(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 May 2010 (20.05.2010)

PCT

(10) International Publication Number
WO 2010/057155 A1

(51) International Patent Classification:
A01N 43/04 (2006.01)

(21) International Application Number:
PCT/US2009/064719

(22) International Filing Date:
17 November 2009 (17.11.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/115,287 17 November 2008 (17.11.2008) US
61/115,365 17 November 2008 (17.11.2008) US
61/115,348 17 November 2008 (17.11.2008) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: RELEASABLE CATIONIC LIPIDS FOR NUCLEIC ACIDS DELIVERY SYSTEMS

(57) **Abstract**: The present invention is directed to releasable cationic lipids and nanoparticle compositions for the delivery of nucleic acids and methods of modulating an expression of a target gene using the same. In particular, the invention relates to cationic lipids including an acid labile linker, and nanoparticle compositions containing the same.

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RELEASABLE CATIONIC LIPIDS FOR NUCLEIC ACIDS DELIVERY SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of priority from U.S. Provisional Patent Application Serial Nos. 61/115,287, 61/115,365, and 61/115,348, filed November 17, 2008, the contents of each of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Therapy using nucleic acids has been proposed for treating various diseases. One such proposed nucleic acid therapy is antisense therapy, wherein therapeutic genes can selectively modulate gene expression associated with disease and minimize side effects that may be associated with other therapeutic approaches to treating disease.

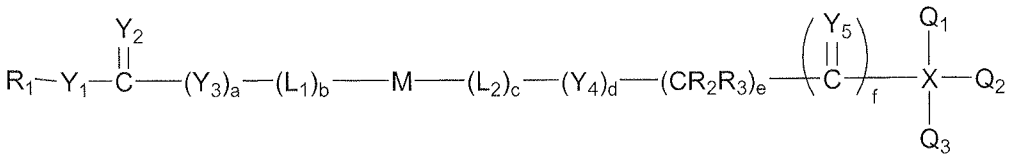
Therapy using nucleic acids has, however, heretofor been limited due to challenges associated with delivery and stability of such therapeutic nucleic acids. Several gene delivery systems have been proposed to overcome the above-noted challenges and effectively introduce therapeutic genes into a target area, such as cancer cells or other cells or tissues, *in vitro* and *in vivo*. One such attempt to improve delivery and enhance cellular uptake of therapeutic genes has employed liposomes as a delivery vehicle. Unfortunately, currently available liposomes do not effectively deliver oligonucleotides into the body, although some progress has been made in the delivery of plasmids.

In spite of the previous attempts and advances, there continues to be a need to provide improved nucleic acids delivery systems. The present invention addresses this need.

SUMMARY OF THE INVENTION

The present invention provides releasable cationic lipids including an acid labile linker and nanoparticle compositions containing the same for nucleic acids delivery. Polynucleic acids, such as oligonucleotides, are encapsulated within nanoparticle complexes containing a mixture of a cationic lipid, a fusogenic lipid, and a PEG lipid.

In accordance with this aspect of the invention, the releasable cationic lipids for the delivery of nucleic acids (i.e., oligonucleotides) have Formula (I):



(I)

wherein

R_1 is cholesterol or an analog thereof;

Y_1 is O, S or NR_4;

Y_2 and Y_5 are independently O, S or NR_5;

Y_{3-4} are independently O, S or NR_6;

L_{1-2} are independently selected bifunctional linkers;

M is an acid labile linker;

(a), (d) and (f) are independently 0 or 1;

(b), (c) and (e) are independently 0 or positive integers;

X is C, N or P;

Q_1 is H, C_{1-6} alkyl, NH_2, or $-(L_{11})_{d1}-R_{11}$;

Q_2 is H, C_{1-6} alkyl, NH_2, or $-(L_{12})_{d2}-R_{12}$;

Q_3 is a lone electron pair, (=O), H, C_{1-6} alkyl, NH_2, or $-(L_{13})_{d3}-R_{13}$;

provided that

(i) when X is C, Q_3 is not a lone electron pair or (=O);

(ii) when X is N, Q_3 is a lone electron pair; and

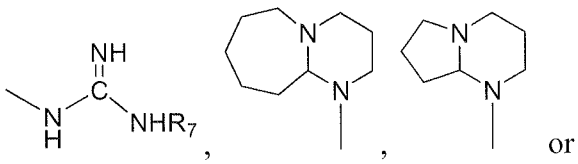
(iii) when X is P, Q_3 is (=O), and (f) is 0,

wherein

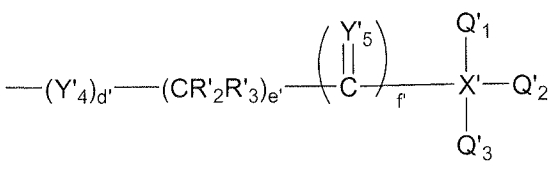
L_{11}, L_{12} and L_{13} are independently selected bifunctional spacers;

(d1), (d2) and (d3) are independently 0 or positive integers;

R_{11}, R_{12} and R_{13} are independently hydrogen, NH_2,



, , or



;

wherein

Y'_4 is O, S, or NR'_6;

Y'_5 are independently O, S or NR'_5;

(d') and (f') are independently 0 or 1;

(e') is 0 or a positive integer;

X' is C, N or P;

Q'_1 is H, C_{1-6} alkyl, NH_2, or $-(L'_{11})_{d'1}-R'_{11}$;

Q'_2 is H, C_{1-6} alkyl, NH_2, or $-(L'_{12})_{d'2}-R'_{12}$;

Q'_3 is a lone electron pair, (=O), H, C_{1-6} alkyl, NH_2, or $-(L_{13})_{d'3}-R'_{13}$;

provided that

(i) when X' is C, Q'_3 is not a lone electron pair or (=O);

(ii) when X' is N, Q'_3 is a lone electron pair; and

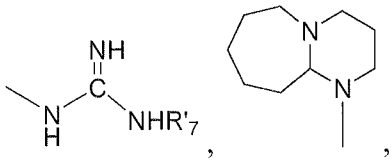
(iii) when X' is P, Q'_3 is (=O) and (f') is 0,

wherein

L'_{11}, L'_{12} and L'_{13} are independently selected bifunctional spacers;

(d'1), (d'2) and (d'3) are independently 0 or positive integers ;

R'_{11}, R'_{12} and R'_{13} are independently hydrogen, NH_2,



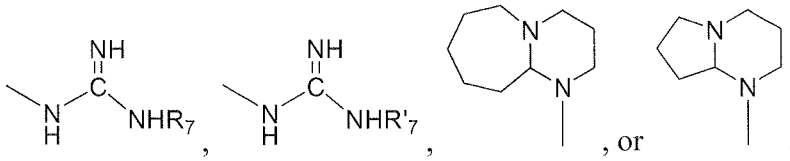
, , or ;

R_{2-6}, R'_{2-3} and R'_{5-6} are independently selected from among hydrogen, hydroxyl, amine, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl; and

R_7, and R'_7 are independently selected from among hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-}

$_6$ substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl,

provided that at least one of Q_{1-3} and Q'_{1-3} includes



, or .

The present invention also provides nanoparticle compositions for nucleic acids delivery.

According to the present invention, the nanoparticle compositions for the delivery of nucleic acids (i.e., an oligonucleotide) include:

(i) a compound of Formula (I);

(ii) a fusogenic lipid; and

(iii) a PEG lipid.

In another aspect of the present invention, there are provided methods of delivering nucleic acids (preferably, oligonucleotides) to a cell or tissue, *in vivo* and *in vitro*. Oligonucleotides introduced by the methods described herein can modulate the expression of a target gene.

Another aspect of the present invention provides methods of inhibiting expression of a target gene, i.e., oncogenes and genes associated with disease in mammals, preferably humans. The methods include contacting cells, such as cancer cells or tissues, with a nanoparticle/nanoparticle complex prepared from the nanoparticle composition described herein. The oligonucleotides encapsulated within the nanoparticle are released, which then mediate the down-regulation of mRNA or protein in the cells or tissues being treated. The treatment with the nanoparticle allows modulation of target gene expression (and the attendant benefits associated therewith) in the treatment of malignant disease, such as inhibition of the growth of cancer cells. Such therapies can be carried out as a single treatment or as part of a combination therapy, with one or more useful and/or approved treatments.

In a further aspect, the present invention provides methods of making the compounds of Formula (I) as well as nanoparticles containing the same.

The releasable cationic lipids described herein can neutralize the negative charges of nucleic acids and facilitate cellular uptake of the nanoparticle containing the nucleic acids therein. The cationic lipids herein provide multiple units of cationic moieties per cholesterol

moiety, to provide high efficiency in (i) neutralizing the negative charges of nucleic acids and (ii) forming a tight ionic complex with nucleic acids. This technology is advantageous for the delivery of therapeutic oligonucleotides and the treatment of mammals, i.e., humans, using therapeutic oligonucleotides.

The compounds described herein provide a means to control the size of the nanoparticles by forming multiple ionic complexes with nucleic acids.

The compounds described herein stabilize nanoparticle complexes and nucleic acids therein in biological fluids. Without being bound by any theory, it is believed that the nanoparticle complex enhances the stability of the encapsulated nucleic acids, at least in part by shielding the molecules from nucleases, thereby protecting from degradation.

The cationic lipids described herein allow high efficiency (e.g. above 50%, 70%, preferably above 80%) of nucleic acids (oligonucleotides) loading compared to art-known neutral or negatively charged nanoparticles, which typically have loadings of about or less than 10%. Without being bound by any theory, the high loading can be achieved in part by the fact that the guanidinium groups with high pKa (13-14) in the releasable cationic lipids of Formula (I) described herein form substantially compact zwitter ionic hydrogen bonds with phosphate groups of nucleic acids, thereby enabling more nucleic acids to be effectively packaged into the inner compartment of nanoparticles.

The nanoparticles described herein provide a further advantage over neutral or negatively charged nanoparticles, in that the aggregation or precipitation of nanoparticles is less likely to occur. Without being bound by any theory, the desired property is attributed in part to the fact that the cationic lipids forming hydrogen bonds or electrostatic interaction with nucleic acids are encapsulated within the nanoparticles, and noncationic/fusogenic lipids and PEG lipids surround the releasable cationic lipids and nucleic acids.

The nanoparticles can be prepared in a wide pH range such as from about 2 through about 12. The nanoparticles described herein also can be used clinically at a desirable physiological pH, such as from about 7.2 through about 7.6.

The nanoparticles described herein allow transfection of cells *in vitro* and *in vivo* without the aid of a transfection agent. The high transfection efficiency of the nanoparticles also provides a means to deliver therapeutic nucleic acids into the cells.

The compounds of Formula (I) include an acid labile linker. Such a linker facilitates disruption/destabilization of nanoparticles and endosome in acidic environments. Acidic environments can include both extracellular and intracellular environments. Intracellular acidic environments include, e.g., endosomes within the cytoplasm. Thus, the compounds described herein help release of therapeutic agents contained in nanoparticles and escape from endosomes into the cytoplasm.

The nanoparticle delivery systems described herein also allow sufficient amounts of the therapeutic oligonucleotides to be selectively available at the desired target area, such as cancer cells via EPR (Enhanced Permeation and Retention) effects. The nanoparticle compositions described herein thus improve specific mRNA downregulation in cancer cells or tissues.

According to the present invention, the nanoparticles described herein can deliever one or more, same or different therapeutic agents (e.g., antisense oligonucleotides), thereby attaining synergistic effects in treatment of disease.

Other and further advantages will be apparent from the following description.

For purposes of the present invention, the term "residue" shall be understood to mean that portion of a compound, to which it refers, e.g., cholesterol, etc. that remains after it has undergone a substitution reaction with another compound.

For purposes of the present invention, the term “alkyl” refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. The term “alkyl” also includes alkyl-thio-alkyl, alkoxyalkyl, cycloalkylalkyl, heterocycloalkyl, and C_{1-6} alkylcarbonylalkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from about 1 to 7 carbons, yet more preferably about 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted, the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C_{1-6} hydrocarbonyl, aryl, and amino groups.

For purposes of the present invention, the term “substituted” refers to adding or replacing one or more atoms contained within a functional group or compound with one of the moieties from the group of halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl,

cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C_{1-6} alkylcarbonylalkyl, aryl, and amino groups.

For purposes of the present invention, the term "alkenyl" refers to groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has about 2 to 12 carbons. More preferably, it is a lower alkenyl of from about 2 to 7 carbons, yet more preferably about 2 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted, the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C_{1-6} hydrocarbonyl, aryl, and amino groups.

For purposes of the present invention, the term "alkynyl" refers to groups containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has about 2 to 12 carbons. More preferably, it is a lower alkynyl of from about 2 to 7 carbons, yet more preferably about 2 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted, the substituted group(s) preferably include halo, oxy, azido, nitro, cyano, alkyl, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, trihalomethyl, hydroxyl, mercapto, hydroxy, cyano, alkylsilyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, alkenyl, alkynyl, C_{1-6} hydrocarbonyl, aryl, and amino groups. Examples of "alkynyl" include propargyl, propyne, and 3-hexyne.

For purposes of the present invention, the term "aryl" refers to an aromatic hydrocarbon ring system containing at least one aromatic ring. The aromatic ring can optionally be fused or otherwise attached to other aromatic hydrocarbon rings or non-aromatic hydrocarbon rings. Examples of aryl groups include phenyl, naphthyl, 1,2,3,4-tetrahydronaphthalene and biphenyl. Preferred examples of aryl groups include phenyl and naphthyl.

For purposes of the present invention, the term "cycloalkyl" refers to a C_{3-8} cyclic hydrocarbon. Examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

For purposes of the present invention, the term "cycloalkenyl" refers to a C_{3-8} cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl

include cyclopentenyl, cyclopentadienyl, cyclohexenyl, 1,3-cyclohexadienyl, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

For purposes of the present invention, the term "cycloalkylalkyl" refers to an alklyl group substituted with a C_{3-8} cycloalkyl group. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

For purposes of the present invention, the term "alkoxy" refers to an alkyl group of indicated number of carbon atoms attached to the parent molecular moiety through an oxygen bridge. Examples of alkoxy groups include methoxy, ethoxy, propoxy and isopropoxy.

For purposes of the present invention, an "alkylaryl" group refers to an aryl group substituted with an alkyl group.

For purposes of the present invention, an "aralkyl" group refers to an alkyl group substituted with an aryl group.

For purposes of the present invention, the term "alkoxyalkyl" group refers to an alkyl group substituted with an alkoxy group.

For purposes of the present invention, the term "alkyl-thio-alkyl" refers to an alkyl-S-alkyl thioether, for example methylthiomethyl or methylthioethyl.

For purposes of the present invention, the term "amino" refers to a nitrogen containing group, as is known in the art, derived from ammonia by the replacement of one or more hydrogen radicals by organic radicals. For example, the terms "acylamino" and "alkylamino" refer to specific N-substituted organic radicals with acyl and alkyl substituent groups respectively.

For purposes of the present invention, the term "alkylcarbonyl" refers to a carbonyl group substituted with alkyl group.

For purposes of the present invention, the term "halogen' or "halo" refers to fluorine, chlorine, bromine, and iodine.

For purposes of the present invention, the term "heterocycloalkyl" refers to a non-aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heterocycloalkyl ring can be optionally fused to or otherwise attached to other heterocycloalkyl rings and/or non-aromatic hydrocarbon rings. Preferred heterocycloalkyl groups have from 3 to 7 members. Examples of heterocycloalkyl groups include piperazine,

morpholine, piperidine, tetrahydrofuran, pyrrolidine, and pyrazole. Preferred heterocycloalkyl groups include piperidinyl, piperazinyl, morpholinyl, and pyrrolidinyl.

For purposes of the present invention, the term "heteroaryl" refers to an aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heteroaryl ring can be fused or otherwise attached to one or more heteroaryl rings, aromatic or non-aromatic hydrocarbon rings or heterocycloalkyl rings. Examples of heteroaryl groups include pyridine, furan, thiophene, 5,6,7,8-tetrahydroisoquinoline and pyrimidine. Preferred examples of heteroaryl groups include thienyl, benzothienyl, pyridyl, quinolyl, pyrazinyl, pyrimidyl, imidazolyl, benzimidazolyl, furanyl, benzofuranyl, thiazolyl, benzothiazolyl, isoxazolyl, oxadiazolyl, isothiazolyl, benzisothiazolyl, triazolyl, tetrazolyl, pyrrolyl, indolyl, pyrazolyl, and benzopyrazolyl.

For purposes of the present invention, the term "heteroatom" refers to nitrogen, oxygen, and sulfur.

In some embodiments, substituted alkyls include carboxyalkyls, aminoalkyls, dialkylaminos, hydroxyalkyls and mercaptoalkyls; substituted alkenyls include carboxyalkenyls, aminoalkenyls, dialkenylaminos, hydroxyalkenyls and mercaptoalkenyls; substituted alkynyls include carboxyalkynyls, aminoalkynyls, dialkynylaminos, hydroxyalkynyls and mercaptoalkynyls; substituted cycloalkyls include moieties such as 4-chlorocyclohexyl; aryls include moieties such as naphthyl; substituted aryls include moieties such as 3-bromo phenyl; aralkyls include moieties such as tolyl; heteroalkyls include moieties such as ethylthiophene; substituted heteroaryls include moieties such as 3-methoxythiophene; alkoxy includes moieties such as methoxy; and phenoxy includes moieties such as 3-nitrophenoxy. Halo shall be understood to include fluoro, chloro, iodo and bromo.

For purposes of the present invention, "positive integer" shall be understood to include an integer equal to or greater than 1 and as will be understood by those of ordinary skill to be within the realm of reasonableness by the artisan of ordinary skill.

For purposes of the present invention, the term "linked" shall be understood to include covalent (preferably) or noncovalent attachment of one group to another, i.e., as a result of a chemical reaction.

The terms "effective amounts" and "sufficient amounts" for purposes of the present invention shall mean an amount which achieves a desired effect or therapeutic effect, as is understood by those of ordinary skill in the art.

The term "nanoparticle" and/or "nanoparticle complex" formed using the nanoparticle composition described herein refers to a lipid-based nanocomplex. The nanoparticle contains nucleic acids such as oligonucleotides encapsulated in a mixture of a cationic lipid, a fusogenic lipid, and a PEG lipid. Alternatively, the nanoparticle can be formed without nucleic acids.

For purposes of the present invention, the term "therapeutic oligonucleotide" refers to an oligonucleotide used as a pharmaceutical or diagnostic agent.

For purposes of the present invention, "modulation of gene expression" shall be understood as broadly including down-regulation or up-regulation of any types of genes, preferably associated with cancer and inflammation, compared to a gene expression observed in the absence of the treatment with the nanoparticle described herein, regardless of the route of administration.

For purposes of the present invention, "inhibition of expression of a target gene" shall be understood to mean that mRNA expression or the amount of protein translated are reduced or attenuated when compared to that observed in the absence of the treatment with the nanoparticle described herein. Suitable assays of such inhibition include, e.g., examination of protein or mRNA levels using techniques known to those of ordinary skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of ordinary skill in the art. The treated conditions can be confirmed by, for example, decrease in mRNA levels in cells, preferably cancer cells or tissues.

Broadly speaking, successful inhibition or treatment shall be deemed to occur when the desired response is obtained. For example, successful inhibition or treatment can be defined by obtaining, e.g., 10% or higher (i.e., 20% 30%, 40%) downregulation of genes associated with tumor growth inhibition. Alternatively, successful treatment can be defined by obtaining at least 20%, preferably 30% or more preferably 40 % or higher (i.e., 50% or 80%) decrease in oncogene mRNA levels in cancer cells or tissues, including other clinical markers contemplated by the artisan in the field, when compared to that observed in the absence of the treatment with the nanoparticle described herein.

Further, the use of singular terms for convenience in description is in no way intended to be so limiting. Thus, for example, reference to a composition comprising an oligonucleotide, a cholesterol analog, a cationic lipid, a fusogenic lipid, a PEG lipid, etc., refers to one or more molecules of that oligonucleotide, cholesterol analog, cationic lipid, fuosogenic lipid, PEG lipid, etc. It is also contemplated that the oligonucleotide can be of the same or different kind of gene. It is also to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically illustrates a reaction scheme of preparing compound **12**, as described in Examples 6-12.

FIG. 2 schematically illustrates a reaction scheme of preparing compound **29**, as described in Examples 13-18.

FIG. 3 schematically illustrates a reaction scheme of preparing compound **31**, as described in Examples 19-20.

FIG. 4 schematically illustrates a reaction scheme of preparing compound **49**, as described in Examples 21-26.

FIG. 5 schematically illustrates a reaction scheme of preparing compound **54**, as described in Examples 27-30.

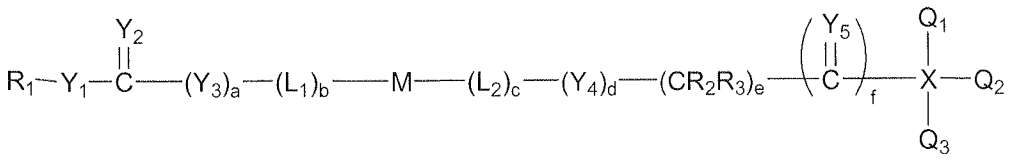
DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention, there are provided releasable lipids containing multiple cationic moieties. According to the present invetion, there are provided nanoparticle compositions containing the same for the delivery of nucleic acids. The nanoparticle composition may contain (i) a compound of Formula (I); (ii) a fusogenic lipid; and (iii) a PEG lipid. The nucleic acids contemplated include oligonucleotides or plasmids, and preferably oligonucleotides. The nanoparticles prepared by using the nanoparticle compositions described herein include nucleic acids encapsulated in the lipid carrier.

A. Releasable Cationic Lipids of Formula (I)

1. Overview

In accordance with the present invention, there are provided a compound of Formula (I):



(I)

wherein

R_1 is cholesterol or an analog thereof;

Y_1 is O, S or NR_4, preferably O;

Y_2 and Y_5 are independently O, S or NR_5, preferably O;

Y_{3-4} are independently O, S or NR_6, preferably O or NR_6;

L_{1-2} are independently selected bifunctional linkers;

M is an acid labile linker;

(a), (d) and (f) are independently zero or 1;

(b), (c) and (e) are independently zero or positive integers, preferably zero or an integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6);

X is C, N or P;

Q_1 is H, C_{1-6} alkyl (e.g, methyl, ethyl, propyl), NH_2, or $-(L_{11})_{d1}-R_{11}$;

Q_2 is H, C_{1-6} alkyl(e.g, methyl, ethyl, propyl), NH_2, or $-(L_{12})_{d2}-R_{12}$;

Q_3 is a lone electron pair, (=O), H, C_{1-6} alkyl (e.g, methyl, ethyl, propyl), NH_2, or $-(L_{13})_{d3}-R_{13}$;

provided that

(i) when X is C, Q_3 is not a lone electron pair or (=O);

(ii) when X is N, Q_3 is a lone electron pair; and

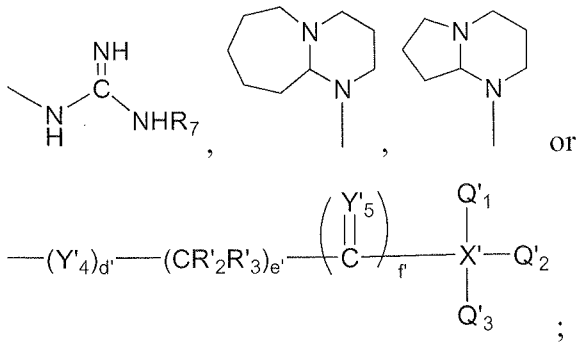
(iii) when X is P, Q_3 is (=O), and (f) is 0,

wherein

L_{11}, L_{12} and L_{13} are independently selected bifunctional spacers;

(d1), (d2) and (d3) are independently zero or positive integers, preferably zero or an integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6), and more preferably, zero, 1, 2, 3, 4;

R_{11}, R_{12} and R_{13} are independently hydrogen, NH_2,



wherein

Y'_4 is O, S, or NR'_6, preferably O or NR'_6;

Y'_5 are independently O, S or NR'_5, preferably O;

(d') and (f') are independently zero or 1;

(e') is zero or a positive integer, preferably zero or an integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6);

X' is C, N or P;

Q'_1 is H, C_{1-6} alkyl (e.g, methyl, ethyl, propyl), NH_2, or $-(L'_{11})_{d'1}-R'_{11}$;

Q'_2 is H, C_{1-6} alkyl (e.g, methyl, ethyl, propyl), NH_2, or $-(L'_{12})_{d'2}-R'_{12}$;

Q'_3 is a lone electron pair, (=O), H, C_{1-6} alkyl (e.g, methyl, ethyl, propyl), NH_2, or $-(L_{13})_{d'3}-R'_{13}$;

provided that

(i) when X' is C, Q'_3 is not a lone electron pair or (=O);

(ii) when X' is N, Q'_3 is a lone electron pair; and

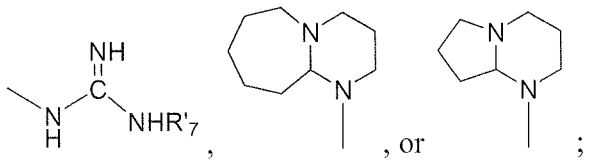
(iii) when X' is P, Q'_3 is (=O) and (f') is 0,

wherein

L'_{11}, L'_{12} and L'_{13} are independently selected bifunctional spacers;

(d'1), (d'2) and (d'3) are independently zero or positive integers, preferably zero or an integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6) ;

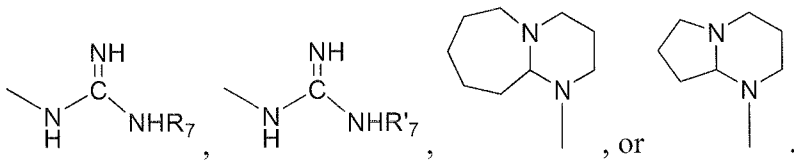
R'_{11}, R'_{12} and R'_{13} are independently hydrogen, NH_2,



R_{2-3}, and R'_{2-3} are independently selected from among hydrogen, amine, hydroxyl, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl, preferably, hydrogen, hydroxyl, amine, methyl, ethyl and propyl; and

R_{4-7}, and R'_{5-7} are independently selected from among hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl, preferably, hydrogen, methyl, ethyl and propyl,

provided that at least one or more (e.g., one, two, three) of Q_{1-3} and Q'_{1-3} includes



L_1 and L_2 in each occurrence are independently the same or different when (b) or (c) is equal to or greater than 2.

$-C(R_2R_3)-$ and $-C(R_2R_3)-$, in each occurrence are independently the same or different when (e) or (e') is equal to or greater than 2.

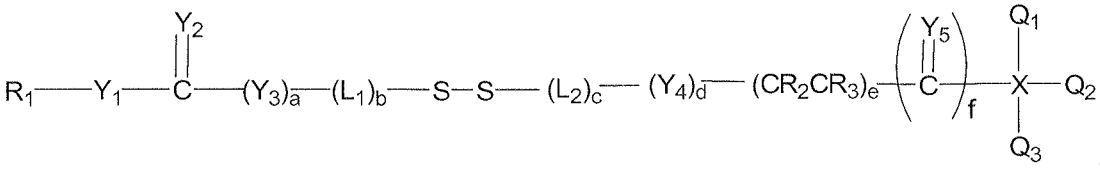
L_{11}, L_{12} and L_{13} in each occurrence are independently the same or different when (d1), (d2) or (d3) is equal to or greater than 2.

L'_{11}, L'_{12} and L'_{13} in each occurrence are independently the same or different when each (d'1), (d'2) or (d'3) is equal to or greater than 2.

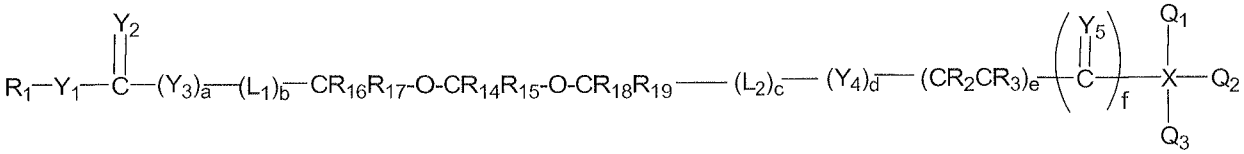
The combinations of the bifunctional linkers and the bifuntional spacers contemplated within the scope of the present invention include those in which combinations of variables and substituents of the linker and spacer groups are permissible so that such combinations result in stable compounds of Formula (I). For example, the combinations of values and substituents do not permit oxygen, nitrogen or carbonyl to be positioned directly adjacent to S-S or imine.

In one preferred aspect, M is -S-S-, $-CR_{16}R_{17}$-O-$CR_{14}R_{15}$-O-$CR_{18}R_{19}$-, or -N=CR_{10}- or -CR_{10}=N-.

In certain embodiments, the releasable cationic lipids have Formula (Ia):



In certain embodiments, the releasable cationic lipids have Formula (Ib):



wherein

R_{14-15} are independently selected from among hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy, substituted and arylcarbonyloxy; preferably R_{14} and R_{15} are selected from among hydrogen, C_{1-6} alkyls, C_{3-8} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls, substituted aryls and aralkyls, preferably, hydrogen, methyl, ethyl or propyl; and

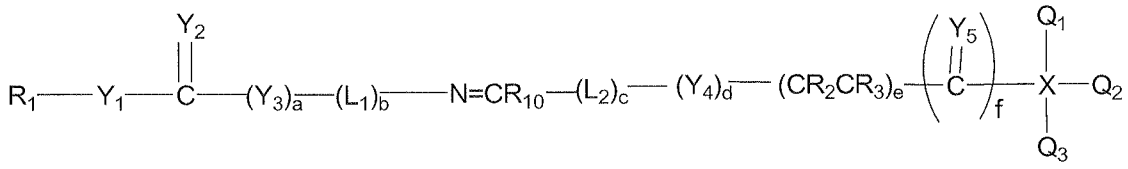
R_{16-19} are independently selected from among hydrogen, hydroxyl, amine, substituted amine, azido, carboxy, cyano, halo, hydroxyl, nitro, silyl ether, sulfonyl, mercapto, C_{1-6} alkylmercapto, arylmercapto, substituted arylmercapto, substituted C_{1-6} alkylthio, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy, substituted and arylcarbonyloxy, preferably, hydrogen, methyl, ethyl or propyl.

Preferably, both R_{14} and R_{15} are not simultaneously hydrogen.

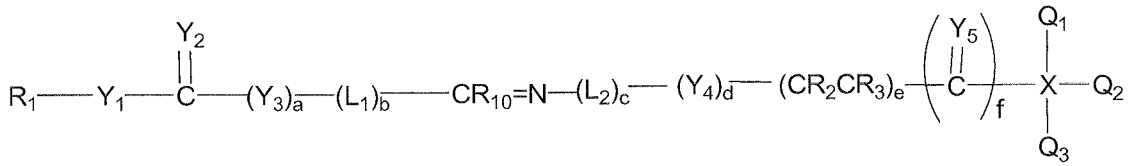
In one preferred embodiment, R_{14} and R_{15} are selected from among hydrogen, C_{1-6} alkyls, C_{3-8} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls, substituted aryls and aralkyls.

More preferably, both R_{14} and R_{15} are selected from among C_{1-6} alkyls (methyl, ethyl, propyl) and C_{3-8} branched alkyls. In one particular embodiment, both R_{14} and R_{15} are methyl.

In certain embodiments, the releasable cationic lipids have Formulas (Ic) or (Ic'):



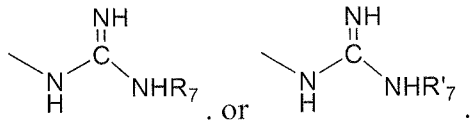
or



wherein

R_{10} is hydrogen, C_{1-6} alkyl, C_{3-8} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{3-8} substituted cycloalkyl, aryl or substituted aryl, preferably, hydrogen, methyl, ethyl, or propyl.

In one preferred aspect, the compounds of Formula (I) include two or more:



. or

.

In another preferred aspect, the compounds of Formula (I) include two or more of R_{11}, R_{12} and R_{13}.

In one preferred embodiment, Y_1 is oxygen.

In another preferred embodiment, both Y_2 and Y_5 are oxygen.

In one embodiment, both (d1) and (d2) are not simultaneously zero.

In another embodiment, (d1), (d2), (d3), (d'1), (d'2) and (d'3) are not simultaneously zero.

The releasable cationic lipids of Formula (I) described herein can carry a net positive or neutral charge at a selected pH, such as pH<13 (e.g. pH 6-12, pH 6-8).

