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[Continued on next page]

(54) Title: PROCESSES FOR PREPARING LIPIDS

Figure 1: SILENCING OF FVII WITH FORMULATION COMPRISING OPTICALLY PURE DLin-K-DMA

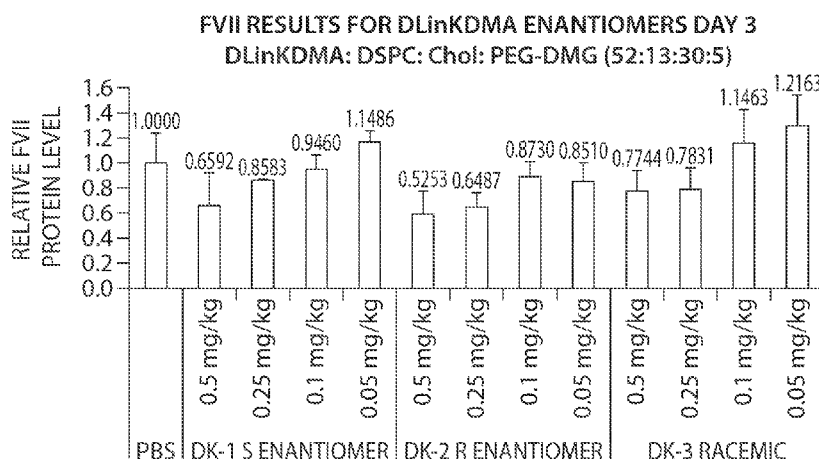


Fig. 1

(57) **Abstract:** The present invention provides compositions and methods for the delivery of therapeutic agents to cells. In particular, these include novel lipids and nucleic acid-lipid particles that provide efficient encapsulation of nucleic acids and efficient delivery of the encapsulated nucleic acid to cells in vivo. The compositions of the present invention are highly potent, thereby allowing effective knock-down of specific target protein at relatively low doses. The compositions and methods of the present invention are less toxic and provide a greater therapeutic index compared to compositions and methods previously known in the art.



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PROCESSES FOR PREPARING LIPIDS

CLAIM OF PRIORITY

Technical Field

5 This application claims priority to U.S.S.N. 61/107,998, filed October 23, 2008, the contents of which are entirely incorporated herein by reference.

BACKGROUND

Technical Field

10 The present invention relates to the field of therapeutic agent delivery using lipid particles. In particular, the present invention provides cationic lipids and lipid particles comprising these lipids, which are advantageous for the *in vivo* delivery of nucleic acids, as well as nucleic acid-lipid particle compositions suitable for *in vivo* therapeutic use. Additionally, the present invention provides methods of making these compositions, as
15 well as methods of introducing nucleic acids into cells using these compositions, *e.g.*, for the treatment of various disease conditions.

Description of the Related Art

 Therapeutic nucleic acids include, *e.g.*, small interfering RNA (siRNA), micro
20 RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, and immune stimulating nucleic acids. These nucleic acids act via a variety of mechanisms. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). Following introduction of siRNA or miRNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a
25 protein termed RISC. The sense strand of the siRNA or miRNA is displaced from the RISC complex providing a template within RISC that can recognize and bind mRNA with a complementary sequence to that of the bound siRNA or miRNA. Having bound the complementary mRNA the RISC complex cleaves the mRNA and releases the cleaved strands. RNAi can provide down-regulation of specific proteins by targeting specific
30 destruction of the corresponding mRNA that encodes for protein synthesis.

The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both *in vitro* and *in vivo* models. In addition, siRNA constructs
5 are currently being evaluated in clinical studies.

However, two problems currently faced by siRNA or miRNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind RISC when administered systemically as the free siRNA or miRNA. These double-stranded constructs can be
10 stabilized by incorporation of chemically modified nucleotide linkers within the molecule, for example, phosphothioate groups. However, these chemical modifications provide only limited protection from nuclease digestion and may decrease the activity of the construct. Intracellular delivery of siRNA or miRNA can be facilitated by use of carrier systems such as polymers, cationic liposomes or by chemical modification of the construct, for example
15 by the covalent attachment of cholesterol molecules [reference]. However, improved delivery systems are required to increase the potency of siRNA and miRNA molecules and reduce or eliminate the requirement for chemical modification.

Antisense oligonucleotides and ribozymes can also inhibit mRNA translation into protein. In the case of antisense constructs, these single stranded
20 deoxynucleic acids have a complementary sequence to that of the target protein mRNA and can bind to the mRNA by Watson-Crick base pairing. This binding either prevents translation of the target mRNA and/or triggers RNase H degradation of the mRNA transcripts. Consequently, antisense oligonucleotides have tremendous potential for specificity of action (*i.e.*, down-regulation of a specific disease-related protein). To date,
25 these compounds have shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* 14:376-387 (1996)). Antisense can also affect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of several antisense drugs are currently underway. Targets for these drugs include the bcl2 and apolipoprotein B
30 genes and mRNA products.

Immune-stimulating nucleic acids include deoxyribonucleic acids and ribonucleic acids. In the case of deoxyribonucleic acids, certain sequences or motifs have been shown to illicit immune stimulation in mammals. These sequences or motifs include the CpG motif, pyrimidine-rich sequences and palindromic sequences. It is believed that the CpG motif in deoxyribonucleic acids is specifically recognized by an endosomal receptor, toll-like receptor 9 (TLR-9), which then triggers both the innate and acquired immune stimulation pathway. Certain immune stimulating ribonucleic acid sequences have also been reported. It is believed that these RNA sequences trigger immune activation by binding to toll-like receptors 6 and 7 (TLR-6 and TLR-7). In addition, double-stranded RNA is also reported to be immune stimulating and is believed to activate via binding to TLR-3.

One well known problem with the use of therapeutic nucleic acids relates to the stability of the phosphodiester internucleotide linkage and the susceptibility of this linker to nucleases. The presence of exonucleases and endonucleases in serum results in the rapid digestion of nucleic acids possessing phosphodiester linkers and, hence, therapeutic nucleic acids can have very short half-lives in the presence of serum or within cells. (Zelphati, O., *et al.*, *Antisense. Res. Dev.* 3:323-338 (1993); and Thierry, A.R., *et al.*, pp147-161 in *Gene Regulation: Biology of Antisense RNA and DNA* (Eds. Erickson, RP and Izant, JG; Raven Press, NY (1992)). Therapeutic nucleic acid being currently being developed do not employ the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

This problem has been partially overcome by chemical modifications that reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (*e.g.*, using phosphorothioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (*e.g.*, 5-propynyl-pyrimidines), or at the sugar (*e.g.*, 2'-modified sugars) (Uhlmann E., *et al.* *Antisense: Chemical Modifications. Encyclopedia of Cancer*, Vol. X., pp 64-81 Academic Press Inc. (1997)). Others have attempted to improve stability using 2'-5' sugar linkages (*see, e.g.*, US Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven

entirely satisfactory, and *in vivo* free therapeutic nucleic acids still have only limited efficacy.

In addition, as noted above relating to siRNA and miRNA, problems remain with the limited ability of therapeutic nucleic acids to cross cellular membranes (see, 5 Vlassov, *et al.*, *Biochim. Biophys. Acta* 1197:95-1082 (1994)) and in the problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al.*, *Antisense Nucl. Acid Drug Des.* 4:201-206 (1994)).

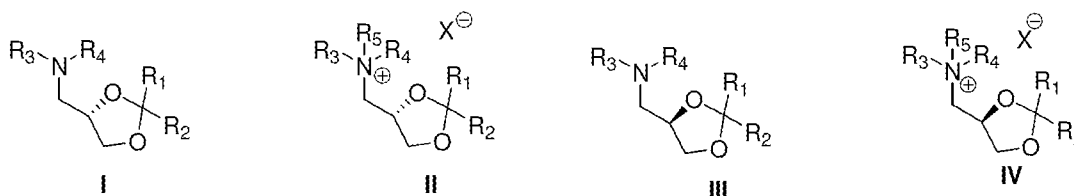
To attempt to improve efficacy, investigators have also employed lipid- 10 based carrier systems to deliver chemically modified or unmodified therapeutic nucleic acids. In Zelphati, O and Szoka, F.C., *J. Contr. Rel.* 41:99-119 (1996), the authors refer to the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes, and cationic lipid/antisense aggregates. Similarly siRNA has been administered systemically in cationic liposomes, and these nucleic acid-lipid particles have 15 been reported to provide improved down-regulation of target proteins in mammals including non-human primates (Zimmermann *et al.*, *Nature* 441: 111-114 (2006)).

In spite of this progress, there remains a need in the art for improved lipid-therapeutic nucleic acid compositions that are suitable for general therapeutic use. Preferably, these compositions would encapsulate nucleic acids with high-efficiency, have 20 high drug:lipid ratios, protect the encapsulated nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the encapsulated nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to 25 the patient. The present invention provides such compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids into cells, including for the treatment of diseases.

BRIEF SUMMARY

The present invention provides novel amino lipids, as well as lipid particles comprising the same. These lipid particles may further comprise an active agent and be used according to related methods of the invention to deliver the active agent to a cell.

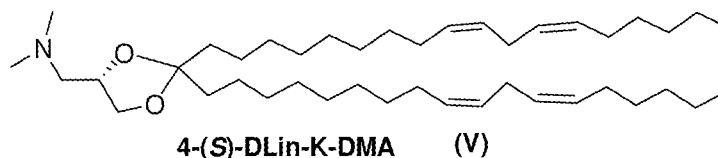
- 5 In one embodiment, the present invention provides an amino lipid having a structure selected from the group consisting of:



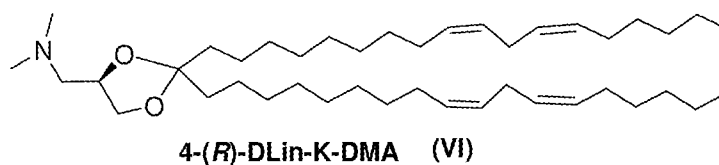
- wherein R_1 and R_2 are independently selected from the group consisting of optionally substituted C_{10} - C_{20} alkyl, optionally substituted C_{10} - C_{20} alkenyl, optionally substituted C_{10} - C_{20} alkynyl or optionally substituted C_{10} - C_{20} acyl; R_3 and R_4 are independently selected from the group consisting of: H, optionally substituted C_1 - C_6 alkyl and optionally substituted C_1 - C_6 alkylamino. In one example, the substituted group is one or more imidazole rings at the terminus. The aminopropan-diol backbones are derived from either 2-(*S*) or 2-(*R*)-3-dialkylamino-1,2-diol.
- 10 15

- It has been found that cationic lipids comprising alkyl chains with at least two or three sites of unsaturation, are particularly useful for forming lipid nucleic acid particles with increased membrane fluidity. In some embodiments, either R_1 or R_2 comprises at least two sites of unsaturation. In some embodiments, both of R_1 and R_2 comprise at least two sites of unsaturation.
- 20

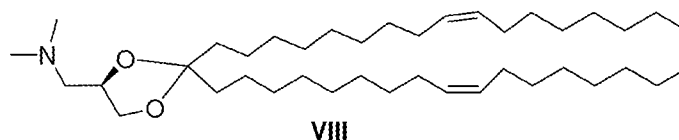
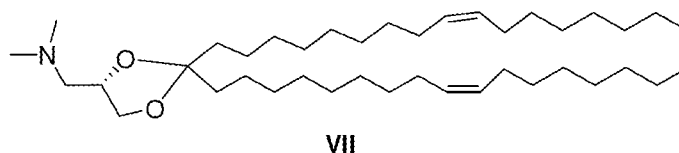
In some embodiments, R_1 and R_2 are both the same, i.e., R_1 and R_2 are both linoleyl (C_{18}) or R_1 and R_2 are both heptadeca-9-enyl. In one embodiment, the cationic lipid is selected from 4-(*S*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (V) and 4-(*R*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (VI).



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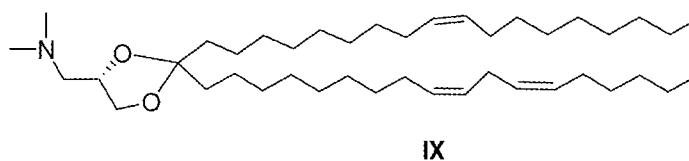


In another preferred embodiment the cationic lipid is selected from 4-(*S*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VII**) and 4-(*R*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VIII**).

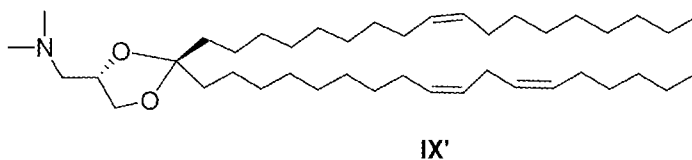


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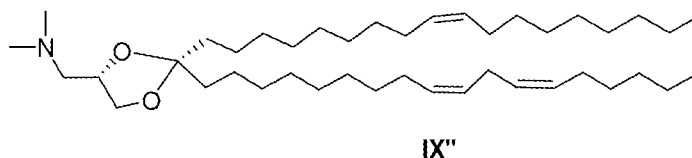
In some embodiments, one of R^1 and R^2 comprises at least one site of unsaturation and other comprises at least two sites of unsaturation. In one preferred embodiment, cationic lipid is 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**IX**).



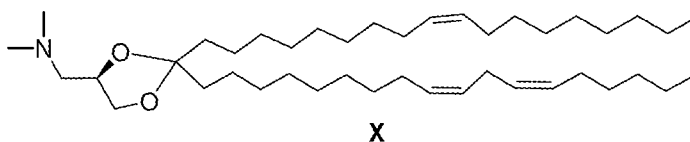
In one embodiment, the cationic lipid has the structure



or

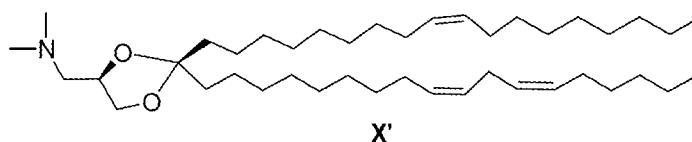


In another preferred embodiment, the cationic lipid is 4-(*R*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**X**).

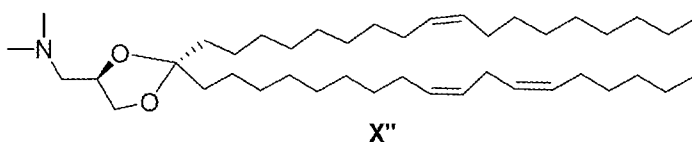


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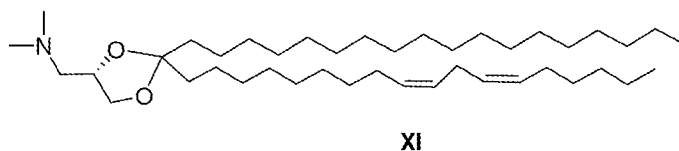
In an even more preferred embodiment, the cationic lipid has the structure



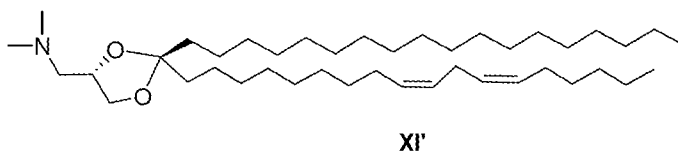
or



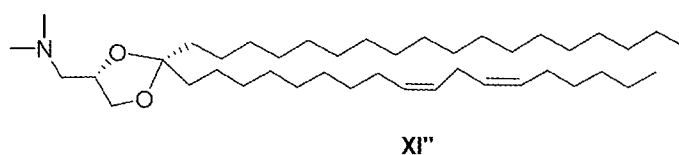
- 10 In one embodiment, one of R₁ and R₂ is optionally substituted C₁₀-C₂₀ alkenyl while the other is an optionally substituted C₁₀-C₂₀ alkyl. In one preferred embodiment the cationic lipid is 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadecyl-[1,3]dioxolan-4-ylmethyl)amine (**XI**).



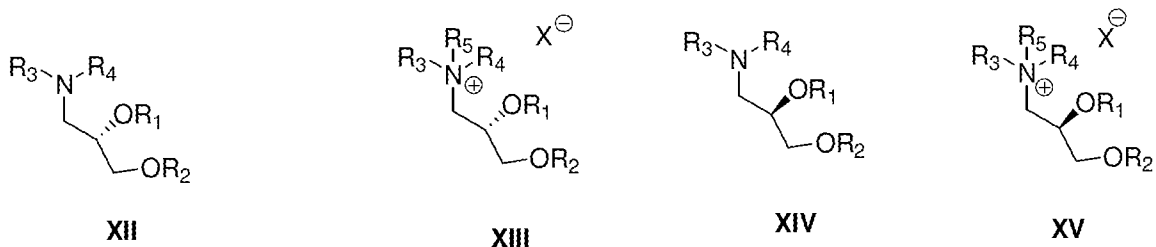
In an even more preferred embodiment, the cationic lipid has the structure



or



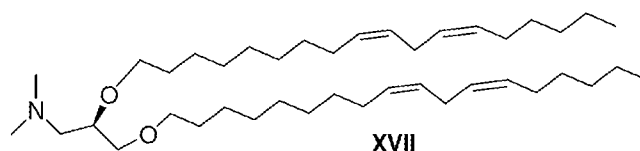
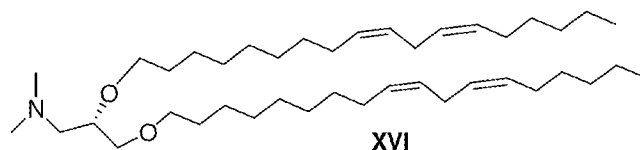
- 5 In another aspect, the invention features a method of evaluating a composition that includes an agent, e.g., a therapeutic agent or diagnostic agent, and an optically pure amino lipid selected from the following:



- 10 wherein R_1 and R_2 are independently selected from the group consisting of optionally substituted C_{10} - C_{20} alkyl, optionally substituted C_{10} - C_{20} alkenyl, optionally substituted C_{10} - C_{20} alkynyl or optionally substituted C_{10} - C_{20} acyl; R_3 and R_4 are independently selected from the group consisting of: H, optionally substituted C_1 - C_6 alkyl and optionally substituted C_1 - C_6 alkylamino. In one example, the substituted group is one or more
- 15 imidazole rings at the terminus.

In some embodiments, R_1 and R_2 are both the same, i.e., R_1 and R_2 are both linoleyl (C_{18}). In some embodiments R_3 and R_4 are independently selected from C_1 - C_6 alkylamino optionally substituted with one or more imidazole rings at the terminus.

In one embodiment, the cationic lipid is selected from 2-(*S*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVI**) and 2-(*R*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVII**).



In further related embodiments, the present invention includes a lipid particle comprising one or more of the above amino lipids of the present invention. In certain embodiments, the particle further comprises a neutral lipid and a lipid capable of reducing particle aggregation. In one particular embodiment, the lipid particle consists essentially of: (i) optically pure DLin-K-DMA (**V** or **VI**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% DLin-K-DMA:5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In one embodiment, the lipid particle consists essentially of: (i) 4-(*S*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VII**) or 4-(*R*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VIII**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid (**VII** or **VIII**):5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In another embodiment, the lipid particle consists essentially of: (i) 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**IX**) or 4-(*R*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**X**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about

20-60% amino lipid (**IX** or **X**):5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In one preferred embodiment, the lipid particle consists essentially of: (i) amino lipid selected from **IX'**, **IX''**, **X'** or **X''**; (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **IX'**, **IX''**, **X'** or **X''**: 5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In another embodiment, the lipid particle consists essentially of: (i) an amino lipid selected from **XII**, **XIII**, **XIV** or **XV**; (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **XII**, **XIII**, **XIV** or **XV**:5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In a preferred embodiment, the lipid particle consists essentially of: (i) 2-(*S*)-((2,3-bis-octadeca-9,12-dienyoxo-propyl)-dimethylamine (**XVI**) or 2-(*R*)-((2,3-bis-octadeca-9,12-dienyoxo-propyl)-dimethylamine (**XVII**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **XVI** or **XVII**:5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In additional related embodiments, the present invention includes lipid particles of the invention that further comprise a therapeutic agent. In one embodiment, the therapeutic agent is a nucleic acid. In various embodiments, the nucleic acid is a plasmid, an immunostimulatory oligonucleotide, a siRNA, a microRNA, an antisense oligonucleotide, or a ribozyme.

In yet another related embodiment, the present invention includes a pharmaceutical composition comprising a lipid particle of the present invention and a pharmaceutically acceptable excipient, carrier, or diluent.

The present invention further includes, in other related embodiments, a method of modulating the expression of a polypeptide by a cell, comprising providing to a cell a lipid particle or pharmaceutical composition of the present invention. In particular embodiments, the lipid particle comprises a therapeutic agent selected from an siRNA, a

microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the

5 polypeptide is reduced. In another embodiment, the nucleic acid is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

In yet a further related embodiment, the present invention includes a method of treating a disease or disorder characterized by overexpression of a polypeptide in a

10 subject, comprising providing to the subject a lipid particle or pharmaceutical composition of the present invention, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that

15 encodes the polypeptide, or a complement thereof.

In another related embodiment, the present invention includes a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject the pharmaceutical composition of the present invention, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a

20 functional variant or fragment thereof.

In a further embodiment, the present invention includes a method of inducing an immune response in a subject, comprising providing to the subject the pharmaceutical composition of the present invention, wherein the therapeutic agent is an immunostimulatory oligonucleotide. In particular embodiments, the pharmaceutical

25 composition is provided to the patient in combination with a vaccine or antigen.

In a related embodiment, the present invention includes a vaccine comprising the lipid particle of the present invention and an antigen associated with a disease or pathogen. In one embodiment, the lipid particle comprises an immunostimulatory nucleic acid or oligonucleotide. In a particular embodiment, the

antigen is a tumor antigen. In another embodiment, the antigen is a viral antigen, a bacterial antigen, or a parasitic antigen.

The present invention further includes methods of preparing the lipid particles and pharmaceutical compositions of the present invention, as well as kits useful
5 in the preparation of these lipid particle and pharmaceutical compositions.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 a bar graph depicting the silencing of FVII using formulation comprising optically pure DLin-K-DMA.

10 Figure 2 is a bar graph depicting the silencing of FVII using formulations 5, 5a-1 and 5a-2.

DETAILED DESCRIPTION

The present invention is based, in part, upon the discovery of cationic lipids that provide advantages when used in lipid particles for the *in vivo* delivery of a therapeutic
15 agent. In particular, as illustrated by the accompanying Examples, the present invention provides nucleic acid-lipid particle compositions comprising a cationic lipid according to the present invention that provide increased activity of the nucleic acid and improved tolerability of the compositions *in vivo*, resulting in a significant increase in therapeutic index as compared to lipid-nucleic acid particle compositions previously described.
20 Additionally compositions and methods of use are disclosed that provided for amelioration of the toxicity observed with certain therapeutic nucleic acid-lipid particles.

In certain embodiments, the present invention specifically provides for improved compositions for the delivery of siRNA molecules. It is shown herein that these compositions are effective in down-regulating the protein levels and/or mRNA levels of
25 target proteins. Furthermore, it is shown that the activity of these improved compositions is dependent on the presence of a certain cationic lipids and that the molar ratio of cationic lipid in the formulation can influence activity.

The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic
30 agents to cells, both *in vitro* or *in vivo*. Accordingly, the present invention provides

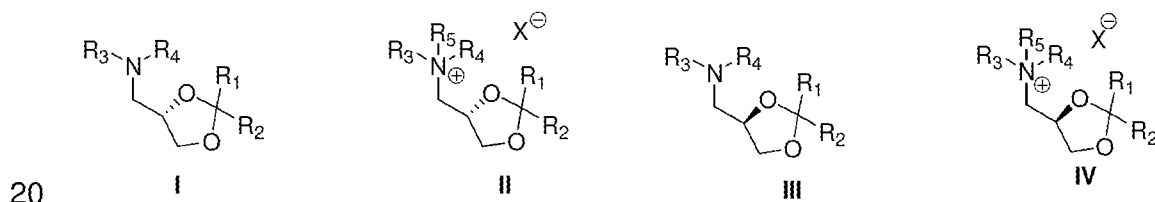
methods of treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle of the present invention associated with a suitable therapeutic agent.

As described herein, the lipid particles of the present invention are particularly useful for the delivery of nucleic acids, including, *e.g.*, siRNA molecules and plasmids. Therefore, the lipid particles and compositions of the present invention may be used to modulate the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a lipid particle of the present invention associated with a nucleic acid that reduces target gene expression (*e.g.*, an siRNA) or a nucleic acid that may be used to increase expression of a desired protein (*e.g.*, a plasmid encoding the desired protein).

Various exemplary embodiments of the cationic lipids of the present invention, as well as lipid particles and compositions comprising the same, and their use to deliver therapeutic agents and modulate gene and protein expression are described in further detail below.

A. Amino Lipids

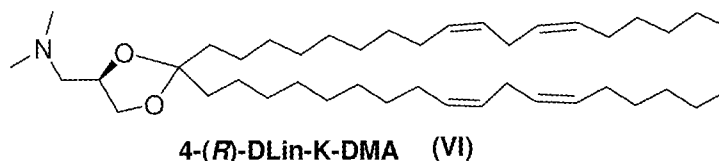
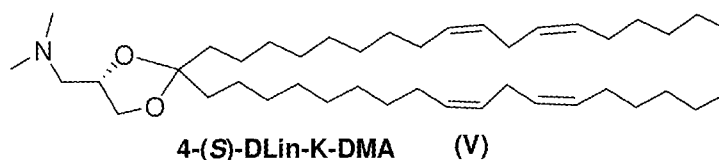
The present invention provides novel optically pure amino lipids that are advantageously used in lipid particles of the present invention for the *in vivo* delivery of therapeutic agents to cells, including optically pure amino lipids having the following structures.



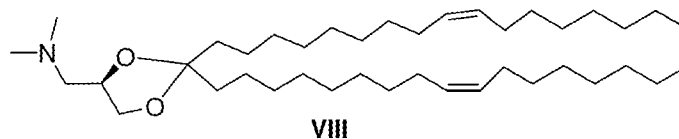
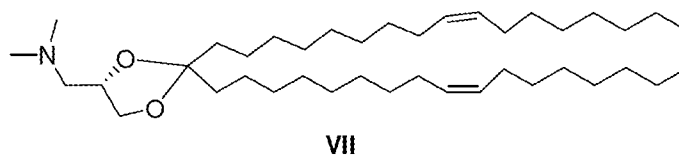
wherein R₁ and R₂ are independently selected from the group consisting of optionally substituted C₁₀-C₂₀ alkyl, optionally substituted C₁₀-C₂₀ alkenyl, optionally substituted C₁₀-C₂₀ alkynyl or optionally substituted C₁₀-C₂₀ acyl; R₃ and R₄ are independently selected from the group consisting of: H, optionally substituted C₁-C₆ alkyl and optionally substituted C₁-C₆ alkylamino. In one example, the substituted group is one or more imidazole rings at the terminus. The aminopropan-diol backbones are derived from either 2-(*S*) or 2-(*R*)-3-dialkylamino-1,2-diol.

It has been found that cationic lipids comprising alkyl chains with at least two or three sites of unsaturation, are particularly useful for forming lipid nucleic acid particles with increased membrane fluidity. In some embodiments, either R_1 or R_2 comprises at least two sites of unsaturation. In some embodiments, both of R_1 and R_2 comprise at least two sites of unsaturation.

In some embodiments, R_1 and R_2 are both the same, i.e., R_1 and R_2 are both linoleyl (C18) or R_1 and R_2 are both heptadeca-9-enyl. In one embodiment, the cationic lipid is selected from 4-(*S*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**V**) and 4-(*R*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VI**).

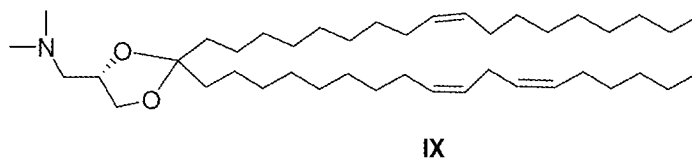


In another preferred embodiment the cationic lipid is selected from 4-(*S*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VII**) and 4-(*R*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VIII**).

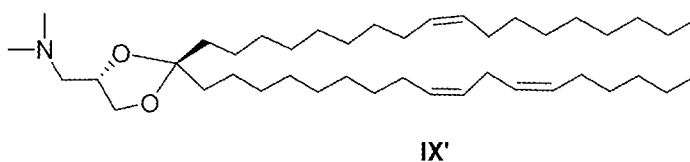


In some embodiments, one of R^1 and R^2 comprises at least one site of unsaturation and other comprises at least two sites of unsaturation. In one preferred embodiment, cationic lipid is 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**IX**).

