-23	IL-23 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	72.4	0.211	98
IL-24	IL-24 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	63.5	0.157	98
MC3	MC3 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	63.6	0.100	100
SM-102	SM-102 / DSPC / Chol / DMG-PEG2000	50 / 10 / 38.5 / 1.5	87.9	0.052	97

Note: E.E. % is defined as the encapsulation efficiency of the mRNA inside the LNP nanoparticle.

- 10 Figure 13 reveals the IgG1 titers at day 35 as determined by ELISA and expressed as the reciprocate of the dilution of the sera that provides signal that is at least 3 times higher than the background $\pm 3SD$.
- Figure 14 reveals the IgG2a titers at day 35 as determined by ELISA and expressed as the reciprocate of the dilution of the sera that provides signal that is at least 3 times higher than the
- 15 background $\pm 3SD$.

CLAIMS

1. An ionizable lipid represented by formula (I)



5 wherein

R_1 and R_2 are each independently selected from -H, -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl; wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R_1 and R_2 together is at least 8;

R_3 and R_4 are each independently -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -halo, -C₁₋₆alkyl, -C₃₋₆cycloalkyl, OH, -OC₁₋₆alkyl, -SC₁₋₆alkyl, and -NH₂; -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and independently substituted with a phenyl substituent; or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

R_5 is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆'';

R_6 and R_6'' are each independently selected from -H, and -C₁₋₆alkyl;

or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;

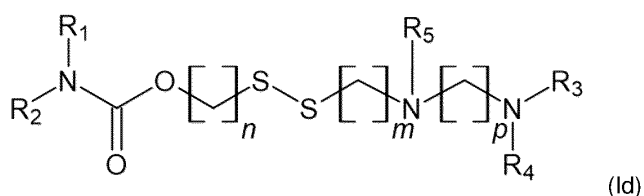
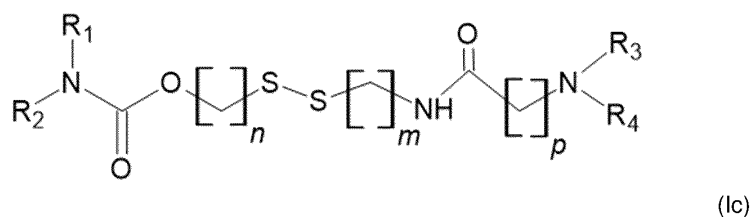
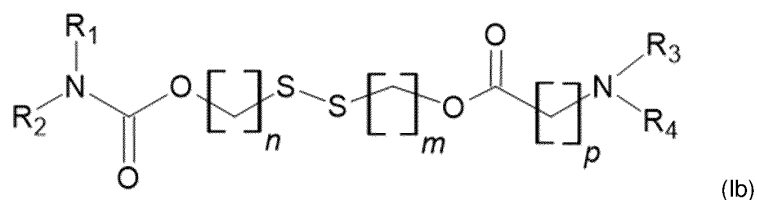
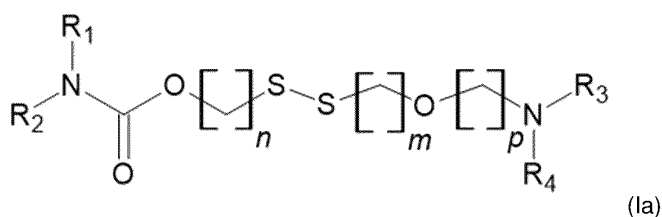
-88-

m, n and p are each independently an integer selected from 1, 2, 3 and 4;

Y is selected from -O-, -OC(O)-, -C(O)O-, -NR₅-, -NH-C(O)-, and -C(O)-NH-.

2. An ionizable lipid as defined in claim 1 and being represented by any one of formula (Ia), (Ib),

5 (Ic) or (Id);



10 wherein

R₁ and R₂ are each independently selected from -H, -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl;

wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl and -C₂₋₂₀alkynyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-

15 C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -

20 OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂

together is at least 8;

- 5 R₃ and R₄ are each independently -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -halo, -C₁₋₆alkyl, -C₃₋₆cycloalkyl, OH, -O-C₁₋₆alkyl, -S-C₁₋₆alkyl, and -NH₂; -C₁₋₆alkyl; wherein each of said -C₁₋₆alkyl is optionally and independently substituted with a phenyl substituent; or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- 10 R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆";
- R₆ and R₆" are each independently selected from -H, and -C₁₋₆alkyl;
- or R₆ and R₆" taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- 15 m, n and p are each independently an integer selected from 1, 2, 3 and 4.