2. Bifunctional Linkers: L_1 and L_2 groups

According to the present invention, L_1 includes, but is not limited to:

$-(CR_{21}R_{22})_{t1}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}Y_{17}-(CR_{23}R_{24})_{t2}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22}CR_{23}R_{24}Y_{17})_{t1}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22}CR_{23}R_{24}Y_{17})_{t1}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-[(CR_{21}R_{22}CR_{23}R_{24})_{t2}Y_{17}]_{t3}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}-[(CR_{23}R_{24})_{t2}Y_{17}]_{t3}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24})_{t2}-$,

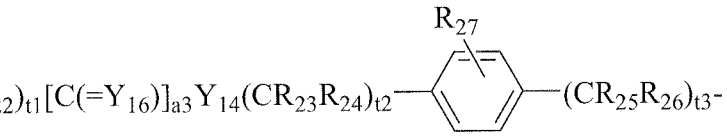
$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24})_{t2}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24})_{t2}-Y_{15}-(CR_{23}R_{24})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24})_{t2}-Y_{15}-(CR_{23}R_{24})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24}CR_{25}R_{26}Y_{19})_{t2}(CR_{27}CR_{28})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24}CR_{25}R_{26}Y_{19})_{t2}(CR_{27}CR_{28})_{t3}-$, and



,

wherein:

Y_{16} is O, NR_{28}, or S, preferably O;

Y_{14-15} and Y_{17-19} are independently O, NR_{29}, or S, preferably O or NR_{29};

R_{21-27} are independently selected from among hydrogen, hydroxyl, amine, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl or propyl;

R_{28-29} are independently selected from among hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl or propyl;

(t1), (t2), (t3) and (t4) are independently zero or positive integers, preferably zero or a positive integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6); and

(a2) and (a3) are independently zero or 1.

The bifunctional L_1 linkers contemplated within the scope of the present invention include those in which combinations of substituents and variables are permissible so that such combinations result in stable compounds of Formula (I). For example, when (a3) is zero, Y_{17} is not linked directly to Y_{14}.

For purposes of the present invention, when values for bifunctional linkers are positive integers equal to or greater than 2, the same or different bifunctional linkers can be employed.

R_{21}-R_{28}, in each occurrence, are independently the same or different when (t1), (t2), (t3) or (t4) is independently equal to or greater than 2.

In one embodiment, Y_{14-15} and Y_{17-19} are O or NH; and R_{21-29} are independently hydrogen or methyl.

In another embodiment, Y_{16} is O; Y_{14-15} and Y_{17-19} are O or NH; and R_{21-29} are hydrogen.

In certain embodiments, L_1 is independently selected from among:

$-(CH_2)_{t1}-[C(=O)]_{a3}-$,

$-(CH_2)_{t1}Y_{17}-(CH_2)_{t2}-(Y_{18})_{a2}-[C(=O)]_{a3}-$,

$-(CH_2CH_2Y_{17})_{t1}-[C(=O)]_{a3}-$,

$-(CH_2CH_2Y_{17})_{t1}(CH_2)_{t4}-(Y_{18})_{a2}-[C(=O)]_{a3}-$,

$-[(CH_2CH_2)_{t2}Y_{17}]_{t3}(CH_2)_{t4}-(Y_{18})_{a2}-[C(=O)]_{a3}-$,

$-(CH_2)_{t1}-[(CH_2)_{t2}Y_{17}]_{t3}(CH_2)_{t4}-(Y_{18})_{a2}-[C(=O)]_{a3}-$,

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}(CH_2)_{t2}-$,

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}Y_{14}(CH_2)_{t2}-$,

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}(CH_2)_{t2}-Y_{15}-(CH_2)_{t3}-$,

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}Y_{14}(CH_2)_{t2}-Y_{15}-(CH_2)_{t3}-$,

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}(CH_2CH_2Y_{19})_{t2}(CH_2)_{t3}-$, and

$-(CH_2)_{t1}(Y_{17})_{a2}[C(=O)]_{a3}Y_{14}(CH_2CH_2Y_{19})_{t2}(CH_2)_{t3}-$,

wherein

Y_{14-15} and Y_{17-19} are independently O, or NH;

(t1), (t2), (t3), and (t4) are independently zero or positive integers, preferably zero or positive integers of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6); and

(a2) and (a3) are independently zero or 1.

Y_{17}, in each occurrence, is the same or different, when (t1) or (t3) is equal to or greater than 2.

Y_{19}, in each occurrence, is the same or different, when (t2) is equal to or greater than 2.

In a further embodiment and/or alternative embodiments, illustrative examples of the L_1 group are selected from among:

$-CH_2-$, $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,$-NH(CH_2)-$,

$-CH(NH_2)CH_2-$,

$-(CH_2)_4-C(=O)-$, $-(CH_2)_5-C(=O)-$, $-(CH_2)_6-C(=O)-$,

$-CH_2CH_2O-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_2-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_3-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_2-C(=O)-$,

$-CH_2CH_2O-CH_2CH_2NH-C(=O)-$,

$-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-CH_2CH_2O-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-CH_2CH_2O-CH_2C(=O)-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2C(=O)-$,

$-(CH_2)_4-C(=O)NH-$, $-(CH_2)_5-C(=O)NH-$, $-(CH_2)_6-C(=O)NH-$,

$-CH_2CH_2O-CH_2O-C(=O)-NH-$,

$-(CH_2CH_2O)_2-CH_2O-C(=O)-NH-$,

$-(CH_2CH_2O)_3-CH_2O-C(=O)-NH-$,

$-(CH_2CH_2O)_2-C(=O)-NH-$,

$-CH_2CH_2O-CH_2CH_2NH-C(=O)-NH-$,

$-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-NH-$,

$-CH_2-O-CH_2CH_2O-CH_2CH_2NH-C(=O)-NH-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-NH-$,

$-CH_2-O-CH_2CH_2O-CH_2C(=O)-NH-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2C(=O)-NH-$,

$-(CH_2CH_2O)_2-$, $-CH_2CH_2O-CH_2O-$.

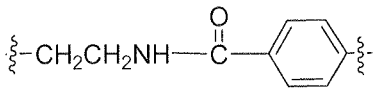
$-(CH_2CH_2O)_2-CH_2CH_2NH-$, $-(CH_2CH_2O)_3-CH_2CH_2NH-$,

$-CH_2CH_2O-CH_2CH_2NH-$,

$-CH_2-O-CH_2CH_2O-CH_2CH_2NH-$,

-CH_2-O-$(CH_2CH_2O)_2$-CH_2CH_2NH-,

-CH_2-O-CH_2CH_2O-, -CH_2-O-$(CH_2CH_2O)_2$-,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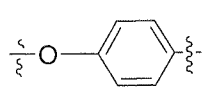
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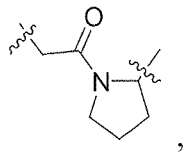
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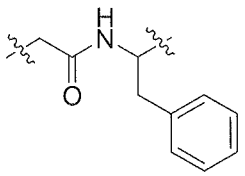
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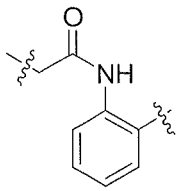

,

-C(=O)NH$(CH_2)_2$- , -CH_2C(=O)NH$(CH_2)_2$- ,

-C(=O)NH$(CH_2)_3$- , -CH_2C(=O)NH$(CH_2)_3$- ,

-C(=O)NH$(CH_2)_4$- , -CH_2C(=O)NH$(CH_2)_4$- ,

-C(=O)NH$(CH_2)_5$- , -CH_2C(=O)NH$(CH_2)_5$- ,

-C(=O)NH$(CH_2)_6$- , -CH_2C(=O)NH$(CH_2)_6$- ,

-C(=O)O$(CH_2)_2$- , -CH_2C(=O)O$(CH_2)_2$- ,

-C(=O)O$(CH_2)_3$- , -CH_2C(=O)O$(CH_2)_3$- ,

-C(=O)O$(CH_2)_4$- , -CH_2C(=O)O$(CH_2)_4$- ,

-C(=O)O$(CH_2)_5$- , -CH_2C(=O)O$(CH_2)_5$- ,

-C(=O)O$(CH_2)_6$- , -CH_2C(=O)O$(CH_2)_6$- ,

-$(CH_2CH_2)_2$NHC(=O)NH$(CH_2)_2$- ,

-$(CH_2CH_2)_2$NHC(=O)NH$(CH_2)_3$- ,

-$(CH_2CH_2)_2$NHC(=O)NH$(CH_2)_4$- ,

-$(CH_2CH_2)_2$NHC(=O)NH$(CH_2)_5$- ,

-$(CH_2CH_2)_2$NHC(=O)NH$(CH_2)_6$- ,

-$(CH_2CH_2)_2$NHC(=O)O$(CH_2)_2$- ,

-$(CH_2CH_2)_2$NHC(=O)O$(CH_2)_3$- ,

-$(CH_2CH_2)_2$NHC(=O)O$(CH_2)_4$- ,

-$(CH_2CH_2)_2$NHC(=O)O$(CH_2)_5$- ,

-$(CH_2CH_2)_2$NHC(=O)O$(CH_2)_6$- ,

-$(CH_2CH_2)_2$NHC(=O)$(CH_2)_2$- ,

-$(CH_2CH_2)_2$NHC(=O)$(CH_2)_3$- ,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_4-$,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_5-$, and

$-(CH_2CH_2)_2NHC(=O)(CH_2)_6-$.

In certain embodiments, L_2 inclues, but is not limited to:

$-(CR'_{21}R'_{22})_{t'1}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'2}-$,

$-(CR'_{21}R'_{22})_{t'1}Y'_{14}-(CR'_{23}R'_{24})_{t'2}-(Y'_{15})_{a'2}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'3}-$,

$-(CR'_{21}R'_{22}CR'_{23}R'_{24}Y'_{14})_{t'1}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'2}-$,

$-(CR'_{21}R'_{22}CR'_{23}R'_{24}Y'_{14})_{t'1}(CR'_{25}R'_{26})_{t'2}-(Y'_{15})_{a'2}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'3}-$,

$-[(CR'_{21}R'_{22}CR'_{23}R'_{24})_{t'2}Y'_{14}]_{t'1}(CR'_{25}R'_{26})_{t'2}-(Y'_{15})_{a'2}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}-[(CR'_{23}R'_{24})_{t'2}Y'_{14}]_{t'2}(CR'_{25}R'_{26})_{t'3}-(Y'_{15})_{a'2}-[C(=Y'_{16})]_{a'3}(CR'_{27}CR'_{28})_{t'4}-$

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24})_{t'2}-$,

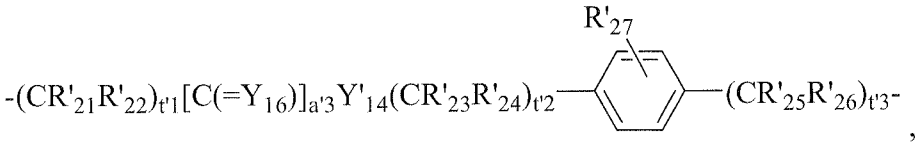
$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{15}(CR'_{23}R'_{24})_{t'2}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24})_{t'2}-Y'_{15}-(CR'_{23}R'_{24})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{14}(CR'_{23}R'_{24})_{t'2}-Y'_{15}-(CR'_{23}R'_{24})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24}CR'_{25}R'_{26}Y'_{15})_{t'2}(CR'_{27}CR'_{28})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{17}(CR'_{23}R'_{24}CR'_{25}R'_{26}Y'_{15})_{t'2}(CR'_{27}CR'_{28})_{t'3}-$, and



,

wherein:

Y'_{16} is O, NR'_{28}, or S, preferably O;

Y'_{14-15} and Y'_{17} are independently O, NR'_{29}, or S, preferably O or NR'_{29};

R'_{21-27} are independently selected from among hydrogen, hydroxyl, amine, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl, or propyl;

R'_{28-29} are independently selected from among hydrogen, hydroxyl, amine, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl, or propyl;

(t'1), (t'2), (t'3) and (t'4) are independently zero or positive integers, preferably zero or a positive integer of from about 1 to about 10 (e.g., 01, 2, 3, 4, 5, 6); and

(a'2) and (a'3) are independently zero or 1.

The bifunctional L_2 linkers contemplated within the scope of the present invention include those in which combinations of variables and substituents of the linkers groups are permissible so that such combinations result in stable compounds of Formula (I). For example, when (a'3) is zero, Y'_{14} is not linked directly to Y'_{14} or Y'_{17}.

For purposes of the present invention, when values for bifunctional L_2 linkers are positive integers equal to or greater than 2, the same or different bifunctional linkers can be employed.

In one embodiment, $Y'_{14\text{-}15}$ and Y'_{17} are O or NH; and $R'_{21\text{-}29}$ are independently hydrogen or methyl.

In another embodiment, Y'_{16} is O; $Y'_{14\text{-}15}$ and Y'_{17} are O or NH; and $R'_{21\text{-}29}$ are hydrogen.

In certain embodiments, L_2 is selected from among:

$-(CH_2)_{t'1}-[C(=O)]_{a'3}(CH_2)_{t'2}-$,

$-(CH_2)_{t'1}Y'_{14}-(CH_2)_{t'2}-(Y'_{15})_{a'2}-[C(=O)]_{a'3}(CH_2)_{t'3}-$,

$-(CH_2CH_2Y'_{14})_{t'1}-[C(=O)]_{a'3}(CH_2)_{t'2}-$,

$-(CH_2CH_2Y'_{14})_{t'1}(CH_2)_{t'2}-(Y'_{15})_{a'2}-[C(=O)]_{a'3}(CH_2)_{t'3}-$,

$-[(CH_2CH_2)_{t'2}Y'_{14}]_{t'1}(CH_2)_{t'2}-(Y'_{15})_{a'2}-[C(=O)]_{a'3}(CH_2)_{t'3}-$,

$-(CH_2)_{t'1}-[(CH_2)_{t'2}Y'_{14}]_{t'2}(CH_2)_{t'3}-(Y'_{15})_{a'2}-[C(=O)]_{a'3}(CH_2)_{t'4}-$,

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}(CH_2)_{t'2}-$,

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}Y'_{15}(CH_2)_{t'2}-$,

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}(CH_2)_{t'2}-Y'_{15}-(CH_2)_{t'3}-$,

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}Y'_{14}(CH_2)_{t'2}-Y'_{15}-(CH_2)_{t'3}-$,

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}(CH_2CH_2Y'_{15})_{t'2}(CH_2)_{t'3}-$, and

$-(CH_2)_{t'1}(Y'_{14})_{a'2}[C(=O)]_{a'3}Y'_{17}(CH_2CH_2Y'_{15})_{t'2}(CH_2)_{t'3}-$,

wherein

$Y'_{14\text{-}15}$ and Y'_{17} are independently O, or NH;

(t'1), (t'2), (t'3), and (t'4) are independently zero or positive integers, preferably 0 or positive integers of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6); and

(a'2) and (a'3) are independently zero or 1.

Y'_{14}, in each occurrence, is the same or different, when (t'1) or (t'2) is equal to or greater than 2.

Y'_{15}, in each occurrence, is the same or different, when (t'2) is equal to or greater than 2.

In a further embodiment and/or alternative embodiments, illustrative examples of the L_2 group are selected from among:

$-CH_2-$, $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,$-NH(CH_2)-$,

$-CH(NH_2)CH_2-$,

$-O(CH_2)_2-$, $-C(=O)O(CH_2)_3-$, $-C(=O)NH(CH_2)_3-$,

$-C(=O)(CH_2)_2-$, $-C(=O)(CH_2)_3-$,

$-CH_2-C(=O)-O(CH_2)_3-$,

$-CH_2-C(=O)-NH(CH_2)_3-$,

$-CH_2-OC(=O)-O(CH_2)_3-$,

$-CH_2-OC(=O)-NH(CH_2)_3-$,

$-(CH_2)_2-C(=O)-O(CH_2)_3-$,

$-(CH_2)_2-C(=O)-NH(CH_2)_3-$,

$-CH_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,

$-CH_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,

$-(CH_2)_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,

$-(CH_2)_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,

$-CH_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$,

$-(CH_2)_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$,

$-(CH_2CH_2O)_2-$, $-CH_2CH_2O-CH_2O-$.

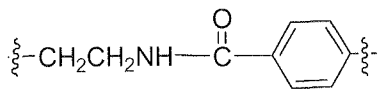
$-(CH_2CH_2O)_2-CH_2CH_2NH-$, $-(CH_2CH_2O)_3-CH_2CH_2NH-$,

$-CH_2CH_2O-CH_2CH_2NH-$,

$-CH_2-O-CH_2CH_2O-CH_2CH_2NH-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2CH_2NH-$,

$-CH_2-O-CH_2CH_2O-$, $-CH_2-O-(CH_2CH_2O)_2-$,



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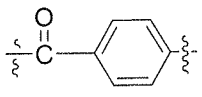

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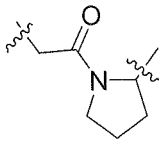

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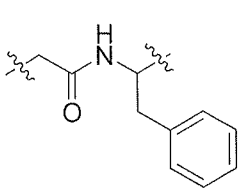
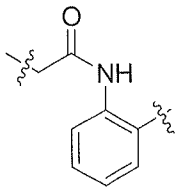



$-(CH_2)_2NHC(=O)-(CH_2CH_2O)_2-$,

$-C(=O)NH(CH_2)_2-$, $-CH_2C(=O)NH(CH_2)_2-$,

$-C(=O)NH(CH_2)_3-$, $-CH_2C(=O)NH(CH_2)_3-$,

$-C(=O)NH(CH_2)_4-$, $-CH_2C(=O)NH(CH_2)_4-$,

$-C(=O)NH(CH_2)_5-$, $-CH_2C(=O)NH(CH_2)_5-$,

$-C(=O)NH(CH_2)_6-$, $-CH_2C(=O)NH(CH_2)_6-$,

$-C(=O)O(CH_2)_2-$, $-CH_2C(=O)O(CH_2)_2-$,

$-C(=O)O(CH_2)_3-$, $-CH_2C(=O)O(CH_2)_3-$,

$-C(=O)O(CH_2)_4-$, $-CH_2C(=O)O(CH_2)_4-$,

$-C(=O)O(CH_2)_5-$, $-CH_2C(=O)O(CH_2)_5-$,

$-C(=O)O(CH_2)_6-$, $-CH_2C(=O)O(CH_2)_6-$,

$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_2-$,

$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_3-$,

$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_4-$,

$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_5-$,

$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_6-$,

$-(CH_2CH_2)_2NHC(=O)O(CH_2)_2-$,

$-(CH_2CH_2)_2NHC(=O)O(CH_2)_3-$,

$-(CH_2CH_2)_2NHC(=O)O(CH_2)_4-$,

$-(CH_2CH_2)_2NHC(=O)O(CH_2)_5-$,

$-(CH_2CH_2)_2NHC(=O)O(CH_2)_6-$,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_2-$,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_3-$,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_4-$,

$-(CH_2CH_2)_2NHC(=O)(CH_2)_5-$, and

$-(CH_2CH_2)_2NHC(=O)(CH_2)_6-$.

In a further embodiment and/or alternative embodiments, the bifunctional linkers L_1 and L_2 can be a spacer having a substituted saturated or unsaturated, branched or linear, C_{3-50} alkyl (i.e., C_{3-40} alkyl, C_{3-20} alkyl, C_{3-15} alkyl, C_{3-10} alkyl, etc.), wherein optionally one or more carbons are replaced with NR_6, O, S or C(=Y), (preferably O or NH), but not exceeding 70% (i.e., less than 60%, 50%, 40%, 30%, 20%, 10%) of the carbons being replaced.

3. Bifunctional Spacers L_{11-13} and L'_{11-13}

According to the present invention, the bifunctional spacers L_{11-13} and L'_{11-13} are terminal bifunctional linkers which can be connected to cationic moieties, such as guanidinium, DBU, DBN, etc. The bifunctional linkers L_{11-13} and L'_{11-13} are independently selected from among:

$-(CR_{31}R_{32})_{q1}-$; and

$-Y_{26}(CR_{31}R_{32})_{q1}-$,

wherein:

Y_{26} is O, NR_{33}, or S, preferably O or NR_{33};

R_{31-32} are independently selected from among hydrogen, OH, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl or propyl;

R_{33} is selected from among hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, preferably, hydrogen, methyl, ethyl or propyl; and

(q1) is zero or a positive integer, preferably zero or an integer of from about 1 to about 10 (e.g., 1, 2, 3, 4, 5, 6).

The bifunctional spacers contemplated within the scope of the present invention include those in which combinations of substituents and variables are permissible so that such combinations result in stable compounds of Formula (I).

R_{31} and R_{32}, in each occurrence, are independently the same or different when (q1) is equal to or greater than 2.

In one preferred embodiment, R'_{31-33} are hydrogen or methyl.

In a further and/or alternative embodiments, $L_{11\text{-}13}$ and $L'_{11\text{-}13}$ is independently selected from among:

$-CH_2-$,$-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,

$-O(CH_2)_2-$, $-O(CH_2)_3-$, $-O(CH_2)_4-$, $-O(CH_2)_5-$, $-O(CH_2)_6-$,

$-(CH_2CH_2O)-CH_2CH_2-$,

$-(CH_2CH_2O)_2-CH_2CH_2-$,

$-C(=O)O(CH_2)_3-$, $-C(=O)NH(CH_2)_3-$,

$-C(=O)(CH_2)_2-$, $-C(=O)(CH_2)_3-$,

$-CH_2-C(=O)-O(CH_2)_3-$,

$-CH_2-C(=O)-NH(CH_2)_3-$,

$-CH_2-OC(=O)-O(CH_2)_3-$,

$-CH_2-OC(=O)-NH(CH_2)_3-$,

$-(CH_2)_2-C(=O)-O(CH_2)_3-$,

$-(CH_2)_2-C(=O)-NH(CH_2)_3-$,

$-CH_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,

$-CH_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,

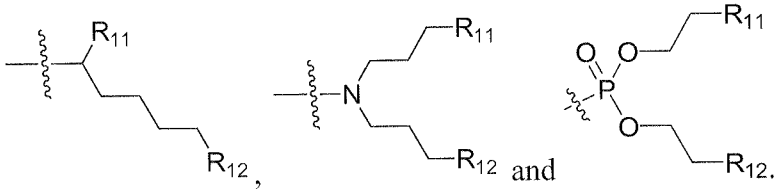
$-(CH_2)_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,

$-(CH_2)_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,

$-CH_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$, and

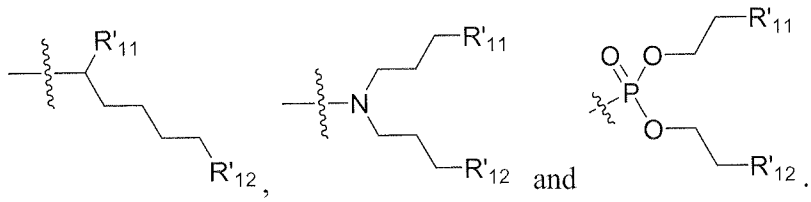
$-(CH_2)_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$.

According to the present invention, some examples of the $X(Q_1)(Q_2)(Q_3)$ moiety include:



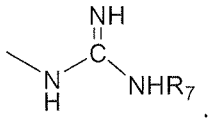
and

Some examples of the $X'(Q'_1)(Q'_2)(Q'_3)$ moiety include:

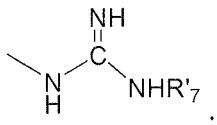


and

In one preferred embodiment, both R_{11} and R_{12} include:



Preferably, both R'_{11} and R'_{12} include:



B. Preparation of Compounds of Formula (I)

Synthesis of representative, specific compounds, is set forth in the Examples. Generally, however, the compounds of the present invention can be prepared in several fashions. The methods of preparing compounds of Formula (I) described herein include reacting an amine-functionalized cholesterol (functionalized cholesterol) with 1H-pyrazole-1-carboxamidine to provide a guanidinium moiety. The amine linked to cholesterol can be a primary and/or secondary amine and the amines in 1H-pyrazole-1-carboxamidine can be unsubstituted or substituted.

In one embodiment, the methods of preparing compounds of Formula (I) described herein include reacting a cholesterol derivative having a disulfide bond with an amine-containing moiety, followed by conversion of the amine to a guanidinium to provide cationic lipids having a disulfide bond.

In another embodiment, the methods of preparing compounds of Formula (I) described herein include reacting a cholesterol derivative having a ketal bond with an amine-containing moiety, followed by conversion of the amine to a guanidinium to provide cationic lipids having a ketal or acetal moiety.

In yet another embodiment, the methods of preparing compounds of Formula (I) described herein include reacting a cholesterol derivative having an aldehyde with an amine-containing moiety to form an imine, followed by conversion of the amine to a guanidinium to provide cationic lipids having an imine moiety.

One illustrative example of preparing cholesteryl cationic lipids containing a disulfide bond is shown in FIG. 1. First, cholesterol is reacted with an amine-protected cysteine containing 2-nitropyridyl disulfide group to form a cholesteryl cysteine ester (compound **3**) in the presence of a coupling agent (EDC) and a base (DMAP). The 2-nitropyridyl disulfide group

of the ester is reacted with a bifunctional spacer containing a thiol group and an amine-protecting group to form a disulfide bond. Removal of the amine protecting group of the bifunctional spacer, followed by conjugation with a branching moiety having terminal amines provides an amine-functionalized cholesterol. The terminal amines of the amine-functionalized cholesterol are treated with 1H-pyrazole-1-carboxaimidine to provide cationic lipids containing a disulfide bond.

Another illustrative example of preparing cholesteryl cationic lipids containing a ketal-containing linker is shown in FIGs. 2 and 3. A bifunctional linker containing a ketal bond (compound **23**) is prepared. One of the diamines of the ketal-containing bifunctional linker is protected with ethyl trifluoroacetate. An activated cholesterol carbonate such as cholesteryl chloroformate, cholesteryl NHS carbonate, or cholesteryl PNP carbonate, is reacted with the other nucleophile amine in the bifunctional linker, followed by deprotection of trifluoroacetamide group to prepare a cholesterol derivative with a terminal amine. The terminal amine is further reacted with lysine to prepare a cholesterol derivative with a branching moiety (compound **30**). The amines on the branching moiety of the cholesterol derivative are reacted with 1H-pyrazole-1-carboxamidine to provide cholesteryl cationic lipids containing a ketal group.

Yet another illustrative example of preparing cholesteryl cationic lipids including an imine linker is shown in FIG. 4. A bifunctional linker containing an amine and protected amines (compound **44**) is prepared from compounds **41** and **42** in two steps. An activated cholesterol carbonate such as cholesteryl chloroformate, cholesteryl NHS carbonate, or cholesteryl PNP carbonate, is reacted with an aldehyde containing compound (e.g. 3-methoxy-4-hydroxybenzaldehyde) to provide a cholesteryl derivative containing an aldehyde. The nucleophilic amine of the bifunctional linker is reacted with the cholesteryl derivative containing aldehyde to form an imine bond, followed by an amine deprotection in a mild basic condition to provide a cholesteryl derivative containing terminal amines. The terminal amines are reacted with 1H-pyrazole-1-carboxamidine to provide cholesteryl cationic lipids containing an imine group.

According to the present invention, the methods can employ alternative art-known techniques to prepare the compounds of Formula (I) without undue experimentation.

Attachment of an amine-containing compound to cholesterol can be carried out using standard organic synthetic techniques in the presence of a base, using coupling agents known to those of ordinary skill in the art such as 1,3-diisopropylcarbodiimide (DIPC), dialkyl carbodiimides, 2-halo-1-alkylpyridinium halides, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), propane phosphonic acid cyclic anhydride (PPACA) and phenyl dichlorophosphates.

In a further embodiment, when cholesterol or amine-containing compound is activated with a leaving group such as NHS, PNP, or chloroformate, a coupling agent is not required and the reaction proceeds in the presence of a base.

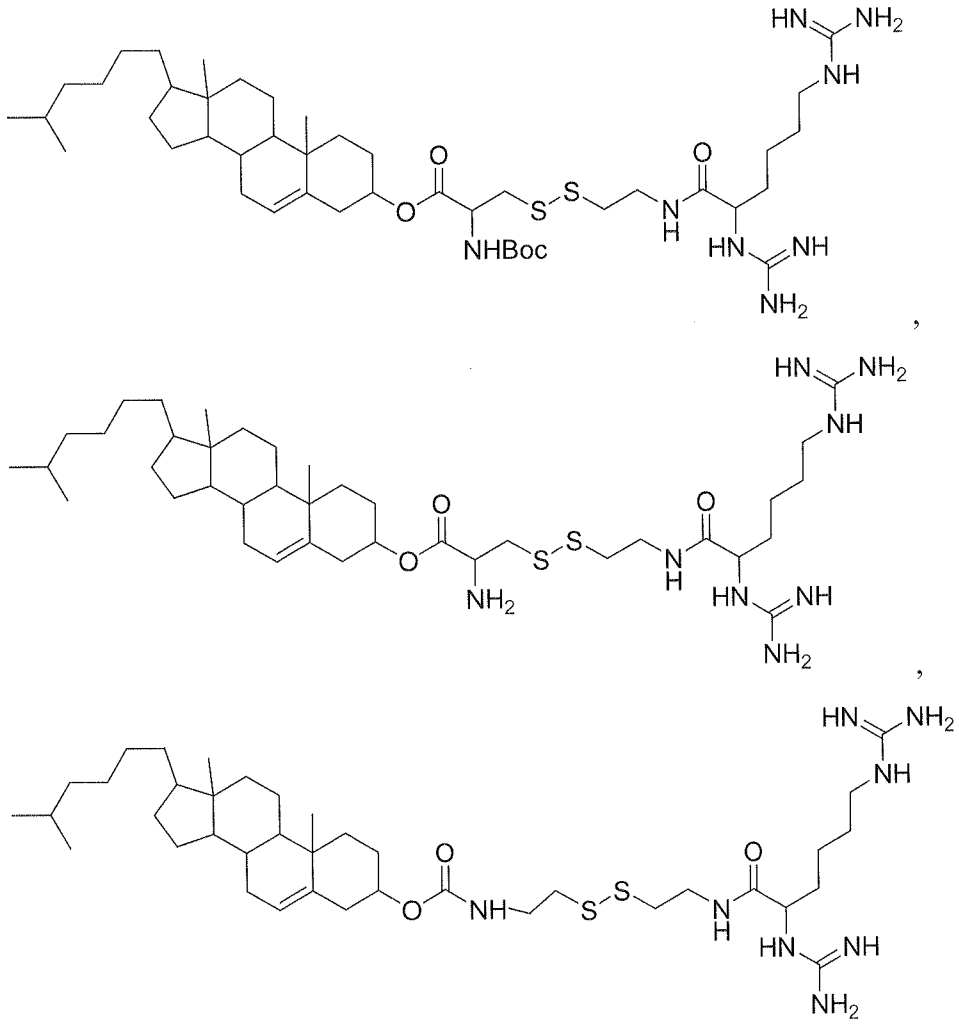
Generally, the compounds of Formula (I) described herein are preferably prepared by reacting an activated cholesterol with an amine-containing nucleophile in the presence of a base such as DMAP or DIEA. Preferably, the reaction is carried out in an inert solvent such as methylene chloride, chloroform, toluene, DMF or mixtures thereof. The reaction is also preferably conducted in the presence of a base, such as DMAP, DIEA, pyridine, triethylamine, etc. at a temperature of from -4 °C to about 70 °C (e.g. -4 °C to about 50 °C). In one preferred embodiment, the reaction is performed at a temperature of from 0 °C to about 25 °C or 0 °C to about room temperature.

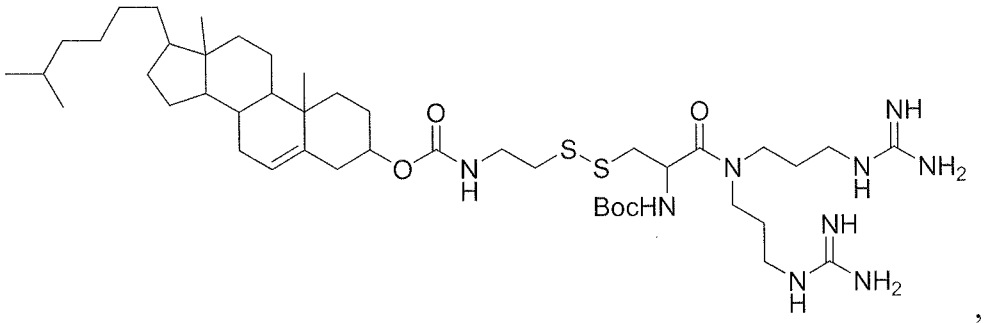
Removal of a protecting group from an amine-containing compound can be carried out with a strong acid such as trifluoroacetic acid (TFA), HCl, sulfuric acid, etc., or catalytic hydrogenation, radical reaction, etc. Alternatively, removal of an amine protecting group can be carried out with a base such as piperidine. In one embodiment, deprotection of Boc group is carried out with HCl solution in dioxane. In another embodiment, deprotection of Fmoc group is carried out with piperidine. The deprotection reaction can be carried out at a temperature from -4 °C to about 50 °C. Preferably, the reaction is carried out at a temperature from 0 °C to about 25 °C or to room temperature. In another embodiment, the deprotection of Boc group is carried out at room temperature.