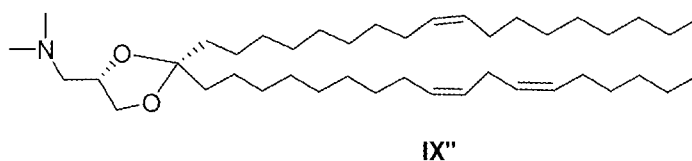
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In one embodiment, the cationic lipid has the structure

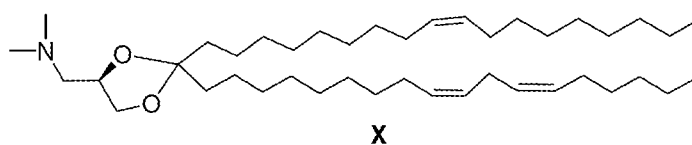


or

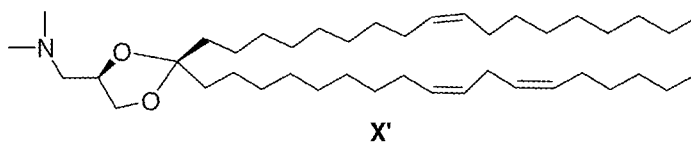


In another preferred embodiment, the cationic lipid is 4-(*R*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**X**).

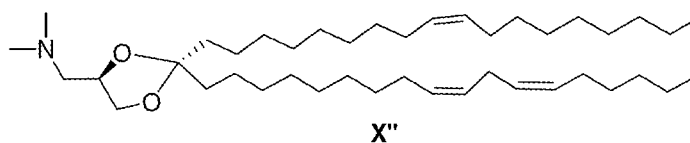
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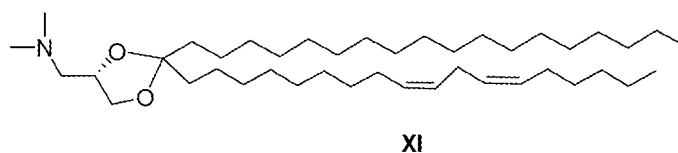
In an even more preferred embodiment, the amino lipid has the structure



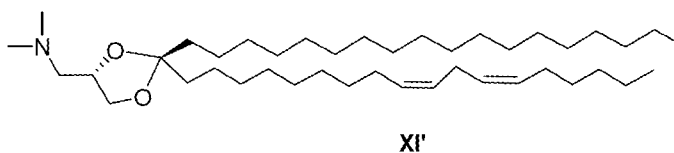
or



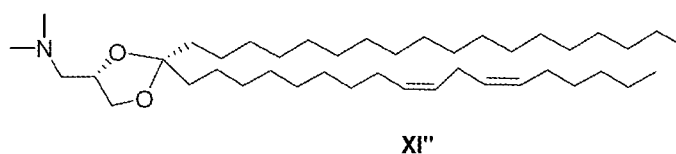
- In one embodiment, only one of R_1 and R_2 comprises at least two sites of unsaturation while the other one has no unsaturation. In one preferred embodiment the cationic lipid is
- 5 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadecyl-[1,3]dioxolan-4-ylmethyl)amine (**XI**).



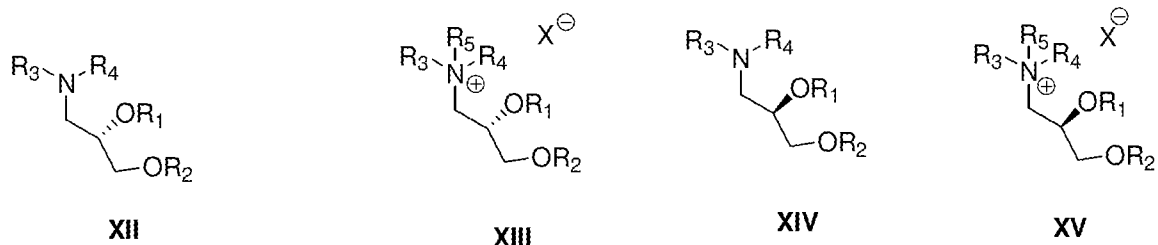
In an even more preferred embodiment, the cationic lipid has the structure



or



- 10 In another aspect, the invention features a method of evaluating a composition that includes an agent, e.g., a therapeutic agent or diagnostic agent, and an optically pure amino lipid selected from the following:

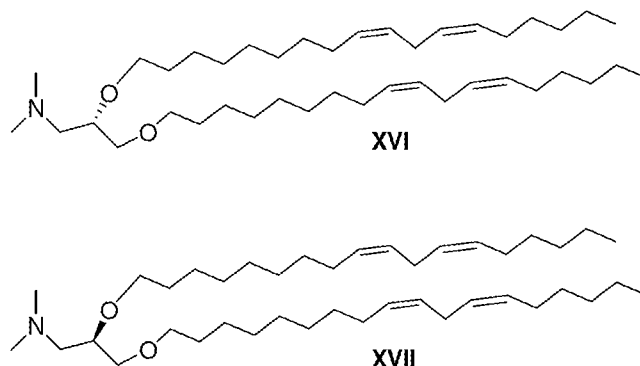


wherein R_1 and R_2 are independently selected from the group consisting of optionally substituted C_{10} - C_{20} alkyl, optionally substituted C_{10} - C_{20} alkenyl, optionally substituted C_{10} - C_{20} alkynyl or optionally substituted C_{10} - C_{20} acyl; R_3 and R_4 are independently selected from the group consisting of: H, optionally substituted C_1 - C_6 alkyl and optionally substituted C_1 - C_6 alkylamino. In one example, the substituted group is one or more imidazole rings at the terminus.

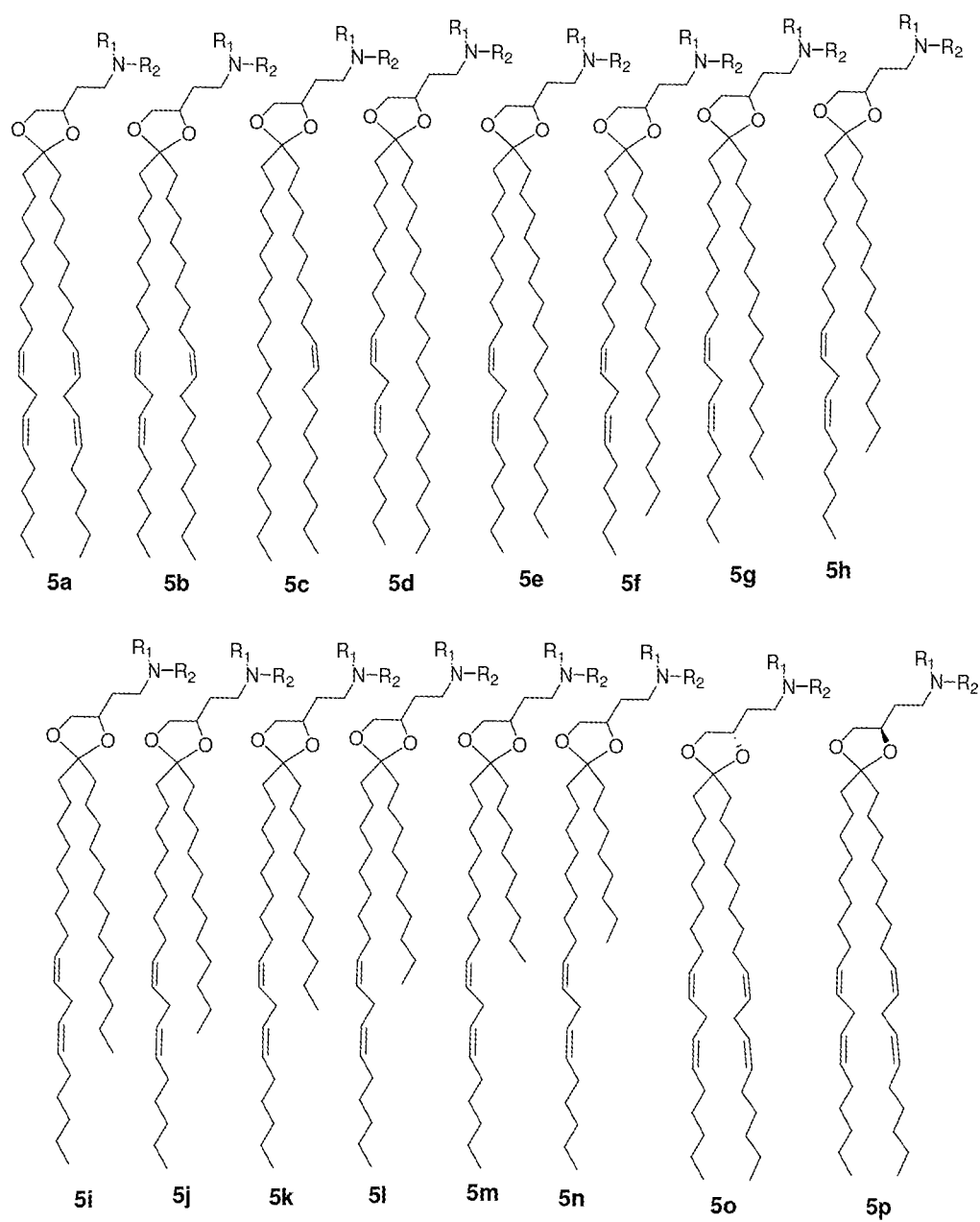
In some embodiments, R_1 and R_2 are both the same, i.e., R_1 and R_2 are both linoleyl (C_{18}).
 In some embodiments R_3 and R_4 are independently selected from C_1 - C_6 alkylamino optionally substituted with one or more imidazole rings at the terminus.

In one embodiment, the cationic lipid is selected from 2-(*S*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVI**) and 2-(*R*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVII**).

15



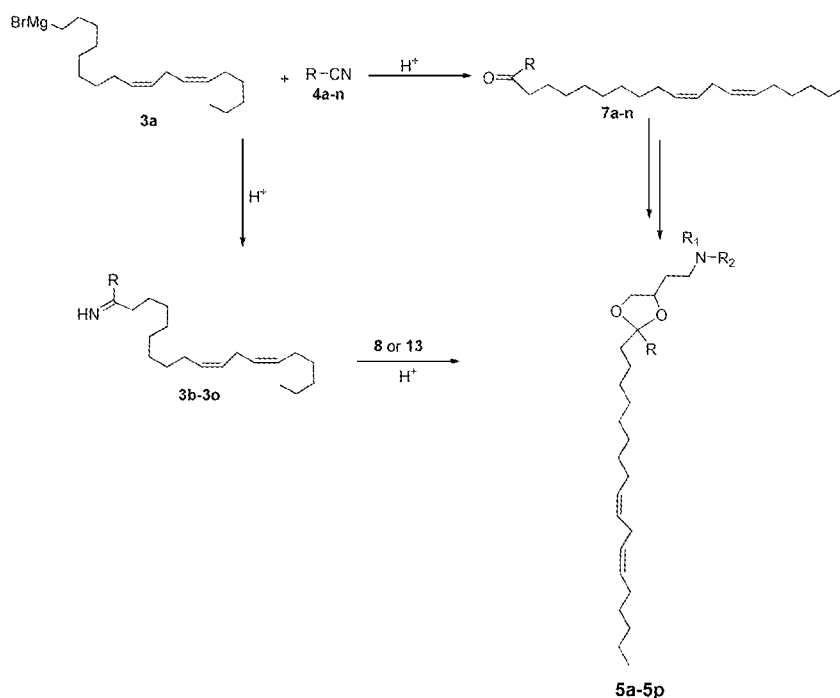
In one embodiment, the cationic lipid is selected from lipids 5a-5p:



$R_1 = \text{H or Me and } R_2 = \text{H or Me}$

Synthetic methods:

5 General chemical methodology for making cyclic lipids 5a-5p



The term “aliphatic” refers to non-aromatic moiety that may contain any combination of carbon atoms, hydrogen atoms, halogen atoms, oxygen, nitrogen or other atoms, and optionally contain one or more units of unsaturation, e.g., double and/or triple bonds. An aliphatic group may be straight chained, branched or cyclic and preferably contains between about 1 and about 24 carbon atoms, more typically between about 1 and about 12 carbon atoms. In addition to aliphatic hydrocarbon groups, aliphatic groups include, for example, polyalkoxyalkyls, such as polyalkylene glycols, polyamines, and polyimines, for example. Such aliphatic groups may be further substituted.

10

“Alkyl” means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, *sec*-butyl, isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

15

“Alkenyl” means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

“Alkynyl” means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butyne, 2-butyne, 1-pentyne, 2-pentyne, 3-methyl-1 butyne, and the like.

“Acyl” means any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. For example, -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl are acyl groups.

“Heterocycle” means a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

The terms “optionally substituted alkyl”, “optionally substituted alkenyl”, “optionally substituted alkynyl”, “optionally substituted acyl”, and “optionally substituted heterocycle” means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, -CN, -OR^x, -NR^xR^y, -NR^xC(=O)R^y, -NR^xSO₂R^y, -C(=O)R^x, -C(=O)OR^x, -C(=O)NR^xR^y, -SO_nR^x and

-SO_nNR^xR^y, wherein n is 0, 1 or 2, R^x and R^y are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, -OR^x, heterocycle, -NR^xR^y, -NR^xC(=O)R^y, -NR^xSO₂R^y, -C(=O)R^x, -C(=O)OR^x, -C(=O)NR^xR^y, -SO_nR^x and
 5 -SO_nNR^xR^y.

“Halogen” means fluoro, chloro, bromo and iodo.

In some embodiments, the methods of the invention may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (*see, for example*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, Green, T.W. et. al., Wiley-
 10 Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some
 15 embodiments an “alcohol protecting group” is used. An “alcohol protecting group” is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

The compounds of the present invention may be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples.

In particular embodiments, the amino lipids of the present invention are cationic
 20 lipids. As used herein, the term “amino lipid” is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) that may be protonated to form a cationic lipid at physiological pH.

Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substituents are different
 25 (*e.g.*, N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R₁ and R₂ are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing unsaturated fatty acids with carbon
 30 chain lengths in the range of C₁₀ to C₂₀ are preferred. Other scaffolds can also be used to

separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid.

Suitable scaffolds are known to those of skill in the art.

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

In certain embodiments, protonatable lipids according to the invention have a pKa of the protonatable group in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at a lower pH formulation stage,
15 while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4. One of the benefits of this pKa is that at least some nucleic acid associated with the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

20 B. Lipid Particles

The present invention also provides lipid particles comprising one or more of the amino lipids described above. Lipid particles include, but are not limited to, liposomes. As used herein, a liposome is a structure having lipid-containing membranes enclosing an aqueous interior. Liposomes may have one or more lipid membranes. The
25 invention contemplates both single-layered liposomes, which are referred to as unilamellar, and multi-layered liposomes, which are referred to as multilamellar. When complexed with nucleic acids, lipid particles may also be lipoplexes, which are composed of cationic lipid bilayers sandwiched between DNA layers, as described, *e.g.*, in Felgner, *Scientific American*.

The lipid particles of the present invention may further comprise one or more additional lipids and/or other components such as cholesterol. Other lipids may be included in the liposome compositions of the present invention for a variety of purposes, such as to prevent lipid oxidation or to attach ligands onto the liposome surface. Any of a
5 number of lipids may be present in liposomes of the present invention, including amphipathic, neutral, cationic, and anionic lipids. Such lipids can be used alone or in combination. Specific examples of additional lipid components that may be present are described below.

Additional components that may be present in a lipid particle of the present
10 invention include bilayer stabilizing components such as polyamide oligomers (*see, e.g.*, U.S. Patent No. 6,320,017), peptides, proteins, detergents, lipid-derivatives, such as PEG coupled to phosphatidylethanolamine and PEG conjugated to ceramides (*see*, U.S. Patent No. 5,885,613).

In particular embodiments, the lipid particles include one or more of a
15 second amino lipid or cationic lipid, a neutral lipid, a sterol, and a lipid selected to reduce aggregation of lipid particles during formation, which may result from steric stabilization of particles which prevents charge-induced aggregation during formation.

Examples of lipids that reduce aggregation of particles during formation include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and
20 polyamide oligomers ("PAO") such as (described in US Pat. No. 6,320,017). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids for use as in the methods and compositions of the invention. ATTA-lipids are described, *e.g.*, in U.S. Patent No. 6,320,017, and PEG-lipid conjugates are described, *e.g.*, in U.S. Patent Nos.
25 5,820,873, 5,534,499 and 5,885,613. Typically, the concentration of the lipid component selected to reduce aggregation is about 1 to 15% (by mole percent of lipids).

Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the present invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable
30 PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic

acid, PEG-ceramide conjugates (*e.g.*, PEG-CerC14 or PEG-CerC20) which are described in co-pending USSN 08/486,214, incorporated herein by reference, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Particularly preferred are PEG-modified diacylglycerols and dialkylglycerols.

- 5 In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mePEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days.
- 10 Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 mins. in some assays. As illustrated in US Pat. Application SN 08/486,214, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of
- 15 these features may be useful for the invention. For some therapeutic applications it may be preferable for the PEG-modified lipid to be rapidly lost from the nucleic acid-lipid particle *in vivo* and hence the PEG-modified lipid will possess relatively short lipid anchors. In other therapeutic applications it may be preferable for the nucleic acid-lipid particle to exhibit a longer plasma circulation lifetime and hence the PEG-modified lipid will possess
- 20 relatively longer lipid anchors.

 It should be noted that aggregation preventing compounds do not necessarily require lipid conjugation to function properly. Free PEG or free ATTA in solution may be sufficient to prevent aggregation. If the particles are stable after formulation, the PEG or ATTA can be dialyzed away before administration to a subject.

- 25 Neutral lipids, when present in the lipid particle, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebroside. The selection of neutral lipids for use in the particles described
- 30 herein is generally guided by consideration of, *e.g.*, liposome size and stability of the

liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (*i.e.*, diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of
 5 embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C₁₀ to C₂₀ are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C₁₀ to C₂₀ are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used in the present invention are DOPE, DSPC, POPC,
 10 or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin, dihydrosphingomyeline, or phospholipids with other head groups, such as serine and inositol.

The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle
 15 preparation. A preferred sterol is cholesterol.

Other cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in lipid particles of the present invention. Such cationic lipids include, but are not limited to, N,N-dioleoyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleoyloxy)propyl-
 20 N,N,N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleoyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3-
 (N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleoyloxy)propyl)-N-2-(sperminecarboxamido)ethyl-N,N-dimethylammonium
 25 trifluoroacetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N, N-dimethyl-2,3-dioleoyloxypropylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE").
 Additionally, a number of commercial preparations of cationic lipids can be used, such as,
 30 *e.g.*, LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and

LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL). In particular embodiments, a cationic lipid is an amino lipid.

Anionic lipids suitable for use in lipid particles of the present invention include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, 5 diacylphosphatidic acid, N-dodecanoyl phosphatidylethanolamine, N-succinyl phosphatidylethanolamine, N-glutaryl phosphatidylethanolamine, lysylphosphatidylglycerol, and other anionic modifying groups joined to neutral lipids.

In numerous embodiments, amphipathic lipids are included in lipid particles of the present invention. "Amphipathic lipids" refer to any suitable material, wherein the 10 hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Such compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids. Representative phospholipids include sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoylloleoyl 15 phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, or dilinoleoylphosphatidylcholine. Other phosphorus-lacking compounds, such as sphingolipids, glycosphingolipid families, diacylglycerols, and α -acyloxyacids, can also be used. Additionally, such amphipathic lipids can be readily 20 mixed with other lipids, such as triglycerides and sterols.

Also suitable for inclusion in the lipid particles of the present invention are programmable fusion lipids. Such lipid particles have little tendency to fuse with cell membranes and deliver their payload until a given signal event occurs. This allows the lipid particle to distribute more evenly after injection into an organism or disease site 25 before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or time. In the latter case, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the lipid particle membrane over time. By the time the lipid particle is suitably distributed in the body, it has lost sufficient cloaking agent so as to be fusogenic.

With other signal events, it is desirable to choose a signal that is associated with the disease site or target cell, such as increased temperature at a site of inflammation.

In certain embodiments, it is desirable to target the lipid particles of this invention using targeting moieties that are specific to a cell type or tissue. Targeting of lipid particles using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (*e.g.*, riboflavin) and monoclonal antibodies, has been previously described (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044). The targeting moieties can comprise the entire protein or fragments thereof. Targeting mechanisms generally require that the targeting agents be positioned on the surface of the lipid particle in such a manner that the target moiety is available for interaction with the target, for example, a cell surface receptor. A variety of different targeting agents and methods are known and available in the art, including those described, *e.g.*, in Sapra, P. and Allen, TM, *Prog. Lipid Res.* 42(5):439-62 (2003); and Abra, RM *et al.*, *J. Liposome Res.* 12:1-3, (2002).

The use of lipid particles, *i.e.*, liposomes, with a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG) chains, for targeting has been proposed (Allen, *et al.*, *Biochimica et Biophysica Acta* 1237: 99-108 (1995); DeFrees, *et al.*, *Journal of the American Chemistry Society* 118: 6101-6104 (1996); Blume, *et al.*, *Biochimica et Biophysica Acta* 1149: 180-184 (1993); Klivanov, *et al.*, *Journal of Liposome Research* 2: 321-334 (1992); U.S. Patent No. 5,013,556; Zalipsky, *Bioconjugate Chemistry* 4: 296-299 (1993); Zalipsky, *FEBS Letters* 353: 71-74 (1994); Zalipsky, in *Stealth Liposomes* Chapter 9 (Lasic and Martin, Eds) CRC Press, Boca Raton FL (1995). In one approach, a ligand, such as an antibody, for targeting the lipid particle is linked to the polar head group of lipids forming the lipid particle. In another approach, the targeting ligand is attached to the distal ends of the PEG chains forming the hydrophilic polymer coating (Klivanov, *et al.*, *Journal of Liposome Research* 2: 321-334 (1992); Kirpotin *et al.*, *FEBS Letters* 388: 115-118 (1996)).

Standard methods for coupling the target agents can be used. For example, phosphatidylethanolamine, which can be activated for attachment of target agents, or derivatized lipophilic compounds, such as lipid-derivatized bleomycin, can be used. Antibody-targeted liposomes can be constructed using, for instance, liposomes that

incorporate protein A (*see*, Renneisen, *et al.*, *J. Bio. Chem.*, 265:16337-16342 (1990) and Leonetti, *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 87:2448-2451 (1990). Other examples of antibody conjugation are disclosed in U.S. Patent No. 6,027,726, the teachings of which are incorporated herein by reference. Examples of targeting moieties can also include other

5 proteins, specific to cellular components, including antigens associated with neoplasms or tumors. Proteins used as targeting moieties can be attached to the liposomes via covalent bonds (*see*, Heath, *Covalent Attachment of Proteins to Liposomes*, 149 *Methods in Enzymology* 111-119 (Academic Press, Inc. 1987)). Other targeting methods include the biotin-avidin system.

10 In one exemplary embodiment, the lipid particle comprises a mixture of an amino lipid of the present invention, neutral lipids (other than an amino lipid), a sterol (*e.g.*, cholesterol) and a PEG-modified lipid (*e.g.*, a PEG-DMG, PEG-C-DOMG or PEG-DMA). In certain embodiments, the lipid mixture consists of or consists essentially of an amino lipid of the present invention, a neutral lipid, cholesterol, and a PEG-modified lipid. In

15 further preferred embodiments, the lipid particle consists of or consists essentially of the above lipid mixture in molar ratios of about 20-70% amino lipid: 5-45% neutral lipid:20-55% cholesterol:0.5-15% PEG-modified lipid.

In particular embodiments, the lipid particle consists of or consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol, and either PEG-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG, PEG-C-DOMG or PEG-DMA. In

20 particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-DMA/DSPC/Chol/PEG-DMG or DLin-K-DMA/DSPC/Chol/PEG-C-DOMG or DLin-K-DMA/DSPC/Chol/PEG-DMA) or 35/15/40/10 mol% DLin-K-DMA/DSPC/Chol/PEG-DMG or DLin-K-DMA/DSPC/Chol/PEG-C-DOMG or DLin-K-DMA/DSPC/Chol/PEG-

25 DMA. In one embodiment the optically pure DLin-K-DMA is 4-(*S*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (V). In a most preferred embodiment the optically pure DLin-K-DMA is 4-(*R*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (VI). In another group of embodiments, the neutral lipid in these

30 compositions is replaced with POPC, DOPE or SM.

In one embodiment, the lipid particle consists essentially of: (i) 4-(*S*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VII**) or 4-(*R*)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine (**VIII**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid (**VII** or **VIII**):5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In another embodiment, the lipid particle consists essentially of: (i) 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**IX**) or 4-(*R*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine (**X**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid (**IX** or **X**):5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In one preferred embodiment, the lipid particle consists essentially of: (i) amino lipid selected from **IX'**, **IX''**, **X'** or **X''**; (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **IX'**, **IX''**, **X'** or **X''**: 5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In another embodiment, the lipid particle consists essentially of: (i) amino lipid selected from **XII**, **XIII**, **XIV** or **XV**; (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **XII**, **XIII**, **XIV** or **XV**:5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

In a preferred embodiment, the lipid particle consists essentially of: (i) 2-(*S*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVI**) or 2-(*R*)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine (**XVII**); (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM; (iii) cholesterol; and (iv) PEG-DMG, PEG-C-DOMG or PEG-DMA, in a molar ratio of about 20-60% amino lipid selected from **XVI** or **XVII**:5-25% neutral lipid:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA.

30

C. Therapeutic Agent-Lipid Particle Compositions and Formulations

The present invention includes compositions comprising a lipid particle of the present invention and an active agent, wherein the active agent is associated with the lipid particle. In particular embodiments, the active agent is a therapeutic agent. In particular embodiments, the active agent is encapsulated within an aqueous interior of the lipid particle. In other embodiments, the active agent is present within one or more lipid layers of the lipid particle. In other embodiments, the active agent is bound to the exterior or interior lipid surface of a lipid particle.

“Fully encapsulated” as used herein indicates that the nucleic acid in the particles is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA. In a fully encapsulated system, preferably less than 25% of particle nucleic acid is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than 10% and most preferably less than 5% of the particle nucleic acid is degraded. Alternatively, full encapsulation may be determined by an Oligreen[®] assay. Oligreen[®] is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA in solution (available from Invitrogen Corporation, Carlsbad, CA). Fully encapsulated also suggests that the particles are serum stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

Active agents, as used herein, include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be biological, physiological, or cosmetic, for example. Active agents may be any type of molecule or compound, including *e.g.*, nucleic acids, peptides and polypeptides, including, *e.g.*, antibodies, such as, *e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, and Primatized[™] antibodies, cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell surface receptors and their ligands; hormones; and small molecules, including small organic molecules or compounds.

In one embodiment, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves

or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative lacks therapeutic activity.

- 5 In various embodiments, therapeutic agents include any therapeutically effective agent or drug, such as anti-inflammatory compounds, anti-depressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs, *e.g.*, anti-arrhythmic agents, vasoconstrictors, hormones, and steroids.
- 10 In certain embodiments, the therapeutic agent is an oncology drug, which may also be referred to as an anti-tumor drug, an anti-cancer drug, a tumor drug, an antineoplastic agent, or the like. Examples of oncology drugs that may be used according to the invention include, but are not limited to, adriamycin, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, azathioprine, bexarotene,
- 15 biCNU, bleomycin, busulfan intravenous, busulfan oral, capecitabine (Xeloda), carboplatin, carmustine, CCNU, celecoxib, chlorambucil, cisplatin, cladribine, cyclosporin A, cytarabine, cytosine arabinoside, daunorubicin, cytoxan, daunorubicin, dexamethasone, dexrazoxane, dodetaxel, doxorubicin, doxorubicin, DTIC, epirubicin, estramustine, etoposide phosphate, etoposide and VP-16, exemestane, FK506, fludarabine, fluorouracil,
- 20 5-FU, gemcitabine (Gemzar), gemtuzumab-ozogamicin, goserelin acetate, hydrea, hydroxyurea, idarubicin, ifosfamide, imatinib mesylate, interferon, irinotecan (Camptostar, CPT-111), letrozole, leucovorin, leustatin, leuprolide, levamisole, litretinoin, megastrol, melphalan, L-PAM, mesna, methotrexate, methoxsalen, mithramycin, mitomycin, mitoxantrone, nitrogen mustard, paclitaxel, pamidronate, Pegademase, pentostatin,
- 25 porfimer sodium, prednisone, rituxan, streptozocin, STI-571, tamoxifen, taxotere, temozolamide, teniposide, VM-26, topotecan (Hycamtin), toremifene, tretinoin, ATRA, valrubicin, velban, vinblastine, vincristine, VP16, and vinorelbine. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors and camptothecins.

1. Nucleic Acid-Lipid Particles

In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle. In particular embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein,
5 the term "nucleic acid" is meant to include any oligonucleotide or polynucleotide. Fragments containing up to 50 nucleotides are generally termed oligonucleotides, and longer fragments are called polynucleotides. In particular embodiments, oligonucleotides of the present invention are 20-50 nucleotides in length.

In the context of this invention, the terms "polynucleotide" and
10 "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native
15 forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Oligonucleotides are classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5' and 3' carbons of this sugar to form an
20 alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-
25 RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA include siRNA and other RNA interference reagents. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, microRNA, and triplex-forming oligonucleotides.

Nucleic acids of the present invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to 100
5 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 50 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length.