- 20 3. An ionizable lipid as defined in any one of claims 1 to 2; wherein

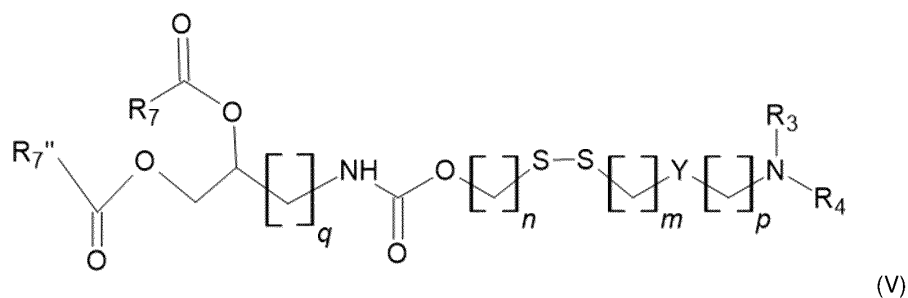
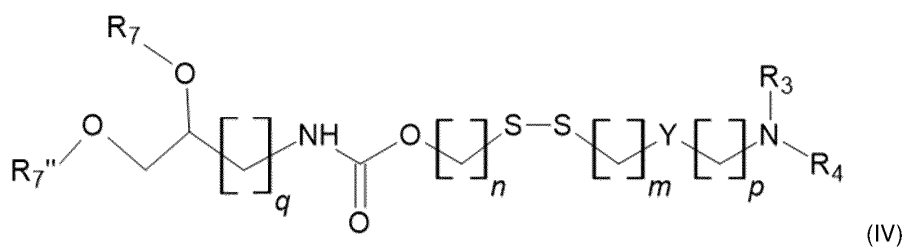
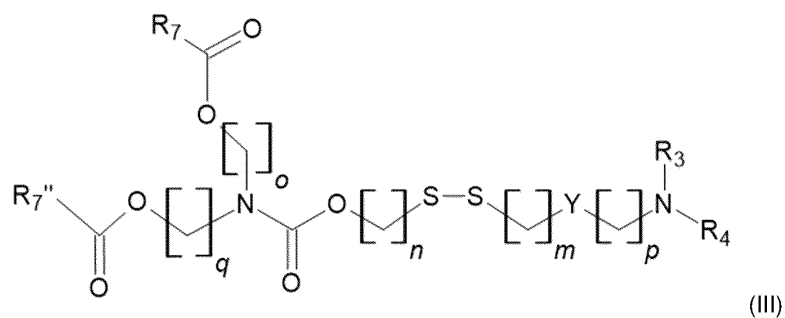
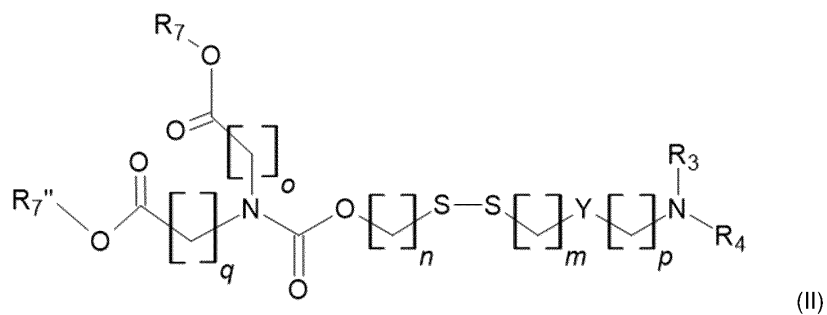
- R₁ and R₂ are each independently selected from -H, -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl; wherein each of said -C₁₋₂₀alkyl, and -C₂₋₂₀alkenyl optionally further comprises one or more heteroatoms and/or is optionally and independently substituted with from 1-3 substituents independently selected from -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C₁₋₂₀alkyl, and -C(O)O-C₂₋₂₀alkenyl;; wherein each of said -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -C(O)O-C₁₋₂₀alkyl, and -C(O)O-C₂₋₂₀alkenyl optionally comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋₂₀alkenyl, -OC(O)-C₂₋₂₀alkynyl, -C(O)O-C₁₋₂₀alkyl, -C(O)O-C₂₋₂₀alkenyl, and -C(O)O-C₂₋₂₀alkynyl; and wherein the total number of C atoms in R₁ and R₂ together is at least 8;
- 25 R₃ and R₄ are each independently -C₁₋₆alkyl;
- or R₃ and R₄ taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from -C₁₋₆alkyl, and -C₁₋₆alkyl-OH;
- 30 R₅ is -H or -C₁₋₆alkyl; wherein said -C₁₋₆alkyl may optionally be substituted with -NR₆R₆";
- R₆ and R₆" are each independently selected from -H, and -C₁₋₆alkyl;
- or R₆ and R₆" taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;
- 35