Conversion of an amine to a guanidinium moiety is carried out by reacting an amine linked to cholesterol (e.g., the amines of compound **9**) with 1H-pyrazole-1-carboxamidine in an inert solvent such as methylene chloride, chloroform, DMF or mixtures thereof. Other reagents, such as N-BOC-1H-Pyrazole-1-carboxamidine or N,N'-Di-(tert-butoxycarbonyl)thiourea and a coupling reagent can be also used to convert the amine to a guanidine moiety.

Coupling agents known to those of ordinary skill in the art, such as 1,3-diisopropylcarbodiimide (DIPC), dialkyl carbodiimides, 2-halo-1-alkylpyridinium halides, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), propane phosphonic acid cyclic anhydride (PPACA) and phenyl dichlorophosphates, can be employed in the preparation of cationic lipids described herein. The reaction preferably is conducted in the presence of a base, such as DMAP, DIEA, pyridine, triethylamine, etc. at a temperature from -4 °C to about 50 °C. In one preferred embodiment, the reaction is performed at a temperature from 0 °C to about 25 °C or to room temperature.

Some representative embodiments prepared by the methods described herein include, but are not limited to:





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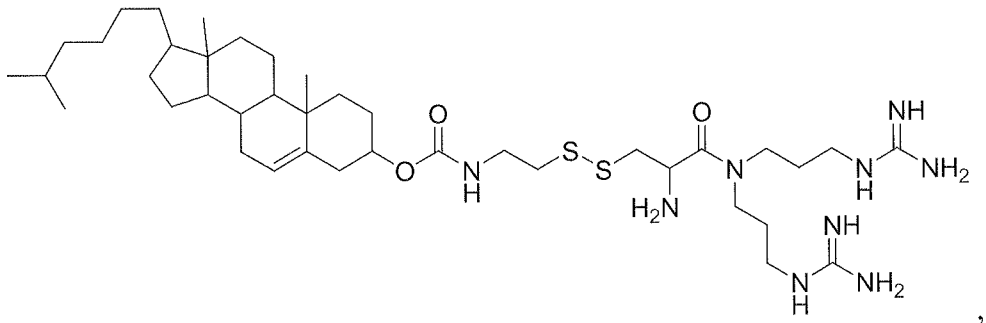

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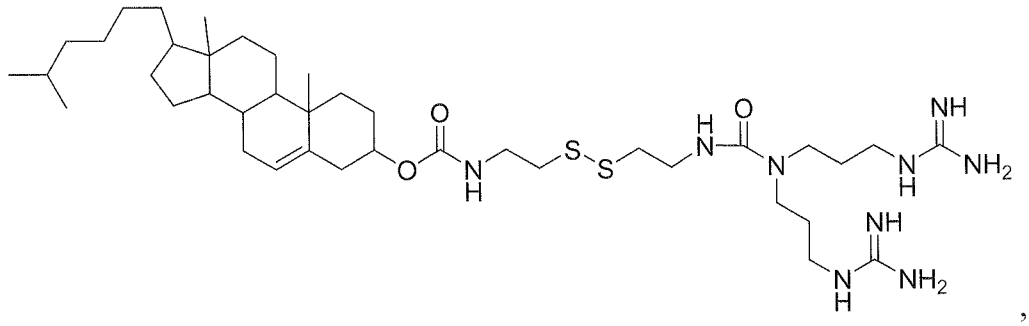

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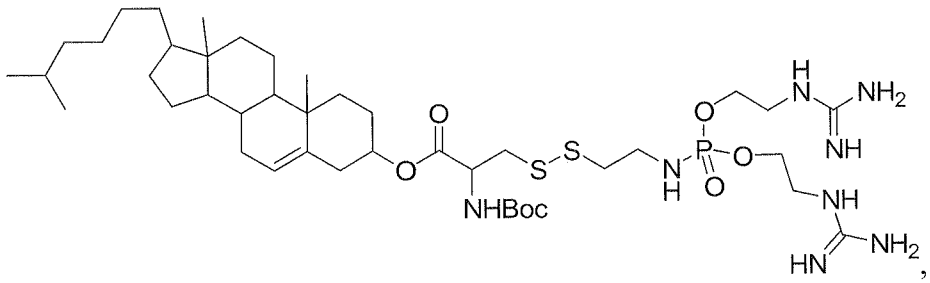

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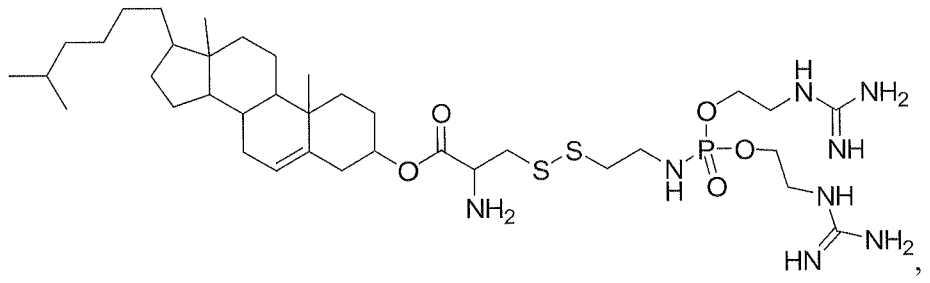

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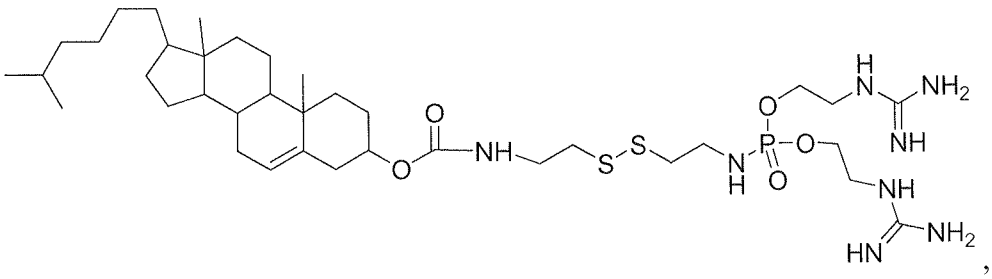

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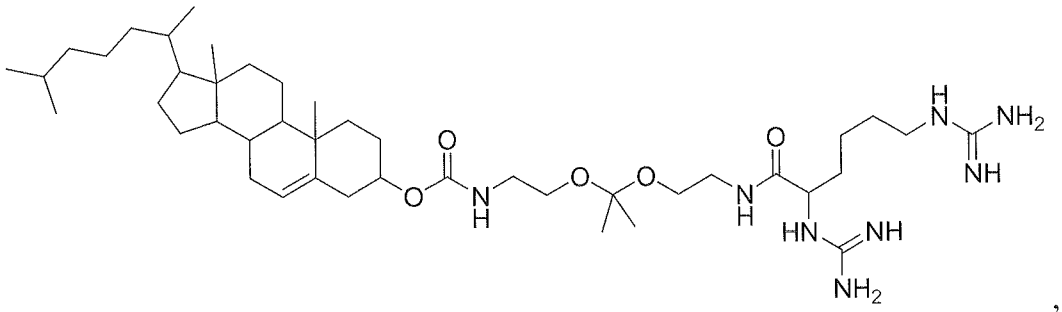

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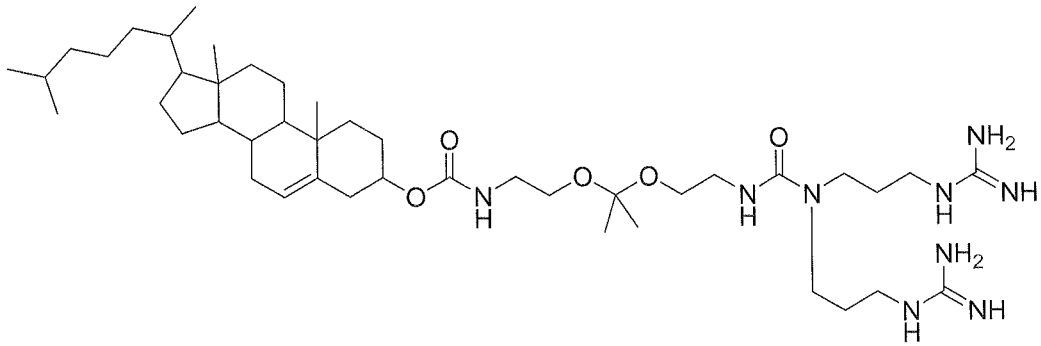

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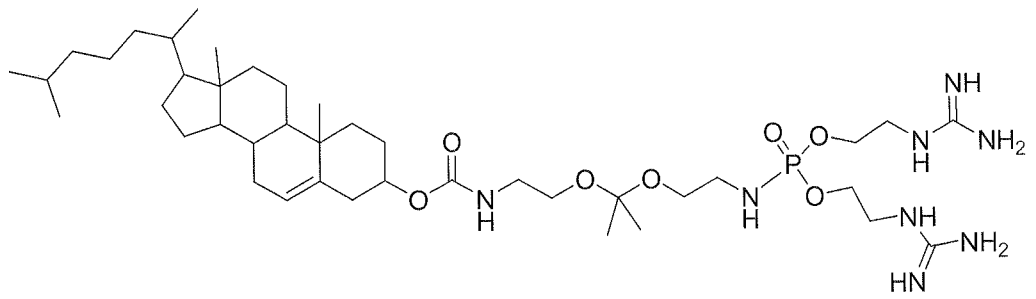

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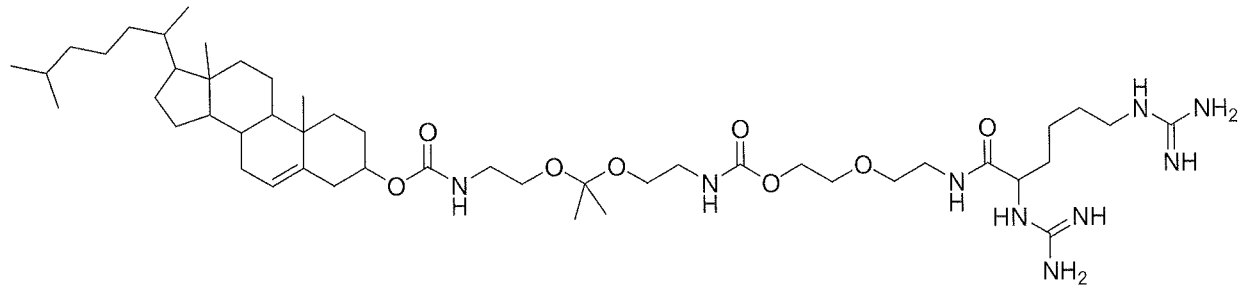

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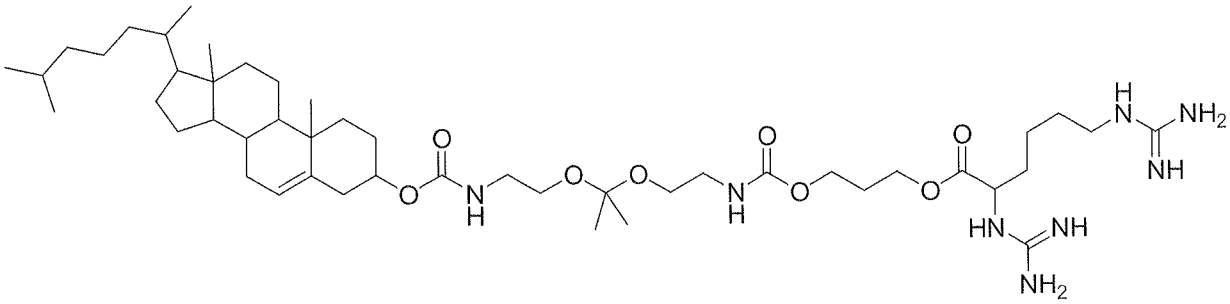

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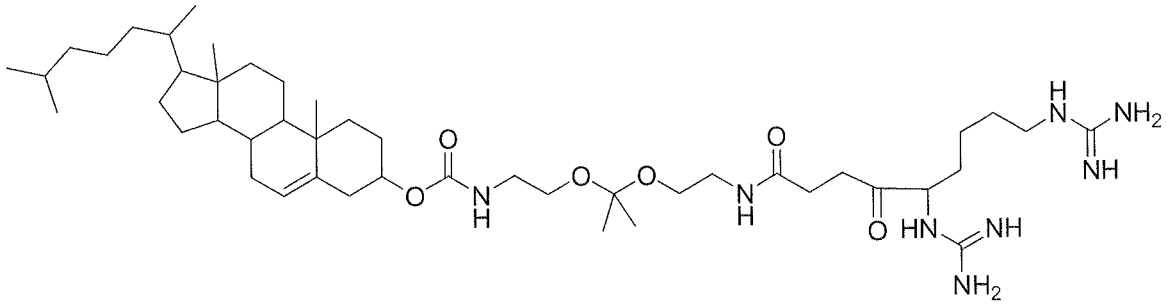

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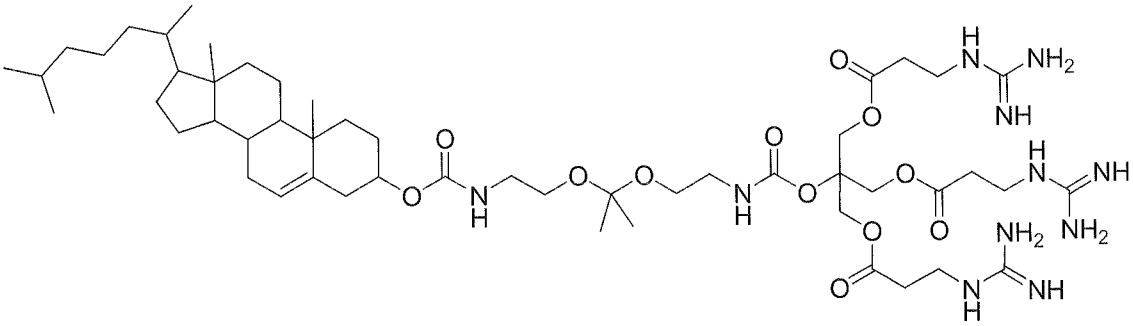

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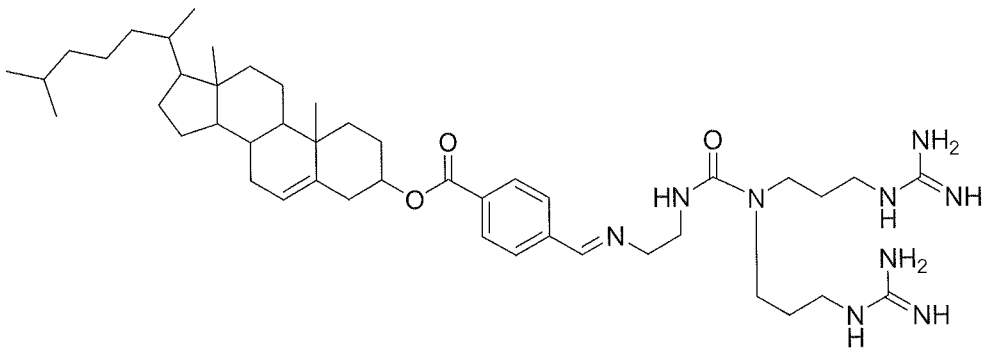

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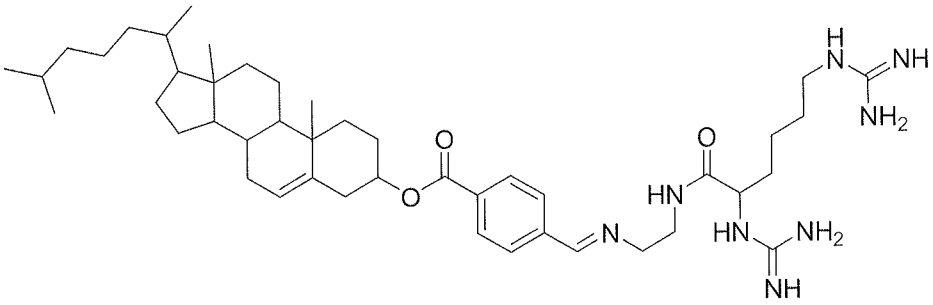

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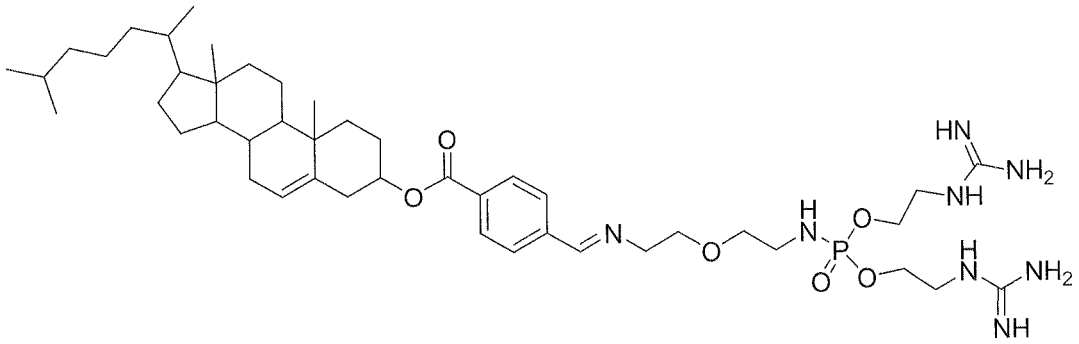

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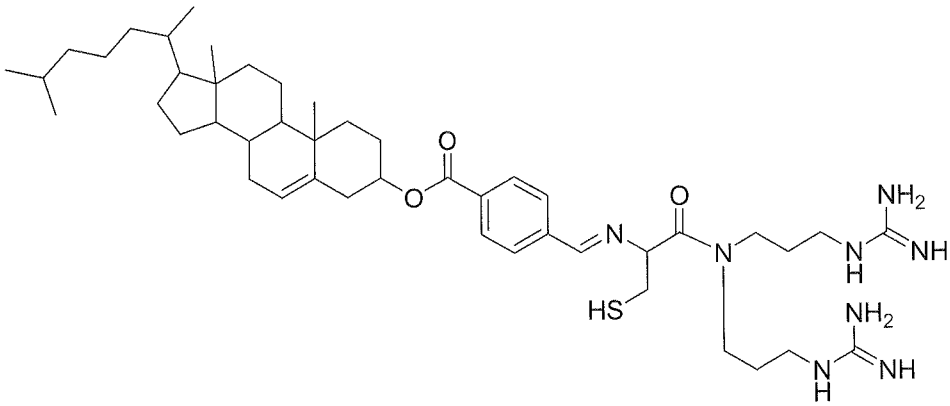

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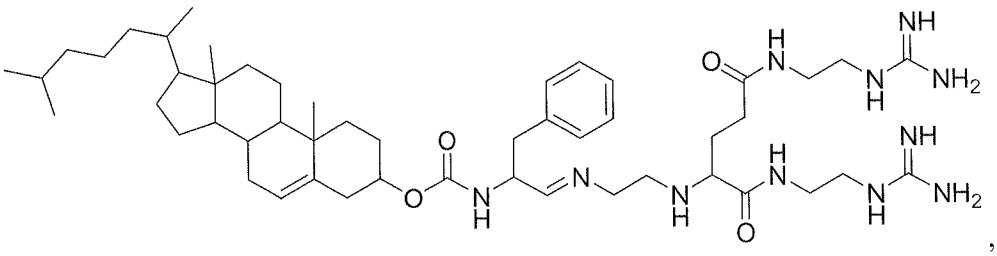

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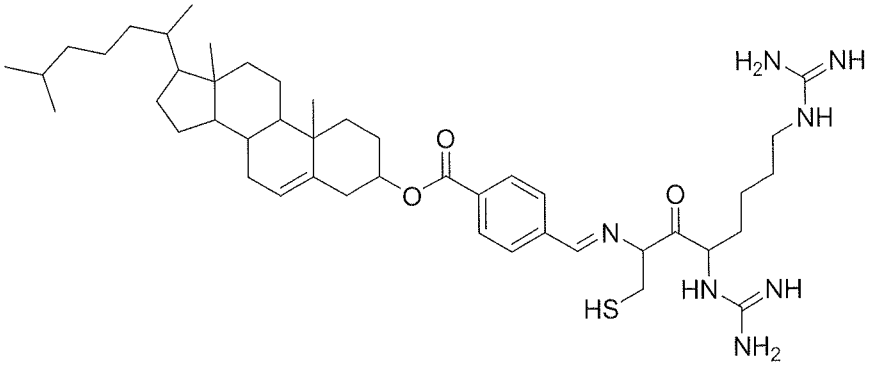

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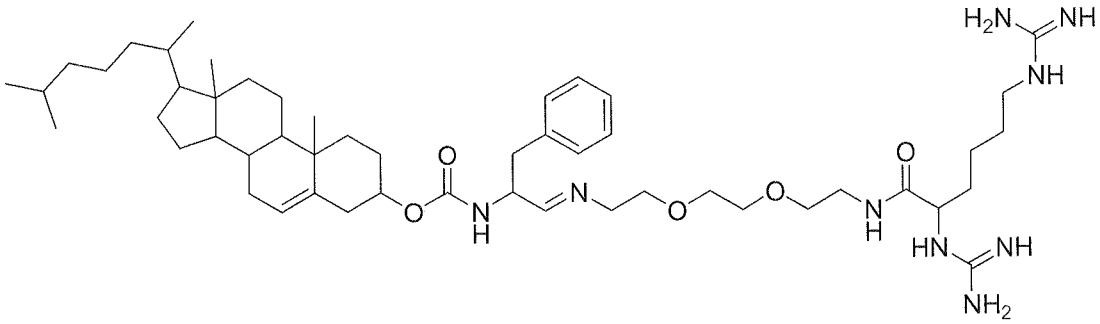

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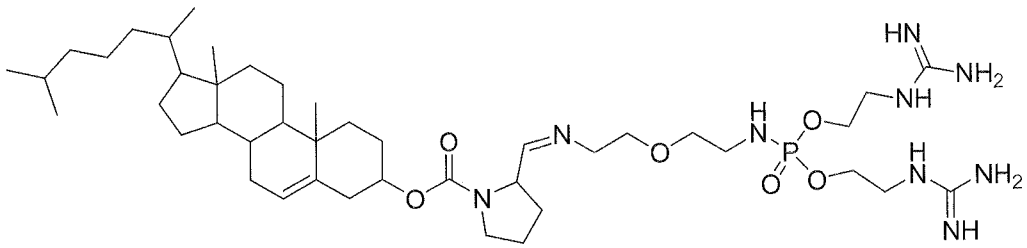

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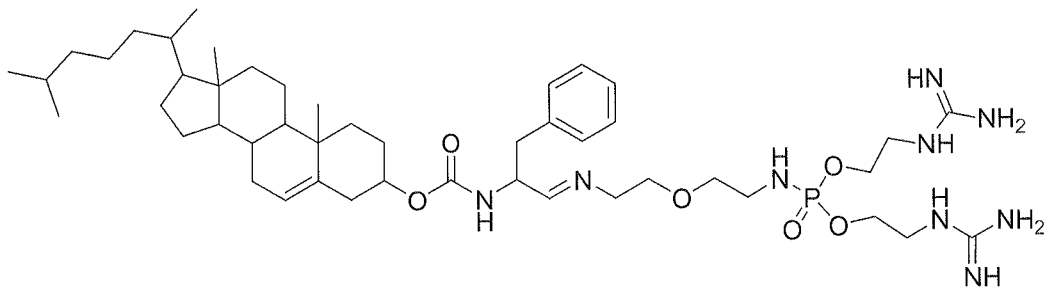

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C. Nanoparticle Compositions/Formulations

1. Overview

In one aspect of the invention, the nanoparticle composition contains a cationic lipid.

According to the present invention the nanoparticle composition contains a compound of

10 Formula (I), a fusogenic lipid, and a PEG-lipid.

In one preferred aspect, the nanoparticle composition includes cholesterol.

In a further aspect of the present invention, the nanoparticle composition described herein may contain additional art-known cationic lipids. The nanoparticle composition containing a mixture of different fusogenic lipids (non-cationic lipids) and/or a mixture of different PEG-lipids are also contemplated.

In another aspect, the nanoparticle composition contains cationic lipids including compounds of Formula (I) in a molar ratio ranging from about 10% to about 99.9% of the total lipid (pharmaceutical carrier) present in the nanoparticle composition.

The cationic lipid component can range from about 2% to about 60%, from about 5% to about 50%, from about 10% to about 45%, from about 15% to about 25%, or from about 30% to about 40% of the total lipid present in the nanoparticle composition.

In one embodiment, the cationic lipid is present in amounts of from about 15 to about 25 % (i.e., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25%) of the total lipid present in the nanoparticle composition.

According to the present invention, the nanoparticle compositions can contain a total fusogenic/non-cationic lipid, including cholesterol and/or noncholesterol-based fusogenic lipid, in a molar ratio of from about 20% to about 85%, from about 25% to about 85%, from about 60% to about 80% (e.g., 65, 75, 78, or 80%) of the total lipid present in the nanoparticle composition. In one embodiment, the total fusogenic/non-cationic lipid is about 80% of the total lipid present in the nanoparticle composition.

In certain embodiments, a noncholesterol-based fusogenic/non-cationic lipid is present in a molar ratio of from about 25 to about 78% (25, 35, 47, 60, or 78%), or from about 60 to about 78% of the total lipid present in the nanoparticle composition. In one embodiment, a noncholesterol-based fusogenic/non-cationic lipid is about 60% of the total lipid present in the nanoparticle composition.

In certain embodiments, the nanoparticle composition includes cholesterol in addition to non-cholesterol fusogenic lipid, in a molar ratio ranging from about 0% to about 60%, from about 10% to about 60%, or from about 20% to about 50% (e.g., 20, 30, 40 or 50%) of the total lipid present in the nanoparticle composition. In one embodiment, cholesterol is about 20% of the total lipid present in the nanoparticle composition.

In certain embodiments, the PEG-lipid contained in the nanoparticle composition ranges in a molar ratio of from about 0.5 % to about 20 %, from about 1.5% to about 18% of the total

lipid present in the nanoparticle composition. In one embodiment of the nanoparticle composition, the PEG lipid is included in a molar ratio of from about 2% to about 10% (e.g., 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the total lipid. For example, a total PEG lipid is about 2% of the total lipid present in the nanoparticle composition.

5

2. Cationic Lipids

In one aspect of the invention, compounds of Formula (I) are included in a nanoparticle composition.

In a further aspect of the invention, the nanoparticle composition described herein can
10 include additional art-known cationic lipids. Additional suitable lipids contemplated include for example:

N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA);

1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane or N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP);

15 1,2-bis(dimyrstoyloxy)-3-3-(trimethylammonia)propane (DMTAP);

1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide or N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE);

dimethyldioctadecylammonium bromide or N,N-distearyl-N,N-dimethylammonium bromide (DDAB);

20 3-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol (DC-Cholesterol);

3β-[N',N'-diguanidinoethyl-aminoethane)carbamoyl cholesterol (BGTC);

2-(2-(3-(bis(3-aminopropyl)amino)propylamino)acetamido)-N,N-ditetradecylacetamide (RPR209120);

1,2-dialkenoyl-sn-glycero-3-ethylphosphocholines (i.e., 1,2-dioleoyl-sn-glycero-3-
25 ethylphosphocholine, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine and 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine);

tetramethyltetrapalmitoyl spermine (TMTPS);

tetramethyltetraoleyl spermine (TMTOS);

tetramethlytetralauryl spermine (TMTLS);

30 tetramethyltetramyristyl spermine (TMTMS);

tetramethyldioleyl spermine (TMDOS);

2,5-bis(3-aminopropylamino)-N-(2-(dioctadecylamino)-2-oxoethyl) pentanamide (DOGS);

2,5-bis(3-aminopropylamino)-N-(2-(di(Z)-octadeca-9-dienylamino)-2-oxoethy-l) pentanamide (DOGS-9-en);

2,5-bis(3-aminopropylamino)-N-(2-(di(9Z,12Z)-octadeca-9,12-dienylamino)-2-oxoethyl) pentanamide (DLinGS);

N4-Spermine cholesteryl carbamate (GL-67);

(9Z,9'Z)-2-(2,5-bis(3-aminopropylamino)pentanamido)propane-1,3-diyl-dioctadec-9-enoate (DOSPER);

2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA);

1,2-dimyristoyl-3-trimethylammonium-propane; 1,2-distearoyl-3-trimethylammonium-propane;

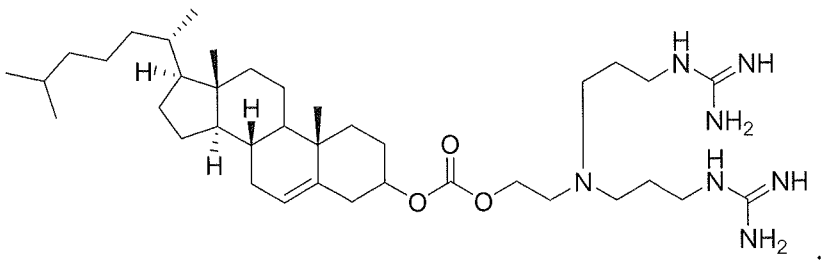
dioctadecyldimethylammonium (DODMA);

distearyldimethylammonium (DSDMA);

N,N-dioleyl-N,N-dimethylammonium chloride (DODAC); pharmaceutically acceptable salts and mixtures thereof.

Details of cationic lipids are also described in US2007/0293449 and U.S. Pat. Nos. 4,897,355; 5,279,833; 6,733,777; 6,376,248; 5,736,392; 5,686,958; 5,334,761; 5,459,127; 2005/0064595; 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992.

In a further embodiment, the nanoparticle compositions described herein can contain cationic lipids described in PCT/US09/52396, the contents of which are incorporated herein by reference. For example, the nanoparticle compositions described herein can include a mixture of compounds of Formula (I) and the following:



Additionally, commercially available preparations including cationic lipids can be used: for example, LIPOFECTIN® (cationic liposomes containing DOTMA and DOPE, from

GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (cationic liposomes containing DOSPA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); and TRANSFECTAM® (cationic liposomes containing DOGS from Promega Corp., Madison, Wisconsin, USA).

5

3. Fusogenic/Non-cationic Lipids

According to the present invention, the nanoparticle composition can contain a fusogenic lipid. The fusogenic lipids include non-cationic lipids such as neutral uncharged, zwitter ionic and anionic lipids. For purposes of the present invention, the terms "fusogenic lipid" and "non-
10 cationic lipids" are interchangeable.

Neutral lipids include a lipid that exists either in an uncharged or neutral zwitter ionic form at a selected pH, preferably at physiological pH. Examples of such lipids include diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides and diacylglycerols.

15 Anionic lipids include a lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and neutral lipids modified with other anionic
20 modifying groups.

Many fusogenic lipids include amphipathic lipids generally having a hydrophobic moiety and a polar head group, and can form vesicles in aqueous solution.

Fusogenic lipids contemplated include naturally-occurring and synthetic phospholipids and related lipids.