In particular embodiments, an oligonucleotide (or a strand thereof) of the
10 present invention specifically hybridizes to or is complementary to a target polynucleotide. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically
15 hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological
20 conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, in other embodiments, this oligonucleotide includes 1, 2, or 3 base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

RNA Interference Nucleic Acids

25 In particular embodiments, nucleic acid-lipid particles of the present invention are associated with RNA interference (RNAi) molecules. RNA interference methods using RNAi molecules may be used to disrupt the expression of a gene or polynucleotide of interest. In the last 5 years small interfering RNA (siRNA) has essentially replaced antisense ODN and ribozymes as the next generation of targeted
30 oligonucleotide drugs under development. SiRNAs are RNA duplexes normally 21-30

nucleotides long that can associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). RISC loaded with siRNA mediates the degradation of homologous mRNA transcripts, therefore siRNA can be designed to knock down protein expression with high specificity. Unlike other antisense technologies, siRNA
5 function through a natural mechanism evolved to control gene expression through non-coding RNA. This is generally considered to be the reason why their activity is more potent *in vitro* and *in vivo* than either antisense ODN or ribozymes. A variety of RNAi reagents, including siRNAs targeting clinically relevant targets, are currently under pharmaceutical development, as described, *e.g.*, in de Fougères, A. *et al.*, Nature
10 Reviews 6:443-453 (2007).

While the first described RNAi molecules were RNA:RNA hybrids comprising both an RNA sense and an RNA antisense strand, it has now been demonstrated that DNA sense:RNA antisense hybrids, RNA sense:DNA antisense hybrids, and DNA:DNA hybrids are capable of mediating RNAi (Lamberton, J.S. and Christian,
15 A.T., (2003) Molecular Biotechnology 24:111-119). Thus, the invention includes the use of RNAi molecules comprising any of these different types of double-stranded molecules. In addition, it is understood that RNAi molecules may be used and introduced to cells in a variety of forms. Accordingly, as used herein, RNAi molecules encompasses any and all molecules capable of inducing an RNAi response in cells, including, but not limited to,
20 double-stranded polynucleotides comprising two separate strands, *i.e.* a sense strand and an antisense strand, *e.g.*, small interfering RNA (siRNA); polynucleotides comprising a hairpin loop of complementary sequences, which forms a double-stranded region, *e.g.*, shRNAi molecules, and expression vectors that express one or more polynucleotides capable of forming a double-stranded polynucleotide alone or in combination with another
25 polynucleotide.

RNA interference (RNAi) may be used to specifically inhibit expression of target polynucleotides. Double-stranded RNA-mediated suppression of gene and nucleic acid expression may be accomplished according to the invention by introducing dsRNA, siRNA or shRNA into cells or organisms. SiRNA may be double-stranded RNA, or a
30 hybrid molecule comprising both RNA and DNA, *e.g.*, one RNA strand and one DNA

strand. It has been demonstrated that the direct introduction of siRNAs to a cell can trigger RNAi in mammalian cells (Elshabir, S.M., *et al.* Nature 411:494-498 (2001)).

Furthermore, suppression in mammalian cells occurred at the RNA level and was specific for the targeted genes, with a strong correlation between RNA and protein suppression

5 (Caplen, N. *et al.*, Proc. Natl. Acad. Sci. USA 98:9746-9747 (2001)). In addition, it was shown that a wide variety of cell lines, including HeLa S3, COS7, 293, NIH/3T3, A549, HT-29, CHO-K1 and MCF-7 cells, are susceptible to some level of siRNA silencing (Brown, D. *et al.* TechNotes 9(1):1-7, available at <http://www.dot.ambion.dot.com/techlib/tn/91/912.html> (9/1/02)).

10 RNAi molecules targeting specific polynucleotides can be readily prepared according to procedures known in the art. Structural characteristics of effective siRNA molecules have been identified. Elshabir, S.M. *et al.* (2001) Nature 411:494-498 and Elshabir, S.M. *et al.* (2001), EMBO 20:6877-6888. Accordingly, one of skill in the art would understand that a wide variety of different siRNA molecules may be used to target a
15 specific gene or transcript. In certain embodiments, siRNA molecules according to the invention are double-stranded and 16 - 30 or 18 - 25 nucleotides in length, including each integer in between. In one embodiment, an siRNA is 21 nucleotides in length. In certain embodiments, siRNAs have 0-7 nucleotide 3' overhangs or 0-4 nucleotide 5' overhangs. In one embodiment, an siRNA molecule has a two nucleotide 3' overhang. In one
20 embodiment, an siRNA is 21 nucleotides in length with two nucleotide 3' overhangs (*i.e.* they contain a 19 nucleotide complementary region between the sense and antisense strands). In certain embodiments, the overhangs are UU or dTdT 3' overhangs.

Generally, siRNA molecules are completely complementary to one strand of a target DNA molecule, since even single base pair mismatches have been shown to reduce
25 silencing. In other embodiments, siRNAs may have a modified backbone composition, such as, for example, 2'-deoxy- or 2'-O-methyl modifications. However, in preferred embodiments, the entire strand of the siRNA is not made with either 2' deoxy or 2'-O-modified bases.

In one embodiment, siRNA target sites are selected by scanning the target
30 mRNA transcript sequence for the occurrence of AA dinucleotide sequences. Each AA

dinucleotide sequence in combination with the 3' adjacent approximately 19 nucleotides are potential siRNA target sites. In one embodiment, siRNA target sites are preferentially not located within the 5' and 3' untranslated regions (UTRs) or regions near the start codon (within approximately 75 bases), since proteins that bind regulatory regions may interfere with the binding of the siRNP endonuclease complex (Elshabir, S. *et al.* Nature 411:494-498 (2001); Elshabir, S. *et al.* EMBO J. 20:6877-6888 (2001)). In addition, potential target sites may be compared to an appropriate genome database, such as BLASTN 2.0.5, available on the NCBI server at www.ncbi.nlm, and potential target sequences with significant homology to other coding sequences eliminated.

In particular embodiments, short hairpin RNAs constitute the nucleic acid component of nucleic acid-lipid particles of the present invention. Short Hairpin RNA (shRNA) is a form of hairpin RNA capable of sequence-specifically reducing expression of a target gene. Short hairpin RNAs may offer an advantage over siRNAs in suppressing gene expression, as they are generally more stable and less susceptible to degradation in the cellular environment. It has been established that such short hairpin RNA-mediated gene silencing works in a variety of normal and cancer cell lines, and in mammalian cells, including mouse and human cells. Paddison, P. *et al.*, Genes Dev. 16(8):948-58 (2002). Furthermore, transgenic cell lines bearing chromosomal genes that code for engineered shRNAs have been generated. These cells are able to constitutively synthesize shRNAs, thereby facilitating long-lasting or constitutive gene silencing that may be passed on to progeny cells. Paddison, P. *et al.*, Proc. Natl. Acad. Sci. USA 99(3):1443-1448 (2002).

ShRNAs contain a stem loop structure. In certain embodiments, they may contain variable stem lengths, typically from 19 to 29 nucleotides in length, or any number in between. In certain embodiments, hairpins contain 19 to 21 nucleotide stems, while in other embodiments, hairpins contain 27 to 29 nucleotide stems. In certain embodiments, loop size is between 4 to 23 nucleotides in length, although the loop size may be larger than 23 nucleotides without significantly affecting silencing activity. ShRNA molecules may contain mismatches, for example G-U mismatches between the two strands of the shRNA stem without decreasing potency. In fact, in certain embodiments, shRNAs are designed to include one or several G-U pairings in the hairpin stem to stabilize hairpins during

propagation in bacteria, for example. However, complementarity between the portion of the stem that binds to the target mRNA (antisense strand) and the mRNA is typically required, and even a single base pair mismatch in this region may abolish silencing. 5' and 3' overhangs are not required, since they do not appear to be critical for shRNA function, although they may be present (Paddison *et al.* (2002) *Genes & Dev.* 16(8):948-58).

MicroRNAs

Micro RNAs (miRNAs) are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Processed miRNAs are single stranded ~17-25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both. RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes.

The number of miRNA sequences identified to date is large and growing, illustrative examples of which can be found, for example, in: "*miRBase: microRNA sequences, targets and gene nomenclature*" Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. *NAR*, 2006, 34, Database Issue, D140-D144; "*The microRNA Registry*" Griffiths-Jones S. *NAR*, 2004, 32, Database Issue, D109-D111; and also at <http://microrna.sanger.ac.uk/sequences/>.

Antisense Oligonucleotides

In one embodiment, a nucleic acid is an antisense oligonucleotide directed to a target polynucleotide. The term "antisense oligonucleotide" or simply "antisense" is meant to include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. In the case of antisense RNA, they prevent translation of complementary RNA strands by binding to it. Antisense DNA can be used to target a specific, complementary (coding or non-coding) RNA. If binding takes place this DNA/RNA hybrid can be degraded by the enzyme RNase H. In particular embodiment,

antisense oligonucleotides contain from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or
5 where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for
10 inhibiting protein synthesis is well established. For example, the synthesis of polygalacturonase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1),
15 ICAM-1, E-selectin, STK-1, striatal GABA_A receptor and human EGF (Jaskulski *et al.*, Science. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, Brain Res Mol Brain Res. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Furthermore, antisense constructs have also been described that inhibit and can
20 be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a
25 given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m , binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those
30 regions at or near the AUG translation initiation codon and those sequences that are

substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, Nucleic Acids Res. 1997, 25(17):3389-402).

5 Ribozymes

According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr
10 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J Mol Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the
15 requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

At least six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general,
20 enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA.
25 Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nucleic acid molecule may be formed in a hammerhead,
30 hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA

guide sequence) or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.*, Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis B virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec 1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, Proc Natl Acad Sci U S A. 1991 Oct 1;88(19):8826-30; Collins and Olive, Biochemistry. 1993 Mar 23;32(11):2795-9); and an example of the Group I intron is described in U. S. Patent 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference, and synthesized to be tested *in vitro* and *in vivo*, as described therein.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

In one embodiment, the formulations of the invention can be use to silence or modulate a target gene such as but not limited to FVII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFkB gene, STAT3 gene, survivin gene, Her2/Neu gene, topoisomerase I gene, topoisomerase II alpha gene, mutations in the p73 gene, mutations in the p21(WAF1/CIP1) gene, mutations in the p27(KIP1) gene, mutations in the PPM1D gene, mutations in the RAS gene, mutations in the caveolin I gene, mutations in the MIB I gene, mutations in the MTAI gene, mutations in the M68 gene, mutations in tumor suppressor genes, mutations in the p53 tumor suppressor gene, mutations in the p53 family member DN-p63, mutations in the pRb tumor suppressor gene, mutations in the APC1 tumor suppressor gene, mutations in the BRCA1 tumor suppressor gene, mutations in the PTEN tumor suppressor gene, mLL fusion gene, BCR/ABL fusion gene, TEL/AML1 fusion gene, EWS/FLI1 fusion gene, TLS/FUS1 fusion gene, PAX3/FKHR fusion gene, AML1/ETO fusion gene, alpha v-integrin gene, Flt-1 receptor gene, tubulin gene, Human Papilloma Virus gene, a gene required for Human Papilloma Virus replication, Human Immunodeficiency Virus gene, a gene required for Human Immunodeficiency Virus replication, Hepatitis A Virus gene, a gene required for Hepatitis A Virus replication, Hepatitis B Virus gene, a gene required for Hepatitis B Virus replication, Hepatitis C Virus gene, a gene required for Hepatitis C Virus replication, Hepatitis D Virus gene, a gene required for Hepatitis D Virus replication, Hepatitis E Virus gene, a gene required for Hepatitis E Virus replication, Hepatitis F Virus gene, a gene required for Hepatitis F Virus replication, Hepatitis G Virus gene, a gene required for Hepatitis G Virus replication, Hepatitis H Virus gene, a gene required for Hepatitis H Virus replication, Respiratory Syncytial Virus gene, a gene that is required for Respiratory Syncytial Virus replication, Herpes Simplex Virus gene, a gene that is required for Herpes Simplex Virus replication, herpes Cytomegalovirus gene, a gene that is required for herpes Cytomegalovirus replication, herpes Epstein Barr Virus gene, a gene that is required for herpes Epstein Barr Virus replication, Kaposi's Sarcoma-associated Herpes Virus gene, a

gene that is required for Kaposi's Sarcoma-associated Herpes Virus replication, JC Virus gene, human gene that is required for JC Virus replication, myxovirus gene, a gene that is required for myxovirus gene replication, rhinovirus gene, a gene that is required for rhinovirus replication, coronavirus gene, a gene that is required for coronavirus replication,

5 West Nile Virus gene, a gene that is required for West Nile Virus replication, St. Louis Encephalitis gene, a gene that is required for St. Louis Encephalitis replication, Tick-borne encephalitis virus gene, a gene that is required for Tick-borne encephalitis virus replication, Murray Valley encephalitis virus gene, a gene that is required for Murray Valley encephalitis virus replication, dengue virus gene, a gene that is required for dengue virus

10 gene replication, Simian Virus 40 gene, a gene that is required for Simian Virus 40 replication, Human T Cell Lymphotropic Virus gene, a gene that is required for Human T Cell Lymphotropic Virus replication, Moloney-Murine Leukemia Virus gene, a gene that is required for Moloney-Murine Leukemia Virus replication, encephalomyocarditis virus gene, a gene that is required for encephalomyocarditis virus replication, measles virus

15 gene, a gene that is required for measles virus replication, Varicella zoster virus gene, a gene that is required for Varicella zoster virus replication, adenovirus gene, a gene that is required for adenovirus replication, yellow fever virus gene, a gene that is required for yellow fever virus replication, poliovirus gene, a gene that is required for poliovirus replication, poxvirus gene, a gene that is required for poxvirus replication, plasmodium

20 gene, a gene that is required for plasmodium gene replication, Mycobacterium ulcerans gene, a gene that is required for Mycobacterium ulcerans replication, Mycobacterium tuberculosis gene, a gene that is required for Mycobacterium tuberculosis replication, Mycobacterium leprae gene, a gene that is required for Mycobacterium leprae replication, Staphylococcus aureus gene, a gene that is required for Staphylococcus aureus replication,

25 Streptococcus pneumoniae gene, a gene that is required for Streptococcus pneumoniae replication, Streptococcus pyogenes gene, a gene that is required for Streptococcus pyogenes replication, Chlamydia pneumoniae gene, a gene that is required for Chlamydia pneumoniae replication, Mycoplasma pneumoniae gene, a gene that is required for Mycoplasma pneumoniae replication, an integrin gene, a selectin gene, complement system

30 gene, chemokine gene, chemokine receptor gene, GCSF gene, Gro1 gene, Gro2 gene, Gro3

- gene, PF4 gene, MIG gene, Pro-Platelet Basic Protein gene, MIP-1I gene, MIP-1J gene, RANTES gene, MCP-1 gene, MCP-2 gene, MCP-3 gene, CMBKR1 gene, CMBKR2 gene, CMBKR3 gene, CMBKR5v, AIF-1 gene, I-309 gene, a gene to a component of an ion channel, a gene to a neurotransmitter receptor, a gene to a neurotransmitter ligand,
- 5 amyloid-family gene, presenilin gene, HD gene, DRPLA gene, SCA1 gene, SCA2 gene, MJD1 gene, CACNL1A4 gene, SCA7 gene, SCA8 gene, allele gene found in LOH cells, or one allele gene of a polymorphic gene.

Immunostimulatory Oligonucleotides

- 10 Nucleic acids associated with lipid particles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal or other patient. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (see Yamamoto S., et al. (1992) J. Immunol. 148: 4072-4076),
- 15 or CpG motifs, as well as other known ISS features (such as multi-G domains, see WO 96/11266).

- The immune response may be an innate or an adaptive immune response. The immune system is divided into a more innate immune system, and acquired adaptive immune system of vertebrates, the latter of which is further divided into humoral cellular
- 20 components. In particular embodiments, the immune response may be mucosal.

- In particular embodiments, an immunostimulatory nucleic acid is only immunostimulatory when administered in combination with a lipid particle, and is not immunostimulatory when administered in its "free form." According to the present invention, such an oligonucleotide is considered to be immunostimulatory.
- 25 Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target polynucleotide in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally occurring gene or mRNA, but they may still be considered non-sequence specific
- 30 immunostimulatory nucleic acids.

In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in said CpG dinucleotide is methylated. In a specific embodiment, the nucleic acid comprises the sequence 5' TAACGTTGAGGGGCAT 3'. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of said CpG dinucleotides comprises a methylated cytosine.

In one specific embodiment, the nucleic acid comprises the sequence 5' TTCCATGACGTTCTGACGT 3'. In another specific embodiment, the nucleic acid sequence comprises the sequence 5' TCCATGACGTTCTGACGT 3', wherein the two cytosines indicated in bold are methylated. In particular embodiments, the ODN is selected from a group of ODNs consisting of ODN #1, ODN #2, ODN #3, ODN #4, ODN #5, ODN #6, ODN #7, ODN #8, and ODN #9, as shown below.

Table 1. Exemplary Immunostimulatory Oligonucleotides (ODNs)

ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3') .
ODN 1		5'-TAACGTTGAGGGGCAT-3
human c-myc		
* ODN 1m		5'-TAAZGTTGAGGGGCAT-3
ODN 2		5'-TCCATGACGTTCTGACGTT-3
* ODN 2m		5'-TCCATGAZGTTCTGAZGTT-3
ODN 3		5'-TAAGCATACGGGGTGT-3
ODN 5		5'-AACGTT-3
ODN 6		5'-GATGCTGTGTCGGGGTCTCCGGGC-3'

ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3') .
ODN 7		5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'
ODN 7m		5'-TZGTZGTTTTGTZGTTTTGTZGTT-3'
ODN 8		5'-TCCAGGACTTCTCTCAGGTT-3'
ODN 9		5'-TCTCCCAGCGTGCGCCAT-3'
ODN 10 murine Intracellular Adhesion Molecule-1		5'-TGCATCCCCCAGGCCACCAT-3
ODN 11 human Intracellular Adhesion Molecule-1		5'-GCCCAAGCTGGCATCCGTCA-3'
ODN 12 human Intracellular Adhesion Molecule-1		5'-GCCCAAGCTGGCATCCGTCA-3'
ODN 13 human erb- B-2		5'-GGT GCTCACTGC GGC-3'
ODN 14 human c- myc		5'-AACC GTT GAG GGG CAT-3'
ODN 15 human c- myc		5'-TAT GCT GTG CCG GGG TCT TCG GGC- 3'
ODN 16		5'-GTGCCG GGGTCTTCGGGC-3'
ODN 17 human Insulin Growth Factor 1 - Receptor		5'-GGACCCTCCTCCGGAGCC-3'
ODN 18 human Insulin Growth Factor 1 - Receptor		5'-TCC TCC GGA GCC AGA CTT-3'

ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3') .
ODN 19 human Epidermal Growth Factor - Receptor		5'-AAC GTT GAG GGG CAT-3'
ODN 20 Epidermal Growth Factor - Receptor		5'-CCGTGGTCA TGCTCC-3'
ODN 21 human Vascular Endothelial Growth Factor		5'-CAG CCTGGCTCACCG CCTTGG-3'
ODN 22 murine Phosphokinase C - alpha		5'-CAG CCA TGG TTC CCC CCA AC-3'
ODN 23		5'-GTT CTC GCT GGT GAG TTT CA-3'
ODN 24 human Bcl-2		5'-TCT CCCAGCGTGCGCCAT-3'
ODN 25 human C- Raf-s		5'-GTG CTC CAT TGA TGC-3'
ODN #26 human Vascular Endothelial Growth Factor Receptor-1		5'- GAGUUCUGAUGAGGCCGAAAGGCCGAA AGUCUG-3'
ODN #27		5'-RRCGY-3'
ODN # 28		5'-AACGTTGAGGGGCAT-3'
ODN #29		5'-CAACGTTATGGGGAGA-3'
ODN #30 human c- myc		5'-TAACGTTGAGGGGCAT-3'

- "Z" represents a methylated cytosine residue.
- Note: ODN 14 is a 15-mer oligonucleotide and ODN 1 is the same oligonucleotide having a thymidine added onto the 5' end making ODN 1 into a 16-mer. No

difference in biological activity between ODN 14 and ODN 1 has been detected and both exhibit similar immunostimulatory activity (Mui *et al.*, 2001)

Additional specific nucleic acid sequences of oligonucleotides (ODNs) suitable for use in the compositions and methods of the invention are described in Raney *et al.*, Journal of Pharmacology and Experimental Therapeutics, 298:1185-1192 (2001). In 5 certain embodiments, ODNs used in the compositions and methods of the present invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

Nucleic Acid Modifications

10 In the 1990's DNA-based antisense oligodeoxynucleotides (ODN) and ribozymes (RNA) represented an exciting new paradigm for drug design and development, but their application *in vivo* was prevented by endo- and exo- nuclease activity as well as a lack of successful intracellular delivery. The degradation issue was effectively overcome following extensive research into chemical modifications that prevented the 15 oligonucleotide (oligo) drugs from being recognized by nuclease enzymes but did not inhibit their mechanism of action. This research was so successful that antisense ODN drugs in development today remain intact *in vivo* for days compared to minutes for unmodified molecules (Kurreck, J. 2003. Antisense technologies. Improvement through novel chemical modifications. *Eur J Biochem* 270:1628-44). However, intracellular 20 delivery and mechanism of action issues have so far limited antisense ODN and ribozymes from becoming clinical products.

RNA duplexes are inherently more stable to nucleases than single stranded DNA or RNA, and unlike antisense ODN, unmodified siRNA show good activity once they access the cytoplasm. Even so, the chemical modifications developed to stabilize 25 antisense ODN and ribozymes have also been systematically applied to siRNA to determine how much chemical modification can be tolerated and if pharmacokinetic and pharmacodynamic activity can be enhanced. RNA interference by siRNA duplexes requires an antisense and sense strand, which have different functions. Both are necessary to enable the siRNA to enter RISC, but once loaded the two strands separate and the sense 30 strand is degraded whereas the antisense strand remains to guide RISC to the target mRNA.

Entry into RISC is a process that is structurally less stringent than the recognition and cleavage of the target mRNA. Consequently, many different chemical modifications of the sense strand are possible, but only limited changes are tolerated by the antisense strand (Zhang *et al.*, 2006).

5 As is known in the art, a nucleoside is a base-sugar combination. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

15 The nucleic acid that is used in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. Thus, the nucleic acid may be a modified nucleic acid of the type used previously to enhance nuclease resistance and serum stability. Surprisingly, however, acceptable therapeutic products can also be prepared using the method of the invention to formulate lipid-nucleic acid particles from nucleic acids that have no modification to the phosphodiester linkages of natural nucleic acid polymers, and the use of unmodified phosphodiester nucleic acids (*i.e.*, nucleic acids in which all of the linkages are phosphodiester linkages) is one embodiment of the invention.

a. Backbone Modifications

25 Antisense, siRNA and other oligonucleotides useful in this invention include, but are not limited to, oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. Modified oligonucleotide backbones include, for example,

30

phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, phosphoroselenate, methylphosphonate, or O-alkyl phosphotriester linkages, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Particular non-limiting examples of particular modifications that may be present in a nucleic acid according to the present invention are shown in Table 2.

Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

In certain embodiments, modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include, *e.g.*, those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts. Representative United States patents that describe the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677;

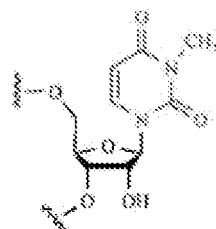
5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

The phosphorothioate backbone modification (Table 2, #1), where a non-bridging oxygen in the phosphodiester bond is replaced by sulfur, is one of the earliest and most common means deployed to stabilize nucleic acid drugs against nuclease degradation. In general, it appears that PS modifications can be made extensively to both siRNA strands without much impact on activity (Kurreck, J., *Eur. J. Biochem.* 270:1628-44, 2003). However, PS oligos are known to avidly associate non-specifically with proteins resulting in toxicity, especially upon i.v. administration. Therefore, the PS modification is usually restricted to one or two bases at the 3' and 5' ends. The boranophosphate linker (Table 2, #2) is a recent modification that is apparently more stable than PS, enhances siRNA activity and has low toxicity (Hall *et al.*, *Nucleic Acids Res.* 32:5991-6000, 2004).

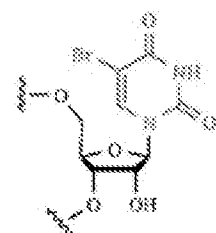
Table 2. Chemical Modifications Applied to siRNA and Other Nucleic Acids

#	Abbrev- iation	Name	Modification Site	Structure
1	PS	Phosphorothioate	Backbone	
2	PB	Boranophosphate	Backbone	

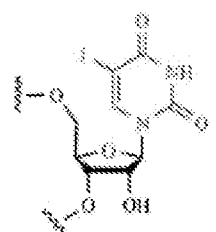
3 N3-MU N3-methyl-uridine Base



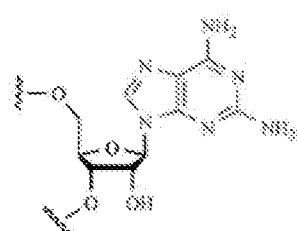
4 5'-BU 5'-bromo-uracil Base



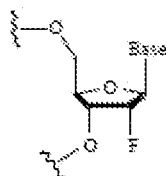
5 5'-IU 5'-iodo-uracil Base



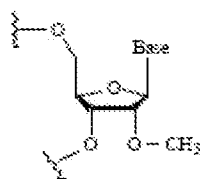
6 2,6-DP 2,6-diaminopurine Base



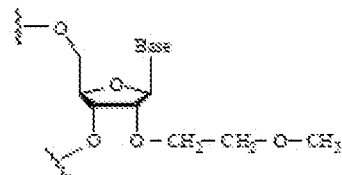
7 2'-F 2'-Fluoro Sugar



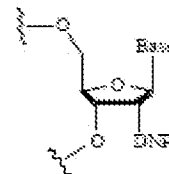
8 2'-OME 2''-O-methyl Sugar



- 9** 2'-O-MOE 2'-O-(2-methoxyethyl) Sugar



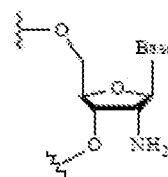
- 10** 2'-DNP 2'-O-(2,4-dinitrophenyl) Sugar



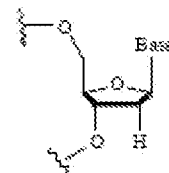
- 11** LNA Locked Nucleic Acid
(methylene bridge connecting the 2'-oxygen with the 4'-carbon of the ribose ring)



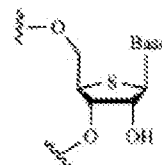
- 12** 2'-Amino 2'-Amino Sugar



- 13** 2'-Deoxy 2'-Deoxy Sugar



- 14** 4'-thio 4'-thio-ribonucleotide Sugar



Other useful nucleic acids derivatives include those nucleic acids molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with -S-, -NH-, -CH₂- and the like. In certain embodiments, the alterations to the antisense, siRNA, or other nucleic acids used will not completely affect the negative

charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense, siRNA, and other nucleic acids in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, in certain embodiments, less than 80% of the nucleic acid

5 linkages are so substituted, or less than 50% of the linkages are so substituted.

b. Base Modifications

Base modifications are less common than those to the backbone and sugar. The modifications shown in 0.3-6 all appear to stabilize siRNA against nucleases and have little effect on activity (Zhang, H.Y., Du, Q., Wahlestedt, C., Liang, Z. 2006. RNA

10 Interference with chemically modified siRNA. *Curr Top Med Chem* 6:893-900).

Accordingly, oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include

15 other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-

20 thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

25 Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention, including 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C.

30 (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*

1993, CRC Press, Boca Raton, pages 276-278). These may be combined, in particular embodiments, with 2'-O-methoxyethyl sugar modifications. United States patents that teach the preparation of certain of these modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as
 5 well as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

c. Sugar Modifications

Most modifications on the sugar group occur at the 2'-OH of the RNA sugar
 10 ring, which provides a convenient chemically reactive site. Manoharan, M. 2004. RNA interference and chemically modified small interfering RNAs. *Curr Opin Chem Biol* 8:570-9; Zhang, H.Y., Du, Q., Wahlestedt, C., Liang, Z. 2006. RNA Interference with chemically modified siRNA. *Curr Top Med Chem* 6:893-900). The 2'-F and 2'-OME (0.7 and 8) are common and both increase stability, the 2'-OME modification does not reduce activity as
 15 long as it is restricted to less than 4 nucleotides per strand (Holen, T., Amarzguioui, M., Babaie, E., Prydz, H. 2003. Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. *Nucleic Acids Res* 31:2401-7). The 2'-O-MOE (0.9) is most effective in siRNA when modified bases are restricted to the middle region of the molecule (Prakash, T.P., Allerson, C.R., Dande, P., Vickers, T.A.,
 20 Sioufi, N., Jarres, R., Baker, B.F., Swayze, E.E., Griffey, R.H., Bhat, B. 2005. Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J Med Chem* 48:4247-53). Other modifications found to stabilize siRNA without loss of activity are shown in 0.10-14.

Modified oligonucleotides may also contain one or more substituted sugar
 25 moieties. For example, the invention includes oligonucleotides that comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)₂ON(CH₃)₂, O(CH₂)_nNH₂, O(CH₂)_nCH₃,
 30 O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10.

Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving
5 group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. One modification includes 2'-methoxyethoxy (2'-O--CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta* 1995, 78, 486-504), *i.e.*, an alkoxyalkoxy group.
10 Other modifications include 2'-dimethylaminooxyethoxy, *i.e.*, a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (2'-DMAEOE).

Additional modifications include 2'-methoxy (2'-O--CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3'
15 terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786;
20 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

In other oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups, although the base units are maintained for hybridization with an appropriate nucleic acid target
25 compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion
30 of the backbone. Representative United States patents that teach the preparation of PNA

compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen *et al.* (*Science*, 1991, 254, 1497-1500).