m, n and p are each independently an integer selected from 1, 2, 3 and 4;

Y is selected from -O-, -OC(O)-, -NR₅-, and -NH-C(O)-.

4. An ionizable lipid as defined in claim 1 and being represented by any one of formula (II), (III),

5 (IV) or (V)



10 wherein

each occurrence of R₇ and R₇' is independently selected from -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl, -C₂₋₂₀alkynyl; wherein each of said -C₁₋₂₀alkyl, -C₂₋₂₀alkenyl, -C₂₋₂₀alkynyl may optionally be substituted with from 1-3 substituents selected from -OH, -OC(O)-C₁₋₂₀alkyl, -OC(O)-C₂₋

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$_{20}$ alkenyl, $-\text{OC}(\text{O})-\text{C}_{2-20}$ alkynyl, $-\text{C}(\text{O})\text{O}-\text{C}_{1-20}$ alkyl, $-\text{C}(\text{O})\text{O}-\text{C}_{2-20}$ alkenyl, $-\text{C}(\text{O})\text{O}-\text{C}_{2-20}$ alkynyl;

and the total number of C atoms in R_7 and R_7'' together is at least 6;

R_3 and R_4 are each independently $-\text{C}_{1-6}$ alkyl; wherein each of said $-\text{C}_{1-6}$ alkyl is optionally and independently substituted with an aryl substituent; wherein each of said aryl is a 5- to 8-

5 membered aromatic cycle optionally comprising 1-3 heteroatoms selected from O, N and S and/or optionally and independently substituted with from 1-3 substituents selected from -

halo, $-\text{C}_{1-6}$ alkyl, $-\text{C}_{3-6}$ cycloalkyl, OH, $-\text{O}-\text{C}_{1-6}$ alkyl, $-\text{S}-\text{C}_{1-6}$ alkyl, and $-\text{NH}_2$; $-\text{C}_{1-6}$ alkyl; wherein each of said $-\text{C}_{1-6}$ alkyl is optionally and independently substituted with a phenyl substituent;

or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered
10 aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N atoms and/or is optionally substituted with from 1-3 substituents selected from $-\text{C}_{1-6}$ alkyl, and $-\text{C}_{1-6}$ alkyl-OH;

R_5 is -H or $-\text{C}_{1-6}$ alkyl; wherein said $-\text{C}_{1-6}$ alkyl may optionally be substituted with $-\text{NR}_6\text{R}_6''$;

R_6 and R_6'' are each independently selected from -H, and $-\text{C}_{1-6}$ alkyl;

15 or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional heteroatoms and/or is optionally substituted with from 1-3 substituents selected from $-\text{C}_{1-6}$ alkyl, and $-\text{C}_{1-6}$ alkyl-OH;

m, n, and p are each independently an integer selected from 1, 2, 3 and 4

20 q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

Y is selected from $-\text{O}-$, $-\text{OC}(\text{O})-$, $-\text{C}(\text{O})\text{O}-$, $-\text{NR}_5-$, $-\text{NH}-\text{C}(\text{O})-$, and $-\text{C}(\text{O})-\text{NH}-$.