25 A non-limiting list of the non-cationic lipids are selected from among phospholipids and nonphosphous lipid-based materials, such as lecithin; lysolecithin; diacylphosphatidylcholine; lysophosphatidylcholine; phosphatidylethanolamine; lysophosphatidylethanolamine; phosphatidylserine; phosphatidylinositol; sphingomyelin; cephalin; ceramide; cardiolipin; phosphatidic acid; phosphatidylglycerol; cerebrosides; dicetylphosphate;

30 1,2-dilauroyl-sn-glycerol (DLG);

1,2-dimyristoyl-sn-glycerol (DMG);

1,2-dipalmitoyl-sn-glycerol (DPG);

1,2-distearoyl-sn-glycerol (DSG);

1,2-dilauroyl-sn-glycero-3-phosphatidic acid (DLPA);

1,2-dimyristoyl-sn-glycero-3-phosphatidic acid (DMPA);

1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA);

1,2-distearoyl-sn-glycero-3-phosphatidic acid (DSPA);

1,2-diarachidoyl-sn-glycero-3-phosphocholine (DAPC);

1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC);

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC);

1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine (DPePC);

1,2-dipalmitoyl-sn-glycero-3-phosphocholine or dipalmitoylphosphatidylcholine (DPPC);

1,2-distearoyl-sn-glycero-3-phosphocholine or distearoylphosphatidylcholine (DSPC);

1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE);

1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine or dimyristoylphosphoethanolamine (DMPE);

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine or dipalmitoylphosphatidyl-ethanolamine (DPPE);

1,2-distearoyl-sn-glycero-3-phosphoethanolamine or distearoylphosphatidyl-ethanolamine (DSPE);

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine or dioleoylphosphatidylethanolamine (DOPE);

1,2-dilauroyl-sn-glycero-3-phosphoglycerol (DLPG);

1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) or 1,2-dimyristoyl-sn-glycero-3-phospho-sn-1-glycerol (DMP-sn-1-G);

1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol or dipalmitoylphosphatidylglycerol (DPPG);

1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) or 1,2-distearoyl-sn-glycero-3-phospho-sn-1-glycerol (DSP-sn-1-G);

1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS);

1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLinoPC);

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine or palmitoyloleoylphosphatidylcholine (POPC);

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG);

1-palmitoyl-2-lyso-sn-glycero-3-phosphocholine (P-lyso-PC);

1-stearoyl-2-lyso-sn-glycero-3-phosphocholine (S-lyso-PC);

diphytanoylphosphatidylethanolamine (DPhPE);

1,2-dioleoyl-sn-glycero-3-phosphocholine or dioleoylphosphatidylcholine (DOPC);

1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC),

dioleoylphosphatidylglycerol (DOPG);

palmitoyloleoylphosphatidylethanolamine (POPE);

dioleoyl- phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal);

16-O-monomethyl PE;

16-O-dimethyl PE;

18-1-trans PE; 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE);

1,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE); and pharmaceutically acceptable salts thereof and mixtures thereof. Details of the fusogenic lipids are described in US Patent Publication Nos. 2007/0293449 and 2006/0051405.

Noncationic lipids include sterols or steroid alcohols such as cholesterol.

Additional non-cationic lipids are, e.g., stearylamine, dodecylamine, hexadecylamine, acetylpalmitate, glycerolricinoleate, hexadecylstereate, isopropylmyristate, amphoteric acrylic polymers, triethanolaminelauryl sulfate, alkylarylsulfate polyethyloxylated fatty acid amides, and dioctadecyldimethyl ammonium bromide.

Anionic lipids contemplated include phosphatidylserine, phosphatidic acid, phosphatidylcholine, platelet-activation factor (PAF), phosphatidylethanolamine, phosphatidyl-DL-glycerol, phosphatidylinositol, phosphatidylinositol, cardiolipin, lysophosphatides, hydrogenated phospholipids, sphingoplipids, gangliosides, phytosphingosine, sphinganines, pharmaceutically acceptable salts and mixtures thereof.

Suitable noncationic lipids useful for the preparation of the nanoparticle composition described herein include diacylphosphatidylcholine (e.g., distearoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and dilinoleoylphosphatidyl-

choline), diacylphosphatidylethanolamine (e.g., dioleoylphosphatidylethanolamine and palmitoyloleoylphosphatidylethanolamine), ceramide or sphingomyelin. The acyl groups in these lipids are preferably fatty acids having saturated and unsaturated carbon chains such as linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, and lauroyl. More preferably, the acyl groups are lauroyl, myristoyl, palmitoyl, stearoyl or oleoyl. Alternatively and/preferably, the fatty acids have saturated and unsaturated C_8-C_{30} (preferably C_{10}-C_{24}) carbon chains.

A variety of phosphatidylcholines useful in the nanoparticle composition described herein includes:

1,2-didecanoyl-sn-glycero-3-phosphocholine (DDPC, C10:0, C10:0);

1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC, C12:0, C12:0);

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, C14:0, C14:0);

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, C16:0, C16:0);

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, C18:0, C18:0);

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, C18:1, C18:1);

1,2-dierucoyl-sn-glycero-3-phosphocholine (DEPC, C22:1, C22:1);

1,2-dieicosapentaenoyl-sn-glycero-3-phosphocholine (EPA-PC, C20:5, C20:5);

1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine (DHA-PC, C22:6, C22:6);

1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC, C14:0, C16:0);

1-myristoyl-2-stearoyl -sn-glycero-3-phosphocholine (MSPC, C14:0, C18:0);

1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (PMPC, C16:0, C14:0);

1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (PSPC, C16:0, C18:0);

1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine (SMPC, C18:0, C14:0);

1-stearoyl-2-palmitoyl -sn-glycero-3-phosphocholine (SPPC, C18:0, C16:0);

1,2-myristoyl-oleoyl-sn-glycero-3-phosphoethanolamine (MOPC, C14:0, C18:0);

1,2-palmitoyl-oleoyl -sn-glycero-3-phosphoethanolamine (POPC, C16:0, C18:1);

1,2-stearoyl-oleoyl -sn-glycero-3-phosphoethanolamine (POPC, C18:0, C18:1), and pharmaceutically acceptable salts thereof and mixtures thereof.

A variety of lysophosphatidylcholine useful in the nanoparticle composition described herein includes:

1-myristoyl-2-lyso-sn-glycero-3-phosphocholine (M-LysoPC, C14:0);

1-malmitoyl-2-lyso-sn-glycero-3-phosphocholine (P-LysoPC, C16:0);

1- stearoyl-2-lyso-sn-glycero-3-phosphocholine (S-LysoPC, C18:0), and pharmaceutically acceptable salts thereof and mixtures thereof. .

A variety of phosphatidylglycerols useful in the nanoparticle composition described herein are selected from among:

hydrogenated soybean phosphatidylglycerol (HSPG);

non-hydrogenated egg phosphatidylgycerol (EPG);

1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG, C14:0, C14:0);

1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG, C16:0, C16:0);

1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG, C18:0, C18:0);

1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG, C18:1, C18:1);

1,2-dierucoyl-sn-glycero-3-phosphoglycerol (DEPG, C22:1, C22:1);

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG, C16:0, C18:1), and pharmaceutically acceptable salts thereof and mixtures thereof.

A variety of phosphatidic acids useful in the nanoparticle composition described herein includes:

1,2-dimyristoyl-sn-glycero-3-phosphatidic acid (DMPA, C14:0, C14:0);

1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA, C16:0, C16:0);

1,2-distearoyl-sn-glycero-3-phosphatidic acid (DSPA, C18:0, C18:0), and pharmaceutically acceptable salts thereof and mixtures thereof.

A variety of phosphatidylethanolamines useful in the nanoparticle composition described herein includes:

hydrogenated soybean phosphatidylethanolamine (HSPE);

non-hydrogenated egg phosphatidylethanolamine (EPE);

1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE, C14:0, C14:0);

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, C16:0, C16:0);

1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE, C18:0, C18:0);

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, C18:1, C18:1);

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DEPE, C22:1, C22:1);

1,2-dierucoyl-sn-glycero-3-phosphoethanolamine (POPE, C16:0, C18:1), and pharmaceutically acceptable salts thereof and mixtures thereof.

A variety of phosphatidylserines useful in the nanoparticle composition described herein includes:

1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS, C14:0, C14:0);

1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS, C16:0, C16:0);

1,2-distearoyl-sn-glycero-3-phospho-L-serine (DSPS, C18:0, C18:0);

1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, C18:1, C18:1);

1-palmitoyl-2-oleoyl-sn-3-phospho-L-serine (POPS, C16:0, C18:1), and pharmaceutically acceptable salts thereof and mixtures thereof.

In one preferred embodiment, suitable neutral lipids useful for the preparation of the nanoparticle composition described herein include, for example,

dioleoylphosphatidylethanolamine (DOPE),

distearoylphosphatidylethanolamine (DSPE),

palmitoyloleoylphosphatidylethanolamine (POPE),

egg phosphatidylcholine (EPC),

dipalmitoylphosphatidylcholine (DPPC),

distearoylphosphatidylcholine (DSPC),

dioleoylphosphatidylcholine (DOPC),

palmitoyloleoylphosphatidylcholine (POPC),

dipalmitoylphosphatidylglycerol (DPPG),

dioleoylphosphatidylglycerol (DOPG),

dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), cholesterol, pharmaceutically acceptable salts and mixtures thereof.

In certain preferred embodiments, the nanoparticle composition described herein includes DSPC, EPC, DOPE, etc, and mixtures thereof.

In a further aspect of the invention, the nanoparticle composition contains non-cationic lipids such as sterol. The nanoparticle composition preferably contains cholesterol or analogs thereof, and more preferably cholesterol.

4. PEG lipids

According to the present invention, the nanoparticle composition described herein contains a PEG lipid. The PEG lipids extend circulation of the nanoparticle described herein and

prevent the premature excretion of the nanoparticles from the body. The PEG lipids reduce the immunogenicity and enhance the stability of the nanoparticles.

The PEG lipids useful in the nanoparticle compositions include PEGylated forms of fusogenic/noncationic lipids. The PEG lipids include, for example, PEG conjugated to diacylglycerol (PEG-DAG), PEG conjugated to diacylglycamides, PEG conjugated to dialkyloxypropyls (PEG-DAA), PEG conjugated to phospholipids such as PEG coupled to phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides (PEG-Cer), PEG conjugated to cholesterol derivatives (PEG-Chol) or mixtures thereof. See U.S. Patent Nos. 5,885,613 and 5,820,873, and US Patent Publication No. 2006/051405, the contents of each of which are incorporated herein by reference.

PEG is generally represented by the structure:

$$-O-(CH_2CH_2O)_n-$$

where (n) is a positive integer from about 5 to about 2300, preferably from about 5 to about 460 so that the polymeric portion of PEG lipid has an average number molecular weight of from about 200 to about 100,000 daltons, preferably from about 200 to about 20,000 daltons. (n) represents the degree of polymerization for the polymer, and is dependent on the molecular weight of the polymer.

In one preferred aspect, the PEG is a polyethylene glycol with a number average molecular weight ranging from about 200 to about 20,000 daltons, more preferably from about 500 to about 10,000 daltons, yet more preferably from about 1,000 to about 5,000 daltons (i.e., about 1,500 to about 3,000 daltons). In one embodiment, the PEG has a molecular weight of about 2,000 daltons. In another embodiment, the PEG has a molecular weight of about 750 daltons.

Alternatively, the polyethylene glycol (PEG) residue portion can be represented by the structure:

$$-Y_{71}-(CH_2CH_2O)_n-CH_2CH_2Y_{71}-,$$

$$-Y_{71}-(CH_2CH_2O)_n-CH_2C(=Y_{72})-Y_{71}-,$$

$$-Y_{71}-C(=Y_{72})-(CH_2)_{a12}-Y_{73}-(CH_2CH_2O)_n-CH_2CH_2-Y_{73}-(CH_2)_{a12}-C(=Y_{72})-Y_{71}- \text{ and}$$

$$-Y_{71}-(CR_{71}R_{72})_{a12}-Y_{73}-(CH_2)_{b12}-O-(CH_2CH_2O)_n-(CH_2)_{b12}-Y_{73}-(CR_{71}R_{72})_{a12}-Y_{71}-,$$

wherein:

Y_{71} and Y_{73} are independently O, S, SO, SO_2, NR_{73} or a bond;

Y_{72} is O, S, or NR_{74}, preferably oxygen;

R_{71-74} are independently selected from among hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy and substituted arylcarbonyloxy, preferably hydrogen, methyl, ethyl or propyl;

(a12) and (b12) are independently zero or positive integers, preferably zero or an integer from about 1 to about 6 (i.e., 1, 2, 3, 4, 5, 6), and more preferably 1 or 2; and

(n) is an integer from about 5 to about 2300, preferably from about 5 to about 460.

The terminal end of PEG can end with H, NH_2, OH, CO_2H, C_{1-6} alkyl (e.g., methyl, ethyl, propyl), C_{1-6} alkoxy, acyl or aryl. In one preferred embodiment, the terminal hydroxyl group of PEG is substituted with a methoxy or methyl group. In one preferred embodiment, the PEG employed in the PEG lipid is methoxy PEG.

The PEG may be directly conjugated to lipids or via a linker moiety. The polymers for conjugation to a lipid structure are converted into a suitably activated polymer, using the activation techniques described in U.S. Patent Nos. 5,122,614 and 5,808,096 and other techniques known in the art without undue experimentation.

Examples of activated PEGs useful for the preparation of a PEG lipid include, for example, methoxypolyethylene glycol-succinate, mPEG-NHS, methoxypolyethylene glycol-succinimidyl succinate, methoxypolyethyleneglycol-acetic acid (mPEG-CH_2COOH), methoxypolyethylene glycol-amine (mPEG-NH_2), and methoxypolyethylene glycol-tresylate (mPEG-TRES).

In certain aspects, polymers having terminal carboxylic acid groups can be used for the preparation of the PEG lipids. Methods of preparing polymers having terminal carboxylic acids in high purity are described in U.S. Patent Application No. 11/328,662, the contents of which are incorporated herein by reference.

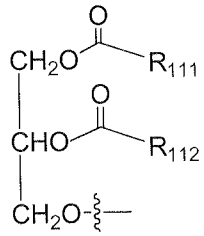
In alternative aspects, polymers having terminal amine groups can be employed to make the PEG-lipids. The methods of preparing polymers containing terminal amines in high purity

are described in U.S. Patent Application Nos. 11/508,507 and 11/537,172, the contents of each of which are incorporated by reference.

PEG and lipids can be bound via a linkage, i.e. a non-ester containing linker moiety or an ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a carbonate (OC(=O)O) linker moiety, a urea linker moiety, an ether linker moiety, a succinyl linker moiety, and combinations thereof. Suitable ester linker moieties include, e.g., succinoyl, phosphate esters (-O-P(=O)(OH)-O-), sulfonate esters, and combinations thereof.

In one embodiment, the nanoparticle composition described herein can include a polyethyleneglycol-diacylglycerol (PEG-DAG) or polyethylene-diacylglycamide. Suitable polyethyleneglycol-diacylglycerol or polyethyleneglycol-diacylglycamide conjugates include a dialkylglycerol or dialkylglycamide group having alkyl chain length independently containing from about C_4 to about C_{30} (preferably from about C_8 to about C_{24}) saturated or unsaturated carbon atoms. The dialkylglycerol or dialkylglycamide group can further include one or more substituted alkyl groups.

The term "diacylglycerol" (DAG) used herein refers to a compound having two fatty acyl chains, R_{111} and R_{112}. The R_{111} and R_{112} have the same or different carbon chain in length of about 4 to about 30 carbons (preferably about 8 to about 24) and are bonded to glycerol by ester linkages. The acyl groups can be saturated or unsaturated with various degrees of unsaturation. DAG has the general formula:



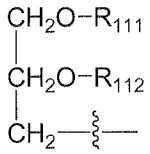
In one preferred embodiment, the PEG-diacylglycerol conjugate is a PEG-dilaurylglycerol (C12), a PEG-dimyristylglycerol (C14, DMG), a PEG-dipalmitoylglycerol (C16, DPG) or a PEG-distearylglycerol (C18, DSG). Those of skill in the art will readily appreciate that other diacylglycerols are also contemplated in the PEG-diacylglycol conjugate. Suitable PEG-diacylglycerol conjugates for use in the present invention, and methods of making and using them, are described in U.S. Patent Publication No. 2003/0077829, and PCT Patent

Application No. CA 02/00669, the contents of each of which are incorporated herein by reference.

Examples of the PEG-diacylglycerol conjugate can be selected from among PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylglycerol (C16), PEG-disterylglycerol (C18). Examples of the PEG-diacylglycamide conjugate includes PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoyl-glycamide (C16), and PEG-disterylglycamide (C18).

In another embodiment, the nanoparticle composition described herein can include a polyethyleneglycol-dialkyloxypropyl conjugates (PEG-DAA).

The term "dialkyloxypropyl" refers to a compound having two alkyl chains, R_{111} and R_{112}. The R_{111} and R_{112} alkyl groups include the same or different carbon chain length between about 4 to about 30 carbons (preferably about 8 to about 24). The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the general formula:



wherein R_{111} and R_{112} alkyl groups are the same or different alkyl groups having from about 4 to about 30 carbons (preferably about 8 to about 24). The alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C12), myristyl (C14), palmityl (C16), stearyl (C18), oleoyl (C18) and icosyl (C20).

In one embodiment, R_{111} and R_{112} are both the same, i.e., R_{111} and R_{112} are both myristyl (C14), both stearyl (C18) or both oleoyl (C18), etc. In another embodiment, R_{111} and R_{112} are different, i.e., R_{111} is myristyl (C14) and R_{112} is stearyl (C18). In a preferred embodiment, the PEG-dialkylpropyl conjugates include the same R_{111} and R_{112}.

In yet another embodiment, the nanoparticle composition described herein can include PEG conjugated to phosphatidylethanolamines (PEG-PE). The phosphatidylethanolaimes useful for the PEG lipid conjugation can contain saturated or unsaturated fatty acids with carbon chain lengths in the range of about 4 to about 30 carbons (preferably about 8 to about 24). Suitable phosphatidylethanolamines include, but are not limited to: dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidylethanolamine (DSPE).

In yet another embodiment, the nanoparticle composition described herein can include PEG conjugated to ceramides (PEG-Cer). Ceramides have only one acyl group. Ceramides can have saturated or unsaturated fatty acids with carbon chain lengths in the range of about 4 to about 30 carbons (preferably about 8 to about 24).

In alternative embodiments, the nanoparticle composition described herein can include PEG conjugated to cholesterol derivatives. The term "cholesterol derivative" means any cholesterol analog containing a cholesterol structure with modification, i.e., substitutions and/or deletions thereof. The term cholesterol derivative herein also includes steroid hormones and bile acids.

Illustrative examples of PEG lipids include N-(carbonyl-methoxypolyethyleneglycol)-1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (^{2kDa} mPEG-DMPE or ^{5kDa} mPEG-DMPE); N-(carbonyl-methoxypolyethyleneglycol)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (^{2kDa} mPEG-DPPE or ^{5kDa} mPEG-DPPE); N-(carbonyl-methoxypolyethyleneglycol)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (^{750Da}mPEG-DSPE, ^{2kDa} mPEG-DSPE, ^{5kDa} mPEG-DSPE); and pharmaceutically acceptable salts therof (i.e., sodium salt) and mixtures thereof.

In certain preferred embodiments, the nanoparticle composition described herein includes a PEG lipid having PEG-DAG or PEG-ceramide, wherein PEG has molecular weight from about 200 to about 20,000, preferably from about 500 to about 10,000, and more preferably from about 1,000 to about 5,000.

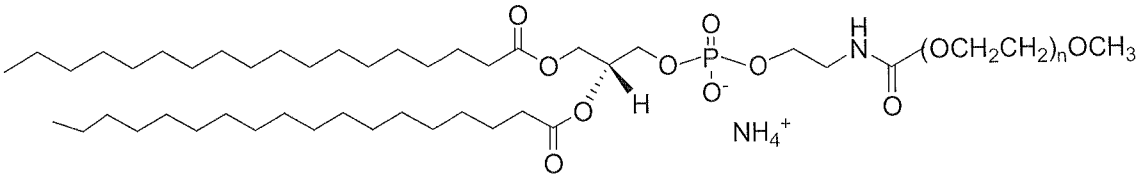
A few illustrative embodiments of PEG-DAG and PEG-ceramide are provided in Table 1.

Table 1.

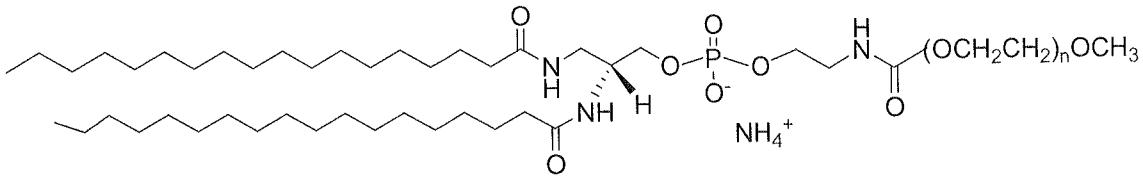
PEG-Lipid	
PEG-DAG	mPEG-diimyristoylglycerol
	mPEG-dipalmitoylglycerol
	mPEG-distearoylglycerol
PEG-Ceramide	mPEG-CerC8
	mPEG-CerC14
	mPEG-CerC16
	mPEG-CerC20

Preferably, the nanoparticle composition described herein includes the PEG lipid selected from among PEG-DSPE, PEG-dipalmitoylglycamide (C16), PEG-Ceramide (C16), etc. and

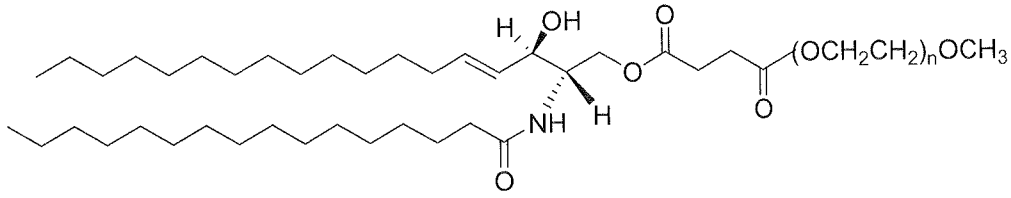
mixtures thereof. The structures of mPEG-DSPE, mPEG-dipalmitoylglycamide (C16), and mPEG-Ceramide (C16) are as follows:



,



and



wherein, (n) is an integer from about 5 to about 2300, preferably from about 5 to about 460.

In one preferred embodiment, (n) is about 45.

In a further embodiment and as an alternative to PAO-based polymers such as PEG, one or more effectively non-antigenic materials such as dextran, polyvinyl alcohols, carbohydrate-based polymers, hydroxypropylmethacrylamide (HPMA), polyalkylene oxides, and/or copolymers thereof can be used. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses, such as hydroxymethylcellulose or hydroxyethylcellulose. See also commonly-assigned U.S. Patent No. 6,153,655, the contents of which are incorporated herein by reference. It will be understood by those of ordinary skill that the same type of activation can be employed as described herein as for PAOs such as PEG. Those of ordinary skill in the art will further realize that the foregoing list is merely illustrative and that all polymeric materials having the qualities described herein are contemplated. For purposes of the present invention, "substantially or effectively non-antigenic" means all materials understood in the art as being nontoxic and not eliciting an appreciable immunogenic response in mammals.

In yet a further embodiment, the nanoparticle described herein can include PEG lipids with a releasable linker such as ketal or imine. Such releasable PEG lipids allow nucleic acids (oligonucleotides) to dissociate from the delivery system after the delivery system enters the cells. Additional details of such releasable PEG lipids are also described in U.S. Provisional Patent Application Nos. 61/115,379 and 61/115,371, entitled "Releasable Polymeric Lipids Based on Imine Moiety For Nucleic Acids Delivery System" and "Releasable Polymeric Lipids Based on Ketal or Acetal Moiety For Nucleic Acids Delivery System" respectively, and PCT Patent Application No. ____, filed on even date, and entitled "Releasable Polymeric Lipids For Nucleic Acids Delivery Systems", the contents of each of which are incorporated herein by reference.

5. Nucleic Acids/Oligonucleotides

The nanoparticle compositions described herein can be used for delivering various nucleic acids into cells or tissues. The nucleic acids include plasmids and oligonucleotides. Preferably, the nanoparticle compositions described herein are used for delivery of oligonucleotides.

In order to more fully appreciate the scope of the present invention, the following terms are defined. The artisan will appreciate that the terms, "nucleic acid" or "nucleotide" apply to deoxyribonucleic acid ("DNA"), ribonucleic acid, ("RNA") whether single-stranded or double-stranded, unless otherwise specified, and to any chemical modifications or analogs thereof, such as, locked nucleic acids (LNA). The artisan will readily understand that by the term "nucleic acid," included are polynucleic acids, derivates, modifications and analogs thereof. An "oligonucleotide" is generally a relatively short polynucleotide, e.g., ranging in size from about 2 to about 200 nucleotides, preferably from about 8 to about 50 nucleotides, more preferably from about 8 to about 30 nucleotides, and yet more preferably from about 8 to about 20 or from about 15 to about 28 in length. The oligonucleotides according to the invention are generally synthetic nucleic acids, and are single stranded, unless otherwise specified. The terms, "polynucleotide" and "polynucleic acid" may also be used synonymously herein.

The oligonucleotides (analogs) are not limited to a single species of oligonucleotide but, instead, are designed to work with a wide variety of such moieties, it being understood that linkers can attach to one or more of the 3'- or 5'- terminals, usually PO_4 or SO_4 groups of a

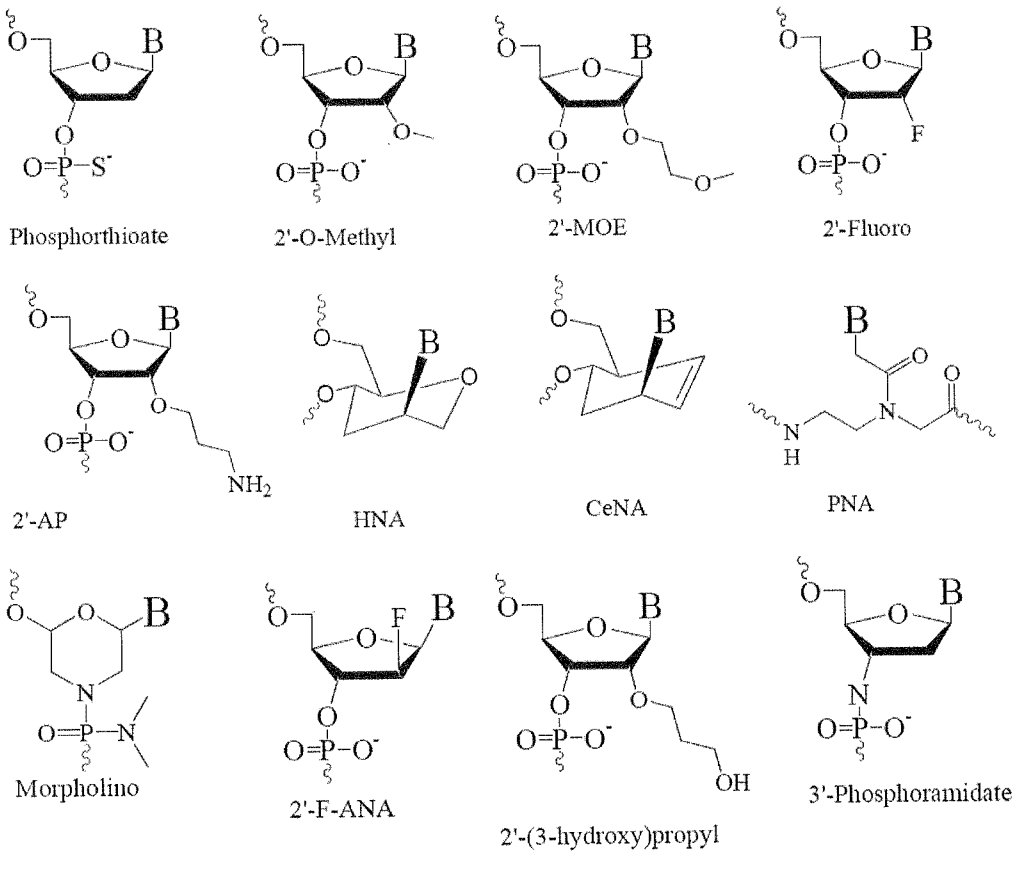
nucleotide. The nucleic acid molecules contemplated can include a phosphorothioate internucleotide linkage modification, sugar modification, nucleic acid base modification and/or phosphate backbone modification. The oligonucleotides can contain natural phosphorodiester backbone or phosphorothioate backbone or any other modified backbone analogues such as LNA (Locked Nucleic Acid), PNA (nucleic acid with peptide backbone), CpG oligomers, and the like, such as those disclosed at Tides 2002, Oligonucleotide and Peptide Technology Conferences, May 6-8, 2002, Las Vegas, NV and Oligonucleotide & Peptide Technologies, 18th & 19th November 2003, Hamburg, Germany, the contents of which are incorporated herein by reference.

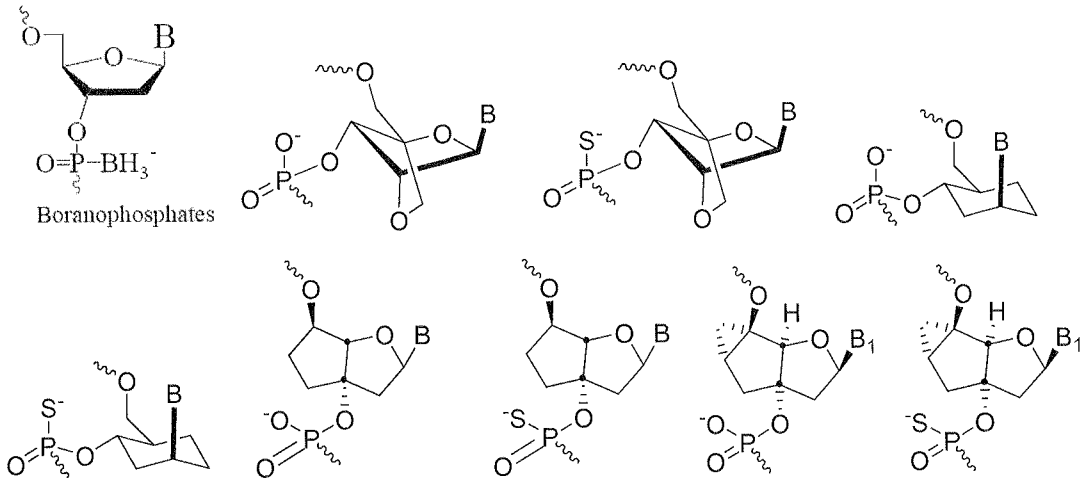
Modifications to the oligonucleotides contemplated by the invention include, for example, the addition or substitution of functional moieties that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and functionality to an oligonucleotide. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodouracil, backbone modifications, methylations, base-pairing combinations such as the isobases isocytidine and isoguanidine, and analogous combinations. Oligonucleotides contemplated within the scope of the present invention can also include 3' and/or 5' cap structure

For purposes of the present invention, "cap structure" shall be understood to mean chemical modifications, which have been incorporated at either terminus of the oligonucleotide. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both termini. A non-limiting example of the 5'-cap includes inverted abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide; 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety. Details are described in WO 97/26270, the contents of which are incorporated by reference herein. The 3'-cap can include for example

4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-aminoalkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide;5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties. See also Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; the contents of which are incorporated by reference herein.

A non-limiting list of nucleoside analogs have the structure:





See more examples of nucleoside analogues described in Freier & Altmann; *Nucl. Acid Res.*, 1997, 25, 4429-4443 and Uhlmann; *Curr. Opinion in Drug Development*, 2000, 3(2), 293-213, the contents of each of which are incorporated herein by reference.

The term "antisense," as used herein, refers to nucleotide sequences which are complementary to a specific DNA or RNA sequence that encodes a gene product or that encodes a control sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. In the normal operation of cellular metabolism, the sense strand of a DNA molecule is the strand that encodes polypeptides and/or other gene products. The sense strand serves as a template for synthesis of a messenger RNA ("mRNA") transcript (an antisense strand) which, in turn, directs synthesis of any encoded gene product. Antisense nucleic acid molecules may be produced by any art-known methods, including synthesis. Once introduced into a cell, this transcribed strand combines with natural sequences produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. The designations "negative" or (-) are also art-known to refer to the antisense strand, and "positive" or (+) are also art-known to refer to the sense strand.

For purposes of the present invention, "complementary" shall be understood to mean that a nucleic acid sequence forms hydrogen bond(s) with another nucleic acid sequence. A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule which can form hydrogen bonds, i.e., Watson-Crick base pairing, with a second nucleic acid sequence, i.e., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary. "Perfectly complementary" means that all the contiguous residues of a nucleic

acid sequence form hydrogen bonds with the same number of contiguous residues in a second nucleic acid sequence.

The nucleic acids (such as one or more same or differen oligonucleotides or oligonucloetide derivatives) useful in the nanoparticle described herein can include from about 5 to about 1000 nucleic acids, and preferably relatively short polynucleotides, *e.g.*, ranging in size preferably from about 8 to about 50 nucleotides in length (e.g., about 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30).

In one aspect of useful nucleic acids encapsulated within the nanoparticle described herein, oligonucleotides and oligodeoxynucleotides with natural phosphorodiester backbone or phosphorothioate backbone or any other modified backbone analogues include:

LNA (Locked Nucleic Acid);

PNA (nucleic acid with peptide backbone);

short interfering RNA (siRNA);

microRNA (miRNA);

nucleic acid with peptide backbone (PNA);

phosphorodiamidate morpholino oligonucleotides (PMO);

tricyclo-DNA;

decoy ODN (double stranded oligonucleotide);

catalytic RNA sequence (RNAi);

ribozymes;

aptamers;

spiegelmers (L-conformational oligonucleotides);

CpG oligomers, and the like, such as those disclosed at:

Tides 2002, Oligonucleotide and Peptide Technology Conferences, May 6-8, 2002, Las Vegas, NV and Oligonucleotide & Peptide Technologies, 18th & 19th November 2003, Hamburg, Germany, the contents of which are incorporated herein by reference.