Particular embodiments of the invention are oligonucleotides with
5 phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--O--CH₂--, --CH₂--N(CH₃)--O--CH₂-- (referred to as a methylene (methylimino) or MMI backbone) --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --O--N(CH₃)--CH₂--CH₂--(wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--) of the above referenced U.S. Pat. No. 5,489,677, and the
10 amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

d. Chimeric Oligonucleotides

It is not necessary for all positions in a given compound to be uniformly
15 modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras," in the context of this invention, are oligonucleotides that contain two or more chemically distinct regions, each made up of at
20 least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, *e.g.*, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for RNase H cleavage.

In one embodiment, a chimeric oligonucleotide comprises at least one
25 region modified to increase target binding affinity. Affinity of an oligonucleotide for its target is routinely determined by measuring the T_m of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the T_m, the greater the affinity of the oligonucleotide for the target. In one embodiment, the region of the oligonucleotide which
30 is modified to increase target mRNA binding affinity comprises at least one nucleotide

modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (*i.e.*, higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. The
5 effect of such increased affinity is to greatly enhance oligonucleotide inhibition of target gene expression.

In another embodiment, a chimeric oligonucleotide comprises a region that acts as a substrate for RNase H. Of course, it is understood that oligonucleotides may include any combination of the various modifications described herein

10 Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such conjugates and methods of preparing the same are known in the art.

Those skilled in the art will realize that for *in vivo* utility, such as
15 therapeutic efficacy, a reasonable rule of thumb is that if a thioated version of the sequence works in the free form, that encapsulated particles of the same sequence, of any chemistry, will also be efficacious. Encapsulated particles may also have a broader range of *in vivo* utilities, showing efficacy in conditions and models not known to be otherwise responsive to antisense therapy. Those skilled in the art know that applying this invention they may
20 find old models which now respond to antisense therapy. Further, they may revisit discarded antisense sequences or chemistries and find efficacy by employing the invention.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied
25 Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

Characteristic of Nucleic Acid-Lipid Particles

In certain embodiments, the present invention relates to methods and compositions for producing lipid-encapsulated nucleic acid particles in which nucleic acids are encapsulated within a lipid layer. Such nucleic acid-lipid particles, incorporating
5 siRNA oligonucleotides, are characterized using a variety of biophysical parameters including: (1) drug to lipid ratio; (2) encapsulation efficiency; and (3) particle size. High drug to lipid ratios, high encapsulation efficiency, good nuclease resistance and serum stability and controllable particle size, generally less than 200 nm in diameter are desirable. In addition, the nature of the nucleic acid polymer is of significance, since the modification
10 of nucleic acids in an effort to impart nuclease resistance adds to the cost of therapeutics while in many cases providing only limited resistance. Unless stated otherwise, these criteria are calculated in this specification as follows:

Nucleic acid to lipid ratio is the amount of nucleic acid in a defined volume of preparation divided by the amount of lipid in the same volume. This may be on a mole
15 per mole basis or on a weight per weight basis, or on a weight per mole basis. For final, administration-ready formulations, the nucleic acid:lipid ratio is calculated after dialysis, chromatography and/or enzyme (*e.g.*, nuclease) digestion has been employed to remove as much of the external nucleic acid as possible;

Encapsulation efficiency refers to the drug to lipid ratio of the starting
20 mixture divided by the drug to lipid ratio of the final, administration competent formulation. This is a measure of relative efficiency. For a measure of absolute efficiency, the total amount of nucleic acid added to the starting mixture that ends up in the administration competent formulation, can also be calculated. The amount of lipid lost during the formulation process may also be calculated. Efficiency is a measure of the
25 wastage and expense of the formulation; and

Size indicates the size (diameter) of the particles formed. Size distribution may be determined using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer. Particles under 200 nm are preferred for distribution to neo-vascularized (leaky) tissues, such as neoplasms and sites of inflammation.

Pharmaceutical Compositions

The lipid particles of present invention, particularly when associated with a therapeutic agent, may be formulated as a pharmaceutical composition, e.g., which further comprises a pharmaceutically acceptable diluent, excipient, or carrier, such as
5 physiological saline or phosphate buffer, selected in accordance with the route of administration and standard pharmaceutical practice.

In particular embodiments, pharmaceutical compositions comprising the lipid-nucleic acid particles of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will
10 be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the
15 compositions can be diluted into pharmaceutically acceptable carriers such as normal saline.

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

The concentration of lipid particle or lipid-nucleic acid particle in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.01%, usually at
30 or at least about 0.05-5% to as much as 10 to 30% by weight and will be selected primarily

by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

As noted above, the lipid-therapeutic agent (*e.g.*, nucleic acid) particels of the invention may include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation. Rather, it may also provide a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

The present invention also provides lipid-therapeutic agent compositions in kit form. The kit will typically be comprised of a container that is compartmentalized for holding the various elements of the kit. The kit will contain the particles or pharmaceutical compositions of the present invention, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In certain embodiments, the particles comprise the active agent, while in other embodiments, they do not.

D. Methods of Manufacture

The methods and compositions of the invention make use of certain cationic lipids, the synthesis, preparation and characterization of which is described below and in the accompanying Examples. In addition, the present invention provides methods of preparing lipid particles, including those associated with a therapeutic agent, *e.g.*, a nucleic acid. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid

encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 3 wt% to about 25 wt%, preferably 5 to 15 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter
5 of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

As described above, several of these cationic lipids are amino lipids that are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above
10 the pK_a . These cationic lipids are termed titratable cationic lipids and can be used in the formulations of the invention using a two-step process. First, lipid vesicles can be formed at the lower pH with titratable cationic lipids and other vesicle components in the presence of nucleic acids. In this manner, the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing
15 the pH of the medium to a level above the pK_a of the titratable cationic lipids present, *i.e.*, to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain
20 toxicities which are associated with cationic liposome preparations. Additional details concerning these uses of such titratable cationic lipids in the formulation of nucleic acid-lipid particles are provided in US Patent 6,287,591 and US Patent 6,858,225, incorporated herein by reference.

It is further noted that the vesicles formed in this manner provide
25 formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles have a size range of from about 30 to about 150 nm, more preferably about 30 to about 90 nm.

Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction
30 at low pH. At acidic pH (e.g. pH 4.0) the vesicle surface is charged and binds a portion of

the nucleic acids through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral buffer (*e.g.*, pH 7.5) the surface of the lipid particle or liposome is neutralized, allowing any external nucleic acid to be removed. More detailed information on the formulation process is provided in various publications (*e.g.*, US Patent 5 6,287,591 and US Patent 6,858,225).

In view of the above, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles, *e.g.*, wherein the 10 encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus 15 providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

In certain embodiments, the mixture of lipids includes at least two lipid components: a first amino lipid component of the present invention that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and 20 neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. In particular embodiments, the amino lipid is a novel cationic lipid of the present invention.

In preparing the nucleic acid-lipid particles of the invention, the mixture of lipids is typically a solution of lipids in an organic solvent. This mixture of lipids can then 25 be dried to form a thin film or lyophilized to form a powder before being hydrated with an aqueous buffer to form liposomes. Alternatively, in a preferred method, the lipid mixture can be solubilized in a water miscible alcohol, such as ethanol, and this ethanolic solution added to an aqueous buffer resulting in spontaneous liposome formation. In most embodiments, the alcohol is used in the form in which it is commercially available. For

example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water. This method is described in more detail in US Patent 5,976,567).

In one exemplary embodiment, the mixture of lipids is a mixture of cationic amino lipids, neutral lipids (other than an amino lipid), a sterol (*e.g.*, cholesterol) and a PEG-modified lipid (*e.g.*, a PEG-DMG, PEG-C-DMG or PEG-DMA) in an alcohol solvent. In preferred embodiments, the lipid mixture consists essentially of a cationic amino lipid, a neutral lipid, cholesterol and a PEG-modified lipid in alcohol, more preferably ethanol. In further preferred embodiments, the first solution consists of the above lipid mixture in molar ratios of about 20-70% amino lipid: 5-45% neutral lipid:20-55% cholesterol:0.5-15% PEG-modified lipid. In still further preferred embodiments, the first solution consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol and PEG-DMG or PEG-DMA, more preferably in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA. In another group of preferred embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

In accordance with the invention, the lipid mixture is combined with a buffered aqueous solution that may contain the nucleic acids. The buffered aqueous solution of is typically a solution in which the buffer has a pH of less than the pK_a of the protonatable lipid in the lipid mixture. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the nucleic acid being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (*see, e.g.*, US Patent 6,287,591 and US Patent 6,858,225). Alternatively, pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of therapeutic nucleic acids is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated nucleic acids. Additionally, the intermediate mixture may also contain some portion of nucleic acids which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

Optionally, the lipid-encapsulated therapeutic agent (*e.g.*, nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by

conventional laser-beam particle size determination. For certain methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

In particular embodiments, methods of the present invention further comprise a step of neutralizing at least some of the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids are removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and nucleic acid release from the surface. The released nucleic acid can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

Optionally the lipid vesicles (*i.e.*, lipid particles) can be formed by hydration in an aqueous buffer and sized using any of the methods described above prior to addition of the nucleic acid. As described above, the aqueous buffer should be of a pH below the pKa of the amino lipid. A solution of the nucleic acids can then be added to these sized, preformed vesicles. To allow encapsulation of nucleic acids into such "pre-formed" vesicles the mixture should contain an alcohol, such as ethanol. In the case of ethanol, it should be present at a concentration of about 20% (w/w) to about 45% (w/w). In addition, it may be necessary to warm the mixture of pre-formed vesicles and nucleic acid in the aqueous buffer-ethanol mixture to a temperature of about 25° C to about 50° C depending on the composition of the lipid vesicles and the nature of the nucleic acid. It will be

apparent to one of ordinary skill in the art that optimization of the encapsulation process to achieve a desired level of nucleic acid in the lipid vesicles will require manipulation of variable such as ethanol concentration and temperature. Examples of suitable conditions for nucleic acid encapsulation are provided in the Examples. Once the nucleic acids are
5 encapsulated within the prefomed vesicles, the external pH can be increased to at least partially neutralize the surface charge. Unencapsulated and surface adsorbed nucleic acids can then be removed as described above.

E. Method of Use

The lipid particles of the present invention may be used to deliver a
10 therapeutic agent to a cell, *in vitro* or *in vivo*. In particular embodiments, the therapeutic agent is a nucleic acid, which is delivered to a cell using a nucleic acid-lipid particles of the present invention. While the following description of various methods of using the lipid particles and related pharmaceutical compositions of the present invention are exemplified by description related to nucleic acid-lipid particles, it is understood that these methods and
15 compositions may be readily adapted for the delivery of any therapeutic agent for the treatment of any disease or disorder that would benefit from such treatment.

In certain embodiments, the present invention provides methods for introducing a nucleic acid into a cell. Preferred nucleic acids for introduction into cells are siRNA, immune-stimulating oligonucleotides, plasmids, antisense and ribozymes. These
20 methods may be carried out by contacting the particles or compositions of the present invention with the cells for a period of time sufficient for intracellular delivery to occur.

The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the nucleic acid-lipid particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer
25 or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. Without intending to be limited with respect to the scope of the invention, it is believed that in the case of particles taken up into the cell by endocytosis the particles then interact with the endosomal membrane, resulting in destabilization of the endosomal membrane, possibly by the formation of non-bilayer phases, resulting in introduction of the
30 encapsulated nucleic acid into the cell cytoplasm. Similarly in the case of direct fusion of

the particles with the cell plasma membrane, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. In certain embodiments, treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures (about 37°C) for periods of time from about 1 to 24 hours, preferably from about 2 to 8 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 20 $\mu\text{g/mL}$, more preferably about 1 $\mu\text{g/mL}$.

Typical applications include using well known procedures to provide intracellular delivery of siRNA to knock down or silence specific cellular targets. Alternatively applications include delivery of DNA or mRNA sequences that code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, *et al.*, *Brit. Med. Bull.* 45(3):630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* 341:102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al.*, *Mol. Pharm.* 41:1023-1033 (1992)).

Alternatively, the compositions of the present invention can also be used for deliver of nucleic acids to cells *in vivo*, using methods which are known to those of skill in the art. With respect to application of the invention for delivery of DNA or mRNA sequences, Zhu, *et al.*, *Science* 261:209-211 (1993), incorporated herein by reference,

describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, *et al.*, *Nature* 362:250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, *et al.*, *Am. J. Med. Sci.* 298:278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

10 For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In particular embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For one example, see Stadler, *et al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubinger, *et al.*, *METHODS IN ENZYMOLOGY*, Academic Press, New York. 101:512-527 (1983); Mannino, *et al.*, *Biotechniques* 6:682-690 (1988); Nicolau, *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.* 6:239-271 (1989), and Behr, *Acc. Chem. Res.* 26:274-278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman *et al.*, U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos *et al.*, U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk *et al.*, U.S. Patent No. 4,522,803; and Fountain *et al.*, U.S. Patent No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical," it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs,

abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrizamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

10 The lipid-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (*see*, Brigham, *et al.*, *Am. J. Sci.* 298(4):278-281 (1989)) or by direct injection at the site of disease (Culver, Human Gene Therapy, MaryAnn Liebert, Inc., Publishers, New York, pp.70-71 (1994)).

 The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

 Dosages for the lipid-therapeutic agent particles of the present invention will depend on the ratio of therapeutic agent to lipid and the administering physician's opinion based on age, weight, and condition of the patient.

20 In one embodiment, the present invention provides a method of modulating the expression of a target polynucleotide or polypeptide. These methods generally comprise contacting a cell with a lipid particle of the present invention that is associated with a nucleic acid capable of modulating the expression of a target polynucleotide or polypeptide. As used herein, the term "modulating" refers to altering the expression of a target polynucleotide or polypeptide. In different embodiments, modulating can mean increasing or enhancing, or it can mean decreasing or reducing. Methods of measuring the level of expression of a target polynucleotide or polypeptide are known and available in the arts and include, e.g., methods employing reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical techniques. In particular embodiments, the level of expression of a target polynucleotide or polypeptide is increased or reduced by at least

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10%, 20%, 30%, 40%, 50%, or greater than 50% as compared to an appropriate control value.

For example, if increased expression of a polypeptide desired, the nucleic acid may be an expression vector that includes a polynucleotide that encodes the desired polypeptide. On the other hand, if reduced expression of a polynucleotide or polypeptide is desired, then the nucleic acid may be, *e.g.*, an antisense oligonucleotide, siRNA, or microRNA that comprises a polynucleotide sequence that specifically hybridizes to a polynucleotide that encodes the target polypeptide, thereby disrupting expression of the target polynucleotide or polypeptide. Alternatively, the nucleic acid may be a plasmid that expresses such an antisense oligonucleotide, siRNA, or microRNA.

In one particular embodiment, the present invention provides a method of modulating the expression of a polypeptide by a cell, comprising providing to a cell a lipid particle that consists of or consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol and PEG-DMG, PEG-C-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is associated with a nucleic acid capable of modulating the expression of the polypeptide. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-DMA/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

In particular embodiments, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced.

In other embodiments, the nucleic acid is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

In related embodiments, the present invention provides a method of treating a disease or disorder characterized by overexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol and PEG-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG, PEG-C-DOMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-DMA/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

In another related embodiment, the present invention includes a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol and PEG-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-DMA/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

The present invention further provides a method of inducing an immune response in a subject, comprising providing to the subject the pharmaceutical composition of the present invention, wherein the therapeutic agent is an immunostimulatory oligonucleotide. In certain embodiments, the immune response is a humoral or mucosal immune response. In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of optically pure DLin-K-DMA (V or VI), DSPC, Chol and PEG-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG, PEG-C-DOMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-DMA/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

In further embodiments, the pharmaceutical composition is provided to the subject in combination with a vaccine or antigen. Thus, the present invention itself provides vaccines comprising a lipid particle of the present invention, which comprises an immunostimulatory oligonucleotide, and is also associated with an antigen to which an immune response is desired. In particular embodiments, the antigen is a tumor antigen or is associated with an infective agent, such as, *e.g.*, a virus, bacteria, or parasite.

A variety of tumor antigens, infections agent antigens, and antigens associated with other disease are well known in the art and examples of these are described in references cited herein. Examples of antigens suitable for use in the present invention include, but are not limited to, polypeptide antigens and DNA antigens. Specific examples of antigens are Hepatitis A, Hepatitis B, small pox, polio, anthrax, influenza, typhus, tetanus, measles, rotavirus, diphtheria, pertussis, tuberculosis, and rubella antigens. In one embodiment, the antigen is a Hepatitis B recombinant antigen. In other aspects, the antigen is a Hepatitis A recombinant antigen. In another aspect, the antigen is a tumor antigen. Examples of such tumor-associated antigens are MUC-1, EBV antigen and antigens associated with Burkitt's lymphoma. In a further aspect, the antigen is a tyrosinase-related protein tumor antigen recombinant antigen. Those of skill in the art will know of other antigens suitable for use in the present invention.

Tumor-associated antigens suitable for use in the subject invention include both mutated and non-mutated molecules that may be indicative of single tumor type, shared among several types of tumors, and/or exclusively expressed or overexpressed in tumor cells in comparison with normal cells. In addition to proteins and glycoproteins, tumor-specific patterns of expression of carbohydrates, gangliosides, glycolipids and mucins have also been documented. Exemplary tumor-associated antigens for use in the subject cancer vaccines include protein products of oncogenes, tumor suppressor genes and other genes with mutations or rearrangements unique to tumor cells, reactivated embryonic gene products, oncofetal antigens, tissue-specific (but not tumor-specific) differentiation antigens, growth factor receptors, cell surface carbohydrate residues, foreign viral proteins and a number of other self proteins.

Specific embodiments of tumor-associated antigens include, *e.g.*, mutated antigens such as the protein products of the Ras p21 protooncogenes, tumor suppressor p53 and BCR-abl oncogenes, as well as CDK4, MUM1, Caspase 8, and Beta catenin; overexpressed antigens such as galectin 4, galectin 9, carbonic anhydrase, Aldolase A, PRAME, Her2/neu, ErbB-2 and KSA, oncofetal antigens such as alpha fetoprotein (AFP), human chorionic gonadotropin (hCG); self antigens such as carcinoembryonic antigen (CEA) and melanocyte differentiation antigens such as Mart 1/Melan A, gp100, gp75, Tyrosinase, TRP1 and TRP2; prostate associated antigens such as PSA, PAP, PSMA, PSM-P1 and PSM-P2; reactivated embryonic gene products such as MAGE 1, MAGE 3, MAGE 4, GAGE 1, GAGE 2, BAGE, RAGE, and other cancer testis antigens such as NY-ESO1, SSX2 and SCP1; mucins such as Muc-1 and Muc-2; gangliosides such as GM2, GD2 and GD3, neutral glycolipids and glycoproteins such as Lewis (y) and globo-H; and glycoproteins such as Tn, Thompson-Freidenreich antigen (TF) and sTn. Also included as tumor-associated antigens herein are whole cell and tumor cell lysates as well as immunogenic portions thereof, as well as immunoglobulin idiotypes expressed on monoclonal proliferations of B lymphocytes for use against B cell lymphomas.

Pathogens include, but are not limited to, infectious agents, *e.g.*, viruses, that infect mammals, and more particularly humans. Examples of infectious virus include, but are not limited to: Retroviridae (*e.g.*, human immunodeficiency viruses, such as HIV-1

(also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (*e.g.*, strains that cause gastroenteritis); Togaviridae (*e.g.*, equine encephalitis viruses, rubella viruses); Flaviridae (*e.g.*, dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (*e.g.*, coronaviruses); Rhabdoviridae (*e.g.*, vesicular stomatitis viruses, rabies viruses); Coronaviridae (*e.g.*, coronaviruses); Rhabdoviridae (*e.g.*, vesicular stomatitis viruses, rabies viruses); Filoviridae (*e.g.*, ebola viruses); Paramyxoviridae (*e.g.*, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (*e.g.*, influenza viruses); Bungaviridae (*e.g.*, Hantaan viruses, bunta viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (*e.g.*, reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (*e.g.*, African swine fever virus); and unclassified viruses (*e.g.*, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses).

Also, gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to Pasteurella species, Staphylococci species, and Streptococcus species. Gram negative bacteria include, but are not limited to, Escherichia coli, Pseudomonas species, and Salmonella species.

Specific examples of infectious bacteria include but are not limited to: Helicobacter pylori, Borelia burgdorferi, Legionella pneumophila, Mycobacteria sps (*e.g.*, M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus (viridans group), Streptococcus faecalis, Streptococcus bovis, Streptococcus

(anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*,
 5 *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israeli*.

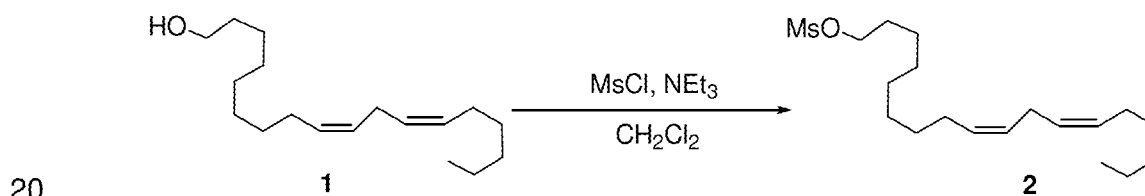
Additional examples of pathogens include, but are not limited to, infectious fungi that infect mammals, and more particularly humans. Examples of infectious fungi include, but are not limited to: *Cryptococcus neoformans*, *Histoplasma capsulatum*,
 10 *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*. Examples of infectious parasites include *Plasmodium* such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*. Other infectious organisms (*i.e.*, protists) include *Toxoplasma gondii*.

15 EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Synthesis of optically pure amino lipids.

Step 1a: Synthesis of methanesulfonic acid octadeca-9, 12-dienyl ester 2

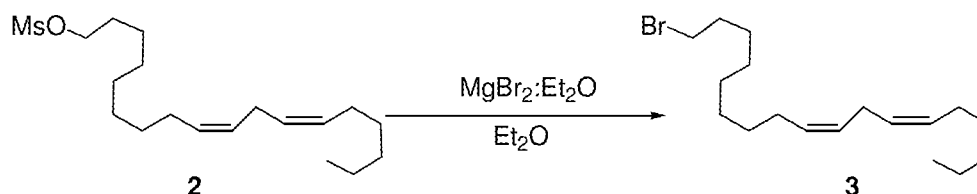


To a solution of the alcohol **1** (26.6 g, 100 mmol) in dichloromethane (100 mL), triethylamine (13.13 g, 130 mmol) was added and this solution was cooled in ice-bath. To this cold solution, a solution of mesyl chloride (12.6 g, 110 mmol) in dichloromethane (60
 25 mL) was added dropwise and after the completion of the addition, the reaction mixture was allowed to warm to ambient temperature and stirred overnight. The TLC of the reaction mixture showed the completion of the reaction. The reaction mixture was diluted with

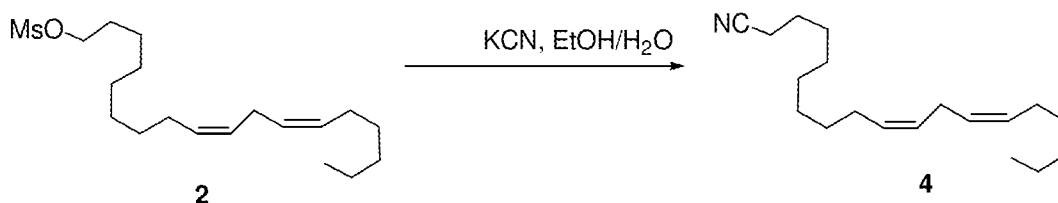
dichloromethane (200 mL), washed with water (200 mL), satd. NaHCO₃ (200 mL), brine (100 mL) and dried (NaSO₄). The organic layer was concentrated to get the crude product which was purified by column chromatography (silica gel) using 0-10% Et₂O in hexanes. The pure product fractions were combined and concentrated to obtain the pure product **2** as colorless oil (30.6 g, 89%). ¹H NMR (CDCl₃, 400 MHz) δ = 5.42-5.21 (m, 4H), 4.20 (t, 2H), 3.06 (s, 3H), 2.79 (t, 2H), 2.19-2.00 (m, 4H), 1.90-1.70 (m, 2H), 1.06-1.18 (m, 18H), 0.88 (t, 3H). ¹³C NMR (CDCl₃) δ = 130.76, 130.54, 128.6, 128.4, 70.67, 37.9, 32.05, 30.12, 29.87, 29.85, 29.68, 29.65, 29.53, 27.72, 27.71, 26.15, 25.94, 23.09, 14.60. MS. Molecular weight calculated for C₁₉H₃₆O₃S, Cal. 344.53, Found 343.52 (M-H).

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Step 1b: Synthesis of 18-Bromo-octadeca-6, 9-diene **3**

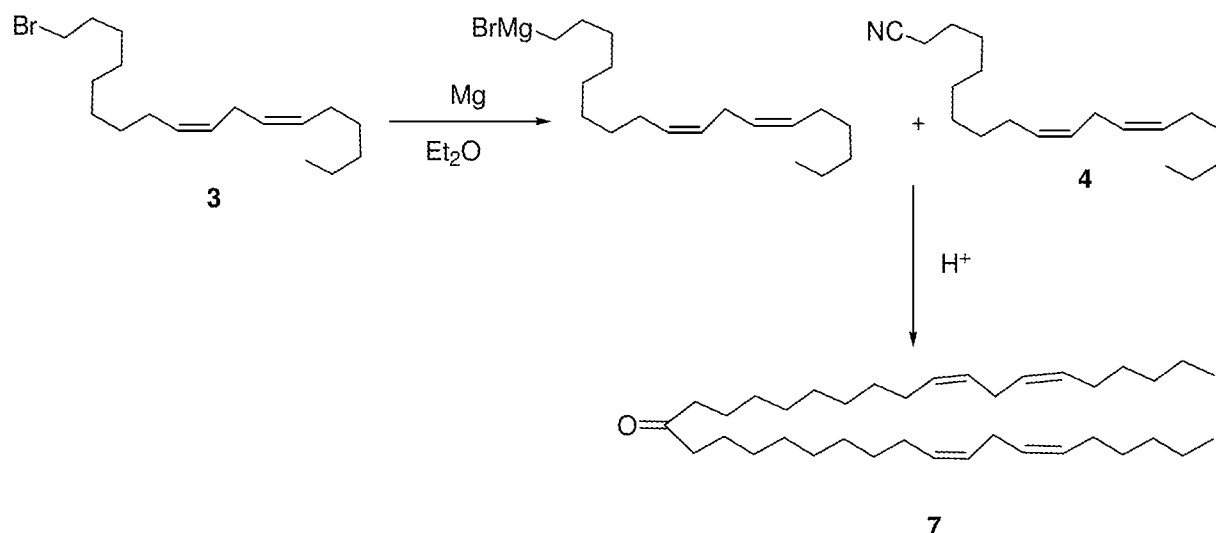


The mesylate (13.44 g, 39 mmol) was dissolved in anhydrous ether (500 mL) and to it the MgBr·Et₂O complex (30.7 g, 118 mmol) was added under argon and the mixture was refluxed under argon for 26 h after which the TLC showed the completion of the reaction. The reaction mixture was diluted with ether (200 mL) and ice-cold water (200 mL) was added to this mixture and the layers were separated. The organic layer was washed with 1% aqueous K₂CO₃ (100 mL), brine (100 mL) and dried (Anhyd. Na₂SO₄). Concentration of the organic layer provided the crude product which was further purified by column chromatography (silica gel) using 0-1% Et₂O in hexanes to isolate the bromide **3** (12.6 g, 94 %) as a colorless oil. ¹H NMR (CDCl₃, 400 MHz) δ = 5.41-5.29 (m, 4H), 4.20 (d, 2H), 3.40 (t, *J* = 7 Hz, 2H), 2.77 (t, *J* = 6.6 Hz, 2H), 2.09-2.02 (m, 4H), 1.88-1.00 (m, 2H), 1.46-1.27 (m, 18H), 0.88 (t, *J* = 3.9 Hz, 3H). ¹³C NMR (CDCl₃) δ = 130.41, 130.25, 128.26, 128.12, 34.17, 33.05, 31.75, 29.82, 29.57, 29.54, 29.39, 28.95, 28.38, 27.42, 27.40, 25.84, 22.79, 14.28.

Step 1c: Synthesis of 18-Cyano-octadeca-6, 9-diene 4

To a solution of the mesylate (3.44 g, 10 mmol) in ethanol (90 mL), a solution of KCN (1.32 g, 20 mmol) in water (10 mL) was added and the mixture was refluxed for 30 min. after which, the TLC of the reaction mixture showed the completion of the reaction after which, ether (200 mL) was added to the reaction mixture followed by the addition of water. The reaction mixture was extracted with ether and the combined organic layers was washed with water (100 mL), brine (200 mL) and dried. Concentration of the organic layer provided the crude product which was purified by column chromatography (0-10 % Et₂O in hexanes). The pure product **4** was isolated as colorless oil (2 g, 74%). ¹H NMR (CDCl₃, 400 MHz) δ = 5.33-5.22 (m, 4H), 2.70 (t, 2H), 2.27-2.23 (m, 2H), 2.00-1.95 (m, 4H), 1.61-1.54 (m, 2H), 1.39-1.20 (m, 18H), 0.82 (t, 3H). ¹³C NMR (CDCl₃) δ = 130.20, 129.96, 128.08, 127.87, 119.78, 70.76, 66.02, 32.52, 29.82, 29.57, 29.33, 29.24, 29.19, 29.12, 28.73, 28.65, 27.20, 27.16, 25.62, 25.37, 22.56, 17.10, 14.06. MS. Molecular weight calculated for C₁₉H₃₃N, Cal. 275.47, Found 276.6 (MH).