5. An ionizable lipid as defined in claim 4; wherein

each occurrence of R_7 and R_7'' is independently selected from $-\text{C}_{1-20}$ alkyl, and $-\text{C}_{2-20}$ alkenyl;

25 wherein each of said $-\text{C}_{1-20}$ alkyl, and $-\text{C}_{2-20}$ alkenyl may optionally be substituted with from 1-3 substituents selected from -OH, $-\text{OC}(\text{O})-\text{C}_{1-20}$ alkyl, $-\text{OC}(\text{O})-\text{C}_{2-20}$ alkenyl, $-\text{C}(\text{O})\text{O}-\text{C}_{1-20}$ alkyl, and $-\text{C}(\text{O})\text{O}-\text{C}_{2-20}$ alkenyl; and the total number of C atoms in R_7 and R_7'' together is at least 6;

R_3 and R_4 are each independently $-\text{C}_{1-6}$ alkyl;

30 or R_3 and R_4 taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle; said heterocycle optionally further comprises one or more additional N or O atoms and/or is optionally substituted with from 1-3 substituents selected from $-\text{C}_{1-6}$ alkyl, and $-\text{C}_{1-6}$ alkyl-OH;

R_5 is -H or $-\text{C}_{1-6}$ alkyl; wherein said $-\text{C}_{1-6}$ alkyl may optionally be substituted with $-\text{NR}_6\text{R}_6''$;

35 R_6 and R_6'' are each independently selected from -H, and $-\text{C}_{1-6}$ alkyl;

or R_6 and R_6'' taken together with the N atom to which they are attached form a 5-10 membered aromatic or non-aromatic heterocycle;

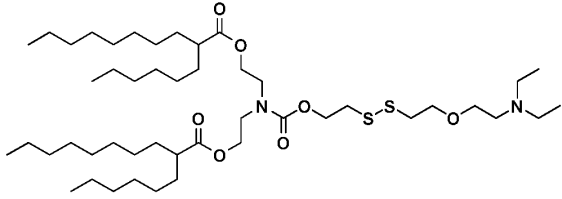
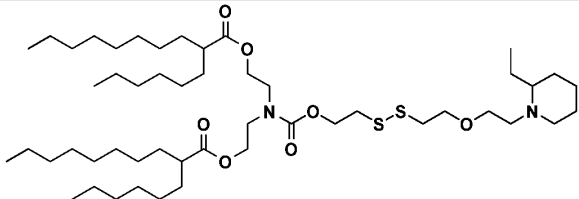
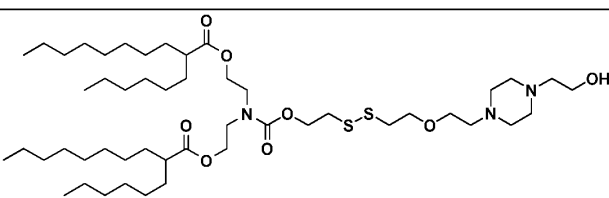
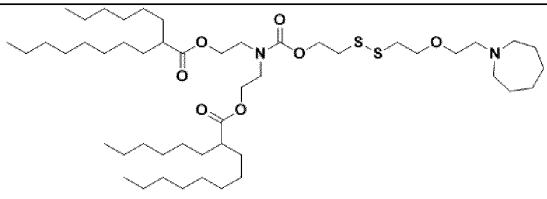
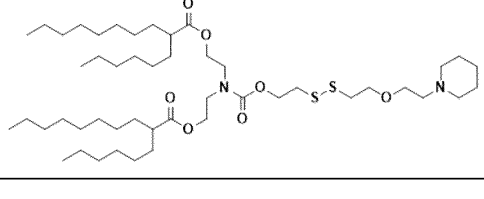
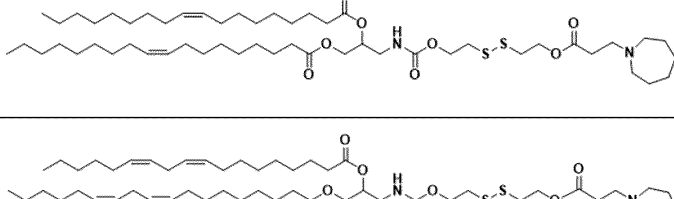
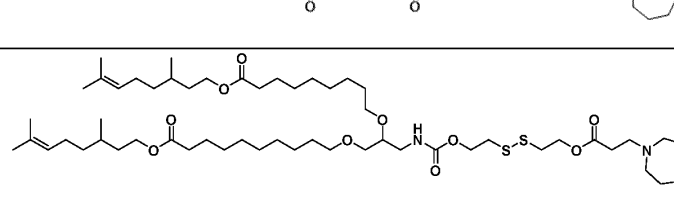
m, n, and p are each independently an integer selected from 1, 2, 3 and 4