In another aspect of the nucleic acids encapsulated within the nanoparticle, oligonucleotides can optionally include any suitable art-known nucleotide analogs and derivatives, including those listed by Table 2, below:

TABLE 2. Representative Nucleotide Analogs And Derivatives

4-acetylcytidine	5-methoxyaminomethyl-2-thiouridine

5-(carboxyhydroxymethyl)uridine	beta, D-mannosylqueuosine
2'-O-methylcytidine	5-methoxycarbonylmethyl-2-thiouridine
5-methoxycarbonylmethyluridine	5-carboxymethylaminomethyl-2-thiouridine
5-methoxyuridine	5-carboxymethylaminomethyluridine
Dihydrouridine	2-methylthio-N6-isopentenyladenosine
2'-O-methylpseudouridine	N-[(9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl]threonine
D-galactosylqueuosine	N-[(9-beta-D-ribofuranosylpurine-6-yl)N-methylcarbamoyl]threonine
2'-O-methylguanosine	uridine-5-oxyacetic acid-methylester
2'-halo-adenosine	2'-halo-cytidine
2'-halo-guanosine	2'-halo-thymine
2'-halo-uridine	2'-halo-methylcytidine
2'-amino-adenosine	2'-amino-cytidine
2'-amino-guanosine	2'-amino-thymine
2'-amino-uridine	2'-amino-methylcytidine
Inosine	uridine-5-oxyacetic acid
N6-isopentenyladenosine	Wybutoxosine
1-methyladenosine	Pseudouridine
1-methylpseudouridine	Queuosine
1-methylguanosine	2-thiocytidine
1-methylinosine	5-methyl-2-thiouridine
2,2-dimethylguanosine	2-thiouridine
2-methyladenosine	4-thiouridine
2-methylguanosine	5-methyluridine
3-methylcytidine	N-[(9-beta-D-ribofuranosylpurine-6-yl)-carbamoyl]threonine
5-methylcytidine	2'-O-methyl-5-methyluridine
N6-methyladenosine	2'-O-methyluridine
7-methylguanosine	Wybutosine

5-methylaminomethyluridine	3-(3-amino-3-carboxy-propyl)uridine
Locked-adenosine	Locked-cytidine
Locked-guanosine	Locked-thymine
Locked-uridine	Locked-methylcytidine

In one preferred aspect, the target oligonucleotides encapsulated in the nanoparticles include, for example, but are not limited to, oncogenes, pro-angiogenesis pathway genes, pro-cell proliferation pathway genes, viral infectious agent genes, and pro-inflammatory pathway genes.

In one preferred embodiment, the oligonucleotide encapsulated within the nanoparticle described herein is involved in targeting tumor cells or downregulating a gene or protein expression associated with tumor cells and/or the resistance of tumor cells to anticancer therapeutics. For example, antisense oligonucleotides for downregulating any art-known cellular proteins associated with cancer, e.g., BCL-2 can be used for the present invention. See U.S. Patent Application No. 10/822,205 filed April 9, 2004, the contents of which are incorporated by reference herein. A non-limiting list of preferred therapeutic oligonucleotides includes antisense bcl-2 oligonucleotides, antisense HIF-1α oligonucleotides, antisense survivin oligonucleotides, antisense ErbB3 oligonucleotides, antisense PIK3CA oligonucleotides, antisense HSP27 oligonucleotides, antisense androgen receptor oligonucleotides, antisense Gli2 oligonucleotides, and antisense beta-catenin oligonucleotides.

More preferably, the oligonucleotides according to the invention described herein include phosphorothioate backbone and LNA.

In one preferred embodiment, the oligonucleotide can be, for example, antisense survivin LNA, antisense ErbB3 LNA, or antisense HIF1-α LNA.

In another preferred embodiment, the oligonucleotide can be, for example, an oligonucleotide that has the same or substantially similar nucleotide sequence as does Genasense® (a/k/a oblimersen sodium, produced by Genta Inc., Berkeley Heights, NJ). Genasense® is an 18-mer phosphorothioate antisense oligonucleotide (SEQ ID NO: 4), that is complementary to the first six codons of the initiating sequence of the human bcl-2 mRNA (human bcl-2 mRNA is art-known, and is described, *e.g.*, as SEQ ID NO: 19 in U.S. Patent No. 6,414,134, incorporated by reference herein).

Preferred embodiments contemplated include:

(i) antisense Survivin LNA oligomer (SEQ ID NO: 1)

$^{m}C_s$-T_s-$^{m}C_s$-A_s-a_s-t_s-c_s-c_s-a_s-t_s-g_s-g_s-$^{m}C_s$-A_s-G_s-c;

where the upper case letter represents LNA, the "s" represents a phosphorothioate backbone;

(ii) antisense Bcl2 siRNA:

SENSE 5'- gcaugcggccucuguuugadTdT-3' (SEQ ID NO: 2)

ANTISENSE 3'- dTdTcguacgccggagacaaacu-5' (SEQ ID NO: 3)

where dT represents DNA;

(iii) Genasense (phosphorothioate antisense oligonucleotide): (SEQ ID NO: 4)

t_s-c_s-t_s-c_s-c_s-c_s-a_s-g_s-c_s-g_s-t_s-g_s-c_s-g_s-c_s-c_s-c_s-a_s-t

where the lower case letter represents DNA and "s" represents phosphorothioate backbone;

(iv) antisense HIF1α LNA oligomer (SEQ ID NO: 5)

$T_sG_sG_sc_sa_sa_sg_sc_sa_st_sc_sc_sT_sG_sT_sa$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(v) antisense ErbB3 LNA oligomer (SEQ ID NO: 6)

$T_sA_sG_sc_sc_st_sg_st_sc_sa_sc_st_st_s{}^{Me}C_sT_s{}^{Me}C_s$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(vi) antisense ErbB3 LNA oligomer (SEQ ID NO: 7)

$G_s{}^{Me}C_sT_sc_sc_sa_sg_sa_sc_sa_st_sc_sa_s{}^{Me}C_sT_s{}^{Me}C$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(vii) antisense PIK3CA LNA oligomer (SEQ ID NO: 8)

$A_sG_s{}^{Me}C_sc_sa_st_st_sc_sa_st_st_sc_sc_sA_s{}^{Me}C_s{}^{Me}C$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(viii) antisense PIK3CA LNA oligomer (SEQ ID NO: 9)

$T_sT_sA_st_st_sg_st_sg_sc_sa_st_sc_st_s{}^{Me}C_sA_sG$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(ix) antisense HSP27 LNA oligomer (SEQ ID NO: 10)

$C_sG_sT_sg_st_sa_st_st_st_sc_sc_sg_sc_sG_sT_sG$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(x) antisense HSP27 LNA oligomer (SEQ ID NO: 11)

$G_sG_s{}^{Me}C_sa_sc_sa_sg_sc_sc_sa_sg_st_sg_sG_s{}^{Me}C_sG$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(xi) antisense Androgen Receptor LNA oligomer (SEQ ID NO: 12)

$^{Me}C_s{}^{Me}C_s{}^{Me}C_sa_sa_sg_sg_sc_sa_sc_st_sg_sc_sA_sG_sA$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(xii) antisense Androgen Receptor LNA oligomer (SEQ ID NO: 13)

$A_s{}^{Me}C_s{}^{Me}C_sa_sa_sg_st_st_st_sc_st_st_sc_sA_sG_s{}^{Me}C$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(xiii) antisense GLI2 LNA oligomer (SEQ ID NO: 14)

$^{Me}C_sT_s{}^{Me}C_sc_st_st_sg_sg_st_sg_sc_sa_sg_sT_s{}^{Me}C_sT$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

(xiv) antisense GLI2 LNA oligomer (SEQ ID NO: 15)

$T_s{}^{Me}C_sA_sg_sa_st_st_sc_sa_sa_sa_sc_s{}^{Me}C_s{}^{Me}C_sA$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone

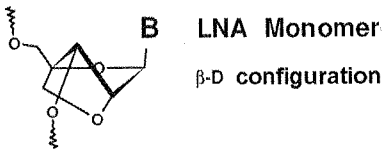
(xv) antisense beta-catenin LNA oligomer (SEQ ID NO: 16)

$G_sT_sG_st_st_sc_st_sa_sc_sa_sc_sc_sc_sa_sT_sT_sA$

where the upper case letter represents LNA and the "s" represents phosphorothioate backbone.

Lower case letters represent DNA units, bold upper case letters represent LNA such as β-D-oxy-LNA units. All cytosine bases in the LNA monomers are 5-methylcytosine. Subscript "s" represents phosphorothioate linkage.

LNA includes 2'-O, 4'-C methylene bicyclonucleotide as shown below:



See detailed description of Survivin LNA disclosed in U.S. Patent Application Serial Nos. 11/272,124, entitled "LNA Oligonucleotides and the Treatment of Cancer" and 10/776,934, entitled "Oligomeric Compounds for the Modulation Survivin Expression", the contents of each of which is incorporated herein by reference. See also U.S. Patent No. 7,589,190 and U.S. Patent Publication No. 2004/0096848 for HIF-1α modulation; U.S. Patent Publication No. 2008/0318894 and PCT/US09/063357 for ErbB3 modulation; U.S. Patent Publication No. 2009/0192110 for PIK3CA modulation; PCT/IB09/052860 for HSP27 modulation; U.S. Patent Publication No. 2009/0181916 for Androgen Receptor modulation; and U.S. Provisional Application No. 61/081,135 and PCT Application No. PCT/IB09/006407, entitled "RNA Antagonists Targeting GLI2"; and U.S. Patent Publication Nos. 2009/0005335 and 2009/0203137 for Beta Catenin modulation; the contents of each which are also incorporated herein by reference. Additional examples of suitable target genes are described in WO 03/74654, PCT/US03/05028, and U.S. Patent Application Ser. No. 10/923,536, the contents of which are incorporated by reference herein.

In a further embodiment, the nanoparticle described herein can include oligonucleotides releasably linked to an endosomal release-promoting group. The endosomal release-promoting groups such as histidine-rich peptides can destabilize/disrupt the endosomal membrane, thereby facilitating cytoplasmic delivery of therapeutic agents. Histidine-rich peptides enhance endosomal release of oligonucleotides to the cytoplasm. Then, the intracellularly released oligonucleotides can translocate to the nucleus. Additional details of oligonucleotide-histidine rich peptide conjugates are described in U.S. Provisional Patent Application Serial Nos. 61/115,350 and 61/115,326 filed November 17, 2008, and PCT Patent Application No. ____,

filed on even date, and entitled "Releasable Conjugates For Nucleic Acids Delivery Systems", the contents of each of which are incorporated herein by reference.

6. Targeting Groups

Optionally/preferably, the nanoparticle compositions described herein further include a targeting ligand for a specific cell or tissue type. The targeting group can be attached to any component of a nanoparticle composition (preferably, fusogenic lipids and PEG-lipids) using a linker molecule, such as an amide, amido, carbonyl, ester, peptide, disulphide, silane, nucleoside, abasic nucleoside, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, phosphate ester, phosphoramidate, thiophosphate, alkylphosphate, maleimidyl linker or photolabile linker. Any known techniques in the art can be used for conjugating a targeting group to any component of the nanoparticle composition without undue experimentation.

For example, targeting agents can be attached to the polymeric portion of PEG lipids to guide the nanoparticles to the target area *in vivo*. The targeted delivery of the nanoparticle described herein enhances the cellular uptake of the nanoparticles encapsulating therapeutic nucleic acids, thereby improving the therapeutic efficacies. In certain aspects, some cell penetrating peptides can be replaced with a variety of targeting peptides for targeted delivery to the tumor site.

In one preferred aspect of the invention, the targeting moiety, such as a single chain antibody (SCA) or single-chain antigen-binding antibody, monoclonal antibody, cell adhesion peptides such as RGD peptides and Selectin, cell penetrating peptides (CPPs) such as TAT, Penetratin and $(Arg)_9$, receptor ligands, targeting carbohydrate molecules or lectins allows nanoparticles to be specifically directed to targeted regions. See *J Pharm Sci.* 2006 Sep; 95(9):1856-72 Cell adhesion molecules for targeted drug delivery, the contents of which are incorporated herein by reference.

Preferred targeting moieties include single-chain antibodies (SCAs) or single-chain variable fragments of antibodies (sFv). The SCA contains domains of antibodies which can bind or recognize specific molecules of targeting tumor cells. In addition to maintaining an antigen binding site, a SCA conjugated to a PEG-lipid can reduce antigenicity and increase the half life of the SCA in the bloodstream.

The terms "single chain antibody" (SCA), "single-chain antigen-binding molecule or antibody" or "single-chain Fv" (sFv) are used interchangeably. The single chain antibody has binding affinity for the antigen. Single chain antibody (SCA) or single-chain Fvs can and have been constructed in several ways. A description of the theory and production of single-chain antigen-binding proteins is found in commonly assigned U.S. Patent Application No. 10/915,069 and U.S. Patent No. 6,824,782, the contents of each of which are incorporated by reference herein.

Typically, SCA or Fv domains can be selected among monoclonal antibodies known by their abbreviations in the literature as 26-10, MOPC 315, 741F8, 520C9, McPC 603, D1.3, murine phOx, human phOx, RFL3.8 sTCR, 1A6, Se155-4,18-2-3,4-4-20,7A4-1, B6.2, CC49,3C2,2c, MA-15C5/$K_{12}G_O$, Ox, etc. (see, Huston, J. S. et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988); Huston, J. S. et al., SIM News 38(4) (Supp):11 (1988); McCartney, J. et al., ICSU Short Reports 10:114 (1990); McCartney, J. E. et al., unpublished results (1990); Nedelman, M. A. et al., J. Nuclear Med. 32 (Supp.):1005 (1991); Huston, J. S. et al., In: Molecular Design and Modeling: Concepts and Applications, Part B, edited by J. J. Langone, Methods in Enzymology 203:46-88 (1991); Huston, J. S. et al., In: Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Epenetos, A. A. (Ed.), London, Chapman & Hall (1993); Bird, R. E. et al., Science 242:423-426 (1988); Bedzyk, W. D. et al., J. Biol. Chem. 265:18615-18620 (1990); Colcher, D. et al., J. Nat. Cancer Inst. 82:1191-1197 (1990); Gibbs, R. A. et al., Proc. Natl. Acad. Sci. USA 88:4001-4004 (1991); Milenic, D. E. et al., Cancer Research 51:6363-6371 (1991); Pantoliano, M. W. et al., Biochemistry 30:10117-10125 (1991); Chaudhary, V. K. et al., Nature 339:394-397 (1989); Chaudhary, V. K. et al., Proc. Natl. Acad. Sci. USA 87:1066-1070 (1990); Batra, J. K. et al., Biochem. Biophys. Res. Comm. 171:1-6 (1990); Batra, J. K. et al., J. Biol. Chem. 265:15198-15202 (1990); Chaudhary, V. K. et al., Proc. Natl. Acad Sci. USA 87:9491-9494 (1990); Batra, J. K. et al., Mol. Cell. Biol. 11:2200-2205 (1991); Brinkmann, U. et al., Proc. Natl. Acad. Sci. USA 88:8616-8620 (1991); Seetharam, S. et al., J. Biol. Chem. 266:17376-17381 (1991); Brinkmann, U. et al., Proc. Natl. Acad. Sci. USA 89:3075-3079 (1992); Glockshuber, R. et al., Biochemistry 29:1362-1367 (1990); Skerra, A. et al., Bio/Technol. 9:273-278 (1991); Pack, P. et al., Biochemistry 31:1579-1534 (1992); Clackson, T. et al., Nature 352:624-628 (1991); Marks, J. D. et al., J. Mol. Biol. 222:581-597 (1991); Iverson, B. L. et al., Science 249:659-662 (1990); Roberts, V. A. et al., Proc. Natl. Acad.

Sci. USA 87:6654-6658 (1990); Condra, J. H. et al., J. Biol. Chem. 265:2292-2295 (1990); Laroche, Y. et al., J. Biol. Chem. 266:16343-16349 (1991); Holvoet, P. et al., J. Biol. Chem. 266:19717-19724 (1991); Anand, N. N. et al., J. Biol. Chem. 266:21874-21879 (1991); Fuchs, P. et al., Biol Technol. 9:1369-1372 (1991); Breitling, F. et al., Gene 104:104-153 (1991); Seehaus, T. et al., Gene 114:235-237 (1992); Takkinen, K. et al., Protein Engng. 4:837-841 (1991); Dreher, M. L. et al., J. Immunol. Methods 139:197-205 (1991); Mottez, E. et al., Eur. J. Immunol. 21:467-471 (1991); Traunecker, A. et al., Proc. Natl. Acad. Sci. USA 88:8646-8650 (1991); Traunecker, A. et al., EMBO J. 10:3655-3659 (1991); Hoo, W. F. S. et al., Proc. Natl. Acad. Sci. USA 89:4759-4763 (1993)). Each of the foregoing publications is incorporated herein by reference.

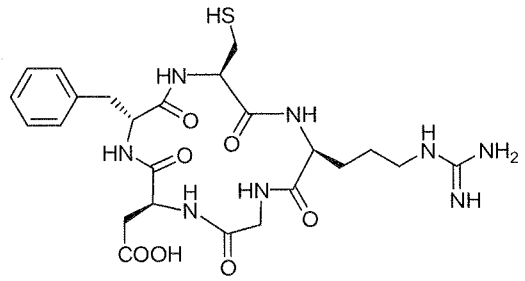
A non-limiting list of targeting groups includes vascular endothelial cell growth factor, FGF2, somatostatin and somatostatin analogs, transferrin, melanotropin, ApoE and ApoE peptides, von Willebrand's Factor and von Willebrand's Factor peptides, adenoviral fiber protein and adenoviral fiber protein peptides, PD1 and PD1 peptides, EGF and EGF peptides, RGD peptides, folate, anisamide, etc. Other optional targeting agents appreciated by artisans in the art can be also employed in the nanoparticles described herein.

In one preferred embodiment, the targeting agents useful for the compounds described herein include single chain antibody (SCA), RGD peptides, selectin, TAT, penetratin, $(Arg)_9$, folic acid, anisamide, etc., and some of the preferred structures of these agents are:

C-TAT: (SEQ ID NO: 17) CYGRKKRRQRRR;

C-$(Arg)_9$: (SEQ ID NO: 18) CRRRRRRRRR;

RGD can be linear or cyclic:



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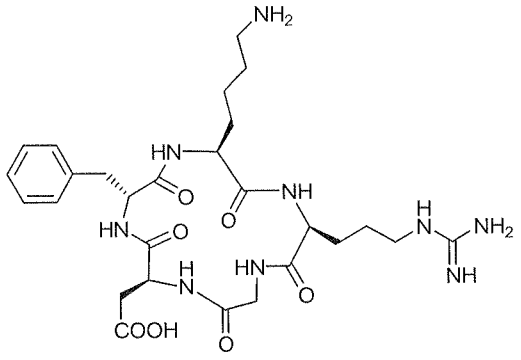
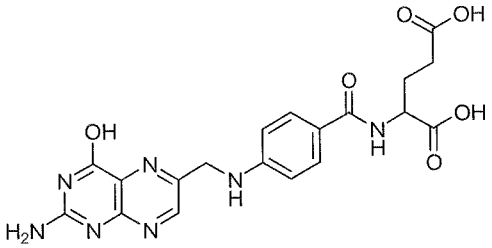


or ;

Folic acid is a residue of



, and

Anisamide is *p*-MeO-Ph-C(=O)OH.

Arg_9 can include a cysteine for conjugating such as CRRRRRRRRR and TAT can add an additional cysteine at the end of the peptide such as CYGRKKRRQRRRC.

For purpose of the current invention, the abbreviations used in the specification and figures represent the following structures.:

(i) C-diTAT (SEQ ID NO: 19) = CYGRKKRRQRRRYGRKKRRQRRR-NH_2;

(ii) Linear RGD (SEQ ID NO: 20) = RGDC ;

(iii) Cyclic RGD (SEQ ID NO: 21 and SEQ ID NO: 22) = c-RGDFC or c-RGDFK;

(iv) RGD-TAT (SEQ ID NO: 23) = CYGRKKRRQRRRGGGRGDS-NH_2 ; and

(v) Arg_9 (SEQ ID NO: 24) = RRRRRRRRR.

Alternatively, the targeting group include sugars and carbohydrates such as galactose, galactosamine, and N-acetyl galactosamine; hormones such as estrogen, testosterone, progesterone, glucocortisone, adrenaline, insulin, glucagon, cortisol, vitamin D, thyroid hormone, retinoic acid, and growth hormones; growth factors such as VEGF, EGF, NGF, and PDGF; neurotransmitters such as GABA, Glutamate, acetylcholine; NOGO; inostitol triphosphate; epinephrine; norepinephrine; Nitric Oxide, peptides, vitamins such as folate and pyridoxine, drugs, antibodies and any other molecule that can interact with a cell surface receptor *in vivo* or *in vitro*.

D. Preparation of Nanoparticles

The nanoparticle described herein can be prepared by any art-known process without undue experimentation.

For example, the nanoparticle can be prepared by providing nucleic acids such as oligonucleotides in an aqueous solution (or an aqueous solution without nucleic acids for comparison study) in a first reservoir, providing an organic lipid solution containing the nanoparticle composition described herein in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution to produce nanoparticles encapsulating the nucleic acids. Details of the process are described in U.S. Patent Publication No. 2004/0142025, the contents of which are incorporated herein by reference.

Alternatively, the nanoparticles described herein can be prepared by using any methods known in the art including, e.g., a detergent dialysis method or a modified reverse-phase method which utilizes organic solvents to provide a single phase during mixing the components. In a detergent dialysis method, nucleic acids (i.e., siRNA) are contacted with a detergent solution of cationic lipids to form a coated nucleic acid complex.

In one embodiment of the invention, the cationic lipids and nucleic acids such as oligonucleotides are combined to produce a charge ratio of from about 1:20 to about 20:1, preferably in a ratio of from about 1:5 to about 5:1, and more preferably in a ratio of from about 1:2 to about 2:1.

In one embodiment of the invention, the cationic lipids and nucleic acids such as oligonucleotides are combined to produce a charge ratio of from about 1:1 to about 20:1, from about 1:1 to about 12:1, and more preferably in a ratio of from about 2:1 to about 6:1. Alternatively, the nitrogen to phoshpate (N/P) ratio of the nanoparticle composition ranges from about 2:1 to about 5:1, (i.e., 2.5:1).

In another embodiment, the nanoparticle described herein can be prepared by using a dual pump system. Generally, the process includes providing an aqueous solution containing nucleic acids in a first reservoir and a lipid solution containing the nanoparticle composition described in a second reservoir. The two solutions are mixed by using a dual pump system to provide nanoparticles. The resulting mixed solution is subsequently diluted with an aqueous buffer and

the nanoparticles formed can be purified and/or isolated by dialysis. The nanoparticles can be further processed to be sterilized by filtering through a 0.22 μm filter.

The nanoparticles containing nucleic acids range from about 5 to about 300 nm in diameter. Preferably, the nanoparticles have a median diameter of less than about 150 nm (e.g., about 50-150 nm), more preferably a diameter of less than about 100 nm, by the measurement using the Dynamic Light Scattering technique (DLS). A majority of the nanoparticles have a median diameter of about 30 to 100 nm (e.g., 59.5, 66, 68, 76, 80, 93, 96 nm), preferably about 60 to about 95 nm. Artisans will appreciate that the measurement using other art-known techniques such as TEM may provide a median diameter number decreased by half, as compared to the DLS technique. The nanoparticles of the present invention are substantially uniform in size as shown by polydispersity.

Optionally, the nanoparticles can be sized by any methods known in the art. The size can be controlled as desired by artisans. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of nanoparticle sizes. Several techniques are available for sizing the nanoparticles to a desired size. See, for example, U.S. Patent No. 4,737,323, the contents of which are incorporated herein by reference.

The present invention provides methods for preparing serum-stable nanoparticles such that nucleic acids (e.g., LNA or siRNA) are encapsulated in a lipid multi-lamellar structure (i.e. a lipid bilayer) and are protected from degradation. The nanoparticles described herein are stable in an aqueous solution. Nucleic acids included in the nanoparticles are protected from nucleases present in the body fluid.

Additionally, the nanoparticles prepared according to the present invention are preferably neutral or positively-charged at physiological pH.

The nanoparticle or nanoparticle complex prepared using the nanoparticle composition described herein includes: (i) a compound of Formula (I); (ii) a neutral lipid/fusogenic lipid; (iii) a PEG-lipid and (iv) nucleic acids such as an oligonucleotide.

In one embodiment, the nanoparticle composition includes a mixture of

a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a compound of Formula (I), a diacylphosphatidylcholine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a compound of Formula (I), a diacylphosphatidylethanolamine, a diacylphosphatidyl-choline, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to ceramide (PEG-Cer), and cholesterol; or

a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), a PEG conjugated to ceramide (PEG-Cer), and cholesterol.

Additional nanoparticle compositions can be prepared by modifying compositions containing art-known cationic lipid(s). Nanoparticle compositions containing a compound of Formula (I) can be modified by adding art-known cationic lipids. See art-known compositions described in Table IV of US Patent Application Publication No. 2008/0020058, the contents of which are incorporated herein by reference.

A non-limiting list of nanoparticle compositions for the preparation of nanoparticles is set forth in Table 3.

Table 3

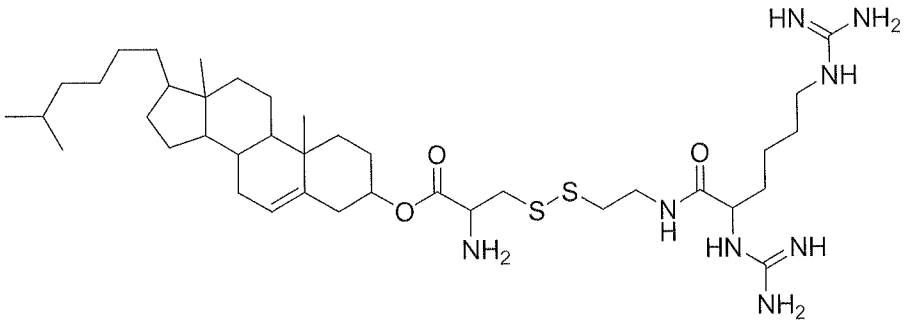
Sample No.	Nanoparticle Composition	Molar Ratio	Oligo
1	Compd of Formula (I) : DOPE: DSPC : Chol : DSPE-PEG	15:15:20:40:10	Oligo-1
2	Compd of Formula (I): DOPE: DSPC: Chol: DSPE-PEG	15:5:20:50:10	Oligo-1
3	Compd of Formula (I): DOPE: DSPC: Chol: DSPE-PEG	25:15:20:30:10	Oligo-1
4	Compd of Formula (I): EPC: Chol: DSPE-PEG	20:47:30: 3	Oligo-1
5	Compd of Formula (I): DOPE: Chol: DSPE-PEG	17:60:20:3	Oligo-1
6	Compd of Formula (I): DOPE: DSPE-PEG	20:78: 2	Oligo-1
7	Compd of Formula (I): DOPE: Chol:C16mPEG-Ceramide	17:60:20:3	Oligo-2
8	Compd of Formula (I): DOPE: Chol: DSPE-PEG: C16mPEG-Ceramide	18:60:20:1:1	Oligo-2

Compound of Formula (I) is: Compounds 12, 31, 49 and 54

In one embodiment, the molar ratio of a compound of Formula (I): DOPE: cholesterol: PEG-DSPE: C16mPEG-Ceramide in the nanoparticle is in a molar ratio of about 18%: 60%: 20%: 1%: 1%, respectively, based the total lipid present in the nanoparticle composition (Sample No. 8).

In another embodiment, the nanoparticle contains a compound of Formula (I), DOPE, cholesterol and C16mPEG-Ceramide in a molar ratio of about 17%: 60%: 20%: 3% of the total lipid present in the nanoparticle composition (Sample No. 7)

These nanoparticle compositions preferably contain releasable cationic lipids having the structure:



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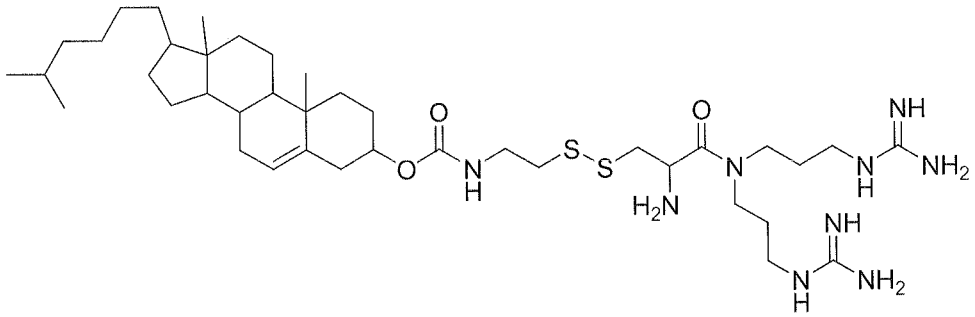

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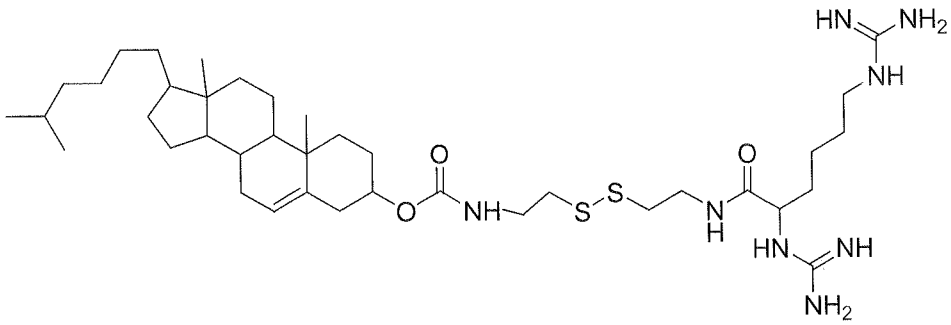

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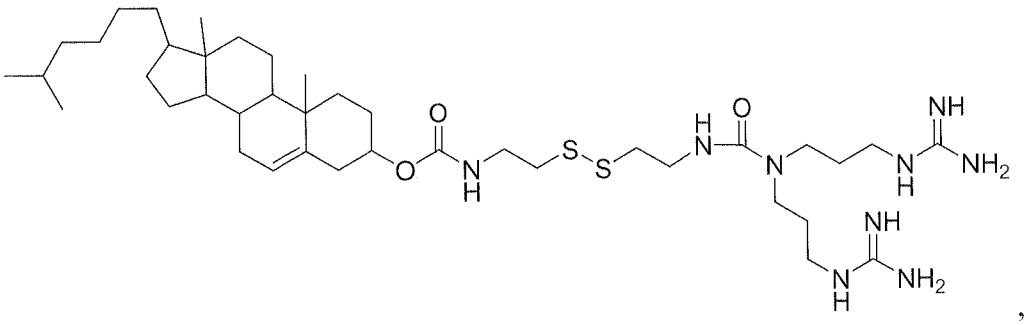

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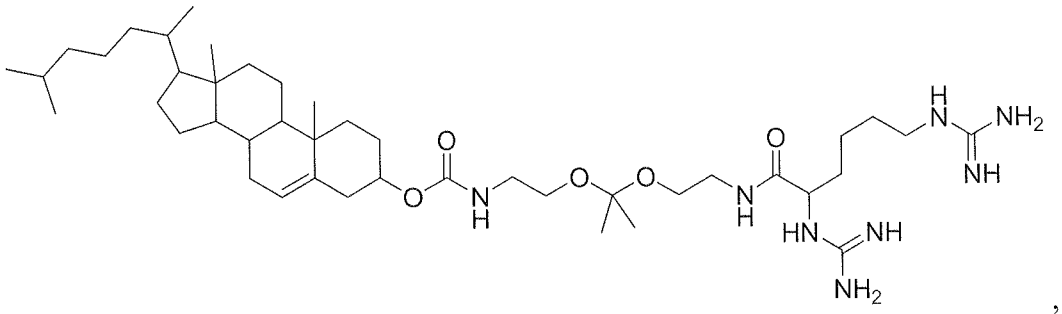

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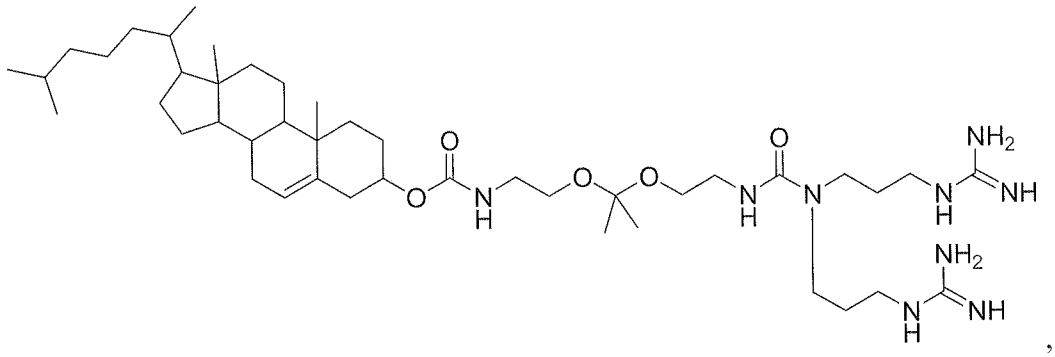

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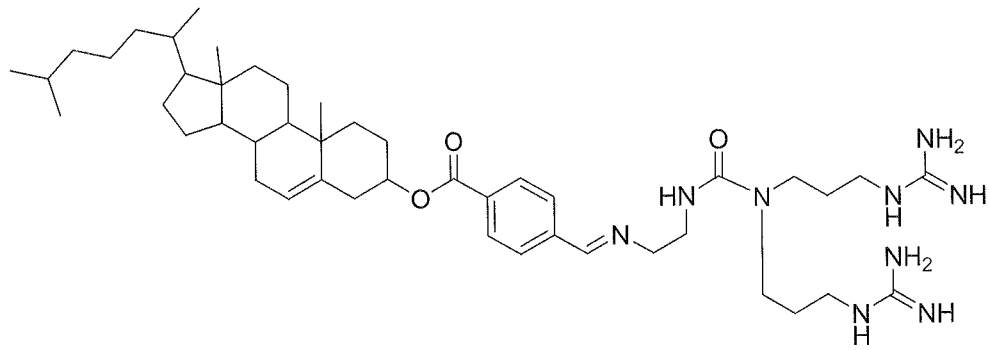

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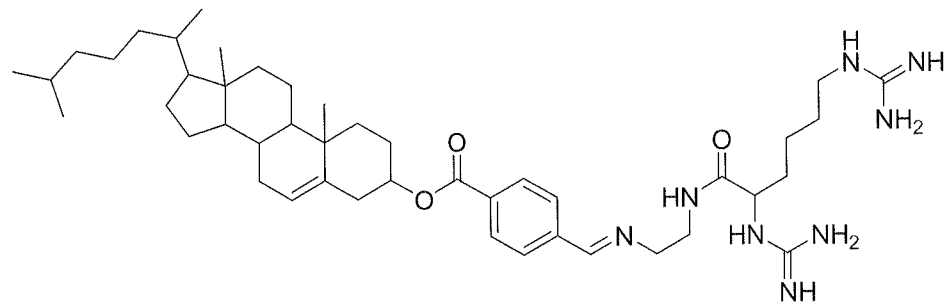

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The molar ratio as used herein refers to the amount relative to the total lipid present in the nanoparticle composition.