Step 1d: Synthesis of Heptatriaconta-6,9,28,31-tetraen-19-one 7

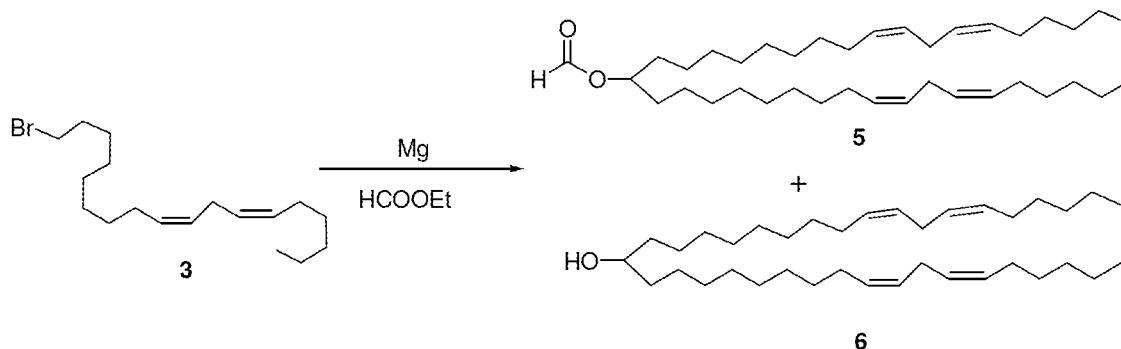


- To a flame dried 500 mL 2NRB flask, freshly activated Mg turnings (0.144 g, 6 mmol)
- 5 were added and the flask was equipped with a magnetic stir bar and a reflux condenser. This set-up was degassed and flushed with argon and 10 mL of anhydrous ether was added to the flask via syringe. The bromide **3** (26.5 g, 80.47 mmol) was dissolved in anhydrous ether (10 mL) and added dropwise via syringe to the flask. An exothermic reaction was noticed (to confirm/accelerate the Grignard reagent formation, 2 mg of iodine was added
- 10 and immediate decolorization was observed confirming the formation of the Grignard reagent) and the ether started refluxing. After the completion of the addition the reaction mixture was kept at 35 °C for 1 h and then cooled in ice bath. The cyanide **4** (1.38 g, 5 mmol) was dissolved in anhydrous ether (20 mL) and added dropwise to the reaction mixture with stirring. An exothermic reaction was observed and the reaction mixture was
- 15 stirred overnight at ambient temperature. The reaction was quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture was treated with aq. H₂SO₄ (10 % by volume, 200 mL) until the solution becomes homogeneous and the layers were separated. The aq. phase was extracted with ether (2x100 mL). The combined ether layers were dried (Na₂SO₄) and concentrated to get the crude product
- 20 which was purified by column (silica gel, 0-10% ether in hexanes) chromatography. The

pure product fractions were evaporated to provide the pure ketone **7** as a colorless oil (2 g, 74%).

In another route, the ketone **7** was synthesized using a two step procedure via the alcohol **6** as follows.

Step 1a(i): Synthesis of Heptatriaconta-6,9,28,31-tetraen-19-ol **7**

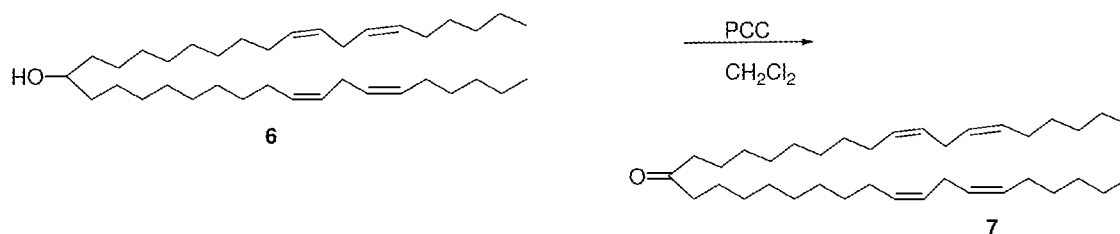


To a flame dried 500 mL RB flask, a freshly activated Mg turnings (2.4 g, 100 mmol) was added and the flask was equipped with a magnetic stir bar, an addition funnel and a reflux condenser. This set-up was degassed and flushed with argon and 10 mL of anhydrous ether was added to the flask via syringe. The bromide **3** (26.5 g, 80.47 mmol) was dissolved in anhydrous ether (50 mL) and added to the addition funnel. About 5 mL of this ether solution was added to the Mg turnings while stirring vigorously. An exothermic reaction was noticed (to confirm/accelerate the Grignard reagent formation, 5 mg of iodine was added and immediate decolorization was observed confirming the formation of the Grignard reagent) and the ether started refluxing. The rest of the solution of the bromide was added dropwise while keeping the reaction under gentle reflux by cooling the flask in water. After the completion of the addition the reaction mixture was kept at 35 °C for 1 h and then cooled in ice bath. Ethyl formate (2.68 g, 36.2 mmol) was dissolved in anhydrous ether (40 mL) and transferred to the addition funnel and added dropwise to the reaction mixture with stirring. An exothermic reaction was observed and the reaction mixture started refluxing. After the initiation of the reaction the rest of the ethereal solution of formate was quickly added as a stream and the reaction mixture was stirred for a further

period of 1 h at ambient temperature. The reaction was quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture was treated with aq. H_2SO_4 (10 % by volume, 300 mL) until the solution becomes homogeneous and the layers were separated. The aq. phase was extracted with ether (2x100 mL). The combined ether layers were dried (Na_2SO_4) and concentrated to get the crude product which was purified by column (silica gel, 0-10% ether in hexanes) chromatography. The slightly less polar fractions were concentrated to get the formate **5** (1.9 g) and the pure product fractions were evaporated to provide the pure product **6** as a colorless oil (14.6 g, 78%).

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Step 1a(ii): Synthesis of Heptatriaconta-6,9,28,31-tetraen-19-one **7**

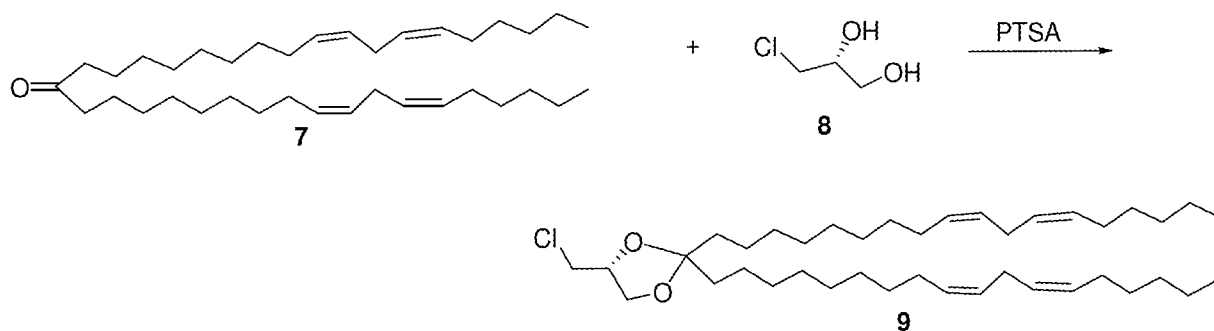


To a solution of the alcohol **6** (3 g, 5.68 mmol) in CH_2Cl_2 (60 mL), freshly activated 4 Å molecular sieves (50 g) were added and to this solution a powdered PCC (4.9 g, 22.7 mmol) was added portionwise over a period of 20 minutes and the mixture was further stirred for 1 hour (**Note:** careful monitoring of the reaction is necessary in order to get good yields since prolonged reaction times leads to lower yields) and the TLC of the reaction mixture was followed every 10 minutes (5% ether in hexanes). After the completion of the reaction, the reaction mixture was filtered through a pad of silica gel and the residue was washed with CH_2Cl_2 (400 mL). The filtrate was concentrated and the thus obtained crude product was further purified by column chromatography (silica gel, 1% Et_2O in hexanes) to isolate the pure product **7** (2.9 g, 97%) as a colorless oil.

25 Example 2:

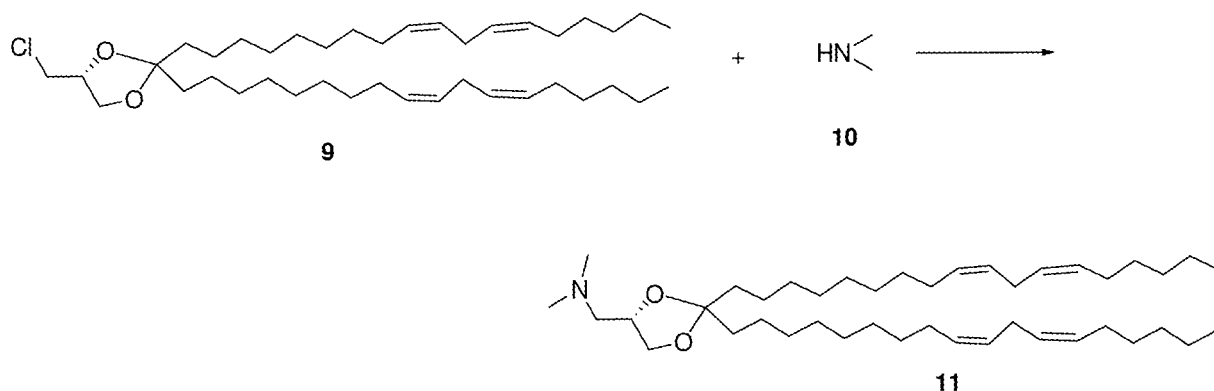
Step 2a: Synthesis of 4-(R)-4-chloromethyl-2,2-di-octadeca-9,12-dienyl-[1,3]dioxolane

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A solution of the ketone **7** (2.7 g, 5.12 mmol), PTSA (10 mg, 0.058 mmol) and diol **8** (1.1 g, 10 mmol) in benzene (250 mL) were heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves and the reflux was continued for 3 days. TLC showed the completion of the reaction. The reaction mixture was washed with satd. NaHCO₃ and the organic layer was washed with brine (100 mL) and concentrated. He thus obtained crude product was further purified by column chromatography (silica gel, 0-2% E₂O in hexanes) gave the pure product **9** (3.07 g, 97 %) as a colorless oil.

Step 2b: Synthesis of 4-(S)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine **11**



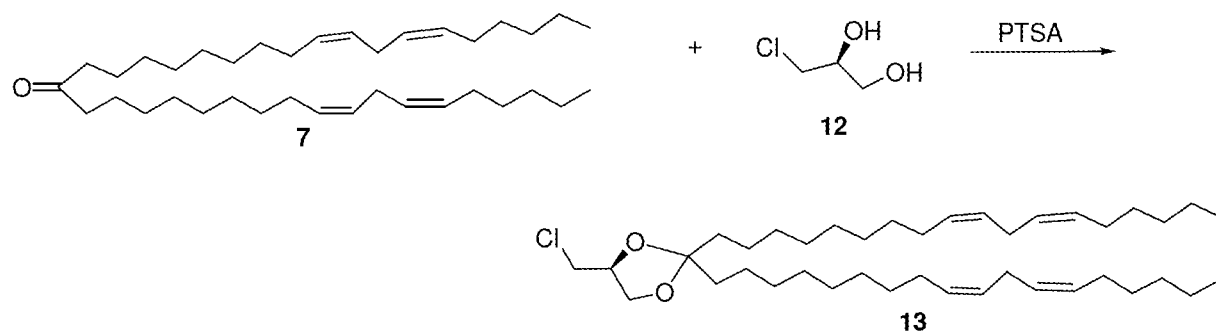
A solution of the ketal **9** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) was heated in a steel bomb at 115°C for 32 h. TLC at this point showed minor amounts of starting material **9** and product **11**. The solvent was removed and the residue was purified

by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **11** (1.72 g, 81%) as a light yellow oil.

In another example the chiral enantiomer of the lipid was synthesized as follows:

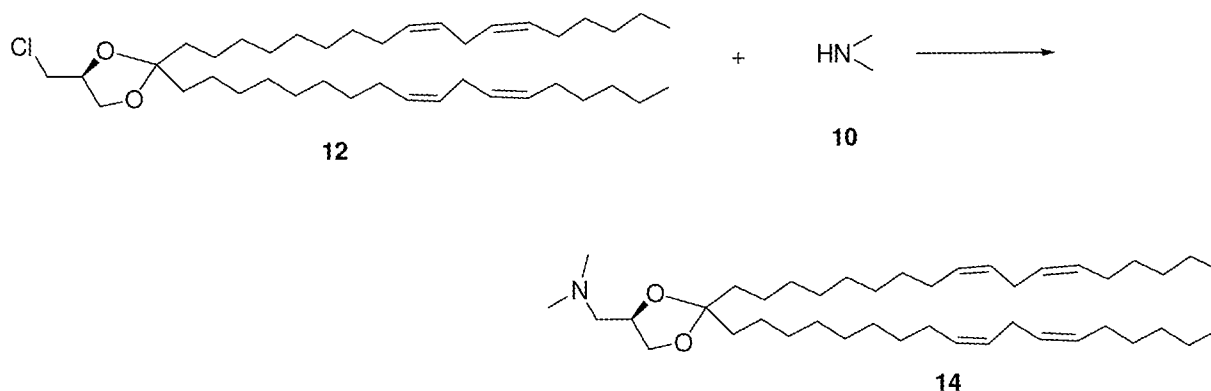
5 Example 3:

Step 3a: Synthesis of 4-(*S*)-4-chloromethyl-2,2-di-octadeca-9,12-dienyl-[1,3]dioxolane **13**



- 10 A solution of compound **7** (2.7 g, 5.12 mmol), PTSA (5 mg, 0.029 mmol) and compound **12** (1 g, 9 mmol) in benzene (250 mL) was heated under reflux with Dean-Stark distillation for 48 h. TLC at this point showed that lots of starting material **7** remained. Further portions of PTSA (10 mg, 0.058 mmol) and compound **12** (1 g, 9 mmol) were added; the Dean-Stark apparatus was swapped for a Soxhlet extractor containing activated 4Å
- 15 molecular sieves and reflux was continued for 48 h. TLC showed mostly product at this point. Work-up by column chromatography gave an inseparable mixture of **13** and **7** (ca. 9:1) (2.7 g, 85 %) as a colorless oil, which was used without further purification in the next step of the reaction.

20 Step 3b: Synthesis of 4-(*R*)-(2,2-diocta-9,12-dienyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine **14**

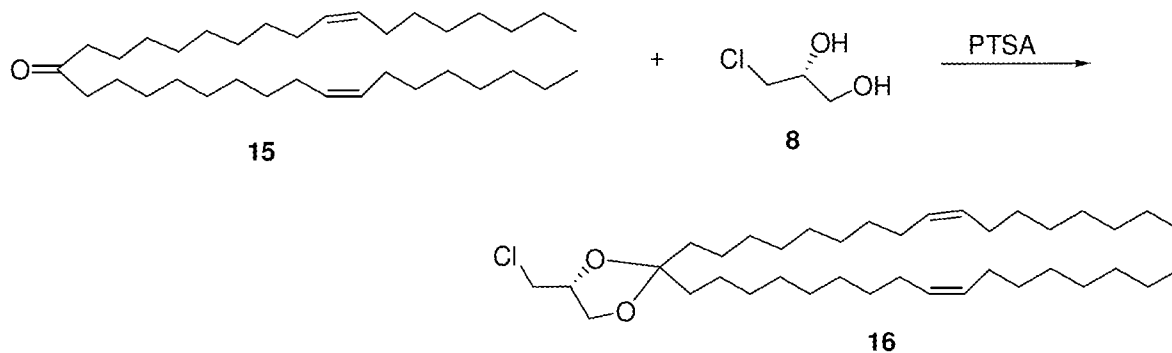


A solution of compound **12** (1.0 g, 1.6 mmol) in 2 M dimethylamine in THF (150 mL) was heated in a steel bomb at 115°C for 16 h. TLC at this point showed a mixture of starting material and product **14**. The solvent was removed and the residue was subjected to column chromatography to afford 0.35 g of pure **14** as a colorless oil.

In another embodiment using the same strategy, diolelyl ketal **17** was also synthesized as follows:

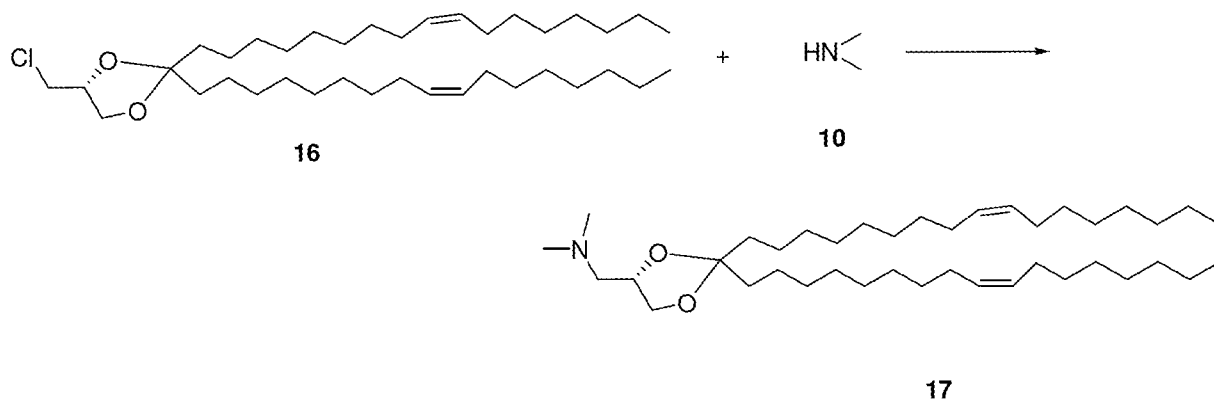
Example 4:

Step 4a: Synthesis of 4-(*R*)-4-chloromethyl-2,2-di-heptadec-9-enyl-[1,3]dioxolane **16**



A solution of compound **15** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **8** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **16** as a colorless oil.

Step 4b: Synthesis of 4-(S)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine 17

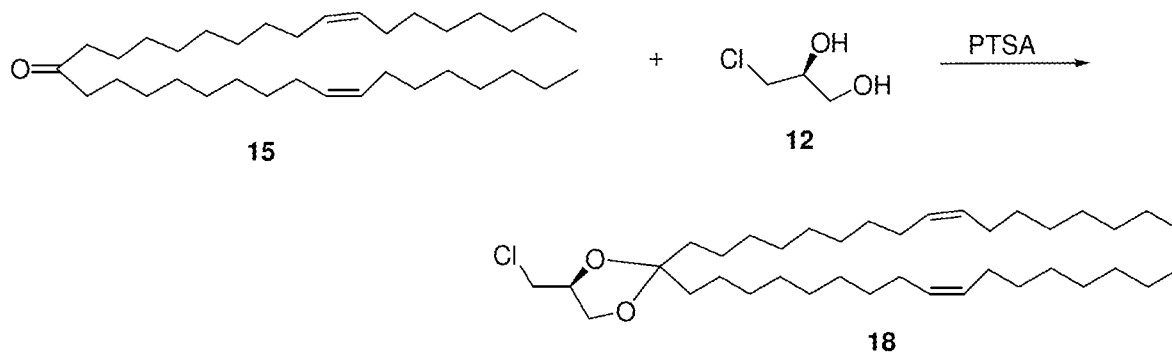


- 5 A solution of the ketal **16** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **16**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **17** as a light yellow oil.

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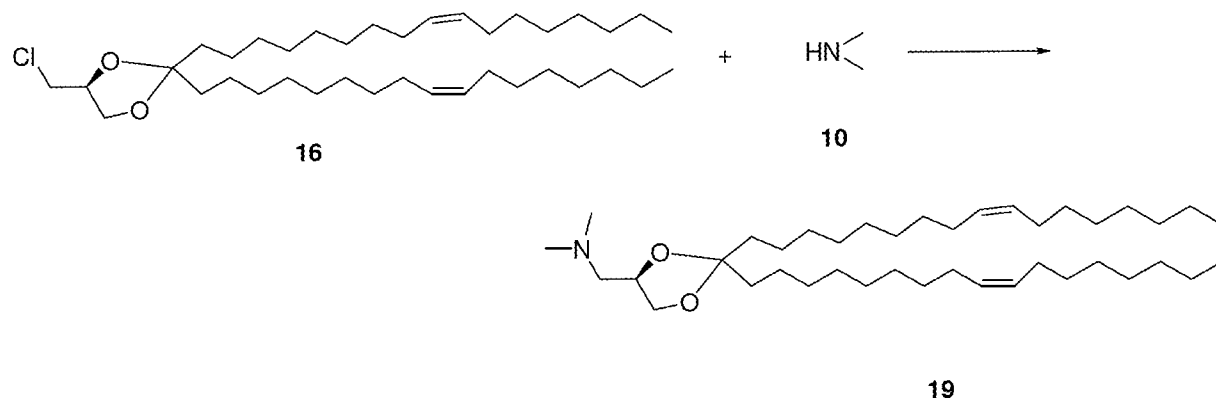
Example 5:

Step 5a: Synthesis of 4-(S)-4-chloromethyl-2,2-di-heptadec-9-enyl-[1,3]dioxolane 18



- 15 A solution of compound **15** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **8** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **18** as a colorless oil.

Step 5b: Synthesis of 4-(S)-(2,2-di-heptadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-dimethylamine 19



5

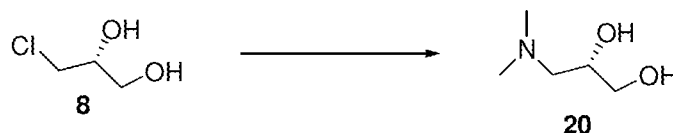
A solution of the ketal **16** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **16**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **19** as a light yellow oil.

10

In another embodiment the homochiral lipids were synthesized with an ether linkage in place of the ketal as follows.

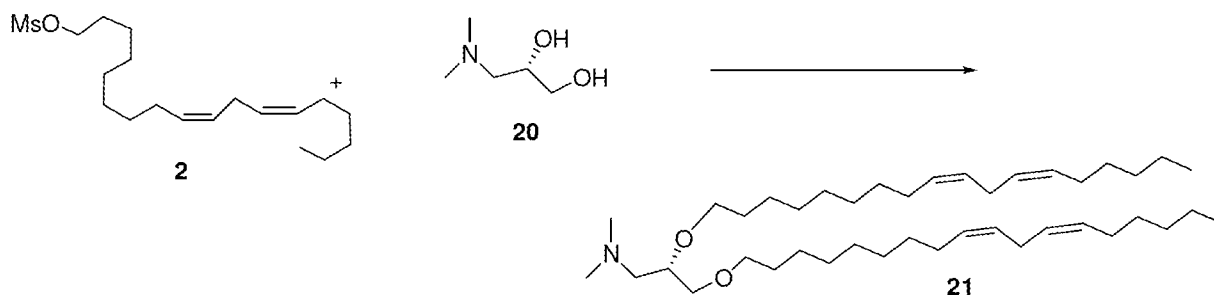
Example 6:

Step 6a: Synthesis of 2-(S)-3-dimethylamino-propane-1,2-diol 20



A solution of the chlorodiol **8** (5 mL, 66.9 mmol) and dimethylamine (32.3 g, 396 mmol) in aqueous sodium hydroxide (20 g in 50 mL of water) was stirred at room temperature in a sealed flask for 24 h. The reaction mixture was diluted with water (100 mL) and extracted with chloroform (3 x 100 mL) and the combined organic layers were concentrated and purified by column chromatography to isolate the pure amine **20** (1 g) as a waxy solid.

20

Step 6b: Synthesis of 2-(S)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine**21**

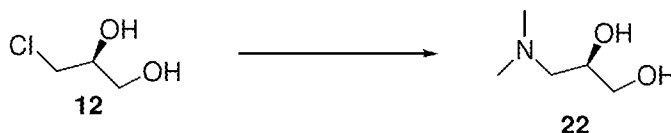
5

A solution of compound **20** (0.9 g, 8.18 mmol) in toluene (100 mL) is treated in portions with NaH (60 % dispersion in mineral oil, 1.6 g, 40 mmol). After stirring for an additional 0.5 h, a solution of compound **2** (7 g, 20 mmol) in toluene (10 mL) was added. The resulting mixture was refluxed for a total of 5 h with monitoring by TLC. Purification by column chromatography provided the pure product **21** (2.7 g, 54%) as a colorless oil.

10

Example 7:

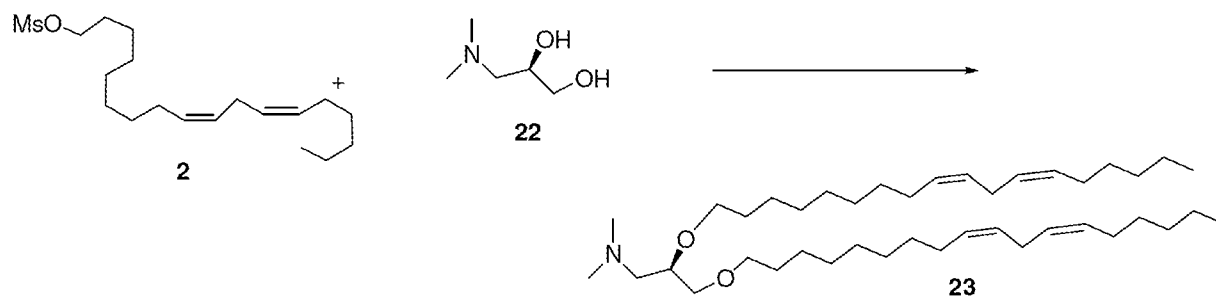
Using a similar procedure the other enantiomer of lipid **21** was synthesized as follows.

Step 7a: Synthesis of 2-(R)-3-dimethylamino-propane-1,2-diol **22**

15

A solution of the chlorodiols **12** (19 g, 172 mmol) and dimethylamine (250 mL of a 5.6 M ethanolic solution) was heated in a steel bomb at 120°C over 18 h. TLC and MS showed no starting material. The solvent was removed and aqueous NaOH (6.9 g, 172 mmol, in 100 mL H₂O) was added. The aqueous layer was washed with DCM (100 mL) then reduced to white residue. The residue was treated with 100 mL of hot EtOAc, and allowed to cool. The mixture was dried by addition of Na₂SO₄, then filtered. The filtrate was subjected to column chromatography to give pure **22** (14.4 g, 70 %) as a light yellow oil.

20

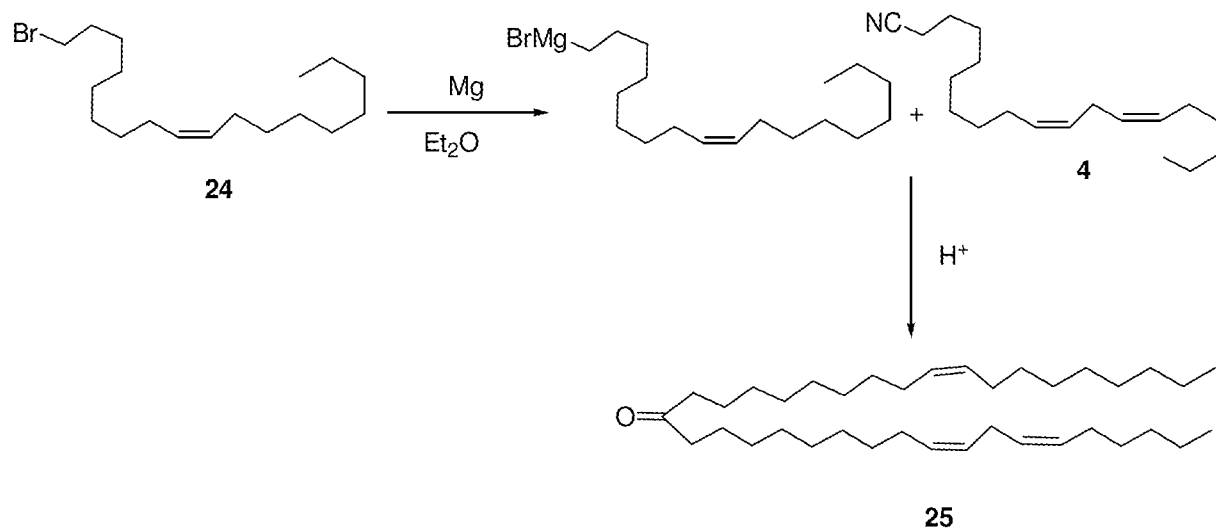
Step 7b: Synthesis of 2-(R)-((2,3-bis-octadeca-9,12-dienyloxy-propyl)-dimethylamine**23**

- 5 A solution of compound **22** () in benzene (50 mL) is treated in portions with NaH (60 % dispersion in mineral oil, 2.67 g, 67 mmol). After stirring for an additional 0.5 h, a solution of compound **2** () in benzene (10 mL) is added. The resulting mixture is refluxed for a total of 5 h with monitoring by TLC. Purification by column chromatography gives g of pure compound **23** as a colorless oil.