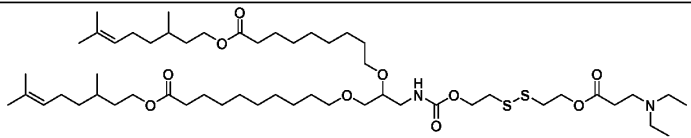
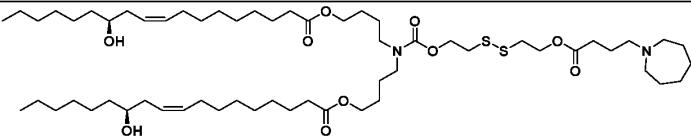
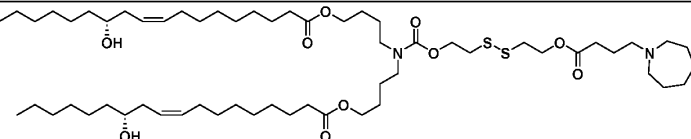
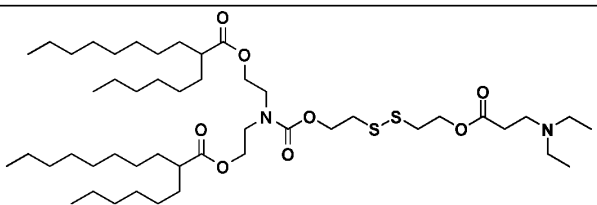
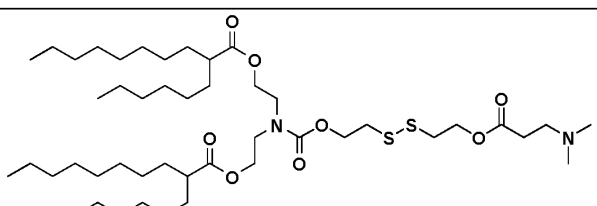
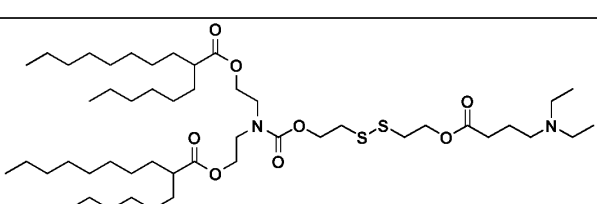
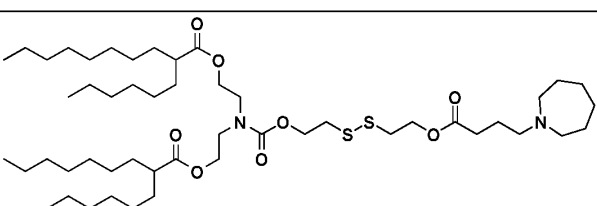
q and o are each independently an integer selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

Y is selected from -O-, -OC(O)-, -NR₅-, and -NH-C(O)-.