E. METHODS OF TREATMENT

The nanoparticles described herein can be employed in the treatment for preventing, inhibiting, reducing or treating any trait, disease or condition that is related to or responds to the levels of target gene expression in a cell or tissue, alone or in combination with other therapies. The methods include administering the nanoparticles described herein to a mammal in need thereof.

One aspect of the present invention provides methods of introducing or delivering therapeutic agents such as nucleic acids/oligonucleotides into a mammalian cell *in vivo* and/or *in vitro*.

The method according to the present invention includes contacting a cell with the compounds described herein. The delivery can be made *in vivo* as part of a suitable pharmaceutical composition or directly to the cells in an *ex vivo* or *in vitro* environment.

The present invention is useful for introducing oligonucleotides to a mammal. The compounds described herein can be administered to a mammal, preferably human.

According to the present invention, the present invention preferably provides methods of inhibiting, or downregulating (or modulating) gene expression in mammalian cells or tissues. The downregulation or inhibition of gene expression can be achieved *in vivo, ex vivo* and/or *in vitro*. The methods include contacting human cells or tissues with nanoparticles encapsulating nucleic acids or administering the nanoparticles to a mammal in need thereof. Once the contacting has occurred, successful inhibition or down-regulation of gene expression such as in mRNA or protein levels shall be deemed to occur when at least about 10%, preferably at least about 20% or higher (e.g., at least about 25%, 30%, 40%, 50%, 60%) is realized *in vivo, ex vivo* or *in vitro* when compared to that observed in the absence of the nanoparticles described herein.

For purposes of the present invention, "inhibiting" or "downregulating" shall be understood to mean that the expression of a target gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits, or activity of one or more protein subunits is reduced when compared to that observed in the absence of the nanoparticles described herein.

In one preferred embodiment, a target gene includes, for example, but is not limited to, oncogenes, pro-angiogenesis pathway genes, pro-cell proliferation pathway genes, viral infectious agent genes, and pro-inflammatory pathway genes.

Preferably, gene expression of a target gene is inhibited in cancer cells or tissues, for example, brain, breast, colorectal, gastric, lung, mouth, pancreatic, prostate, skin or cervical cancer cells. The cancer cells or tissues can be from one or more of the following: solid tumors, lymphomas, small cell lung cancer, acute lymphocytic leukemia (ALL), pancreatic cancer, glioblastoma, ovarian cancer, gastric cancer, breast cancer, colorectal cancer, prostate cancer, cervical cancer, brain tumors, KB cancer, lung cancer, colon cancer, epidermal cancer, etc.

In one particular embodiment, the nanoparticles according to the methods described herein include, for example, antisense bcl-2 oligonucleotides, antisense HIF-1α oligonucleotides, antisense survivin oligonucleotides, antisense ErbB3 oligonucleotides, antisense PIK3CA oligonucleotides, antisense HSP27 oligonucleotides, antisense androgen receptor oligonucleotides, antisense Gli2 oligonucleotides, and antisense beta-catenin oligonucleotides.

According to the present invention, the nanoparticles can include oligonucleotides (SEQ ID NO: 1, SEQ ID NOs 2 and 3, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, and SEQ ID NO: 16 in which each nucleic acid is a naturally occurring or modified nucleic acid) can be used. The therapy contemplated herein uses nucleic acids encapsulated in the aforementioned nanoparticle. In one embodiment, therapeutic nucleotides containing eight or more consecutive antisense nucleotides can be employed in the treatment.

Alternatively, there are also provided methods of treating a mammal. The methods include administering an effective amount of a pharmaceutical composition containing a nanoparticle described herein to a patient in need thereof. The efficacy of the methods would depend upon efficacy of the nucleic acids for the condition being treated. The present invention provides methods of treatment for various medical conditions in mammals. The methods include administering, to the mammal in need of such treatment, an effective amount of a nanoparticle containing encapsulated therapeutic nucleic acids. The nanoparticles described herein are useful for, among other things, treating diseases such as (but not limited to) cancer, inflammatory disease, and autoimmune disease.

In one embodiment, there are also provided methods of treating a patient having a malignancy or cancer, comprising administering an effective amount of a pharmaceutical composition containing the nanoparticle described herein to a patient in need thereof. The

cancer being treated can be one or more of the following: solid tumors, lymphomas, small cell lung cancer, acute lymphocytic leukemia (ALL), pancreatic cancer, glioblastoma, ovarian cancer, gastric cancers, colorectal cancer, prostate cancer, cervical cancer, brain tumors, KB cancer, lung cancer, colon cancer, epidermal cancer, etc. The nanoparticles are useful for treating neoplastic disease, reducing tumor burden, preventing metastasis of neoplasms and preventing recurrences of tumor/neoplastic growths in mammals by downregulating gene expression of a target gene. For example, the nanoparticles are useful in the treatment of metastatic disease (i.e. cancer with metastasis into the liver).

In yet another aspect, the present invention provides methods of inhibiting the growth or proliferation of cancer cells *in vivo* or *in vitro*. The methods include contacting cancer cells with the nanopaticle described herein. In one embodiment, the present invention provides methods of inhibiting the growth of cancer *in vivo* or *in vitro* wherein the cells express ErbB3 gene.

In another aspect, the present invention provides a means to deliver nucleic acids (e.g., antisense ErbB3 LNA oligonucleotides) inside a cancer cell where it can bind to ErbB3 mRNA, e.g., in the nucleus. As a consequence, the ErbB3 protein expression is inhibited, which inhibits the growth of the cancer cells. The methods introduce oligonucleotides (e.g. antisense oligonucleotides including LNA) to cancer cells and reduce target gene (e.g., survivin, HIF-1α or ErbB3) expression in the cancer cells or tissues.

Alternatively, the present invention provides methods of modulating apoptosis in cancer cells. In yet another aspect, there are also provided methods of increasing the sensitivity of cancer cells or tissues to chemotherapeutic agents *in vivo* or *in vitro*.

In yet another aspect, there are provided methods of killing tumor cells *in vivo* or *in vitro*. The methods include introducing the compounds described herein to tumor cells to reduce gene expression such as ErbB3 gene and contacting the tumor cells with an amount of at least one anticancer agent (e.g., a chemotherapeutic agent) sufficient to kill a portion of the tumor cells. Thus, the portion of tumor cells killed can be greater than the portion which would have been killed by the same amount of the chemotherapeutic agent in the absence of the nanoparticles described herein.

In a further aspect of the invention, an anticancer/chemotherapeutic agent can be used in combination, simultaneously or sequentially, with the compounds described herein. The compounds described herein can be administered prior to, or concurrently with, the anticancer

agent, or after the administration of the anticancer agent. Thus, the nanoparticles described herein can be administered prior to, during, or after treatment of the chemotherapeutic agent.

Still further aspects include combining the compound of the present invention described herein with other anticancer therapies for synergistic or additive benefit.

Alternatively, the nanoparticle composition described herein can be used to deliver a pharmaceutically active agent, preferably having a negative charge or a neutral charge to a mammal. The nanoparticle encapsulating pharmaceutically active agents/compounds can be administered to a mammal in need thereof. The pharmaceutically active agents/compounds include small molecular weight molecules. Typically, the pharmaceutically active agents have a molecular weight of less than about 1,500 daltons (i.e., less than 1,000 daltons).

In a further embodiment, the compounds described herein can be used to deliver nucleic acids, a pharmaceutically active agent, or in combination thereof.

In yet a further embodiment, the nanoparticle associated with the treatment can contain a mixture of one or more therapeutic nucleic acids (either the same or different, for example, the same or different oligonucleotides), and/or one or more pharmaceutically active agents for synergistic application.

F. Pharmaceutical Compositions/Formulations of Nanoparticles

Pharmaceutical compositions/formulations including the nanoparticles described herein may be formulated in conjunction with one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen, i.e., whether local or systemic treatment is treated.

Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or injection. Factors for considerations known in the art for preparing proper formulations include, but are not limited to, toxicity and any disadvantages that would prevent the composition or formulation from exerting its effect.

Administration of pharmaceutical compositions of nanoparticles described herein may be oral, pulmonary, topical or parentarel. Topical administration includes, without limitation, administration via the epidermal, transdermal, ophthalmic routes, including via mucous membranes, e.g., including vaginal and rectal delivery. Parenteral administration, including

intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion, is also contemplated.

In one preferred embodiment, the nanoparticles containing therapeutic oligonucleotides are administered intravenously (i.v.) or intraperitoneally (i.p.). Parenteral routes are preferred in many aspects of the invention.

For injection, including, without limitation, intravenous, intramuscular and subcutaneous injection, the nanoparticles of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as physiological saline buffer or polar solvents including, without limitation, a pyrrolidone or dimethylsulfoxide.

The nanoparticles may also be formulated for bolus injection or for continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers. Useful compositions include, without limitation, suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain adjuncts such as suspending, stabilizing and/or dispersing agents. Pharmaceutical compositions for parenteral administration include aqueous solutions of a water soluble form. Aqueous injection suspensions may contain substances that modulate the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers and/or agents that increase the concentration of the nanoparticles in the solution. Alternatively, the nanoparticles may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

For oral administration, the nanoparticles described herein can be formulated by combining the nanoparticles with pharmaceutically acceptable carriers well-known in the art. Such carriers enable the nanoparticles of the invention to be formulated as tablets, pills, lozenges, dragees, capsules, liquids, gels, syrups, pastes, slurries, solutions, suspensions, concentrated solutions and suspensions for diluting in the drinking water of a patient, premixes for dilution in the feed of a patient, and the like, for oral ingestion by a patient. Pharmaceutical preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding other suitable auxiliaries if desired, to obtain tablets or dragee cores. Useful excipients are, in particular, fillers such as sugars (for example, lactose, sucrose, mannitol, or sorbitol), cellulose preparations such as maize starch, wheat starch, rice starch and potato starch and other materials such as gelatin, gum tragacanth,

methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid. A salt such as sodium alginate may also be used.

For administration by inhalation, the nanoparticles of the present invention can conveniently be delivered in the form of an aerosol spray using a pressurized pack or a nebulizer and a suitable propellant.

The nanoparticles may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the nanoparticles may also be formulated as depot preparations. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. A nanoparticle of this invention may be formulated for this route of administration with suitable polymeric or hydrophobic materials (for instance, in an emulsion with a pharmacologically acceptable oil), with ion exchange resins, or as a sparingly soluble derivative such as, without limitation, a sparingly soluble salt.

Additionally, the nanoparticles may be delivered using a sustained-release system, such as semi-permeable matrices of solid hydrophobic polymers containing the nanoparticles. Various sustained-release materials have been established and are well known by those skilled in the art.

In addition, antioxidants and suspending agents can be used in the pharmaceutical compositions of the nanoparticles described herein.

G. Dosages

Determination of doses adequate to inhibit the expression of one or more preselected genes, such as a therapeutically effective amount in the clinical context, is well within the capability of those skilled in the art, especially in light of the disclosure herein.

For any therapeutic nucleic acids used in the methods of the invention, the therapeutically effective amount can be estimated initially from *in vitro* assays. Then, the dosage can be formulated for use in animal models so as to achieve a circulating concentration range that

includes the effective dosage. Such information can then be used to more accurately determine dosages useful in patients.

The amount of the pharmaceutical composition that is administered will depend upon the potency of the nucleic acids included therein. Generally, the amount of the nanoparticles containing nucleic acids used in the treatment is that amount which effectively achieves the desired therapeutic result in mammals. Naturally, the dosages of the various nanoparticles will vary somewhat depending upon the nucleic acids (or pharmaceutically active agents) encapsulated therein (e.g., oligonucleotides). In addition, the dosage, of course, can vary depending upon the dosage form and route of administration. In general, however, the nucleic acids encapsulated in the nanoparticles described herein can be administered in amounts ranging from about 0.1 to about 1 g/kg/week, preferably from about 1 to about 500 mg/kg and more preferably from 1 to about 100 mg/kg (i.e., from about 3 to about 90 mg/kg/dose).

The range set forth above is illustrative and those skilled in the art will determine the optimal dosing based on clinical experience and the treatment indication. Moreover, the exact formulation, route of administration and dosage can be selected by the individual physician in view of the patient's condition. Additionally, toxicity and therapeutic efficacy of the nanoparticles described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals using methods well-known in the art.

Alternatively, an amount of from about 1 mg to about 100 mg/kg/dose (0.1 to 100mg/kg/dose) can be used in the treatment depending on potency of the nucleic acids. Dosage unit forms generally range from about 1 mg to about 60 mg of an active agent, oligonucleotides.

In one embodiment, the treatment of the present invention includes administering the nanoparticles described herein in an amount of from about 1 to about 60 mg/kg/dose (from about 25 to 60 mg/kg/dose, from about 3 to about 20 mg/kg/dose), such as 60, 45, 35, 30, 25, 15, 5 or 3 mg/kg/dose (either in a single or multiple dose regime) to a mammal. For example, the nanoparticles described herein can be administered introvenously in an amount of 5, 25, 30, or 60 mg/kg/dose at q3d x 9. For another example, the treatment protocol includes administering an antisense oligonucleotide in an amount of from about 4 to about 18 mg/kg/dose weekly, or about 4 to about 9.5 mg/kg/dose weekly (e.g., about 8 mg/kg/dose weekly for 3 weeks in a six week cycle).

Alternatively, the delivery of the oligonucleotide encapsulated within the nanoparticles

described herein includes contacting a concentration of oligoncleotides of from about 0.1 to about 1000 μM, preferably from about 10 to about 1500 μM (i.e. from about 10 to about 1000 μM, from about 30 to about 1000 μM) with tumor cells or tissues *in vivo, ex vivo* or *in vitro*.

The compositions may be administered once daily or divided into multiple doses which can be given as part of a multi-week treatment protocol. The precise dose will depend on the stage and severity of the condition, the susceptibility of the disease such as tumor to the nucleic acids, and the individual characteristics of the patient being treated, as will be appreciated by one of ordinary skill in the art.

In all aspects of the invention where nanoparticles are administered, the dosage amount mentioned is based on the amount of oligonucleotide molecules rather than the amount of nanoparticles administered.

It is contemplated that the treatment will be given for one or more days until the desired clinical result is obtained. The exact amount, frequency and period of administration of the nanoparticles encapsulating therapeutic nucleic acids (or pharmaceutically active agents) will vary, of course, depending upon the sex, age and medical condition of the patent as well as the severity of the disease as determined by the attending clinician.

Still further aspects include combining the nanoparticles of the present invention described herein with other anticancer therapies for synergistic or additive benefit.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

In the examples, all synthesis reactions are run under an atmosphere of dry nitrogen or argon. N-(3-aminopropyl)-1,3-propanediamine), BOC-ON, $LiOCl_4$, Cholesterol and 1H-Pyrazole-1-carboxamidine·HCl were purchased from Aldrich. All other reagents and solvents were used without further purification. An LNA Oligo-1 targeting survivin gene, and Oligo-2 targeting ErbB3 gene were prepared in house and their sequences are given in Table 4. The internucleosides linkage is phosphorothioate, ^{m}C represents methylated cytosine, and the upper case letters indicate LNA.

Table 4

LNA Oligo	Sequence

Oligo-1 (SEQ ID NO: 1)	5'- ^{m}CT^{m}CAatccatgg^{m}CAGc -3'
Oligo-2 (SEQ ID NO: 6)	5'- TAGcctgtcactt^{m}CT^{m}C -3'

Following abbreviations may be used throughout the examples such as, LNA (Locked nucleic acid oligonucleotide), BACC (2-[N,N'-di (2-guanidiniumpropyl)]aminoethyl-cholesteryl-carbonate), Chol (cholesterol), DIEA (diisopropylethylamine), DMAP (4-*N,N*-dimethylamino-pyridine), DOPE (L-α-dioleoyl phosphatidylethanolamine, Avanti Polar Lipids, USA or NOF, Japan), DLS (Dynamic Light Scaterring), DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) (NOF, Japan), DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(polyethylene glycol)2000 ammonium salt or sodium salt, Avanti Polar Lipids, USA and NOF, Japan), KD (knowndown), EPC (egg phosphatidylcholine, Avanti Polar Lipids, USA) and C16 mPEG-Ceramide (N-palmitoyl-sphingosine-1-succinyl(methoxypolyethylene glycol)2000, Avanti Polar Lipids, USA). Other abbreviations such as the FAM (6-carboxyfluorescein), FBS (fetal bovine serum), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), DMEM (Dulbecco's Modified Eagle's Medium), MEM (Modified Eagle's Medium), TEAA (tetraethylammonium acetate), TFA (trifluoroacetic acid), RT-qPCR (reverse transcription-quantitative polymerase chain reaction) may be also used.

Example 1. General NMR Method.

^{1}H NMR spectra were obtained at 300 MHz and ^{13}C NMR spectra at 75.46 MHz using a Varian Mercury 300 NMR spectrometer and deuterated chloroform as the solvents unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS).

Example 2. General HPLC Method.

The reaction mixtures and the purity of intermediates and final products are monitored by a Beckman Coulter System Gold® HPLC instrument. It employs a ZORBAX® 300SB C8 reversed phase column (150 × 4.6 mm) or a Phenomenex Jupiter® 300A C18 reversed phase column (150 x 4.6 mm) with a 168 Diode Array UV Detector, using a gradient of 10-90 % of

acetonitrile in 0.05 % TFA at a flow rate of 1 mL/minute or a gradient of 25-35 % acetonitrile in 50 mM TEAA buffer at a flow rate of 1 mL/minute. The anion exchange chromatography was run on AKTA explorer 100A from GE healthcare (Amersham Biosciences) using Poros 50HQ strong anion exchange resin from Applied Biosystems packed in an AP-Empty glass column from Waters. Desalting was achieved by using HiPrep 26/10 desalting columns from Amersham Biosciences. (for PEG-Oligo)

Example 3. General mRNA Down-Regulation Procedure.

The cells are maintained in complete medium (F-12K or DMEM, supplemented with 10% FBS). A 12 well plate containing 2.5×10^5 cells in each well is incubated overnight at 37 °C. Cells are washed once with Opti-MEM® and 400 µL of Opti-MEM® is added per each well. Then, a solution of nanoparticle or Lipofectamine2000® containing oligonucleotide is added to each well. The cells is incubated for 4 hours, followed by addition of 600 µL of media per well, and incubation for 24 hours. After 24 hours of treatment, the intracellular mRNA levels of the target gene, such as human survivin, and a housekeeping gene, such as GAPDH are quantitated by RT-qPCR. The expression levels of mRNA are normalized.

Example 4. General RNA Preparation Procedure.

For the in vitro mRNA down-regulation screen, total RNA is prepared using RNAqueous Kit® (Ambion) following the manufacturer's instruction. The RNA concentrations are determined by $OD_{260\ nm}$ using Nanodrop.

Example 5. General RT-qPCR Procedure.

All the reagents are from Applied Biosystems: High Capacity cDNA Reverse Transcription Kit® (4368813), 20x PCR master mix (4304437), and TaqMan® Gene Expression Assays kits for human GAPDH (Cat. #0612177) and survivin (BIRK5 Hs00153353). 2.0 µg of total RNA is used for cDNA synthesis in a final volume of 50 µL. The reaction is conducted in a PCR thermocycler at 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 secconds and then stored at 4 °C. Real-time PCR is conducted with the program of 50 °C-2 minutes, 95 °C-10

minutes, and 95 °C-15 seconds / 60 °C-1 minute for 40 cycles. For each qPCR reaction, 1 μL of cDNA is used in a final volume of 30 μL.

Example 6. Preparation of Compound 3.

Cholesterol (compound **1**) is reacted with a protected cysteine (compound **2**) in the presence of EDC and DMAP to form a cholesteryl cysteine (compound **3**).

Example 7. Preparation of Compound 5.

Compound **3** and a bifunctional spacer containing a thiol group (compound **4**) are reacted in the presence of DIPEA to provide compound **5** forming a disulfide bond.

Example 8. Preparation of Compound 6.

Compound **5** is treated with piperidine and DMF (1:1) to remove the Fmoc group and to provide compound **6**.

Example 9. Preparation of Compound 8.

Compound **6** is coupled with FmocLys-OH (compound **7**) in the presence of EDC and DMAP to provide compound **8**.

Example 10. Preparation of Compound 9.

Compound **8** is treated with piperidine and DMF (1:1) to remove the Fmoc group and to give compound **9**.

Example 11. Preparation of Compound 11.

To a solution of **9** (1.48 mmol) in 12 mL anhydrous chloroform is added 1H-pyrazole-1-carboxamidine HCl (compound **10**, 0.87 g, 5.9 mmol) followed by DIEA (1.03 mL, 5.9 mmol) at room temperature. The reaction is refluxed for 16 hours. The solution is cooled to room temperature. The mixture is precipitated with 15 mL ACN and crude solids are isolated with centrifuge. The solids are dissolved in 14 mL water/ACN (1:1) solution. After complete dissolution, 14 mL ACN is added to precipitate solids. The solids are centrifuged and dried to yield the product.

Example 12. Preparation of Compound 12

Compound **11** is treated with TFA to remove the BOC group and provide compound **12**.

Example 13. Preparation of Compound 22.

N-(2-hydroxyethyl)phthalimide (**21**, 25 g, 130.8 mmol, 1 eq) was dissolved in 500 mL of dry benzene and azeotroped for 1 hour, removing 125 mL of benzene, followed by cooling to room temperature and addition of *p*-TsOH (0.240 g, 1.26 mmol, 0.0096 eq). The reaction mixture was cooled to 0-5 °C, then added 2-methoxypropene (10.4 g, 13.8 mL, 143.8 mmol, 1.1 eq) through an addition funnel over 15 minutes at 0-5 °C. The reaction mixture was stirred at 0-5 °C for 1 hour, followed by heating to 89-95 °C and azeotroped for 3 hours, removing MeOH/benzene. Following removal of the solvents, the solution was cooled to stop the azeotroping and an equivalent volume of benzene was added. After 3 hours, the reaction mixture was cooled to room temperature and was added 30 mL of TEA and 5 mL of acetic anhydride and allowed to stir overnight at room temperature. The reaction mixture was concentrated *in vacuo* at 35 °C to remove 2/3 volume of benzene and crude products were precipitated with 300 mL of hexane dropwise. The precipitates were filtered and washed with hexane. The solids (8.5 g) were dissolved in 70 mL of toluene at 65 °C and the solution was cooled to 0 °C. The product was collected by centrifugation, washed with hexane, and coevaporated with CCl_4 *in vacuo* to yield 4.9 g of product: ^{13}C NMR δ 24.67, 38.09, 57.88, 100.39, 123.05, 131.92, 133.66, 167.88.

Example 14. Preparation of Compound 23.

Compound **22** (4.9 g, 11.6 mmol) was dissolved in 6 M NaOH (9.1 g of NaOH in 38 mL water) and the solution was refluxed overnight. The resulting solution was cooled to room temperature, then extracted three times with 40 mL of 1:1 (v/v) of chloroform/IPA, dried over anhydrous sodium sulfate, and concentrated *in vacuo* at 35 °C. The solids were suspended twice in hexane and once in CCl_4, and dryed *in vacuo* at 35 °C to obtain the product (1.8g, 95%): ^{13}C NMR δ 24.99, 42.08, 43.81, 62.82, 63.58, 77.41, 99.64.

Example 15. Preparation of Compound 25.

Compound **23** (1.8 g, 11.1 mmol, 1 eq) was dissolved in 36 mL of anhydrous THF, cooled to -78 °C in a dry ice/IPA bath, followed by addition of ethyltrifluoroacetate. The reaction mixture was stirred at room temperature for 1.5 hours before the solvent was removed *in vacuo* by coevaporating with hexane to give crude product. The crude product was purified by column chromatography on deactivated alumina using DCM and MeOH (100:0.1 to 98:2, v/v) to yield 1.30 g of product: ^{13}C NMR δ 24.88, 40.68, 41.11, 42.13, 57.99, 60.26, 62.10, 99.83.

Example 16. Preparation of Compound 27.

Compound **26** (2.88 mmol) and compound **25** (15.0 mmol) are dissolved in 60 mL dry DCM and 8 mL dry DMF. DIEA (0.60g, 0.82 mL. 4.61 mmol, 1.6 eq) is added and the reaction mixture is stirred overnight at room temperature. The resulting reaction solution is concentrated *in vacuo* at room temperature, followed by addition of ether to precipitate solids at 0-5 °C in an ice bath. The solids are filtered and purified by column chromatography to provide compound **27**.

Example 17. Preparation of Compound 28.

Compound **27** is treated with K_2CO_3 to provide compound **28**.

Example 18. Preparation of Compound 29.

Compound **28** is coupled with FmocLys-OH (compound **7**) in the presence of EDC and DMAP to provide compound **29**.

Example 19. Preparation of Compound 30.

Compound **29** is treated with piperidine and DMF (1:1) to remove the Fmoc group to give compound **30**.

Example 20. Preparation of Compound 31.

To a solution of **30** (1.48 mmol) in 12 mL anhydrous chloroform is added 1H-pyrazole-1-carboxamidine HCl (compound **10**, 0.87 g, 5.9 mmol) followed by DIEA (1.03 mL, 5.9 mmol) at room temperature. The reaction is refluxed for 16 hours. The solution is cooled to room temperature. The mixture is precipitated with 15 mL ACN and crude solids are isolated with

centrifuge. The solids are dissolved in 14 mL water/ACN (1:1) solution. After complete dissolution, 14 mL ACN is added to precipitate solids. The solids are centrifuged and dried to yield the product.

Example 21. Preparation of Compound 43.

Compound **41** is reacted with compound **42** in the presence of DIEA to provide compound **43**.

Example 22. Preparation of Compound 44.

Compound **43** is treated with TFA in DCM to provide compound **44**.

Example 23. Preparation of Compound 46.

Cholesteryl chloroformate (compound **26**) is reacted with 2-methoxy-4-hydroxybenazldehyde (compound **45**) in the presence of DIEA to provide compound **46**.

Example 24. Preparation of Compound 47.

Compound **44** and compound **46** are reacted in the presence of molecular sieves to provide compound **47** forming an imine bond.

Example 25. Preparation of Compound 48.

Compound **47** is treated with piperidine and DMF (1:1) to remove the Fmoc group. The reaction is stirred for 30 minutes and then desalted on HiPrep column with water to give compound **48**.

Example 26. Preparation of Compound 49.

To a solution of **48** (1.48 mmol) in 12 mL anhydrous chloroform is added 1H-pyrazole-1-carboxamidine HCl (compound **10**, 0.87 g, 5.9 mmol) followed by DIEA (1.03 mL, 5.9 mmol) at room temperature. The reaction is refluxed for 16 hours. The solution is cooled to room temperature. The mixture is precipitated with 15 mL ACN and crude solids are isolated with centrifuge. The solids are dissolved in 14 mL water/ACN (1:1) solution. After complete

dissolution, 14 mL ACN is added to precipitate solids. The solids are centrifuged and dried to yield the product.

Example 27. Preparation of Compound 51.

TEA (33.6 g, 0.033 mol) was added to a solution of cholesteryl chloroformate (**26**, 5 g, 0.011 mol) in CH_2Cl_2 (200 mL) and DMF (100 mL), followed by addition of cystamine di-hydrochloride (**50**, 25 g, 0.11 mol). The reaction mixture was stirred at room temperature for 5 days. The insoluble residue was filtered and the eluent was concentrated under reduced pressure. The residue was purified by flash column chromatography using 5-10% MeOH in CH_2Cl_2 to yield 0.9 g (14%) of product.

Example 28. Preparation of Compound 52.

Compound **51** is coupled with FmocLys-OH (compound **7**) in the presence of EDC and DMAP to provide compound **52**.

Example 29. Preparation of Compound 53.

Compound **52** is treated with piperidine and DMF (1:1) to remove the Fmoc group to give compound **53**.

Example 30. Preparation of Compound 54.

To a solution of **53** (1.48 mmol) in 12 mL anhydrous chloroform is added 1H-pyrazole-1-carboxamidine HCl (compound **10**, 0.87 g, 5.9 mmol) followed by DIEA (1.03 mL, 5.9 mmol) at room temperature. The reaction is refluxed for 16 hours. The solution is cooled to room temperature. The mixture is precipitated with 15 mL ACN and crude solids are isolated with centrifuge. The solids are dissolved in 14 mL water/ACN (1:1) solution. After complete dissolution, 14 mL ACN is added to precipitate solids. The solids are centrifuged and dried to yield the product.

Example 31. Preparation of Nucleic acids-Nanoparticle Composition

In this example, nanoparticle compositions encapsulating various nucleic acids such as LNA-containing oligonucleotides are prepared. For example, compound **54**, DOPE, Chol,

DSPE-PEG and C_{16}mPEG-Ceramide are mixed at a molar ratio of 18: 60: 20:1:1 in 10 mL of 90% ethanol (total lipid 30 μmole). LNA oligonucleotides (0.4 μmole) are dissolved in 10 mL of 20 mM Tris buffer (pH 7.4-7.6). After being heated to 37 °C, the two solutions are mixed together through a duel syringe pump and the mixed solution is subsequently diluted with 20 mL of 20 mM Tris buffer (300 mM NaCl, pH 7.4-7.6). The mixture is incubated at 37 °C for 30 minutes and dialyzed in 10 mM PBS buffer (138 mM NaCl, 2.7mM KCl, pH 7.4). Stable particles are obtained after the removal of ethanol from the mixture by dialysis. The nanoparticle solution is concentrated by centrifugation. The nanoparticle solution is transferred into a 15 mL centrifugal filter device (Amicon Ultra-15, Millipore, USA). Centrifuge speed is at 3,000 rpm and temperature is at 4 °C during centrifugation. The concentrated suspension is collected after a given time and is sterilized by filtration through a 0.22 μm syringe filter (Millex-GV, Millipore, USA).