10

Example 8:

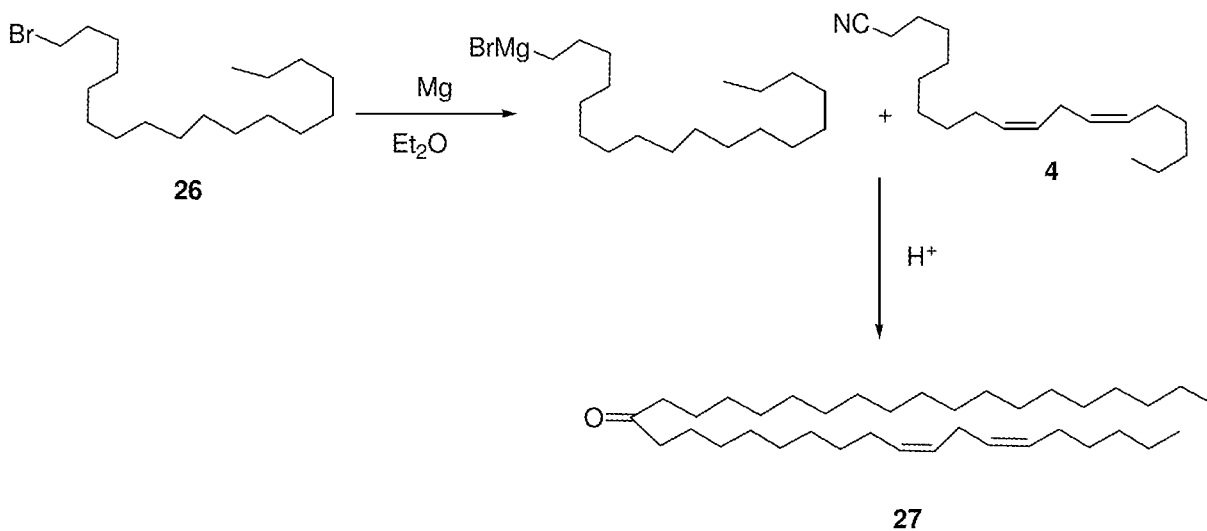
In another embodiment the homochiral ketal with different lipid chains are prepared as follows.

Step 8a: Synthesis of heptatriaconta-6,9,28-trien-19-one

15

To a flame dried 500 mL 2NRB flask, a freshly activated Mg turnings (0.144 g, 6 mmol) is added and the flask is equipped with a magnetic stir bar and a reflux condenser. This set-up is degassed and flushed with argon and 10 mL of anhydrous ether is added to the flask via syringe. The commercially available bromide **24** (26.5 g, 80.4 mmol) is dissolved in anhydrous ether (10 mL) and added dropwise via syringe to the flask. After the completion of the addition the reaction mixture is kept at 35 °C for 1 h and then cooled in ice bath. The cyanide **4** (1.38 g, 5 mmol) is dissolved in anhydrous ether (20 mL) and added dropwise to the reaction mixture with stirring. An exothermic reaction is observed and the reaction mixture is stirred overnight at ambient temperature. The reaction is quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture is treated with aq. H₂SO₄ (10 % by volume, 200 mL) until the solution becomes homogeneous and the layers are separated. The aq. phase is extracted with ether (2x100 mL). The combined ether layers are dried (Na₂SO₄) and concentrated to get the crude product which is purified by column chromatography to provide the pure ketone **25** as a colorless oil.

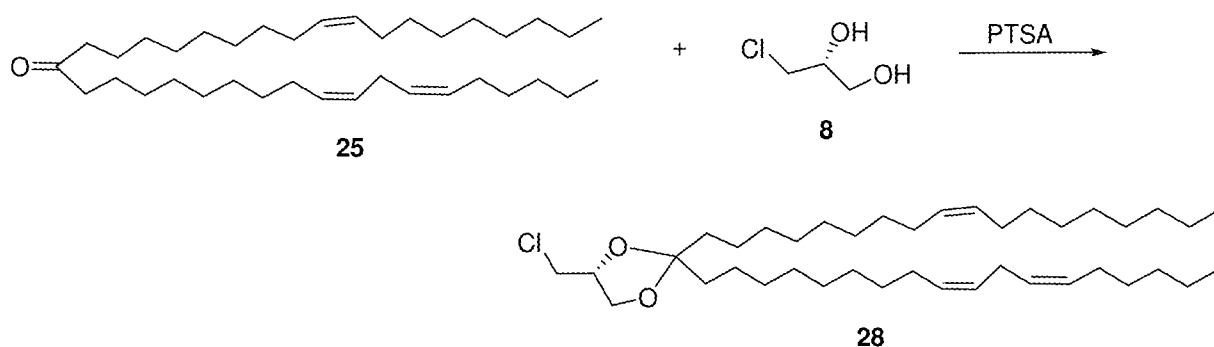
Step 8b: Synthesis of heptatriaconta-6,9-dien-19-one 27



To a flame dried 500 mL 2NRB flask, a freshly activated Mg turnings (0.144 g, 6 mmol) is added and the flask is equipped with a magnetic stir bar and a reflux condenser. This set-up is degassed and flushed with argon and 10 mL of anhydrous ether is added to the flask via

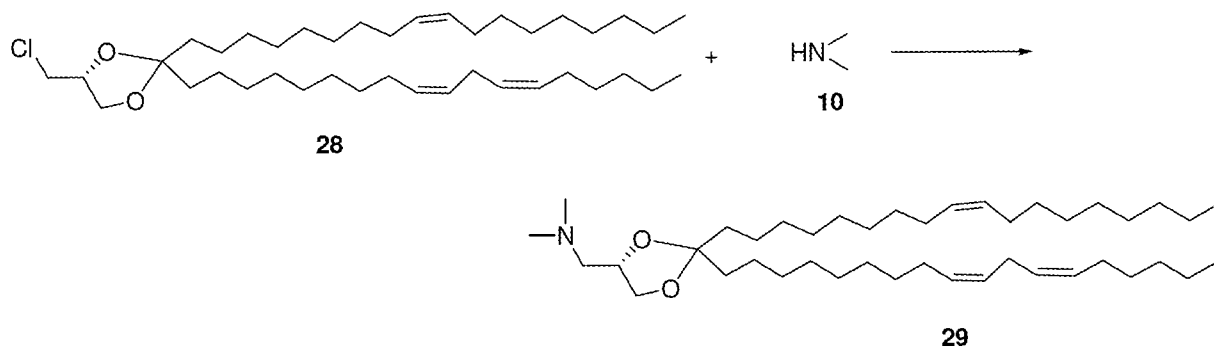
syringe. The commercially available bromide **26** (26.5 g, 80.4 mmol) is dissolved in anhydrous ether (10 mL) and added dropwise via syringe to the flask. After the completion of the addition the reaction mixture is kept at 35 °C for 1 h and then cooled in ice bath. The cyanide **4** (1.38 g, 5 mmol) is dissolved in anhydrous ether (20 mL) and added dropwise to the reaction mixture with stirring. An exothermic reaction is observed and the reaction mixture is stirred overnight at ambient temperature. The reaction is quenched by adding 10 mL of acetone dropwise followed by ice cold water (60 mL). The reaction mixture is treated with aq. H₂SO₄ (10 % by volume, 200 mL) until the solution becomes homogeneous and the layers are separated. The aq. phase is extracted with ether (2x100 mL). The combined ether layers are dried (Na₂SO₄) and concentrated to get the crude product which is purified by column chromatography to provide the pure ketone **27** as a colorless oil.

Step 8c: Synthesis of 4-(R)-4-chloromethyl-2-octadeca-9,12-deenyl-2-octadec-9-enyl-[1,3]dioxolane 28



A solution of compound **25** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **8** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **28** as a colorless oil.

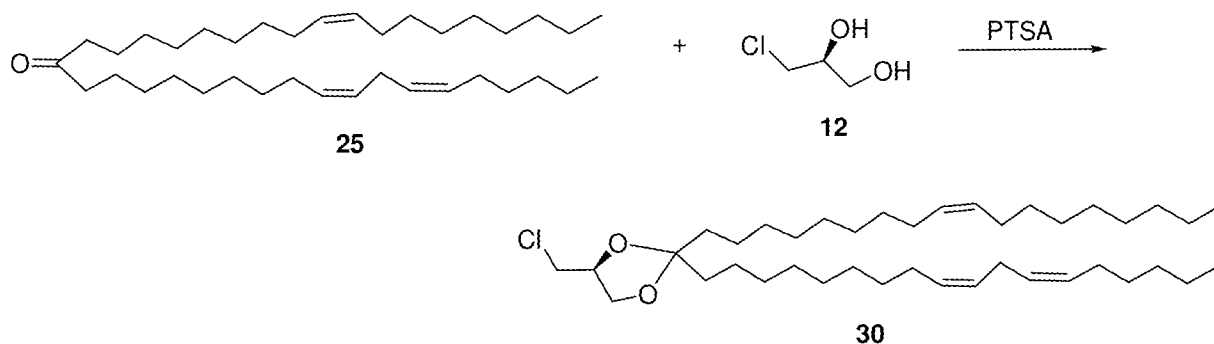
Step 8d: Synthesis of 4-(S)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine 29



A solution of the ketal **28** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **28**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **29** as a light yellow oil.

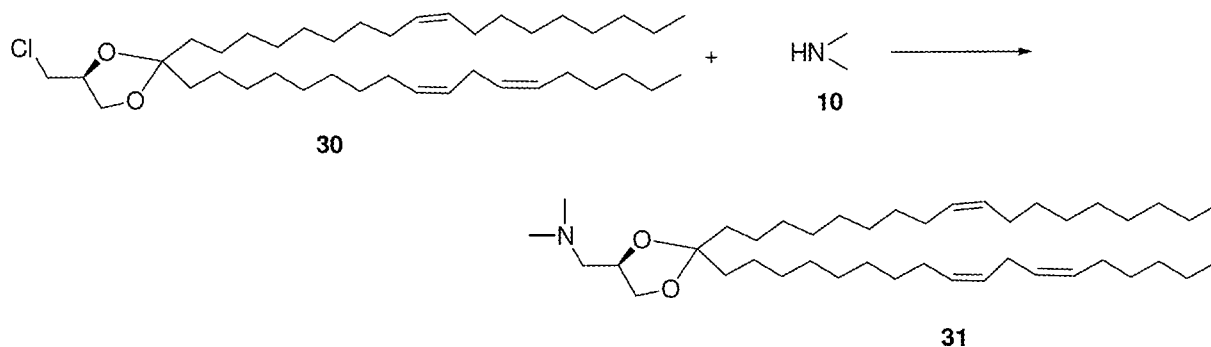
Example 9:

Step 9a: Synthesis of 4-(S)-4-chloromethyl-2-octadeca-9,12-deenyl-2-octadec-9-enyl-[1,3]dioxolane 30



A solution of compound **25** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **12** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **30** as a colorless oil.

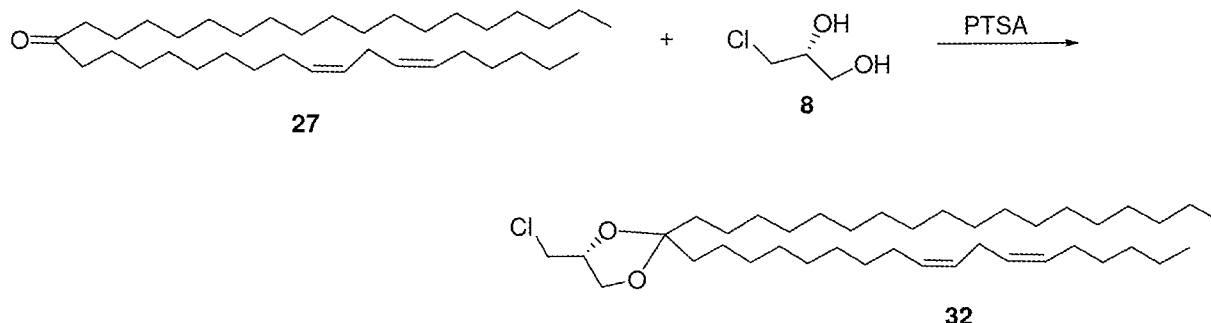
Step 9b: Synthesis of 4-(R)-dimethyl-(2-octadeca-9,12-dienyl-2-octadec-9-enyl-[1,3]dioxolan-4-ylmethyl)-amine 31



A solution of the ketal **30** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **30**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **31** as a light yellow oil.

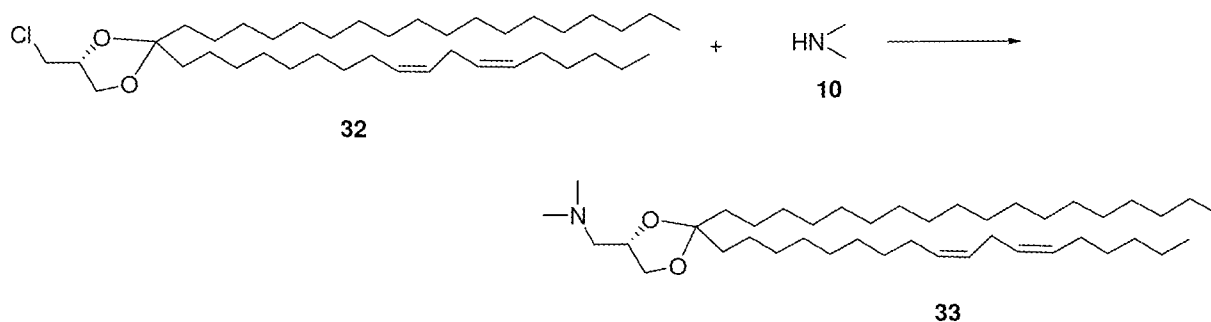
Example 10:

10 Step 10a: Synthesis of 4-(*R*)-4-chloromethyl-2-octadeca-9,12-deenyl-2-octadecyl-[1,3]dioxolane **32**



A solution of compound **27** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **8** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **32** as a colorless oil.

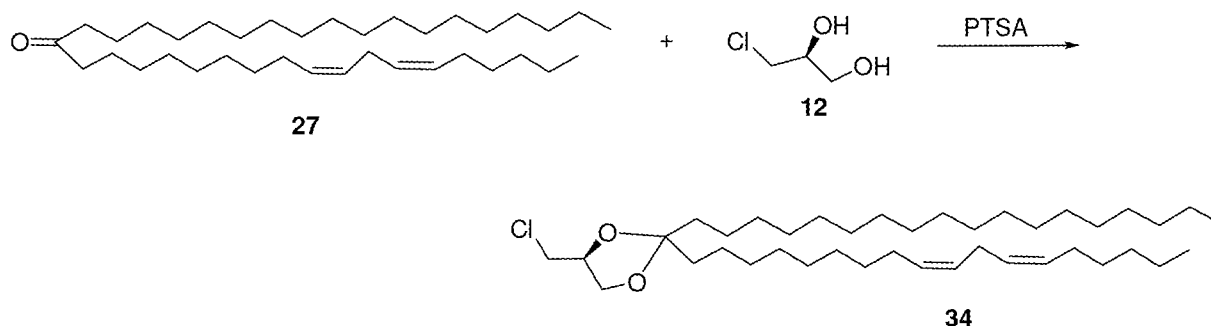
20 Step 10b: Synthesis of 4-(*S*)-dimethyl-(2-octadeca-9,12-dienyl-2-octadecyl-[1,3]dioxolan-4-ylmethyl)amine **33**



A solution of the ketal **32** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **32**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **33** as a light yellow oil.

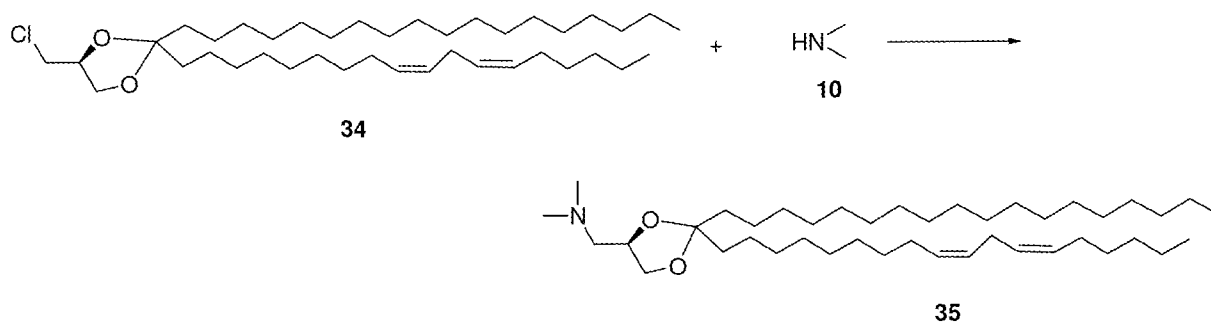
Example 11:

10 Step 11a: Synthesis of 4-(S)-4-chloromethyl-2-octadeca-9,12-deenyl-2-octadecyl-[1,3]dioxolane **34**



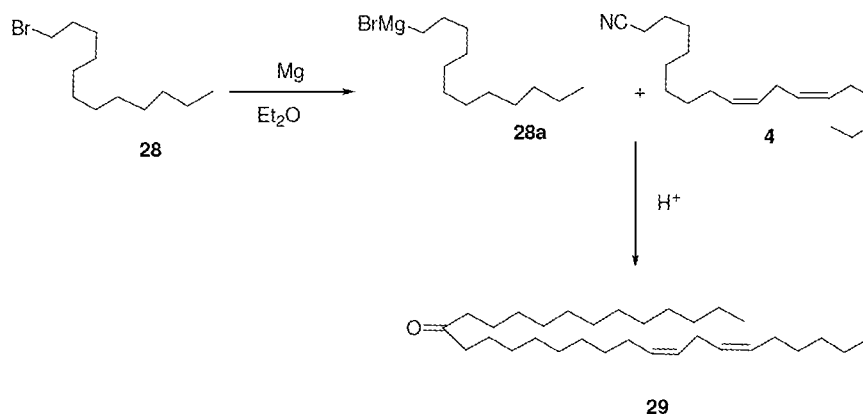
A solution of compound **27** (2.0 g, 4 mmol), PTSA (10 mg, 0.029 mmol) and compound **12** (1 g, 9 mmol) in toluene (250 mL) is heated under reflux with a Soxhlet extractor containing activated 4Å molecular sieves for 48 h. Work-up followed by column chromatography gives the ketal **34** as a colorless oil.

20 Step 11b: Synthesis of 4-(R)-dimethyl-(2-octadeca-9,12-dienyl-2-octadecyl-[1,3]dioxolan-4-yl)methyl)amine **35**



A solution of the ketal **34** (2.0 g, 3.2 mmol) in 2 M dimethylamine in THF (200 mL) is heated in a steel bomb at 115°C for 48 h. TLC showed the complete consumption of starting material **34**. The solvent is removed and the residue purified by column chromatography (silica gel, 0-10% MeOH in EtOAc) to afford the pure product **35** as a light yellow oil.

10 Example 12: Synthesis of Unsymmetrical Ketones with C₁₂ Chain **29**

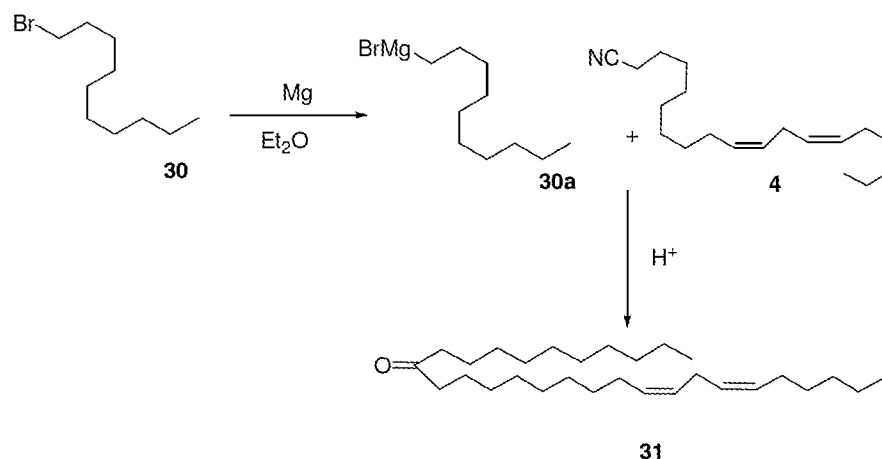


To a dry 50 ml 2NRB flask, a freshly activated Mg turnings (175 mg, 0.0072 mol) was added and the flask was equipped with a magnetic stir bar and a reflux condenser. This setup was degassed and flushed with nitrogen and 10mL of anhydrous ether was added to the flask via syringe. The bromide **28** (1.5g, 0.006 mol) was dissolved in anhydrous ether (7 ml) and added dropwise via syringe to the flask. An exothermic reaction was noticed (reaction initiated with dibromoethane) and the ether started refluxing. After completion of the addition the reaction mixture was kept at 35°C for 1h and then cooled in ice bath to 10-15°C. The cyanide **4** (1g, 0.0036mol) was dissolved in anhydrous ether (7 mL) and added

dropwise to the reaction with stirring. An exothermic reaction was observed and the reaction mixture was refluxed for 12h and quenched with ammonium chloride solution. It was then treated with 25% HCl solution until the solution becomes homogenous and the layers were separated. The aq phase was extracted with ether. The combined ether layers were dried and concentrated to get the crude product which was purified by column chromatography. The pure product fractions were evaporated to provide the pure ketone **29** as colorless oil. Yield: 0.65 g (26%). $^1\text{H-NMR}$ (δ ppm): 5.388-5.302 (m, 4H), 2.77 – 2.74 (t, 2H), 2.38 – 2.34 (t, 4H), 2.04-2.01 (m, 4H), 1.34 – 1.18 (m, 36H), 0.89 – 0.85 (m 6H). IR (cm^{-1}): 3009, 2920, 2851, 1711 (C=O), 1466, 1376, 1261.

10

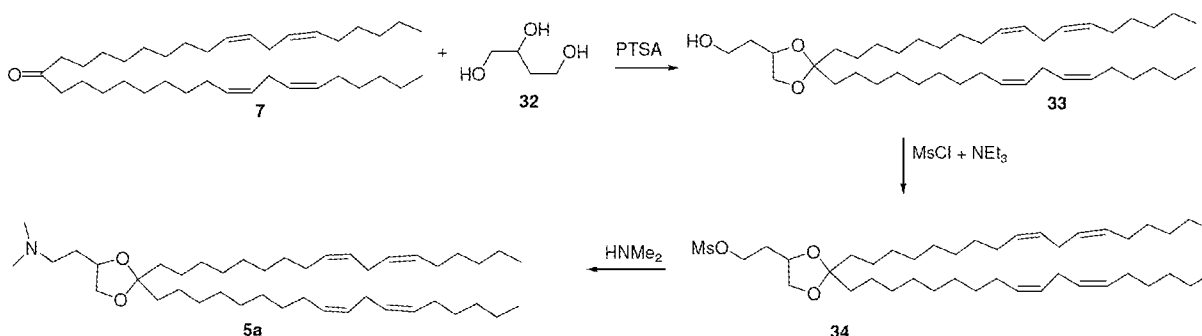
Example 13: Synthesis of Unsymmetrical Ketones with C10 Chain 31



- 15 To a dry 50 ml 2NRB flask, a freshly activated Mg turnings (266 mg, 0.0109 mol) was added and the flask was equipped with a magnetic stir bar and a reflux condenser. This setup was degassed and flushed with nitrogen and 10mL of anhydrous ether was added to the flask via syringe. The bromide (2.43 g, 0.0109 mol) was dissolved in anhydrous ether (7 ml) and added dropwise via syringe to the flask. An exothermic reaction was noticed
- 20 (reaction initiated with dibromoethane) and the ether started refluxing. After completion of the addition the reaction mixture was kept at 35°C for 1h and then cooled in ice bath to $10-15^\circ\text{C}$. The cyanide (1 g, 0.0036 mol) was dissolved in anhydrous ether (7 mL) and added dropwise to the reaction with stirring. An exothermic reaction was observed and the

reaction mixture was stirred at ambient temperature for 2 hr. THF (4ml) was added to the reaction mixture and it was warmed to 45-50⁰ C for 4 hr till the cyano derivative was complete consumed. The reaction was quenched by adding 3mL of acetone dropwise followed by ice cold water. The reaction mixture was treated with 25% HCl solution until the solution becomes homogenous and the layers were separated. The aq. phase was extracted with ether. The combined ether layers were dried and concentrated to get the crude product which was purified by column chromatography. The pure product fractions were evaporated to provide the pure ketone as colorless oil. Yield: 0.93 gms (61%). ¹H-NMR (δ ppm): 5.37-5.302 (m, 4H), 2.77 – 2.74 (t, 2H), 2.38 – 2.34 (t, 4H), 2.05-2.00 (m, 4H), 1.55 – 1.52 (m, 2H), 1.35 – 1.24 (m, 34H), 0.89 – 0.84 (m 6H). IR (cm⁻¹): 3009, 2925, 2854, 1717 (C=O), 1465, 1376.

Example 14: Process 1 for making the cationic lipid 5a



Step 14a: Preparation of Compound 33

A mixture of compound 32 (10.6 g, 100 mmol), compound 7 (10.54 g, 20 mmol) and PTSA (0.1 eq) was heated under toluene reflux with Soxhlet extractor containing activated 4Å molecular sieves for 3 h. Removal of solvent then column purification (silica gel, 0-30% EtOAc in hexanes) gave compound 33 (11 g, 90 %) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 5.45 – 5.24 (m, 8H), 4.30 – 4.17 (m, 1H), 4.08 (dd, *J* = 7.8, 6.1, 1H), 3.80 (dd, *J* = 10.6, 5.0, 3H), 3.53 (t, *J* = 8.0, 1H), 2.77 (t, *J* = 6.4, 5H), 2.29 – 2.18 (m, 1H), 2.05 (q, *J* = 6.7, 9H), 1.86 – 1.74 (m, 2H), 1.59 (dd, *J* = 18.3, 9.7, 5H), 1.42 – 1.18 (m, 43H), 0.89 (t, *J* = 6.8, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 130.39, 130.36, 130.35, 128.14, 112.80, 77.54, 77.22, 76.90, 75.74, 70.14, 61.08, 37.97, 37.50, 35.56, 31.74, 30.14, 30.13, 29.88, 29.80, 29.73, 29.57, 29.53, 27.45, 27.41, 25.84, 24.20, 24.00, 22.79, 14.30.

Step 14b: Preparation of Compound 34

To an ice-cold solution of compound **33** (10.5 g, 17 mmol) and NEt₃ (5 mL) in DCM (100 mL) a solution of MsCl (2.96 g, 20.5 mmol) in DCM (20 mL) was added dropwise with stirring. After 1 h at r.t., aqueous workup gave a pale yellow oil of **34** which was column purified (silica gel, 0-30% EtOAc in hexanes) to provide the pure mesylate (11.1 g, 94%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 5.44 – 5.26 (m, 8H), 4.37 (m, 2H), 4.26 – 4.13 (m, 1H), 4.10 (m, 1H), 3.53 (m, 1H), 3.02 (s, 3H), 2.76 (d, *J* = 6.4, 4H), 2.05 (d, *J* = 6.9, 10H), 1.55 (s, 4H), 1.29 (d, *J* = 9.8, 34H), 0.88 (t, *J* = 6.9, 6H). Electrospray MS (+ve): Molecular weight for C₄₂H₇₆O₅S (M + H)⁺ Calc. 693.5, Found 693.4.