6. An ionizable lipid as defined in any one of claims 1 to 5, and being selected from the list comprising:

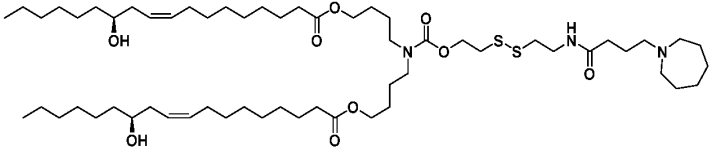
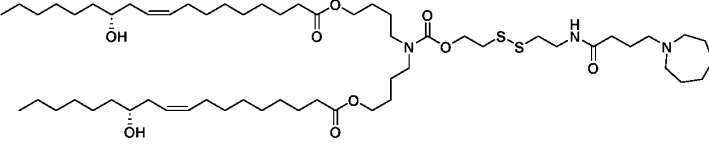
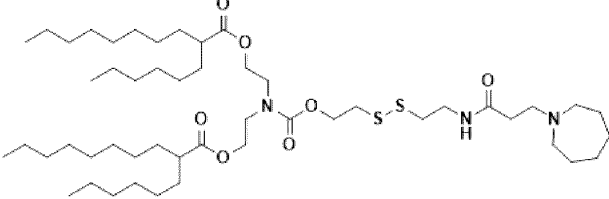
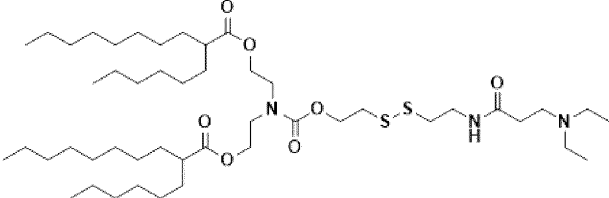
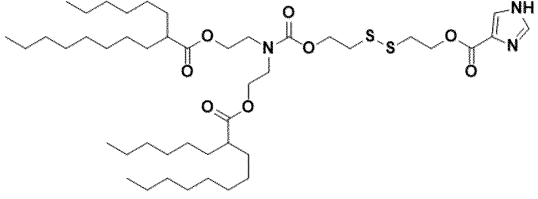
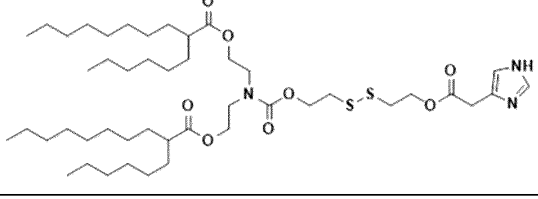
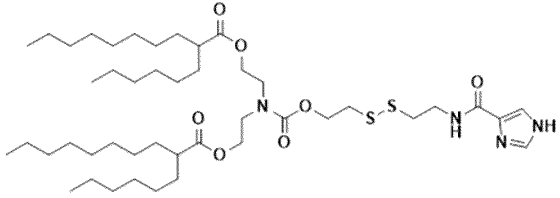
Lipid Name	Chemical Structure
IL-1	
IL-2	
IL-3	
IL-4	
IL-5	
IL-6	
IL-7	
IL-8	

IL-9	
IL-10	
IL-11	
IL-12	
IL-13	
IL-14	
IL-15	
IL-16	

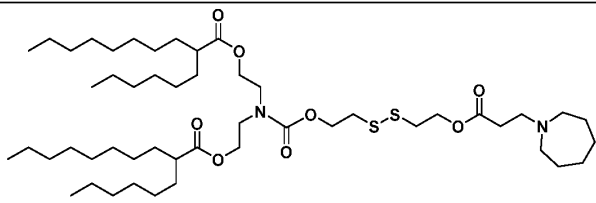
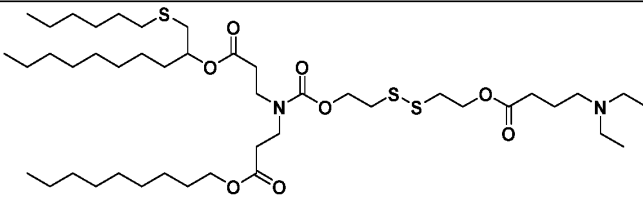
IL-17	
IL-18	
IL-19	
IL-20	
IL-21	
IL-22	
IL-23	

IL-24	
IL-25	
IL-26	
IL-27	
IL-28	
IL-29	
IL-30	

IL-31	
IL-32	
IL-33	
IL-34	
IL-35	
IL-36	
IL-37	
IL-38	

IL-39	
IL-40	
IL-41	
IL-42	
IL-43	
IL-44	
IL-45	

-98-

IL-46	
IL-47	

7. An ionizable lipid as defined in any one of claims 1 to 3; wherein the total number of C atoms in R₁ and R₂ together is at least 14.

5 8. An ionizable lipid as defined in any one of claims 4 to 5; wherein the total number of C atoms in R₇ and R₇' together is at least 12.

9. A lipid nanoparticle or lipid nanoparticle composition comprising an ionizable lipid as defined in any one of claims 1 to 8.

10

10. The lipid nanoparticle or lipid nanoparticle composition according to claim 9, further comprising a phospholipid, a sterol and/or a PEG lipid.

11. The lipid nanoparticle or lipid nanoparticle composition according to any one of claims 9 to 10, further comprising an active agent, in particular a nucleic acid, preferably mRNA.

15

12. Use of an ionizable lipid as defined in any one of claims 1 to 10 in the manufacture of a lipid nanoparticle or lipid nanoparticle composition.

20 13. A pharmaceutical composition comprising a lipid nanoparticle or lipid nanoparticle composition as defined in any one of claims 9 to 11, and a pharmaceutically acceptable agent.

14. A pharmaceutical composition as defined in claim 13 for use in medicine.

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