The diameter and polydispersity of nanoparticle are measured at 25 ° in water (Sigma) as a medium on a Plus 90 Particle Size Analyzer Dynamic Light Scattering Instrument (Brookhaven, New York).

Encapsulation efficiency of LNA oligonucleotides is determined by UV-VIS (Agilent 8453). The background UV-vis spectrum is obtained by scanning solution, which is a mixed solution composed of PBS buffer saline (250 μL), methanol (625 μL) and chloroform (250 μL). In order to determine the encapsulated nucleic acids concentration, methanol (625 μL) and chloroform (250 μL) are added to PBS buffer saline nanoparticle suspension (250 μL). After mixing, a clear solution is obtained and this solution is sonicated for 2 minutes before measuring absorbance at 260 nm. The encapsulated nucleic acid concentration and loading efficiency is calculated according to equations (1) and (2):

$$C_{en}\ (\mu g / ml) = A_{260} \times OD_{260}\ \text{unit}\ (\mu g / mL) \times \text{dilution factor}\ (\mu L / \mu L) \text{----------(1)}$$

where the dilution factor is given by the assay volume (μL) divided by the sample stock volume (μL).

$$\text{Encapsulation efficiency}\ (\%) = [C_{en} / C_{initial}] \times 100 \text{ ----------(2)}$$

where C_{en} is the nucleic acid (i.e., LNA oligonucleotide) concentration encapsulated in nanoparticle suspension after purification, and $C_{initial}$ is the initial nucleic acid (LNA

oligonucleotide) concentration before the formation of the nanoparticle suspension. Examples of various nanoparticle compositions are summarized in Tables 5 and 6.

Table 5.

Sample No.	Nanoparticle Composition	Molar Ratio	Oligo
1	Cpd of Formula (I): DOPE: DSPC : Chol : PEG-DSPE	15:15:20:40:10	Oligo-1
2	Cpd of Formula (I): DOPE: DSPC: Chol: PEG-DSPE	15:5:20:50:10	Oligo-1
3	Cpd of Formula (I): DOPE: DSPC: Chol: PEG-DSPE	25:15:20:30:10	Oligo-1
4	Cpd of Formula (I): EPC: Chol: PEG-DSPE	20:47:30: 3	Oligo-1
5	Cpd of Formula (I): DOPE: Chol: PEG-DSPE	17:60:20:3	Oligo-1
6	Cpd of Formula (I): DOPE: PEG-DSPE	20:78: 2	Oligo-1
7	Cpd of Formula (I): DOPE: Chol:C16mPEG-Ceramide	17:60:20:3	Oligo-2
8	Cpd of Formula (I): DOPE: Chol: PEG-DSPE: C16mPEG-Ceramide	18:60:20:1:1	Oligo-2

Table 6.

Sample No.	Nanoparticle Composition	Molar Ratio	Oligo
NP1	Cpd of Formula (I): DOPE: Chol: PEG-DSPE: C16mPEG-Ceramide	18:60:20:1:1	Oligo-2
NP2	Cpd of Formula (I): DOPE: Chol: PEG-DSPE: C16mPEG-Ceramide	18:60:20:1:1	FAM-Oligo-2
NP3	Cpd of Formula (I): DOPE: Chol: PEG-DSPE: C16mPEG-Ceramide	18:60:20:1:1	none

*Compounds of Formula (I): compound 12, compound 31, compound 49 and compound 54.

Example 32. Nanoparticle Stability

Nanoparticle stability is defined as their capability to retain the structural integrity in PBS buffer at 4 °C over time. The colloidal stability of nanoparticles is evaluated by monitoring changes in the mean diameter over time. Nanoparticles prepared by Sample No. NP1 in Table 6 are dispersed in 10 mM PBS buffer (138 mM NaCl, 2.7 mM KCl, pH 7.4) and stored at 4 °C. At a given time point, about 20-50 μL of the nanoparticle suspension is taken and diluted with pure water up to 2 mL. The sizes of nanoparticles are measured by DLS at 25 °C.

Example 33. *In vitro* Nanoparticle Cellular Uptake

The efficiency of cellular uptake of nucleic acids (LNA oligonucleotide Oilgo-2) encapsulated in the nanoparticle described herein is evaluated in human cancer cells such as prostate cancer cells (15PC3 cell line). Nanoparticles of Sample NP2 are prepared using the

method described in Example 31. LNA oligonucleotides (Oligo-2) are labeled with FAM for fluorescent microscopy studies.

The nanoparticles are evaluated in the 15PC3 cell line. The cells are maintained in a complete medium (DMEM, supplemented with 10% FBS). A 12 well plate containing 2.5×10^5 cells in each well is incubated overnight at 37 °C. The cells are washed once with Opti-MEM and 400 mL of Opti-MEM is added to each well. Then, the cells are treated with a nanoparticle solution of Sample No. NP2 (200 nM) encapsulating nucleic acids (FAM-modified Oligo 2) or a solution of free nucleic acids without the nanoparticles (naked FAM-modified Oligo 2) as a control. The cells are incubated for 24 hours at 37°C. The cells are washed with PBS five times, and then stained with 300 mL of Hoechst solution (2 mg / mL) per well for 30 minutes, followed by washing with PBS 5 times. The cells are fixed with pre-cooled (-20 °C) 70% EtOH at -20 °C for 20 minutes. The cells are inspected under a fluorescent microscope to evaluate the efficiency of cellular uptake of nucleic acids encapsulated within the nanoparticle described herein.

Example 34. *In vitro* Efficacy of Nanoparticles on mRNA Down-regulation in a Variety of Human Cancer Cells

The efficacy of the nanoparticles described herein is evaluated in a variety of cancer cells, for example, human epideram cancer cells (A431), human gastric cancer cells (N87), human lung cancer cells (A549, HCC827, or H1581), human prostate cancer cells (15PC3, LNCaP, PC3, CWR22, DU145), human breast cancer cells (MCF7, SKBR3), colon cancer cells (SW480), pancreatic cancer cells (BxPC3), and melanoma (518A2). The cells are treated with one of the following: nanoparticles encapsulating antisense ErbB3 oligonucleotides (Sample NP1), or empty placebo nanoparticles (Sample No. NP3). The *in vitro* efficacy of each of the nanoparticles on downregulation of ErbB3 expression is measured by the procedures described in Example 3.

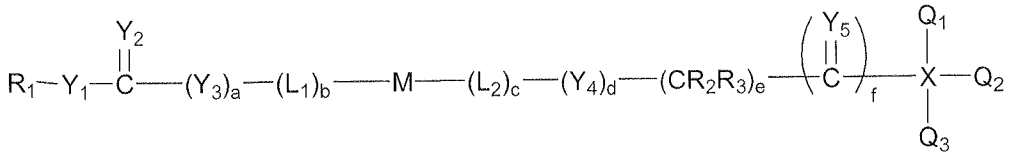
Example 35. Effects of Nanoparticles on mRNA Down-regulation in Tumor and Liver of Human Prostate Cancer Xenografted Mice Model

The *in vivo* efficacy of nanoparticles described herein is evaluated in human prostate cancer xenografted mice. The 15PC3 human prostate tumors are established in nude mice by subcutaneous injection of 5×10^6 cells/mouse into the right auxiliary flank. When tumors reach

the average volume of 100 mm^3, the mice are randomly grouped 5 mice per group. The mice of each group are treated with nanoparticle encapsulating antisense ErbB3 oligonucleotides (Sample NP1) or corresponding naked oligonucleotides (Oligo 2). The nanoparticles are given intravenously (i.v.) at 15 mg/kg/dose, 5 mg/kg/dose, 1 mg/kg/dose, or 0.5 mg/kg/dose at q3d x 4 (or q3d x10). The dosage amount is based on the amount of oligonucleotides in the nanoparticles. The naked oligonucleotides are given intraperitoneally (i.p.) at 30 mg/kg/dose or intravenously at 25 mg/kg/dose or 45 mg/kg/dose at q3d x 4 for 12 days. The mice are sacrificed twenty four hours after the final dose. Plasma samples are collected from the mice and stored at -20 °C. Tumor and liver samples are also collected from the mice. The samples are analyzed for mRNA KD in the tumors and livers. The survival of the animals is observed.

We claim:

1. A compound of Formula (I):



(I)

wherein

R_1 is cholesterol or an analog thereof;

Y_1 is O, S or NR_4;

Y_2 and Y_5 are independently O, S or NR_5;

Y_{3-4} are independently O, S or NR_6;

L_{1-2} are independently selected bifunctional linkers;

M is an acid labile linker;

(a), (d) and (f) are independently 0 or 1;

(b), (c) and (e) are independently 0 or positive integers;

X is C, N or P;

Q_1 is H, C_{1-6} alkyl, NH_2, or $-(L_{11})_{d1}-R_{11}$;

Q_2 is H, C_{1-6} alkyl, NH_2, or $-(L_{12})_{d2}-R_{12}$;

Q_3 is a lone electron pair, (=O), H, C_{1-6} alkyl, NH_2, or $-(L_{13})_{d3}-R_{13}$;

provided that

(i) when X is C, Q_3 is not a lone electron pair or (=O);

(ii) when X is N, Q_3 is a lone electron pair; and

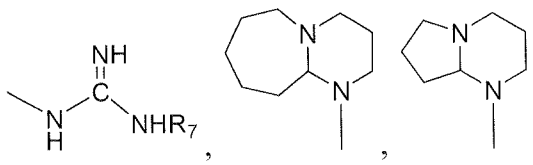
(iii) when X is P, Q_3 is (=O), and (f) is 0,

wherein

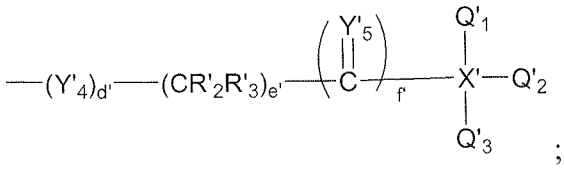
L_{11}, L_{12} and L_{13} are independently selected bifunctional spacers;

(d1), (d2) and (d3) are independently 0 or positive integers;

R_{11}, R_{12} and R_{13} are independently hydrogen, NH_2,



or



;

wherein

Y'_4 is O, S, or NR'_6;

Y'_5 are independently O, S or NR'_5;

(d') and (f') are independently 0 or 1;

(e') is 0 or a positive integer;

X' is C, N or P;

Q'_1 is H, C_{1-6} alkyl, NH_2, or $-(L'_{11})_{d'1}-R'_{11}$;

Q'_2 is H, C_{1-6} alkyl, NH_2, or $-(L'_{12})_{d'2}-R'_{12}$;

Q'_3 is a lone electron pair, (=O), H, C_{1-6} alkyl, NH_2, or $-(L_{13})_{d'3}-R'_{13}$;

provided that

(i) when X' is C, Q'_3 is not a lone electron pair or (=O);

(ii) when X' is N, Q'_3 is a lone electron pair; and

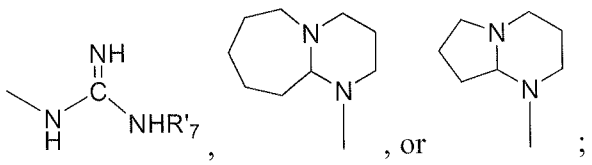
(iii) when X' is P, Q'_3 is (=O) and (f') is 0,

wherein

L'_{11}, L'_{12} and L'_{13} are independently selected bifunctional spacers;

(d'1), (d'2) and (d'3) are independently 0 or positive integers ;

R'_{11}, R'_{12} and R'_{13} are independently hydrogen, NH_2,



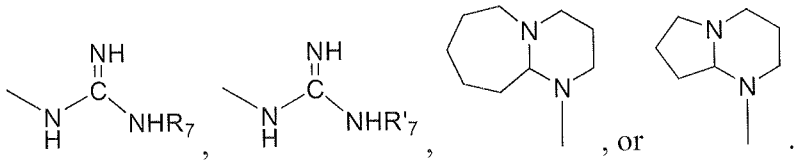
;

R_{2-3}, and R'_{2-3} are independently selected from the group consisting of hydrogen, hydroxyl, amine, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl,

aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl; and

R_{4-7}, and R'_{5-7} are independently selected from the group consisting of hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, and substituted C_{1-6} heteroalkyl,

provided that at least one of Q_{1-3} and Q'_{1-3} includes



2. The compound of claim 1, wherein M is selected from the group consisting of –S-S-, a ketal- or acetal-containing moiety, and an imine-containing moiety.

3. The compound of claim 1, wherein M is –S-S-.

4. The compound of claim 1, wherein M is $-CR_{16}R_{17}\text{-}O\text{-}CR_{14}R_{15}\text{-}O\text{-}CR_{18}R_{19}-$, wherein

R_{14-15} are independently selected from the group consisting of hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy, substituted and arylcarbonyloxy; and

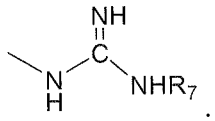
R_{16-19} are independently selected from the group consisting of hydrogen, amine, substituted amine, azido, carboxy, cyano, halo, hydroxyl, nitro, silyl ether, sulfonyl, mercapto, C_{1-6} alkylmercapto, arylmercapto, substituted arylmercapto, substituted C_{1-6} alkylthio, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl,

heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy, substituted and arylcarbonyloxy.

5. The compound of claim 4, wherein R_{14} and R_{15} are selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-8} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls, substituted aryls and aralkyls.

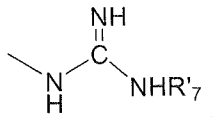
6. The compound of claim 1, wherein M is $-N=CR_{10}-$ or $-CR_{10}=N-$, wherein R_{10} is hydrogen, C_{1-6} alkyl, C_{3-8} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{3-8} substituted cycloalkyl, aryl and substituted aryl.

7. The compound of claim 1, wherein both Q_1 and Q_2 include



.

8. The compound of claim 1, wherein both Q'_1 and Q'_2 include



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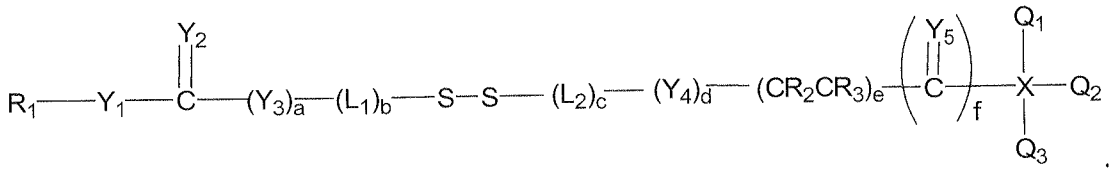
9. The compound of claim 1 having Formula (Ia):



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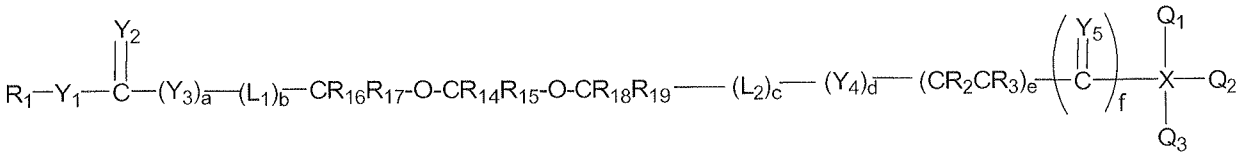
10. The compound of claim 1 having Formula (Ib):



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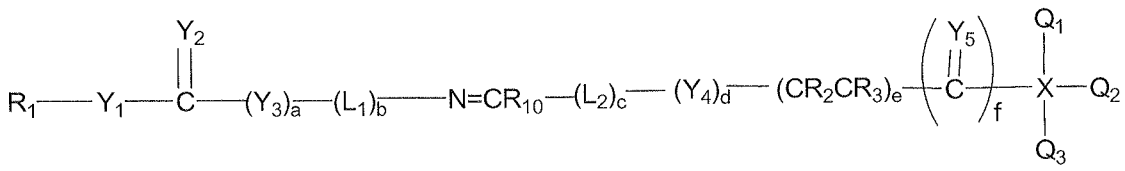
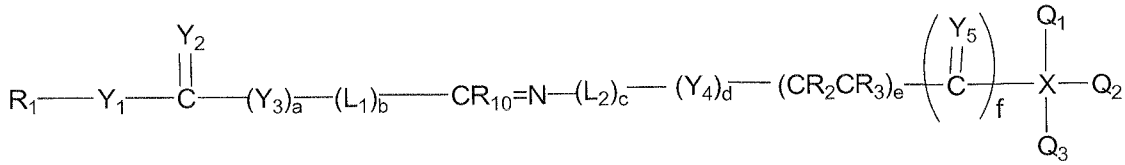
11. The compound of claim 1 having Formula (Ic) or (Ic'):



or



.

12. The compound of claim 1, wherein Y_1 is O.

13. The compound of claim 1, wherein Y_2 is O; and Y_5 is O.

14. The compound of claim 1, wherein L_1 is selected from the group consisting of:

$-(CR_{21}R_{22})_{t1}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}Y_{17}-(CR_{23}R_{24})_{t2}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22}CR_{23}R_{24}Y_{17})_{t1}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22}CR_{23}R_{24}Y_{17})_{t1}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-[(CR_{21}R_{22}CR_{23}R_{24})_{t2}Y_{17}]_{t3}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}-[(CR_{23}R_{24})_{t2}Y_{17}]_{t3}(CR_{25}R_{26})_{t4}-(Y_{18})_{a2}-[C(=Y_{16})]_{a3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24})_{t2}-$,

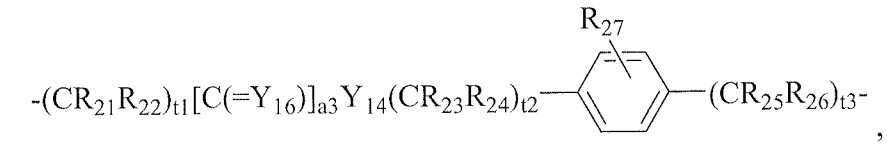
$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24})_{t2}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24})_{t2}-Y_{15}-(CR_{23}R_{24})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24})_{t2}-Y_{15}-(CR_{23}R_{24})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}(CR_{23}R_{24}CR_{25}R_{26}Y_{19})_{t2}(CR_{27}CR_{28})_{t3}-$,

$-(CR_{21}R_{22})_{t1}(Y_{17})_{a2}[C(=Y_{16})]_{a3}Y_{14}(CR_{23}R_{24}CR_{25}R_{26}Y_{19})_{t2}(CR_{27}CR_{28})_{t3}-$, and



wherein:

Y_{16} is O, NR_{28}, or S;

Y_{14-15} and Y_{17-19} are independently O, NR_{29}, or S;

R_{21-27} are independently selected from the group consisting of hydrogen, hydroxyl, amine, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

R_{28-29} are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

(t1), (t2), (t3) and (t4) are independently zero or positive integers; and

(a2) and (a3) are independently zero or 1.

15. The compound of claim 1, wherein L_1 is selected from the group consisting of:

$-CH_2-$, $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,$-NH(CH_2)-$,

$-CH(NH_2)CH_2-$,

$-(CH_2)_4-C(=O)-$, $-(CH_2)_5-C(=O)-$, $-(CH_2)_6-C(=O)-$,

$-CH_2CH_2O-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_2-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_3-CH_2O-C(=O)-$,

$-(CH_2CH_2O)_2-C(=O)-$,

$-CH_2CH_2O-CH_2CH_2NH-C(=O)-$,

$-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-CH_2CH_2O-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2CH_2NH-C(=O)-$,

$-CH_2-O-CH_2CH_2O-CH_2C(=O)-$,

$-CH_2-O-(CH_2CH_2O)_2-CH_2C(=O)-$,

-$(CH_2)_4$-C(=O)NH-, -$(CH_2)_5$-C(=O)NH-,

-$(CH_2)_6$-C(=O)NH-,

-CH_2CH_2O-CH_2O-C(=O)-NH-,

-$(CH_2CH_2O)_2$-CH_2O-C(=O)-NH-,

-$(CH_2CH_2O)_3$-CH_2O-C(=O)-NH-,

-$(CH_2CH_2O)_2$-C(=O)-NH-,

-CH_2CH_2O-CH_2CH_2NH-C(=O)-NH-,

-$(CH_2CH_2O)_2$-CH_2CH_2NH-C(=O)-NH-,

-CH_2-O-CH_2CH_2O-CH_2CH_2NH-C(=O)-NH-,

-CH_2-O-$(CH_2CH_2O)_2$-CH_2CH_2NH-C(=O)-NH-,

-CH_2-O-CH_2CH_2O-CH_2C(=O)-NH-,

-CH_2-O-$(CH_2CH_2O)_2$-CH_2C(=O)-NH-,

-$(CH_2CH_2O)_2$-, -CH_2CH_2O-CH_2O-,

-$(CH_2CH_2O)_2$-CH_2CH_2NH -,

-$(CH_2CH_2O)_3$-CH_2CH_2NH -,

-CH_2CH_2O-CH_2CH_2NH-,

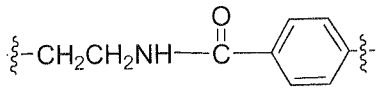
-$(CH_2CH_2O)_2$-CH_2CH_2NH-,

-CH_2-O-CH_2CH_2O-CH_2CH_2NH-,

-CH_2-O-$(CH_2CH_2O)_2$-CH_2CH_2NH-,

-CH_2-O-CH_2CH_2O-,

-CH_2-O-$(CH_2CH_2O)_2$-,



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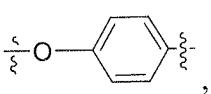

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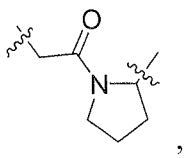

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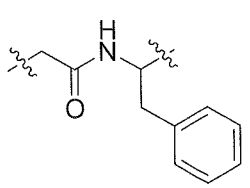

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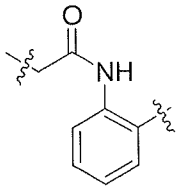


,

-C(=O)NH$(CH_2)_2$- , -CH_2C(=O)NH$(CH_2)_2$- ,

-C(=O)NH$(CH_2)_3$- , -CH_2C(=O)NH$(CH_2)_3$- ,

-C(=O)NH$(CH_2)_4$- , -CH_2C(=O)NH$(CH_2)_4$- ,

-C(=O)NH(CH_2)$_5$- , -CH_2C(=O)NH(CH_2)$_5$- ,
-C(=O)NH(CH_2)$_6$- , -CH_2C(=O)NH(CH_2)$_6$- ,
-C(=O)O(CH_2)$_2$- , -CH_2C(=O)O(CH_2)$_2$- ,
-C(=O)O(CH_2)$_3$- , -CH_2C(=O)O(CH_2)$_3$- ,
-C(=O)O(CH_2)$_4$- , -CH_2C(=O)O(CH_2)$_4$- ,
-C(=O)O(CH_2)$_5$- , -CH_2C(=O)O(CH_2)$_5$- ,
-C(=O)O(CH_2)$_6$- , -CH_2C(=O)O(CH_2)$_6$- ,
-(CH_2CH_2)$_2$NHC(=O)NH(CH_2)$_2$- ,
-(CH_2CH_2)$_2$NHC(=O)NH(CH_2)$_3$- ,
-(CH_2CH_2)$_2$NHC(=O)NH(CH_2)$_4$- ,
-(CH_2CH_2)$_2$NHC(=O)NH(CH_2)$_5$- ,
-(CH_2CH_2)$_2$NHC(=O)NH(CH_2)$_6$- ,
-(CH_2CH_2)$_2$NHC(=O)O(CH_2)$_2$- ,
-(CH_2CH_2)$_2$NHC(=O)O(CH_2)$_3$- ,
-(CH_2CH_2)$_2$NHC(=O)O(CH_2)$_4$- ,
-(CH_2CH_2)$_2$NHC(=O)O(CH_2)$_5$- ,
-(CH_2CH_2)$_2$NHC(=O)O(CH_2)$_6$- ,
-(CH_2CH_2)$_2$NHC(=O)(CH_2)$_2$- ,
-(CH_2CH_2)$_2$NHC(=O)(CH_2)$_3$- ,
-(CH_2CH_2)$_2$NHC(=O)(CH_2)$_4$- ,
-(CH_2CH_2)$_2$NHC(=O)(CH_2)$_5$- , and
-(CH_2CH_2)$_2$NHC(=O)(CH_2)$_6$-.

16. The compound of claim 1, wherein L_2 is independently selected from the group consisting of

-($CR'_{21}R'_{22}$)$_{t'1}$-[C(=Y'_{16})]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'2}$ - ,
-($CR'_{21}R'_{22}$)$_{t'1}$$Y'_{14}$-($CR'_{23}R'_{24}$)$_{t'2}$-($Y'_{15}$)$_{a'2}$-[C(=$Y'_{16}$)]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'3}$ - ,
-($CR'_{21}R'_{22}CR'_{23}R'_{24}Y'_{14}$)$_{t'1}$-[C(=$Y'_{16}$)]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'2}$ - ,
-($CR'_{21}R'_{22}CR'_{23}R'_{24}Y'_{14}$)$_{t'1}$($CR'_{25}R'_{26}$)$_{t'2}$-($Y'_{15}$)$_{a'2}$-[C(=$Y'_{16}$)]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'3}$ - ,
-[($CR'_{21}R'_{22}CR'_{23}R'_{24}$)$_{t'2}$$Y'_{14}$]$_{t'1}$($CR'_{25}R'_{26}$)$_{t'2}$-($Y'_{15}$)$_{a'2}$-[C(=$Y'_{16}$)]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'3}$ - ,
-($CR'_{21}R'_{22}$)$_{t'1}$-[($CR'_{23}R'_{24}$)$_{t'2}$$Y'_{14}$]$_{t'2}$($CR'_{25}R'_{26}$)$_{t'3}$-($Y'_{15}$)$_{a'2}$-[C(=$Y'_{16}$)]$_{a'3}$($CR'_{27}CR'_{28}$)$_{t'4}$ -

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24})_{t'2}-$,

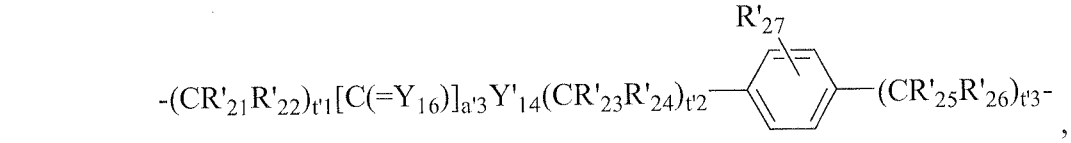
$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{15}(CR'_{23}R'_{24})_{t'2}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24})_{t'2}-Y'_{15}-(CR'_{23}R'_{24})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{14}(CR'_{23}R'_{24})_{t'2}-Y'_{15}-(CR'_{23}R'_{24})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}(CR'_{23}R'_{24}CR'_{25}R'_{26}Y'_{15})_{t'2}(CR'_{27}CR'_{28})_{t'3}-$,

$-(CR'_{21}R'_{22})_{t'1}(Y'_{14})_{a'2}[C(=Y'_{16})]_{a'3}Y'_{17}(CR'_{23}R'_{24}CR'_{25}R'_{26}Y'_{15})_{t'2}(CR'_{27}CR'_{28})_{t'3}-$, and



wherein:

Y'_{16} is O, NR'_{28}, or S;

Y'_{14-15} and Y'_{17} are independently O, NR'_{29}, or S;

R'_{21-27} are independently selected from the group consisting of hydrogen, hydroxyl, amine, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

R'_{28-29} are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

(t'1), (t'2), (t'3) and (t'4) are independently zero or positive integers; and

(a'2) and (a'3) are independently zero or 1.

17. The compound of claim 1, wherein L_2 is independently selected from the group consisting of:

$-CH_2-$, $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,$-(CH_2)NH-$

$-CH_2CH(NH_2)-$,

$-O(CH_2)_2-$, $-C(=O)O(CH_2)_3-$, $-C(=O)NH(CH_2)_3-$,

$-C(=O)(CH_2)_2-$, $-C(=O)(CH_2)_3-$,

$-CH_2-C(=O)-O(CH_2)_3-$,

$-CH_2-C(=O)-NH(CH_2)_3-$,

-CH_2-OC(=O)-O$(CH_2)_3$- ,

-CH_2-OC(=O)-NH$(CH_2)_3$- ,

-$(CH_2)_2$-C(=O)-O$(CH_2)_3$- ,

-$(CH_2)_2$-C(=O)-NH$(CH_2)_3$- ,

-CH_2C(=O)O$(CH_2)_2$-O-$(CH_2)_2$- ,

-CH_2C(=O)NH$(CH_2)_2$-O-$(CH_2)_2$- ,

-$(CH_2)_2$C(=O)O$(CH_2)_2$-O-$(CH_2)_2$- ,

-$(CH_2)_2$C(=O)NH$(CH_2)_2$-O-$(CH_2)_2$- ,

-CH_2C(=O)O$(CH_2CH_2O)_2CH_2CH_2$- ,

-$(CH_2)_2$C(=O)O$(CH_2CH_2O)_2CH_2CH_2$- ,

-$(CH_2CH_2O)_2$-, -CH_2CH_2O-CH_2O-.

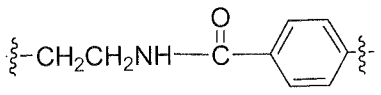
-$(CH_2CH_2O)_2$-CH_2CH_2NH -, -$(CH_2CH_2O)_3$-CH_2CH_2NH -,

-CH_2CH_2O-CH_2CH_2NH-,

-CH_2-O-CH_2CH_2O-CH_2CH_2NH-,

-CH_2-O-$(CH_2CH_2O)_2$-CH_2CH_2NH-,

-CH_2-O-CH_2CH_2O-, -CH_2-O-$(CH_2CH_2O)_2$-,



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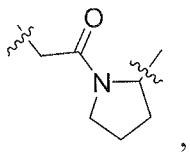

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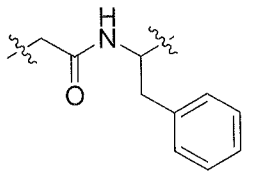

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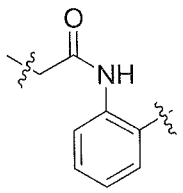


,

-$(CH_2)_2$NHC(=O)-$(CH_2CH_2O)_2$-,

-C(=O)NH$(CH_2)_2$- , -CH_2C(=O)NH$(CH_2)_2$- ,

-C(=O)NH$(CH_2)_3$- , -CH_2C(=O)NH$(CH_2)_3$- ,

-C(=O)NH$(CH_2)_4$- , -CH_2C(=O)NH$(CH_2)_4$- ,

-C(=O)NH$(CH_2)_5$- , -CH_2C(=O)NH$(CH_2)_5$- ,

-C(=O)NH$(CH_2)_6$- , -CH_2C(=O)NH$(CH_2)_6$- ,

-C(=O)O$(CH_2)_2$- , -CH_2C(=O)O$(CH_2)_2$- ,

-C(=O)O$(CH_2)_3$- , -CH_2C(=O)O$(CH_2)_3$- ,

$-C(=O)O(CH_2)_4-$, $-CH_2C(=O)O(CH_2)_4-$,
$-C(=O)O(CH_2)_5-$, $-CH_2C(=O)O(CH_2)_5-$,
$-C(=O)O(CH_2)_6-$, $-CH_2C(=O)O(CH_2)_6-$,
$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_2-$,
$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_3-$,
$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_4-$,
$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_5-$,
$-(CH_2CH_2)_2NHC(=O)NH(CH_2)_6-$,
$-(CH_2CH_2)_2NHC(=O)O(CH_2)_2-$,
$-(CH_2CH_2)_2NHC(=O)O(CH_2)_3-$,
$-(CH_2CH_2)_2NHC(=O)O(CH_2)_4-$,
$-(CH_2CH_2)_2NHC(=O)O(CH_2)_5-$,
$-(CH_2CH_2)_2NHC(=O)O(CH_2)_6-$,
$-(CH_2CH_2)_2NHC(=O)(CH_2)_2-$,
$-(CH_2CH_2)_2NHC(=O)(CH_2)_3-$,
$-(CH_2CH_2)_2NHC(=O)(CH_2)_4-$,
$-(CH_2CH_2)_2NHC(=O)(CH_2)_5-$, and
$-(CH_2CH_2)_2NHC(=O)(CH_2)_6-$.