Step 14c: Preparation of Compound 5a

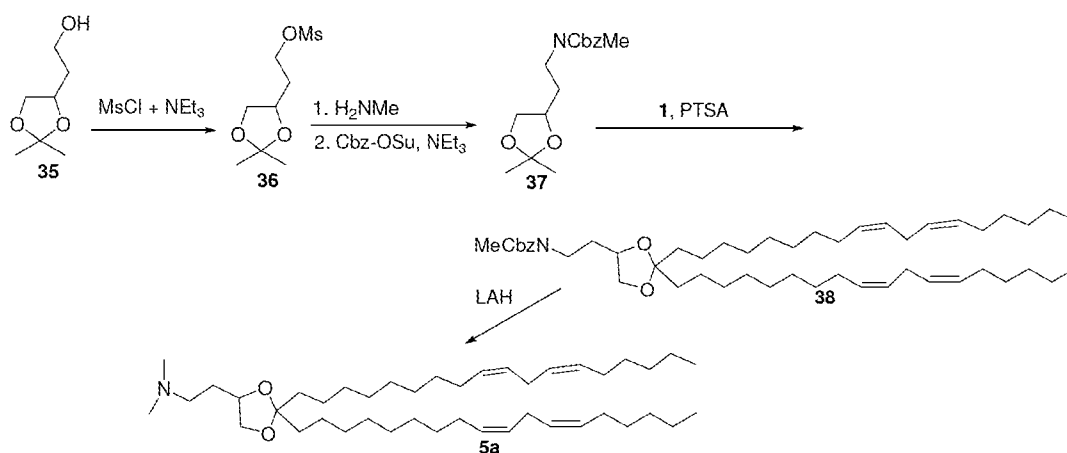
The mesylate **34** (11 g, 15.9 mmol) was dissolved in 400 mL of 2M dimethylamine in THF and the solution was transferred to a Parr pressure reactor and the contents were stirred at 70 °C for 14 h. The reaction mixture was cooled and the TLC of the reaction mixture showed the completion of the reaction. The reaction mixture was concentrated in a rotary evaporator and the thus obtained crude product was purified by column chromatography (silica gel, 0-10% MeOH in dichloromethane) to yield the pure product **5a** (9.4 g, 92%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 5.45 – 5.24 (m, 8H), 4.07 (dt, *J* = 17.3, 6.4, 2H), 3.48 (t, *J* = 7.3, 1H), 2.77 (t, *J* = 6.4, 4H), 2.47 – 2.25 (m, 2H), 2.24 (d, *J* = 10.5, 6H), 2.04 (q, *J* = 6.6, 8H), 1.73 (ddd, *J* = 22.8, 14.5, 7.9, 2H), 1.59 (dt, *J* = 20.0, 9.9, 4H), 1.43 – 1.18 (m, 34H), 0.89 (t, *J* = 6.8, 6H). ¹³C NMR (CDCl₃, 100 MHz) δ = 130.2, 130.1, 128.0, 112.1, 74.8, 70.0, 56.3, 45.5, 37.8, 37.5, 31.8, 31.5, 30.0, 30.0, 29.7, 29.6, 29.6, 29.5, 29.5, 29.3, 29.3, 27.2, 27.2, 25.6, 24.0, 23.7, 22.6, 14.0. Electrospray MS (+ve): Molecular weight for C₄₃H₇₉NO₂ (M + H)⁺ Calc. 642.6, Found 642.6.

Alterntaive 14c: Preparation of Compound 5a

An ethanolic solution of dimethylamine (35% by Wt.) was prepared by passing dimethylamine gas into ethanol (200 proof) under ice cold condition. The mesylate **34** (47 g, 67.8 mmol) was dissolved in this freshly prepared ethanolic solution of dimethylamine

(450 mL) and the solution was transferred to a Parr pressure reactor; the contents were stirred at 70 °C for 16 h. The reaction mixture was cooled to ambient temperature and the TLC of the reaction mixture showed completion of the reaction. The reaction mixture was concentrated in a rotary evaporator and the crude product thus obtained was purified by column chromatography (CombiFlash Rf system, 330 g silica gel cartridge, eluent: 0-10% MeOH in DCM) to yield the pure product **5a** (38 g, 88%) as a glass colored oil. ¹H NMR (400 MHz, CDCl₃): δ 5.45 – 5.24 (m, 8H), 4.07 (dt, *J* = 17.3, 6.4, 2H), 3.48 (t, *J* = 7.3, 1H), 2.77 (t, *J* = 6.4, 4H), 2.47 – 2.25 (m, 2H), 2.24 (d, *J* = 10.5, 6H), 2.04 (q, *J* = 6.6, 8H), 1.73 (dd, *J* = 22.8, 14.5, 7.9, 2H), 1.59 (m, *J* = 20.0, 9.9, 4H), 1.43 – 1.18 (m, 34H), 0.89 (t, *J* = 6.8, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ = 130.2, 130.1, 128.0, 112.1, 74.8, 70.0, 56.3, 45.5, 37.8, 37.5, 31.8, 31.5, 30.0, 30.0, 29.7, 29.6, 29.6, 29.5, 29.5, 29.3, 29.3, 27.2, 27.2, 25.6, 24.0, 23.7, 22.6, 14.0. EI-MS (+ve): MW calc. for C₄₃H₇₉NO₂ (MH⁺): 642.6, found: 642.6.

15 Example 15: Process 2 for making compound 5a



Step 15a: Preparation of Compound 36

MsCl (1.1 eq) was added to an ice-cold stirring solution of compound **11** (5 g, 34.2 mmol) and NEt₃ (1.2 eq) in DCM (10 mL). After 1 h at r.t., aqueous workup gave a pale yellow oil of **36** (7.7 g, quantitative) which was used without further purification. ¹³C NMR (CDCl₃, 100 MHz) δ = 109.2, 72.3, 72.1, 69.1, 67.0, 37.3, 33.4, 26.9, 25.5: Electrospray MS (+ve): Molecular weight for C₈H₁₆O₅S (M + H)⁺ Calc. 225.1, Found 225.0.

Step 15b: Preparation of Compound 37

Compound **36** (3.9 g, 17.4 mmol) was stirred with ethanolic methylamine (33 %, 100 mL) over 72 h. Removal of solvent gave a residue which was treated with Cbz-OSu (1.2 eq) and NEt₃ (3 eq) for 18 h. Aqueous workup then column chromatography gave compound **37** (5.2 g, 98 %).

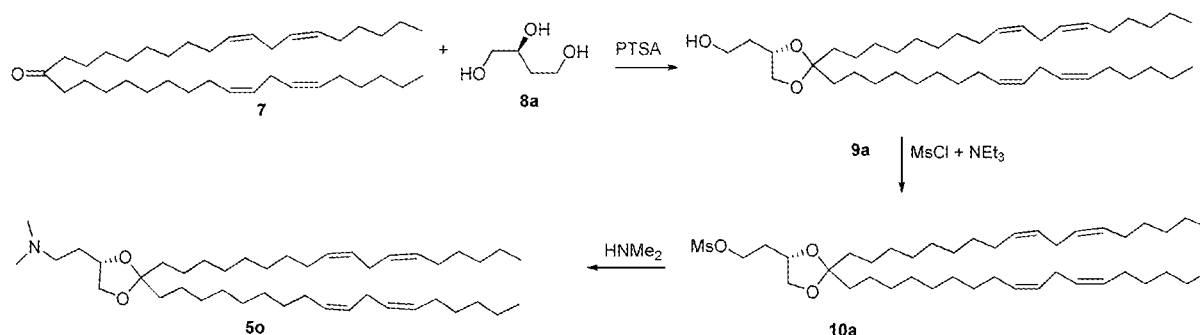
Electrospray MS (+ve): Molecular weight for C₁₆H₂₃NO₄ (M + H)⁺ Calc. 294.2, Found 294.0.

Step 15c: Preparation of Compound 38

A solution of **7** (1 eq), compound **37** (1 eq), and *p*-TSA (0.1 eq) is heated under toluene reflux with Dean-Stark apparatus for 18 h. Removal of solvent then column chromatography gives compound **38** as a colorless oil.

Step 15d: Preparation of Compound 5a

An ice-cooled solution of 1 M LAH (2 eq) in THF is treated dropwise over 0.5 h with a solution of compound **38** (1 eq) in hexane. After addition, the solution is warmed to 40°C for 0.5. The mixture is ice-cooled then hydrolyzed with saturated aqueous Na₂SO₄. Celite is added (5 g) and the resulting mixture is filtered. The filtrate is reduced. Column chromatography affords compound **5a** as colorless oil.

Example 16: Synthesis of Enantiomerically pure ketals 5o**Preparation of 2-((S)-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl) ethanol 9a**

A mixture of freshly distilled alcohol **8a** (40.3 g, 0.38 mol), ketone **7** (40.05 g 76 mmol) and PTSA (0.5 g) was refluxed in toluene (600 mL) using Dean-Stark apparatus for 2 days after which the TLC indicated the complete consumption of the starting ketone. The

reaction mixture was cooled and washed with satd. NaHCO_3 solution (300 mL) and dried over anhyd. Na_2SO_4 and concentrated. The thus obtained crude product was purified by column chromatography (silica gel, 0-30% EtOAc in hexanes) to isolate pure ketal **9a** (39.9 g, 85%) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 5.45 – 5.24 (m, 8H), 4.30 – 4.17 (m, 1H), 4.08 (dd, J = 7.8, 6.1, 1H), 3.80 (dd, J = 10.6, 5.0, 3H), 3.53 (t, J = 8.0, 1H), 2.77 (t, J = 6.4, 5H), 2.29 – 2.18 (m, 1H), 2.05 (q, J = 6.7, 9H), 1.86 – 1.74 (m, 2H), 1.59 (dd, J = 18.3, 9.7, 5H), 1.42 – 1.18 (m, 43H), 0.89 (t, J = 6.8, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 130.39, 130.36, 130.35, 128.14, 112.80, 77.54, 77.22, 76.90, 75.74, 70.14, 61.08, 37.97, 37.50, 35.56, 31.74, 30.14, 30.13, 29.88, 29.80, 29.73, 29.57, 29.53, 27.45, 27.41, 25.84, 24.20, 24.00, 22.79, 14.30.

Preparation of 2-((S)-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)ethyl methanesulfonate 10a

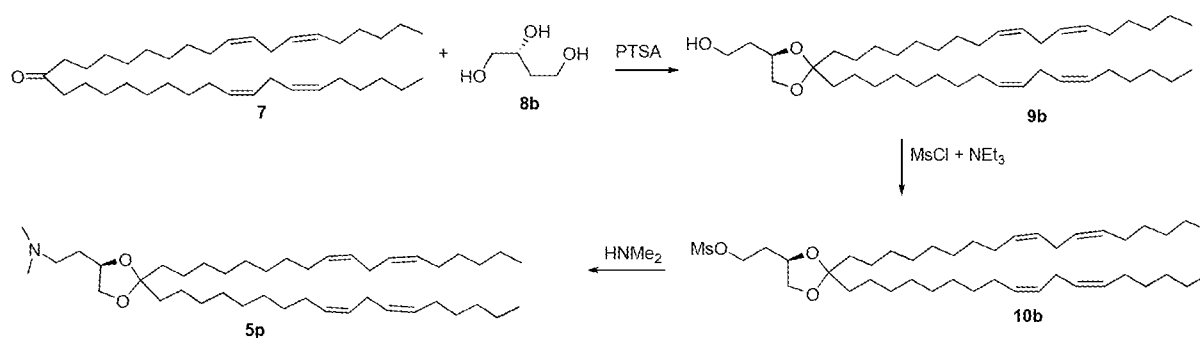
To an ice-cold solution of compound **9a** (47 g, 76.4 mmol) and NEt_3 (20 mL) in DCM (500 mL) a solution of MsCl (13.2 g, 91.7 mmol) in DCM (100 mL) was added dropwise with stirring. After 1 h at r.t., aqueous workup gave a pale yellow oil of **10a** which was column purified (silica gel, 0-30% EtOAc in hexanes) to provide the pure mesylate (47 g, 89 %) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 5.44 – 5.26 (m, 8H), 4.37 (m, 2H), 4.26 – 4.13 (m, 1H), 4.10 (m, 1H), 3.53 (m, 1H), 3.02 (s, 3H), 2.76 (d, J = 6.4, 4H), 2.05 (d, J = 6.9, 10H), 1.55 (s, 4H), 1.29 (d, J = 9.8, 34H), 0.88 (t, J = 6.9, 6H). Electrospray MS (+ve): Molecular weight for $\text{C}_{42}\text{H}_{76}\text{O}_5\text{S}$ ($\text{M} + \text{H}$)⁺ Calc. 693.5, Found 693.4.

Preparation of 2-((S)-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)-N,N-dimethylethanamine 5o

An ethanolic solution of dimethylamine (35% by Wt.) was prepared by passing dimethylamine gas into ethanol (200 proof) under ice cold condition. The mesylate **10a** (47 g, 67.8 mmol) was dissolved in this freshly prepared ethanolic solution of dimethylamine (450 mL) and the solution was transferred to a Parr pressure reactor; the contents were stirred at 70 °C for 16 h. The reaction mixture was cooled to ambient temperature and the TLC of the reaction mixture showed completion of the reaction. The reaction mixture was

concentrated in a rotary evaporator² and the crude product thus obtained was purified by column chromatography (CombiFlash Rf system, 330 g silica gel cartridge, eluent: 0-10% MeOH in DCM) to yield the pure product **5o** (38 g, 88%) as a glass colored oil. ¹H NMR (400 MHz, CDCl₃): δ 5.45 – 5.24 (m, 8H), 4.07 (dt, *J* = 17.3, 6.4, 2H), 3.48 (t, *J* = 7.3, 1H),
 5 2.77 (t, *J* = 6.4, 4H), 2.47 – 2.25 (m, 2H), 2.24 (d, *J* = 10.5, 6H), 2.04 (q, *J* = 6.6, 8H), 1.73 (dd, *J* = 22.8, 14.5, 7.9, 2H), 1.59 (m, *J* = 20.0, 9.9, 4H), 1.43 – 1.18 (m, 34H), 0.89 (t, *J* = 6.8, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ = 130.2, 130.1, 128.0, 112.1, 74.8, 70.0, 56.3, 45.5, 37.8, 37.5, 31.8, 31.5, 30.0, 30.0, 29.7, 29.6, 29.6, 29.5, 29.5, 29.3, 29.3, 27.2, 27.2, 25.6, 24.0, 23.7, 22.6, 14.0; EI-MS (+ve): MW calc. for C₄₃H₇₉NO₂ (MH⁺): 642.6, found:
 10 642.6.

Example 17: Synthesis of enantiomerically pure ketals **5p**



15 Step 17a: Preparation of Compound **9b**

Using a similar procedure to that used for the synthesis of **9**, using optically pure alcohol 2-*R* **8b** (2.02 g, 19 mmol) on treatment with ketone **7** (2 g, 3.8 mmol) and *p*-TSA (0.1 eq) provided the ketal **9b** (1.68 g, 72%) as a colorless oil. The spectral and analytical data was same as that for compound **9** consistent with the structure.

20 Step 17b: Preparation of Compound **10b**

Treatment of the homochiral alcohol **9b** (1.68 g, 2.73 mmol) with mesyl chloride (1.2 eq.) in the presence of triethylamine (3 eq.) provided the mesylate **10b** (1.9 g) in quantitative yield. This product was used as such without further purification in the next step.

Step 17 c: Preparation of Compound **5p**

The mesylate **10b** (1.9 g, 2.7 mmol) was dissolved in 100 mL of 2M dimethylamine in THF and the solution was transferred to a pressure bottle and the contents were stirred at 40 °C for 2 days. The reaction mixture was cooled and the TLC of the reaction mixture showed the completion of the reaction. The reaction mixture was concentrated in a rotary
5 evaporator and the thus obtained crude product is purified by column chromatography (silica gel, 0-10% MeOH in dichloromethane) to yield the pure product **5o** as a colorless oil.

Example 18: Oigonucleotide Synthesis.

10 Synthesis

All oligonucleotides are synthesized on an AKTAoligopilot synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-*O*-dimethoxytrityl N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-
15 dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2--isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the
20 oligonucleotide synthesis. The 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-fluoro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluoro-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite are purchased from (Promega). All phosphoramidites are used at a concentration of 0.2M in acetonitrile (CH₃CN) except for guanosine which is used at 0.2M concentration in 10%
25 THF/ANC (v/v). Coupling/recycling time of 16 minutes is used. The activator is 5-ethyl thiotetrazole (0.75M, American International Chemicals); for the PO-oxidation iodine/water/pyridine is used and for the PS-oxidation PADS (2%) in 2,6-lutidine/ACN (1:1 v/v) is used.

3'-ligand conjugated strands are synthesized using solid support containing the
30 corresponding ligand. For example, the introduction of cholesterol unit in the sequence is

performed from a hydroxyprolinol-cholesterol phosphoramidite. Cholesterol is tethered to *trans*-4-hydroxyprolinol via a 6-aminohexanoate linkage to obtain a hydroxyprolinol-cholesterol moiety. 5'-end Cy-3 and Cy-5.5 (fluorophore) labeled siRNAs are synthesized from the corresponding Quasar-570 (Cy-3) phosphoramidite are purchased from Biosearch Technologies. Conjugation of ligands to 5'-end and or internal position is achieved by using appropriately protected ligand-phosphoramidite building block. An extended 15 min coupling of 0.1 M solution of phosphoramidite in anhydrous CH₃CN in the presence of 5-(ethylthio)-1*H*-tetrazole activator to a solid-support-bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate is carried out using standard iodine-water as reported (1) or by treatment with *tert*-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate is introduced by the oxidation of phosphite to phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent. The cholesterol phosphoramidite is synthesized in house and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite is 16 minutes.

Deprotection I (Nucleobase Deprotection)

After completion of synthesis, the support is transferred to a 100 mL glass bottle (VWR). The oligonucleotide is cleaved from the support with simultaneous deprotection of base and phosphate groups with 80 mL of a mixture of ethanolic ammonia [ammonia: ethanol (3:1)] for 6.5 h at 55°C. The bottle is cooled briefly on ice and then the ethanolic ammonia mixture is filtered into a new 250-mL bottle. The CPG is washed with 2 x 40 mL portions of ethanol/water (1:1 v/v). The volume of the mixture is then reduced to ~ 30 mL by roto-vap. The mixture is then frozen on dry ice and dried under vacuum on a speed vac.

Deprotection II (Removal of 2'-TBDMS group)

The dried residue is resuspended in 26 mL of triethylamine, triethylamine trihydrofluoride (TEA•3HF) or pyridine-HF and DMSO (3:4:6) and heated at 60°C for 90 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction is then quenched with 50 mL of 20 mM sodium acetate and the pH is adjusted to 6.5. Oligonucleotide is stored in a freezer until purification.

Analysis

The oligonucleotides are analyzed by high-performance liquid chromatography (HPLC) prior to purification and selection of buffer and column depends on nature of the sequence
 5 and or conjugated ligand.

HPLC Purification

The ligand-conjugated oligonucleotides are purified by reverse-phase preparative HPLC. The unconjugated oligonucleotides are purified by anion-exchange HPLC on a TSK gel
 10 column packed in house. The buffers are 20 mM sodium phosphate (pH 8.5) in 10% CH₃CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH₃CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides are pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides are diluted in water to 150 µL and then pipetted into special vials for CGE and LC/MS analysis. Compounds are
 15 then analyzed by LC-ESMS and CGE.

siRNA preparation

For the preparation of siRNA, equimolar amounts of sense and antisense strand are heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex
 20 is confirmed by HPLC analysis.

Table 3. siRNA duplexes for Luc and FVII targeting

Duplex		Sequence 5'-3'	Target
1000/2434		CUU ACG CUG AGU ACU UCG AdTdT U*CG AAG fUAC UCA GCG fUAA GdT*dT	Luc
2433/1001		C*UfU ACG CUG AGfU ACU UCG AdT*dT UCG AAG UAC UCA GCG UAA GdTdT	Luc
2433/2434		C*UfU ACG CUG AGfU ACU UCG AdT*dT U*CG AAG fUAC UCA GCG fUAA GdT*dT	Luc
1000/1001		CUU ACG CUG AGU ACU UCG AdTdT	Luc

		UCG AAG UAC UCA GCG UAA GdTdT	
AD-1596		GGAUCAUCUCAAGUCUUACdTdT GUAAGACUUGAGAUGAUCCdTdT	FVII
AD-1661		GGAfUfCAfUfCfUfCAAGfUfCfUfUafCdTsdT GfUAAGAfCfUfUGAGAfUGAfUfCfCdT*dT	

Table 4. Other oligonucleotides.

<i>Number</i>		<i>Sequence 5' to 3'</i>
	<i>siLuc sense</i>	<i>CUUACGCUGAGUACUUCGATT</i>
	<i>siLuc antisense</i>	<i>UCGAAGUACUCAGCGUAAGTT</i>
	<i>siFVII sense</i>	<i>GGAucAucucAAGucuuAcT*T</i>
	<i>siFVII antisense</i>	<i>GuAAGAcuuGAGAuGAuccT*T</i>
	<i>siApoB sense</i>	<i>GGAAUCuuAuAuuuGAUCcA*A</i>
	<i>siApoB antisense</i>	<i>uuGGAUcAAuAuAAGAuUCc*c*U</i>
	<i>siCont sense</i>	<i>cuuAcGcuGAGuAcuucGAT*T</i>
	<i>siCont antisense</i>	<i>UCGAAGuACUcAGCGuAAGT*T</i>
	<i>siGFP sense</i>	<i>CcAcAuGAAGcAGcACGACu*U</i>
	<i>siGFP antisense</i>	<i>AAGUCGUGCUGCUUCAUGUGg*u*C</i>
	<i>siCD45 sense</i>	<i>cuGGcuGAAuuucAGAGcAT*T</i>
	<i>siCD45 antisense</i>	<i>UGCUCUGAAAUUcAGCcAGT*T</i>
	<i>mm-siRSV sense</i>	<i>CGAUUAUAUUACAGGAUGAT*T</i>
	<i>mm-siRSV antisense</i>	<i>UCAUCCUGUAAUAUAAUCGT*T</i>
	<i>siRSV sense</i>	<i>UCCUAGAAUCAAUAAAGGGTT</i>
	<i>siRSV antisense</i>	<i>CCCUUUAUUGAUUCUAGGATT</i>
	<i>antagomir122</i>	<i>a*c*aaacaccauugucacacu*c*c*a-Cholesterol</i>
	<i>mm-antagomir122</i>	<i>a*c*acacaacacugucacauu*c*c*a-Cholesterol</i>
	<i>anti-miR122</i>	<i>a*c*aaacaccauugucacacu*c*c*a</i>
	<i>mm-anti-miR122</i>	<i>a*c*acacaacacugucacauu*c*c*a</i>

2'-O-Me modified nucleotides are in lower case, 2'-Fluoro modified nucleotides are in bold lower case, and phosphorothioate linkages are represented by asterisks. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

5 **Example 19: Serum Stability Assay for siRNA**

A medium throughput assay for initial sequence-based stability selection was performed by the “stains all” approach. To perform the assay, an siRNA duplex is incubated in 90% human serum at 37°C. Samples of the reaction mix are quenched at various time points (at 0, 15, 30, 60, 120, and 240 min) and subjected to electrophoretic analysis. Cleavage of the
10 RNA over the time course provides information regarding the susceptibility of the siRNA duplex to serum nuclease degradation.

A radiolabeled dsRNA and serum stability assay is used to further characterize siRNA cleavage events. First, a siRNA duplex is 5' end-labeled with ³²P on either the sense or antisense strand. The labeled siRNA duplex is incubated with 90% human serum at 37°C
15 and a sample of the solution is removed and quenched at increasing time points. The samples are analyzed by electrophoresis to provide a measure of the stability of the siRNA duplex in serum.

Example 20: Dual Luciferase Gene Silencing Assays

20 In vitro activity of siRNAs is determined using a high-throughput 96-well plate format luciferase silencing assay. Assays are performed in one of two possible formats. In the first format, HeLa SS6 cells are first transiently transfected with plasmids encoding firefly (target) and *renilla* (control) luciferase. DNA transfections are performed using Lipofectamine 2000 (Invitrogen) and the plasmids gWiz-Luc (Aldevron, Fargo, ND) (200
25 ng/well) and pRL-CMV (Promega, Madison, WI) (200 ng/well). After 2 h, the plasmid transfection medium is removed, and the firefly luciferase targeting siRNAs are added to the cells at various concentrations. In the second format, HeLa Dual-luc cells (stably expressing both firefly and *renilla* luciferase) are directly transfected with firefly luciferase targeting siRNAs. siRNA transfections are performed using either TransIT-TKO (Mirus,
30 Madison, WI) or Lipofectamine 2000 according to manufacturer's protocols. After 24 h, cells are analyzed for both firefly and *renilla* luciferase expression using a plate

luminometer (VICTOR², PerkinElmer, Boston, MA) and the Dual-Glo Luciferase Assay kit (Promega). Firefly/*renilla* luciferase expression ratios are used to determine percent gene silencing relative to mock-treated (no siRNA) controls.

5 **Example 21: Factor VII (FVII) *in vitro* Assay**

Cell Seeding for Transfection. Cells are seeded into 96-well plates one day prior to siRNA transfection at a density of 15,000 cells per well in media without antibiotics (150,000 cells/ml media, 100 µl per well).

Standard Transfection Conditions for FVII Stable Cell Line

- 10 • Lipofectamine 2000 at a concentration of 0.5 µL/well is used for transfection in a 96 well plate set-up
- FVII-targeting siRNA or control siRNA is diluted to a concentration of 6 nM in OptiMEM
- siRNA and transfection agent (lipofectamine 2000) are mixed and complex allowed
- 15 to form by incubating 20 minutes at room temperature
- After 20 minutes, 50 µL of complexes (out of total 60 µl volume) added to a single well containing cells that were seeded on the previous day (well already contains 100 µL of growth medium), sample is mixed by gently pipetting up and down; well now contains 150 µL total volume, 1 nM siRNA, 0.5 µL LF 2000 reagent
- 20 • Plate is returned to 37 °C incubator.
- After 24 h, media is removed and replaced with fresh media (100 µL/well)
- 24 hours after media exchange, media supernatant is collected for FVII activity assay
- Levels of Factor VII protein in the supernatant are determined in samples using a
- 25 chromogenic assay (Coaset Factor VII, DiaPharma Group, OH or Biophen FVII, Aniara Corporation, OH) according to manufacturer's protocols

Example 22: FVII and apoB *in vivo* Assay

- C57BL/6 mice (Charles River Labs, MA) and Sprague-Dawley rats (Charles River Labs,
- 30 MA) receive either saline or siRNA in desired formulations via tail vein injection at a

volume of 0.01 mL/g. At various time points post-administration, animals are anesthetized by isoflurane inhalation and blood is collected into serum separator tubes by retroorbital bleed. Serum levels of Factor VII protein are determined in samples using a chromogenic assay (Coaset Factor VII, DiaPharma Group, OH or Biophen FVII, Aniara Corporation, OH) according to manufacturer's protocols. A standard curve is generated using serum collected from saline treated animals. In experiments where liver mRNA levels are assessed at various time points post-administration, animals are sacrificed and livers are harvested and snap frozen in liquid nitrogen. Frozen liver tissue is ground into powder. Tissue lysates are prepared and liver mRNA levels of Factor VII and *apoB* are determined using a branched DNA assay (QuantiGene Assay, Panomics, CA).

Example 23: Cytokine Induction in Hhuman PBMCs

Procedure

- Peripheral blood mononuclear cells (PBMCs) are isolated from human blood
- FVII siRNAs are compared
 - best positive control for IFN- γ direct incubation (DI)
 - siRNA positive control for direct incubation (DI)
 - positive control for siRNA transfection
 - FVII siRNA unmodified AD-1596 and 2'-F modified AD-1661
- direct incubation (500 nM)
- transfection (130 nM) with Lipofectamine2000
- ELISA with supernatants taken after 24h; IFN- γ

Example 24: Binding Affinity and Thermal Stability

Absorbance versus temperature curves are measured at 260 and 280 nm using a DU 800 spectrophotometer (serial number 8001373) with software version 2.0, Build 83. Oligonucleotide concentrations are 4 μ M with concentration of each strand determined from the absorbance at 85 °C and extinction coefficients calculated according to Puglisi and Tinoco (Methods Enzymol, 1989, 180, 304-325). Oligonucleotide solutions are heated

at a rate of 0.5 °C/min in 1 cm path length cells and then cooled to confirm reversibility and lack of evaporation. T_m values are obtained from the absorbance versus temperature curves. Standard deviations should not exceed ± 0.5 °C. Each T_m reported will be an average of two experiments. A plot of absorbance vs. temperature yields thermal denaturation of siRNA
5 duplexes.

Example 25: Cationic Liposome-mediated Delivery *in vitro*

Once synthesized, the cationic lipid is screened for the ability to deliver siRNA to the human cervical cancer cell line HeLa. A HeLa cell line was created that stably expresses
10 both firefly and Renilla luciferase. Efficacy of siRNA delivery by cationic lipid is determined by treating cells with siRNA-lipidoid complexes, prepared using a firefly luciferase-targeting siRNA (siLuc), and then measuring the ratio of firefly to Renilla luciferase expression. In this assay, toxic or other non-specific effects result in reduction of
15 reduction of only firefly luciferase. To facilitate screening throughput, siRNA-lipid complexes are formed by simple mixing of siRNA-cationic lipid solution in microtiter plates.

The protocol was adapted from Anderson, D.G., *et al.*²⁹. HeLa cells, stably expressing firefly luciferase and Renilla luciferase are seeded (14,000 cells/well) into each well of an
20 opaque white 96-well plate (Corning-Costar, Kennebunk, ME, USA) and allowed to attach overnight in growth medium. Growth medium is composed of 90% phenol red-free DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells are transfected with 50 ng of firefly-specific siLuc complexed with cationic lipid at cationic lipid:siRNA ratios of 2.5:1, 5:1, 10:1, and 15:1
25 (wt:wt) to determine the optimum for transfection efficiency. Transfections are performed in quadruplicate.

Working dilutions of each lipid are prepared (at concentrations necessary to yield the different lipid/siRNA weight ratios) in 25 mM sodium acetate buffer (pH 5). The diluted lipid (25 µL) is added to 25 µL of 60 µg/mL siRNA in a well of a 96-well plate. The
30 mixtures are incubated for 20 min to allow for complex formation, and then 30 µL of each

of the cationic lipid/siRNA solutions is added to 200 μ L of media in 96-well polystyrene plates. The growth medium is removed from the cells using a 12-channel aspirating wand (V&P Scientific, San Diego, CA, USA) after which 150 μ L of the cationic lipid/siRNA solution is immediately added. Cells are allowed to grow for 1 day at 37°C, 5% CO₂ and
5 are then analyzed for luciferase expression. Control experiments are performed with Lipofectamine™ 2000, as described by the vendor (Invitrogen, Carlsbad, CA, USA). Firefly and Renilla luciferase expression is analyzed using Dual-Glo assay kits (Promega, Madison, WI, USA). Luminescence is measured using a Victor3™ luminometer (Perkin Elmer, Wellesley, MA, USA). A standard curve for luciferase is generated by titration of
10 luciferase enzyme (Promega) into growth medium in an opaque white 96-well plate.

Example 26: Cationic Lipid-mediated Delivery in Rodent Hepatic Gene Silencing Models

The liver represents an attractive organ for therapeutic intervention, both because of the
15 number of potential hepatic targets as well as the highly-perfused nature of the organ, which may render it more amenable to delivery of exogenous siRNAs. A liver-directed *in vivo* screen is used to identify cationic lipid/siRNA complexes that facilitate high levels of siRNA-mediated gene silencing in hepatocytes, the cells comprising the liver parenchyma. Factor VII, a blood clotting factor, is an ideal target gene for assaying functional siRNA
20 delivery to liver. It is produced specifically in hepatocytes; therefore, gene silencing indicates successful delivery to parenchyma, as opposed to delivery solely to the cells of the reticulo-endothelial system (e.g., Kupffer cells). Furthermore, Factor VII is a secreted protein that can be readily measured in serum, obviating the need to sacrifice animals. Finally, owing to its short half-life (2-5 hours), silencing at the mRNA level is manifest as
25 silencing at the protein level with minimal lag.

All procedures used in animal studies conducted at Alnylam were approved by the Institutional Animal Care and Use Committee (IACUC) and are consistent with local, state, and federal regulations as applicable. Mice will receive two daily i.v. injections of different cationic lipid formulations of siRNA at a dose of 2.5 mg/kg. Factor VII protein levels are
30 quantified 24 h after the second administration. Alternatively, rats are injected with

cationic lipid/siRNA at 1.25, 2.5, 5, and 10 mg/kg. Animals are bled at various time points and sacrificed 48 h after administration. Evaluated are liver factor VII mRNA levels, serum Factor VII protein levels, and prothrombin time.

5 Example 27: Specificity of Cationic Liposome-mediated siRNA Delivery in Rodent Hepatic Gene Silencing Models

All procedures used in animal studies conducted at Alnylam are approved by the Institutional Animal Care and Use Committee (IACUC) and are consistent with local, state, and federal regulations as applicable. To verify the specificity of gene silencing, liver
10 mRNA levels are measured for both Factor VII and another hepatocyte-expressed gene, apolipoprotein B (apoB). Animals will be treated with formulations containing only siFVII or only siapoB and levels of mRNAs transcribed from both genes will be measured. Further, administration of a cationic lipid formulation of a mixture of the two siRNAs will be evaluated as will the effect of a mismatched Factor VII siRNA. These data will show
15 that the observed gene silencing is a direct result of the specific effects of cationic lipid/siRNA on mRNA levels in the liver and that these effects are applicable to multiple hepatocyte-expressed genes.

Example 28: *In vivo* Rodent Factor VII and apoB Silencing Experiments.

20 All procedures used in animal studies conducted at Alnylam are approved by the Institutional Animal Care and Use Committee (IACUC) and are consistent with local, state, and federal regulations as applicable. C57BL/6 mice (Charles River Labs, MA) and Sprague-Dawley rats (Charles River Labs, MA) receive either saline or siRNA in cationic lipid formulations via tail vein injection at a volume of 0.01 mL/g. At various time points
25 post-administration, animals are anesthetized by isofluorane inhalation and blood is collected into serum separator tubes by retroorbital bleed. Serum levels of Factor VII protein are determined in samples using a chromogenic assay (Coaset Factor VII, DiaPharma Group, OH or Biophen FVII, Aniara Corporation, OH) according to manufacturer's protocols. A standard curve is generated using serum collected from
30 saline-treated animals. In experiments where liver mRNA levels are assessed, at various

time points post-administration, animals are sacrificed and livers are harvested and snap frozen in liquid nitrogen. Frozen liver tissue is ground into powder. Tissue lysates are prepared and liver mRNA levels of Factor VII and apoB are determined using a branched DNA assay (QuantiGene Assay, Panomics, CA) ⁸.

5

Example 29: In vivo activities of optically active lipid Compounds

Formulation Method

The efficacy of formulations made with S and R enantiomers, as well as the racemic form, of DLinKDMA lipid composition 52:14:30:5 (DLinKDMA:DSPC:Cholesterol:PEG-DMG) was determined. The formulations were made using the preformed liposome and siRNA mixing method. The liposomes were preformed by addition of lipids DK-S-1, DK-R-2, or DK-Racemic-3 in ethanol to a 100 mM citrate, pH 3.0 aqueous solution to a final ethanol concentration of 35%. The liposomes were then extruded 1× through 2×80 nm extrusion membranes. The siRNA (in 35% ethanol, 100 mM citrate) was added to the extruded liposomes and incubated for 30 min at 37°C. The final formulation was then dialyzed against PBS overnight to remove ethanol using MWCO 10,000.

15

Table 5:

	DK-S-1	DK-R-2	DK-Racemic-3
Particle Size -Post Hydration (nm)	48.8	50.3	57.0
PDI	0.147	0.123	0.140
Particle Size – Final	61.7	62.2	73.1
PDI	0.103	0.074	0.124
% Encapsulation RiboGreen	69.8%	69.3%	61.7%

Experimental Protocol

Experiment number: 08-050

Study Date: 4/18/2008

Title: Comparison of DLinkDMA enantiomers

- 5 Purpose: The objective of this study is to determine efficacy of formulations made with S, R enantiomers as well as the racemic form of DLinkDMA Lipid composition 52:14:30:5 (DLinkDMA:DSPC:Cholesterol:PEG-DMG)

Animals: C57BL6 mice

Target: FVII

- 10 siRNA:1661

Injection volume: variable based on weight

Animals were dosed on April 18 and bleed on April 21.

Table 6:

Group	Number of Animals	Conc. (mg/mL)	Inj. vol. (μL/g)	Dose (mg/kg)	Vehicle*
1	3	0.050	10	0.5	PBS
2	3	0.025	10	0.25	DK-1
3	3	0.010	10	0.1	DK-1
4	3	0.005	10	0.05	DK-1
5	3	0.050	10	0.5	DK-1
6	3	0.025	10	0.25	DK-2
7	3	0.010	10	0.1	DK-2
8	3	0.005	10	0.05	DK-2
9	3	0.050	10	0.5	DK-2
10	3	0.025	10	0.25	DK-3
11	3	0.010	10	0.1	DK-3
12	3	0.005	10	0.05	DK-3
13	3	0.050	10	0.5	DK-3

- 15 * DK-1 is S-enantiomer; DK-2 is R-enantiomer; DK-3 is racemic mixture.

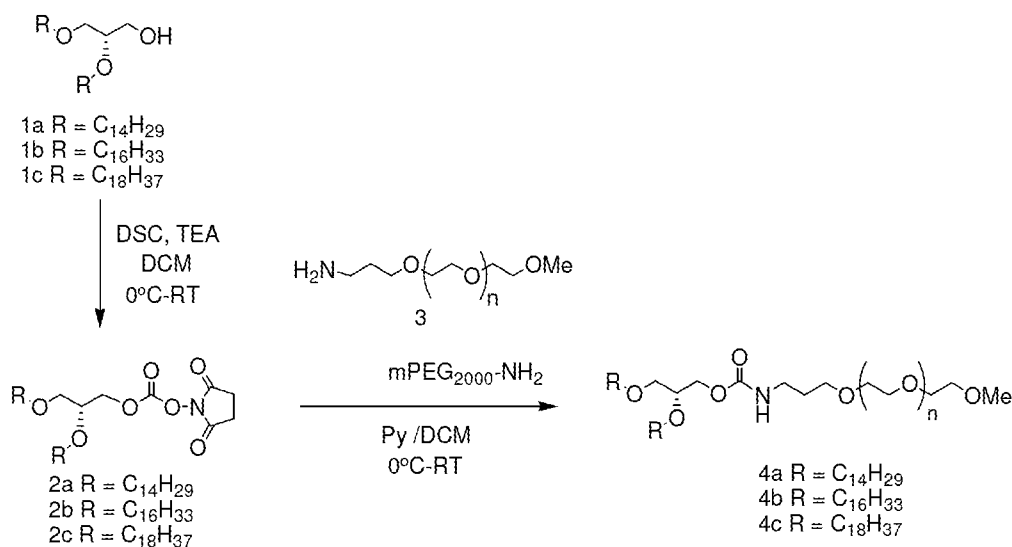
Figure 1 shows the results of using optically pure DLin-K-DMA in the formulation.

Example 30: Synthesis of mPEG2000-1,2-Di-*O*-alkyl-*sn*3-carbomoylglyceride (PEG-DMG)

The PEG-lipids, such as mPEG2000-1,2-Di-*O*-alkyl-*sn*3-carbomoylglyceride (PEG-DMG)

5 were synthesized using the following procedures:

Scheme 1^a



^a Scheme 1: mPEG2000-1,2-Di-*O*-alkyl-*sn*3-carbomoylglyceride

- 10 **Preparation of compound 4a:** 1,2-Di-*O*-tetradecyl-*sn*-glyceride **1a** (30 g, 61.80 mmol) and *N,N'*-succinimidylcarboante (DSC, 23.76 g, 1.5eq) were taken in dichloromethane (DCM, 500 mL) and stirred over an ice water mixture. Triethylamine (25.30 mL, 3eq) was added to stirring solution and subsequently the reaction mixture was allowed to stir overnight at ambient temperature. Progress of the reaction was monitored by TLC. The
- 15 reaction mixture was diluted with DCM (400 mL) and the organic layer was washed with water (2X500 mL), aqueous NaHCO₃ solution (500 mL) followed by standard work-up. Residue obtained was dried at ambient temperature under high vacuum overnight. After drying the crude carbonate **2a** thus obtained was dissolved in dichloromethane (500 mL) and stirred over an ice bath. To the stirring solution mPEG₂₀₀₀-NH₂ (**3**, 103.00 g, 47.20
- 20 mmol, purchased from NOF Corporation, Japan) and anhydrous pyridine (80 mL, excess) were added under argon. In some embodiments, the methoxy-(PEG)*x*-amine has an *x*=

from 45-49, preferably 47-49, and more preferably 49. The reaction mixture was then allowed stir at ambient temperature overnight. Solvents and volatiles were removed under vacuum and the residue was dissolved in DCM (200 mL) and charged on a column of silica gel packed in ethyl acetate. The column was initially eluted with ethyl acetate and subsequently with gradient of 5-10 % methanol in dichloromethane to afford the desired PEG-Lipid **4a** as a white solid (105.30g, 83%). ¹H NMR (CDCl₃, 400 MHz) δ = 5.20-5.12(m, 1H), 4.18-4.01(m, 2H), 3.80-3.70(m, 2H), 3.70-3.20(m, -O-CH₂-CH₂-O-, PEG-CH₂), 2.10-2.01(m, 2H), 1.70-1.60 (m, 2H), 1.56-1.45(m, 4H), 1.31-1.15(m, 48H), 0.84(t, J= 6.5Hz, 6H). MS range found: 2660-2836.

10

Preparation of 4b: 1,2-Di-*O*-hexadecyl-*sn*-glyceride **1b** (1.00 g, 1.848 mmol) and DSC (0.710 g, 1.5eq) were taken together in dichloromethane (20 mL) and cooled down to 0°C in an ice water mixture. Triethylamine (1.00 mL, 3eq) was added to that and stirred overnight. The reaction was followed by TLC, diluted with DCM, washed with water (2 times), NaHCO₃ solution and dried over sodium sulfate. Solvents were removed under reduced pressure and the residue **2b** under high vacuum overnight. This compound was directly used for the next reaction without further purification. MPEG₂₀₀₀-NH₂ **3** (1.50g, 0.687 mmol, purchased from NOF Corporation, Japan) and compound from previous step **2b** (0.702g, 1.5eq) were dissolved in dichloromethane (20 mL) under argon. The reaction was cooled to 0°C. Pyridine (1 mL, excess) was added to that and stirred overnight. The reaction was monitored by TLC. Solvents and volatiles were removed under vacuum and the residue was purified by chromatography (first Ethyl acetate then 5-10% MeOH/DCM as a gradient elution) to get the required compound **4b** as white solid (1.46 g, 76 %). ¹H NMR (CDCl₃, 400 MHz) δ = 5.17(t, J= 5.5Hz, 1H), 4.13(dd, J= 4.00Hz, 11.00 Hz, 1H), 4.05(dd, J= 5.00Hz, 11.00 Hz, 1H), 3.82-3.75(m, 2H), 3.70-3.20(m, -O-CH₂-CH₂-O-, PEG-CH₂), 2.05-1.90(m, 2H), 1.80-1.70 (m, 2H), 1.61-1.45(m, 6H), 1.35-1.17(m, 56H), 0.85(t, J= 6.5Hz, 6H). MS range found: 2716-2892.

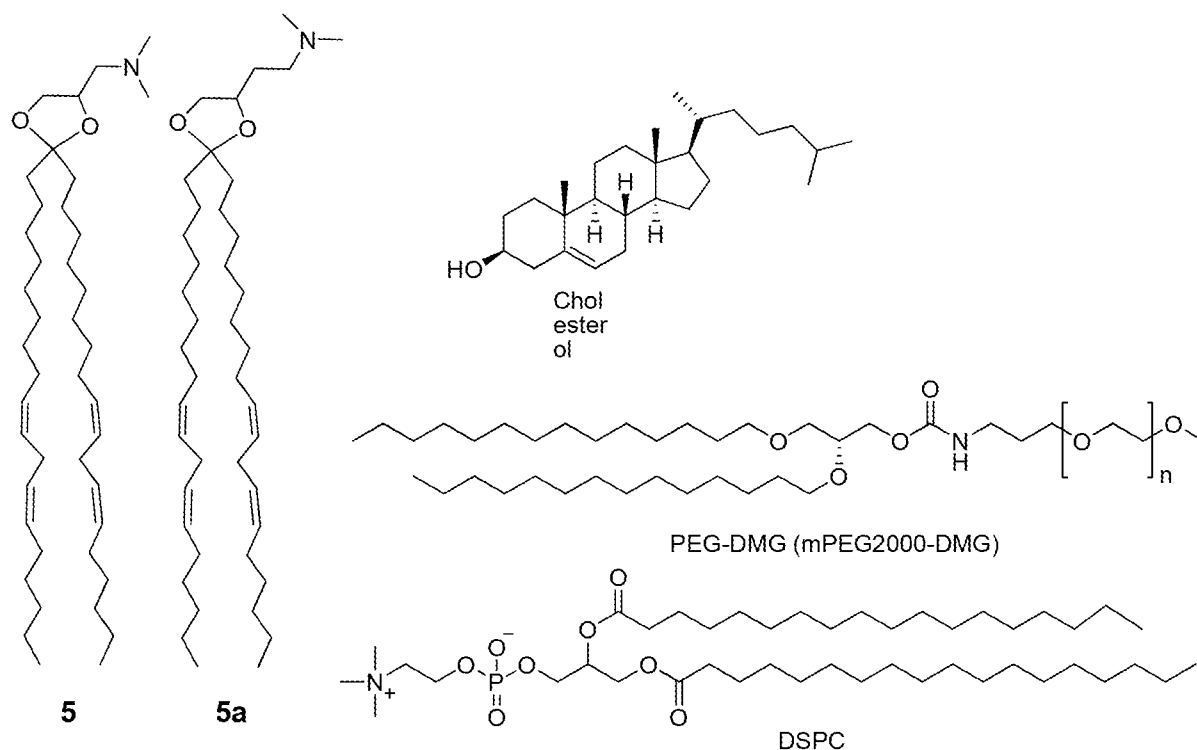
Preparation of 4c: 1,2-Di-*O*-octadecyl-*sn*-glyceride **1c** (4.00 g, 6.70 mmol) and DSC (2.58 g, 1.5eq) were taken together in dichloromethane (60 mL) and cooled down to 0°C in

30

an ice water mixture. Triethylamine (2.75 mL, 3eq) was added to that and stirred overnight. The reaction was followed by TLC, diluted with DCM, washed with water (2 times), NaHCO₃ solution and dried over sodium sulfate. Solvents were removed under reduced pressure and the residue under high vacuum overnight. This compound was directly used
5 for the next reaction with further purification. MPEG₂₀₀₀-NH₂ **3** (1.50g, 0.687 mmol, purchased from NOF Corporation, Japan) and compound from previous step **2c** (0.760g, 1.5eq) were dissolved in dichloromethane (20 mL) under argon. The reaction was cooled to 0°C. Pyridine (1 mL, excess) was added to that and stirred overnight. The reaction was monitored by TLC. Solvents and volatiles were removed under vacuum and the residue
10 was purified by chromatography (first Ethyl acetate then 5-10% MeOH/DCM as a gradient elution) to get the required compound **4 c** as white solid (0.92 g, 48 %). ¹H NMR (CDCl₃, 400 MHz) δ = 5.22-5.15(m, 1H), 4.16(dd, J= 4.00Hz, 11.00 Hz, 1H), 4.06(dd, J= 5.00Hz, 11.00 Hz, 1H), 3.81-3.75(m, 2H), 3.70-3.20(m, -O-CH₂-CH₂-O-, PEG-CH₂), 1.80-1.70 (m, 2H), 1.60-1.48(m, 4H), 1.31-1.15(m, 64H), 0.85(t, J= 6.5Hz, 6H). MS range found: 2774-
15 2948.

Example 31: Liposome Formulations

The cyclic cationic lipids **5** and **5a** -based formulations with two different compositions as described below were prepared, characterized and evaluated *in vivo* by FVII assay.

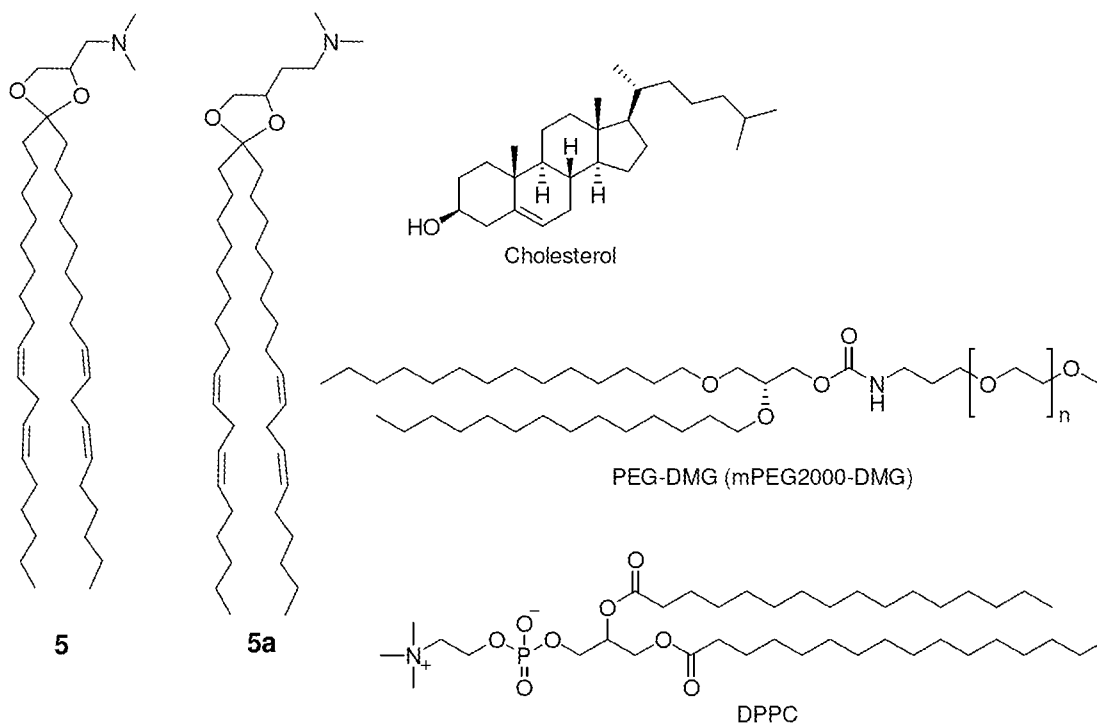


	5 /Chol/PEG-C14/DSPC 52:30:5:13 (Formulation 5-1)	5a /Chol/PEG-DMG/DSPC 52:30:5:13 (Formulation 5a-1)	5a /Chol/PEG-DMG/DSPC 55:35:2:8 (Formulation 5a-2)
Particle size before siRNA conjugation (nm)	90.7	86.2	108
PDI	0.1	0.117	0.17
Particle size final (nm)	95.5	82.6	130
PDI	0.1	0.09	0.09
% Encapsulation, Ribogreen	79	85	91.5

5

Example 31: Liposome Formulations

The above formulation is made with DPPC in place of DSPC with varying amounts and the assays are repeated.



5

	5 /Chol/PEG-C14/DPPC 52:30:5:13 (Formulation 5-1)	5a /Chol/PEG-DMG/DPPC 52:30:5:13 (Formulation 5a-1)	5a /Chol/PEG-DMG/DPPC 55:35:2:8 (Formulation 5a-2)
Particle size before siRNA conjugation (nm)	90.7	86	108
PDI	0.1	0.117	0.17
Particle size final (nm)	94	82	130
PDI	0.1	0.09	0.09
% Encapsulation, Ribogreen	79	85	91

The various embodiments described above can be combined to provide further
 10 embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications

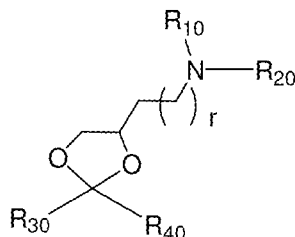
referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

- 5 These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not
- 10 limited by the disclosure.

CLAIMS

We claim:

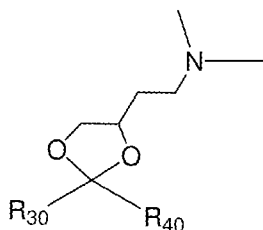
1. A process for preparing lipids of the the following formula:



where R_{10} and R_{20} are independently H or Me, R_{30} and R_{40} are independently aliphatic, and r is 0-10, comprising the steps of a) treating Br-R_{30} with Mg, b) adding $\text{R}_{40}\text{-CN}$ to the reaction mixture, c) quenching with reaction mixture a proton source and d) reacting the product from

step c) with .

2. A process for preparing lipids of the the following formula:



where R_{30} and R_{40} are independently aliphatic, comprising the steps of a) converting the hydroxyl moiety of 2-(2,2-Dimethyl-[1,3]dioxolan-4-yl)-ethanol into a leaving group in the presence of a base; b) treating compound from step a) with $\text{N}\alpha$ -(benzyloxycarbonyloxy)succinimide in the presence of a base and ethanolic amine; c) trans-ketalizing compound from

step b) with ketone in the presence of an acid; and d) reducing the Cbz protecting group with LAH.

3. A lipid formulation comprising about 55 mol % of lipid **5a**, about 8 mol% of DPPC, about 35 mol% of cholesterol, and about 2 mol% of PEG-DMG.
4. A lipid formulation comprising about 52 mol % of lipid **5a**, about 13 mol% of DPPC, about 30 mol% of cholesterol, and about 5 mol% of PEG-DMG.
5. The method of delivering a therapeutic agent to a target gene comprising the lipid formulation of claim 3 or 4.
6. The method of claim 5, wherein the therapeutic agent is an RNA-based construct.
7. The method of claim 6, wherein the RNA-based construct is a dsRNA.
8. The method of claim 5, wherein the target gene is selected from the group consisting of Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFkB gene, STAT3 gene, survivin gene, Her2/Neu gene, topoisomerase I gene, topoisomerase II alpha gene, mutations in the p73 gene, mutations in the p21(WAF1/CIP1) gene, mutations in the p27(KIP1) gene, mutations in the PPM1D gene, mutations in the RAS gene, mutations in the caveolin I gene, mutations in the MIB I gene, mutations in the MTAI gene, mutations in the M68 gene, mutations in tumor suppressor genes, and mutations in the p53 tumor suppressor gene.
9. The method of claim 5, wherein the target gene is Factor VII.
10. The method of claim 5, further comprising comparing expression of the target gene with a preselected reference value.

11. The method of claim 5, wherein the therapeutic agent is an antisense, siRNA, ribozyme or microRNA.
12. The method of claim 9, wherein the method reduces FVII protein or mRNA levels in the blood.
13. The method of claim 9, wherein the method reduces FVII protein or mRNA levels in the liver.

1/1

Figure 1: SILENCING OF FVII WITH FORMULATION COMPRISING OPTICALLY PURE DLink-DMA

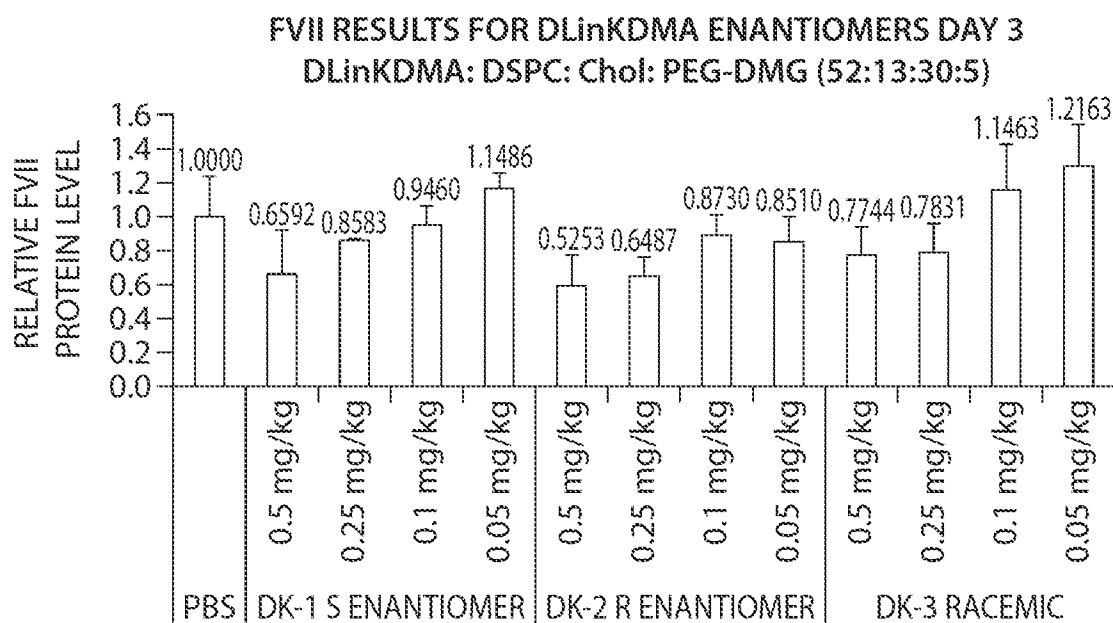


Fig. 1

Figure 2: SILENCING OF FVII WITH FORMULATIONS 5, 5a-1 AND 5a-2

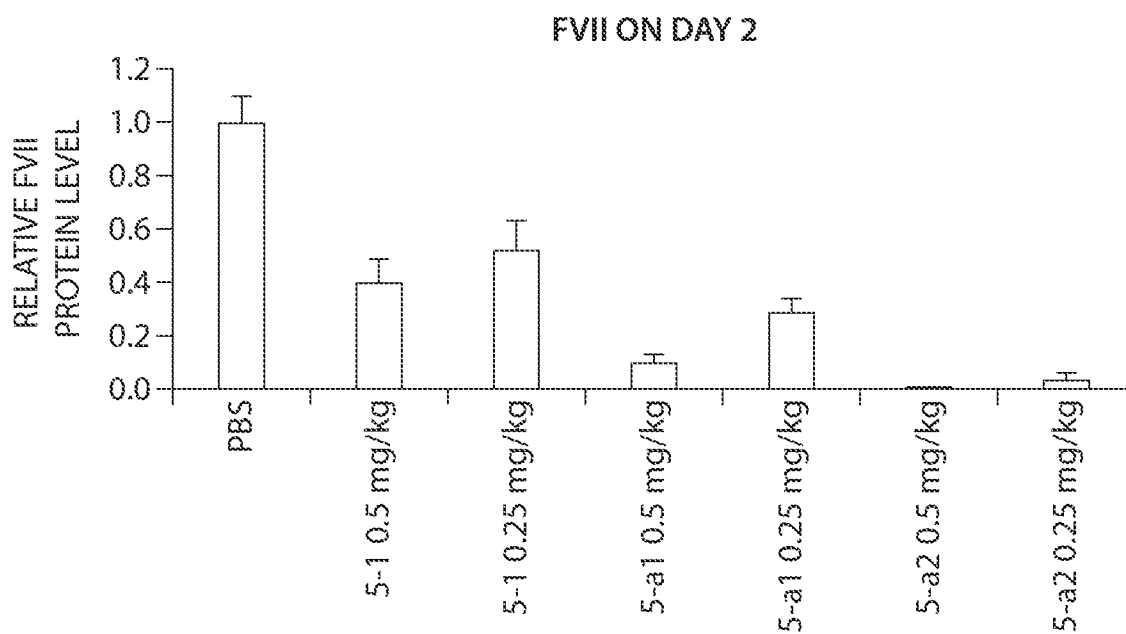


Fig. 2