18. The compound of claim 1, wherein L_{11-13} and L'_{11-13} are independently selected from the group consisting of:

$-(CR_{31}R_{32})_{q1}-$; and

$-Y_{26}(CR_{31}R_{32})_{q1}-$,

wherein:

Y_{26} is O, NR_{33}, or S;

R_{31-32} are independently selected from the group consisting of hydrogen, OH, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy; and

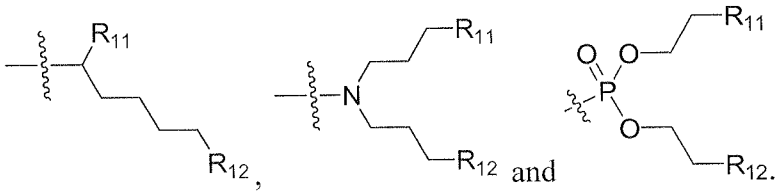
R_{33} is selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy; and

(q1) is zero or a positive integer.

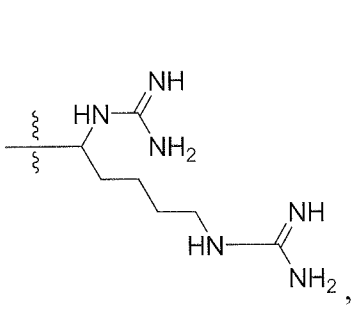
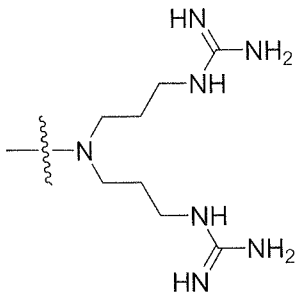
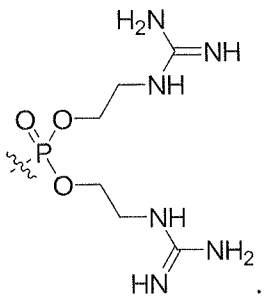
19. The compound of claim 1, wherein $L_{11\text{-}13}$ and $L'_{11\text{-}13}$ are independently selected from the group consisting of

$-CH_2-$,$-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$,
$-O(CH_2)_2-$, $-O(CH_2)_3-$, $-O(CH_2)_4-$, $-O(CH_2)_5-$, $-O(CH_2)_6-$,
$-(CH_2CH_2O)-CH_2CH_2-$,
$-(CH_2CH_2O)_2-CH_2CH_2-$,
$-C(=O)O(CH_2)_3-$, $-C(=O)NH(CH_2)_3-$,
$-C(=O)(CH_2)_2-$, $-C(=O)(CH_2)_3-$,
$-CH_2-C(=O)-O(CH_2)_3-$,
$-CH_2-C(=O)-NH(CH_2)_3-$,
$-CH_2-OC(=O)-O(CH_2)_3-$,
$-CH_2-OC(=O)-NH(CH_2)_3-$,
$-(CH_2)_2-C(=O)-O(CH_2)_3-$,
$-(CH_2)_2-C(=O)-NH(CH_2)_3-$,
$-CH_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,
$-CH_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,
$-(CH_2)_2C(=O)O(CH_2)_2-O-(CH_2)_2-$,
$-(CH_2)_2C(=O)NH(CH_2)_2-O-(CH_2)_2-$,
$-CH_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$, and
$-(CH_2)_2C(=O)O(CH_2CH_2O)_2CH_2CH_2-$.

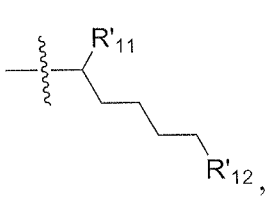
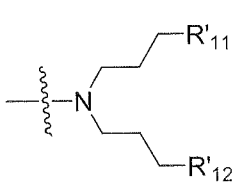
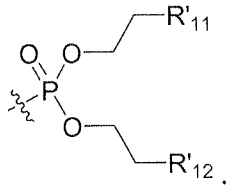
20. The compound of claim 1, wherein the $X(Q_1)(Q_2)(Q_3)$ moiety is selected from the group consisting of:



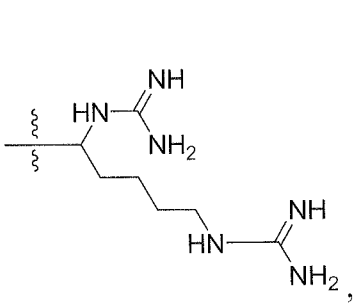
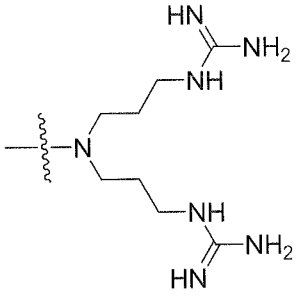
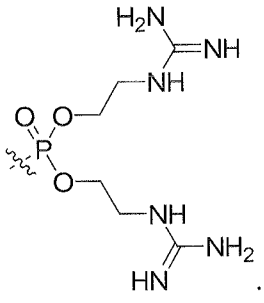
21. The compound of claim 20, wherein the $X(Q_1)(Q_2)(Q_3)$ moiety is

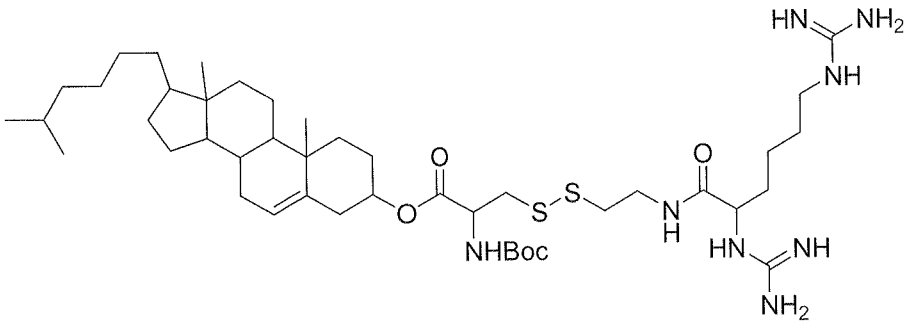
22. The compound of claim 1, wherein the X'(Q'$_1$)(Q'$_2$)(Q'$_3$) moiety is selected from the group consisting of:

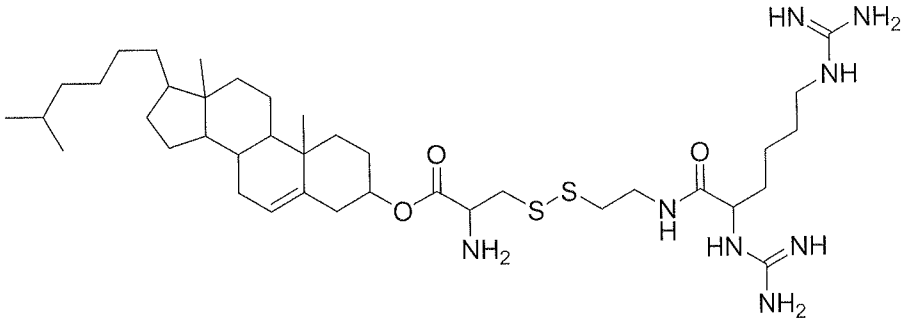




23. The compound of claim 22, wherein the X'(Q'$_1$)(Q'$_2$)(Q'$_3$) moiety is:

24. The compound of claim 1 selected from the group consisting of:





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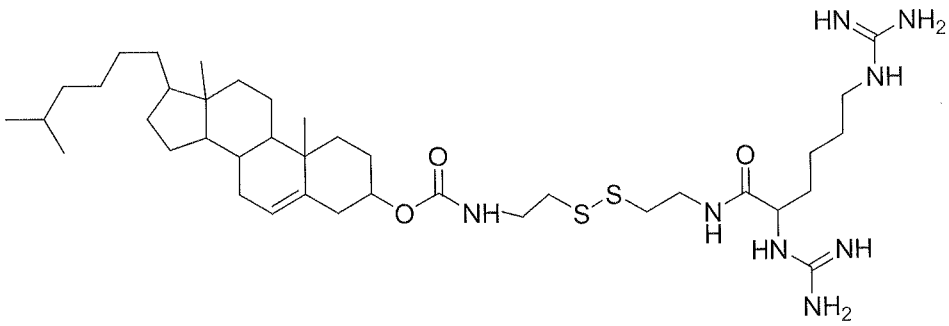

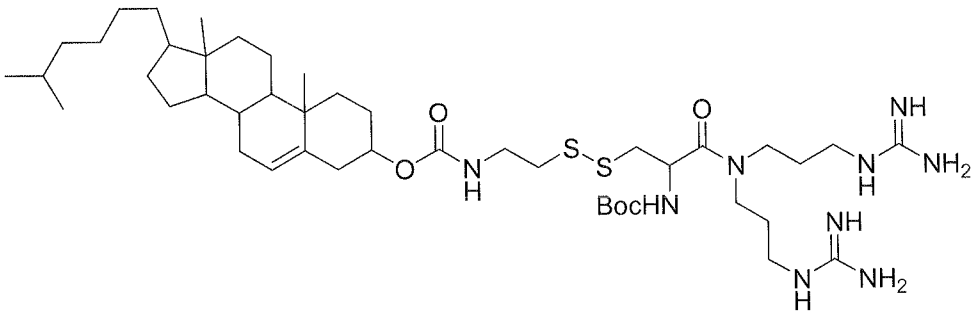

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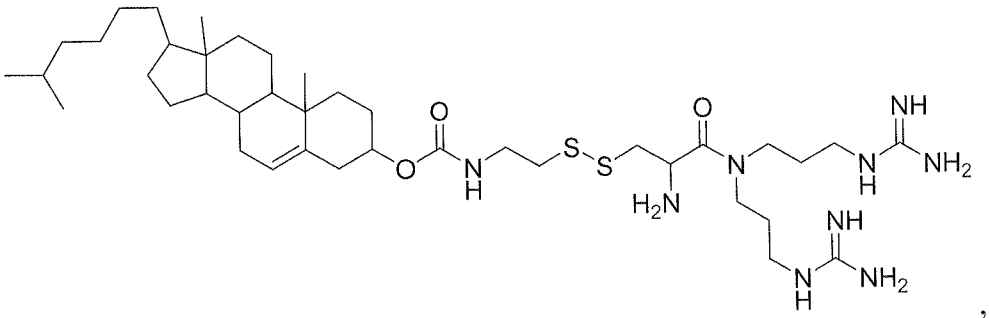

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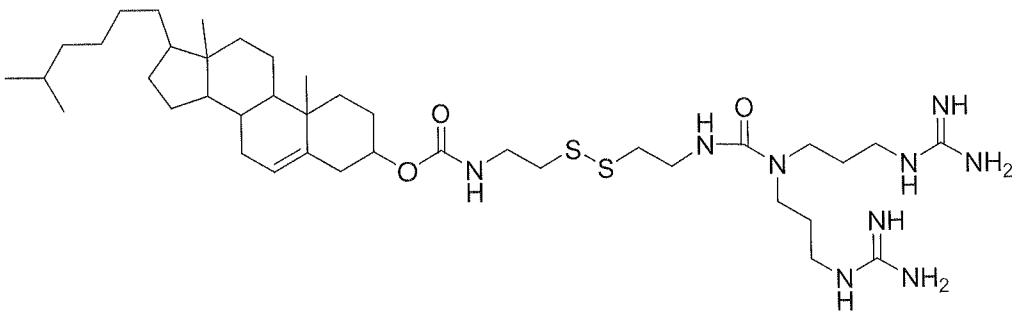

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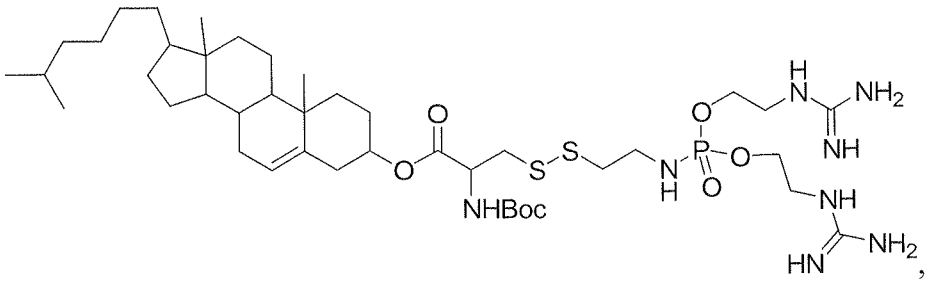

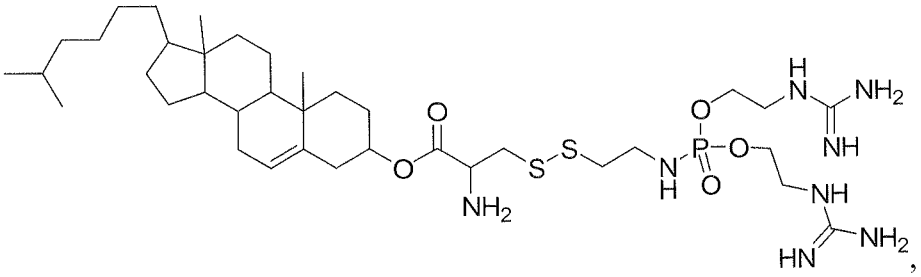

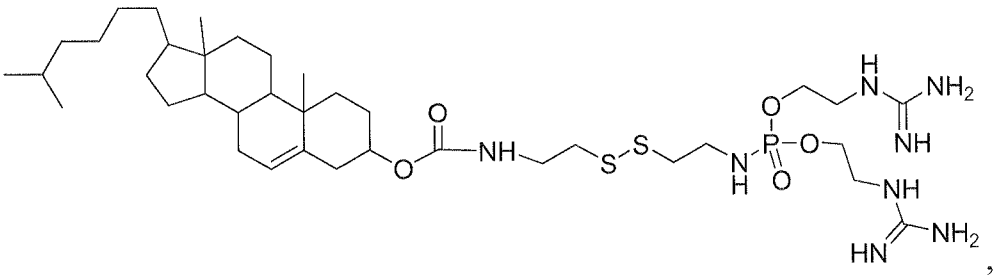

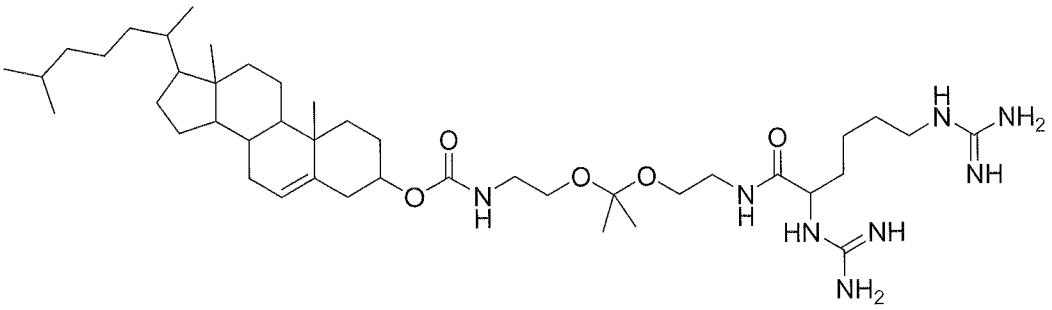

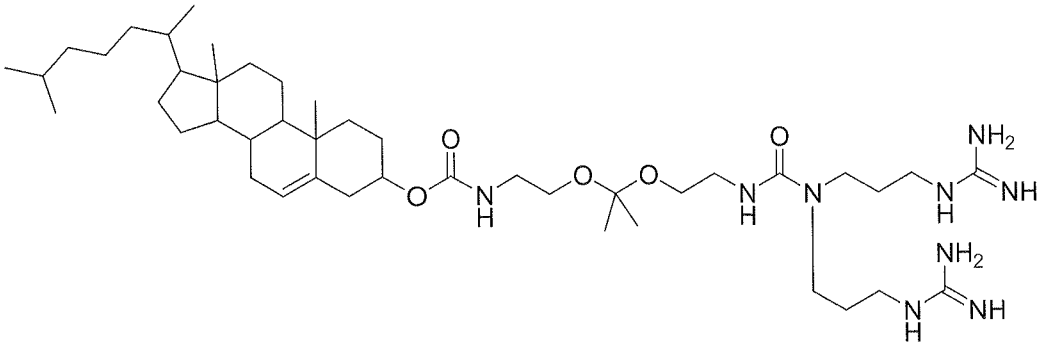

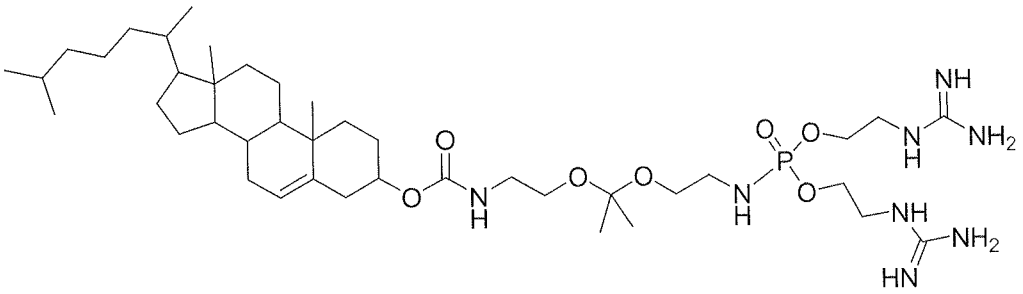

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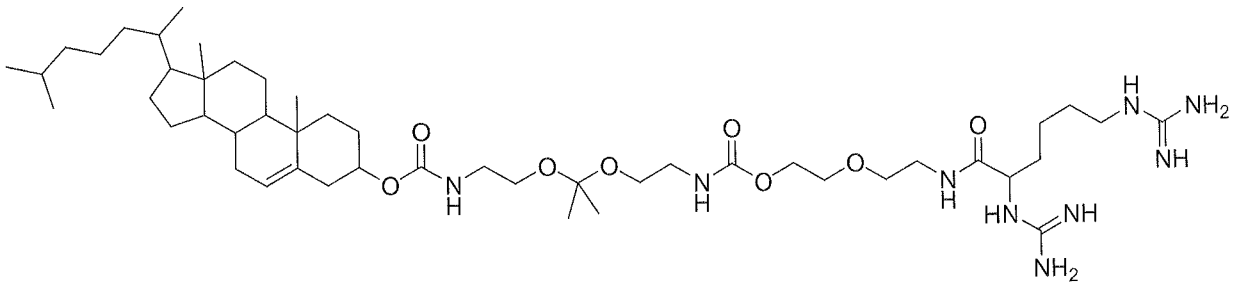

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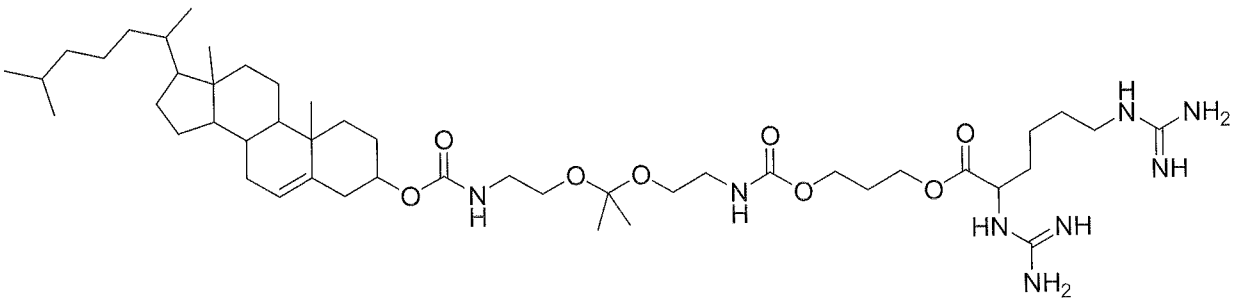

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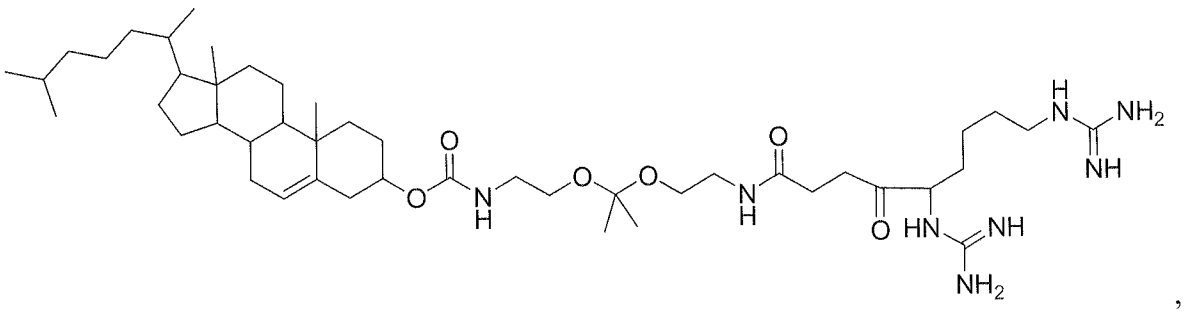

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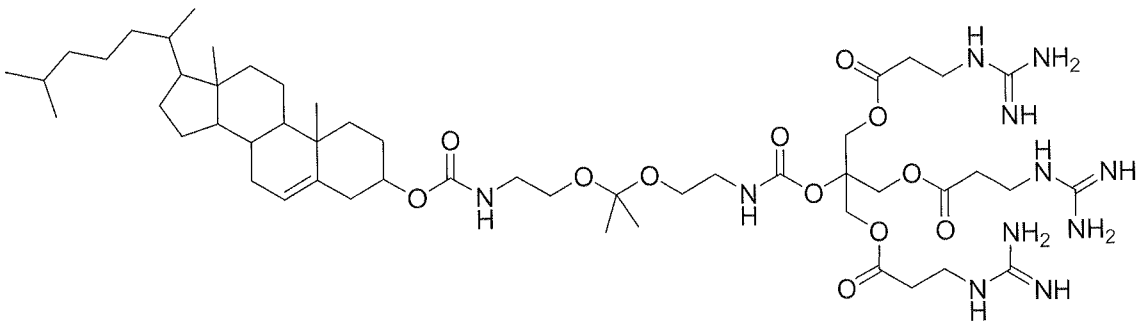

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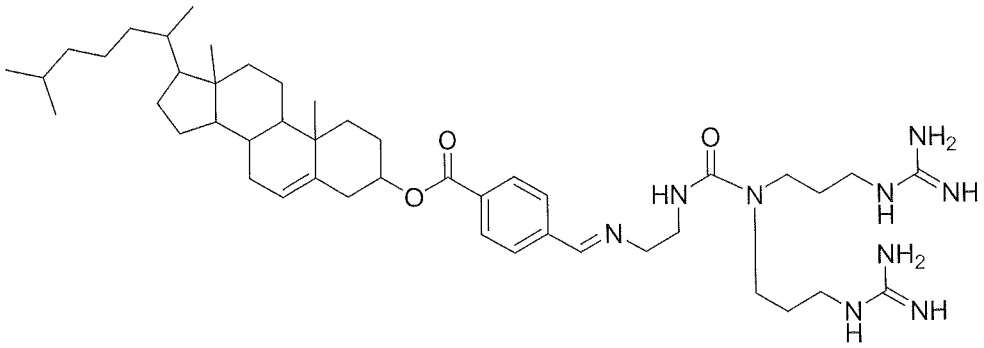

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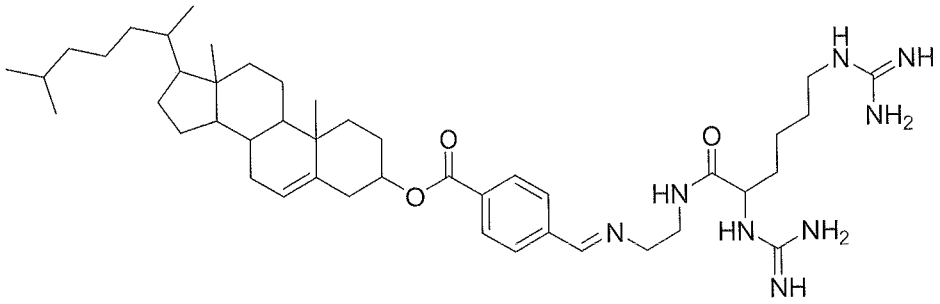

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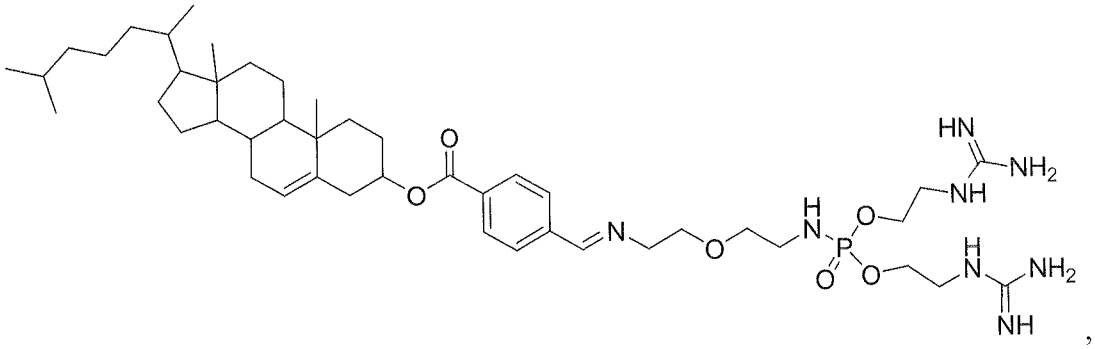

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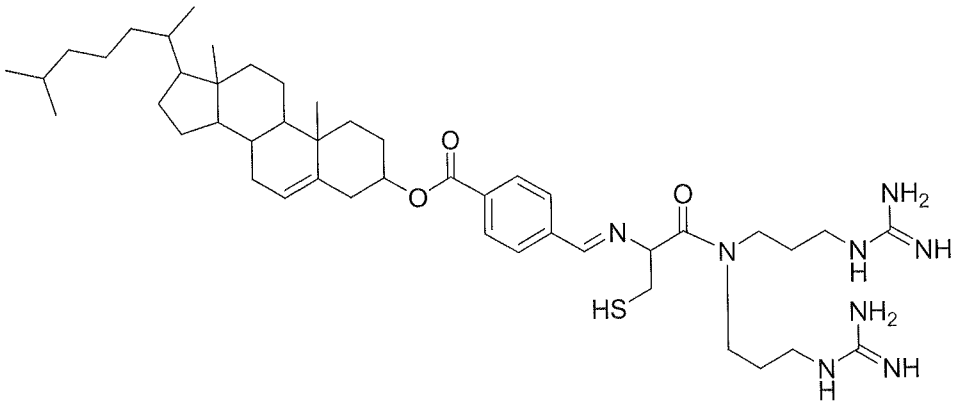

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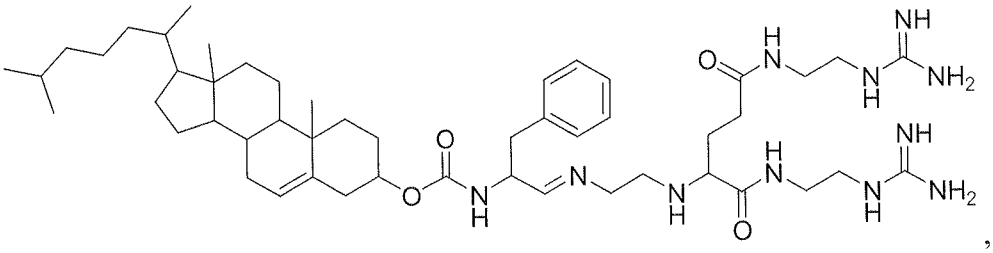

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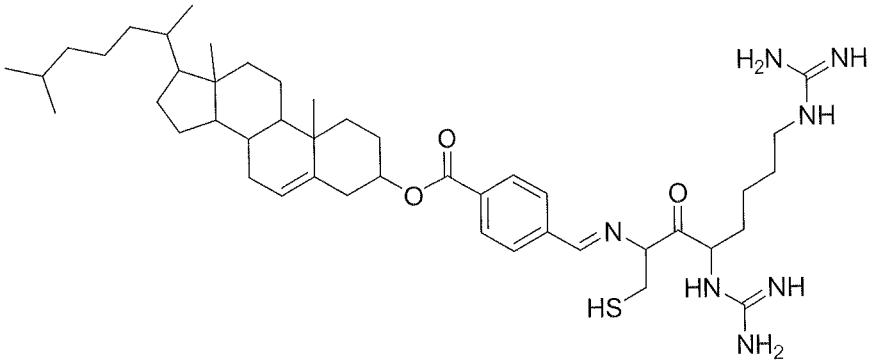

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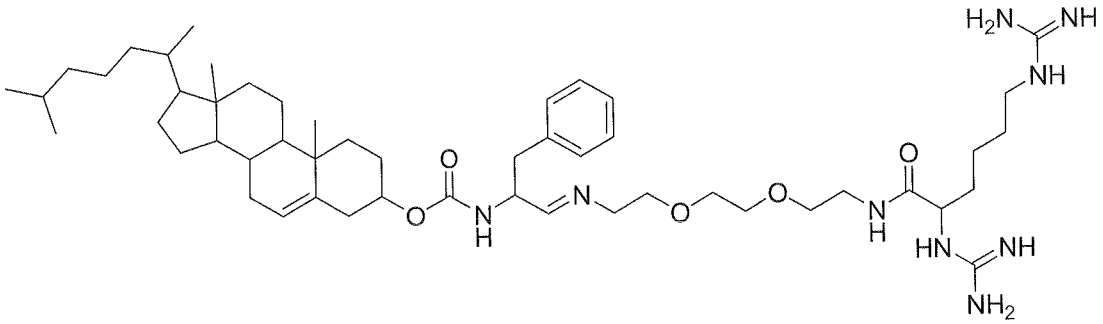

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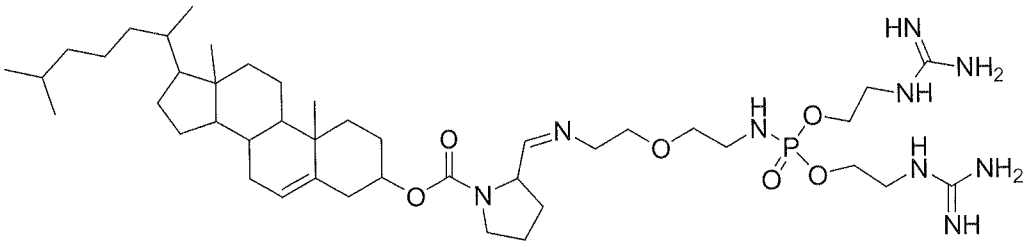

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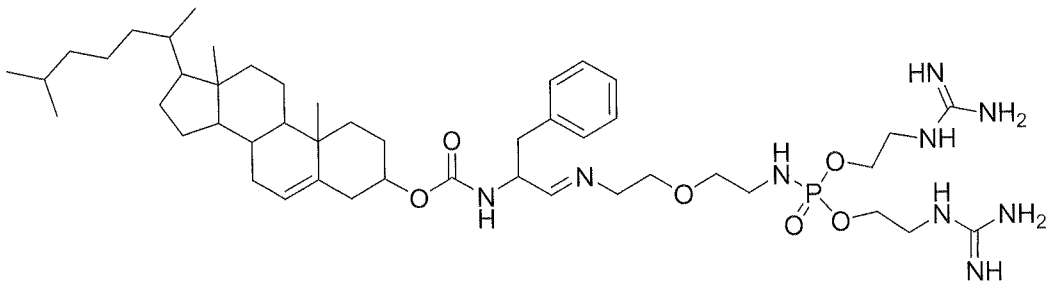

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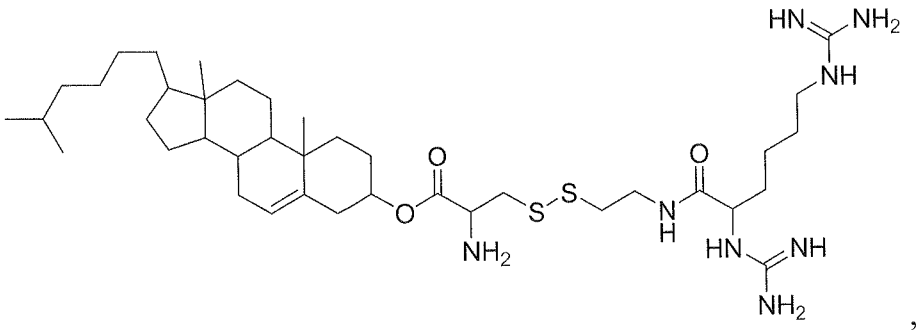
25. A nanoparticle composition comprising a compound of Formula (I) of claim 1.

26. The nanoparticle composition of claim 25, further comprising a fusogenic lipid and a PEG lipid.

27. The nanoparticle composition of claim 25, wherein the compound of Formula (I) is selected from the group consisting of: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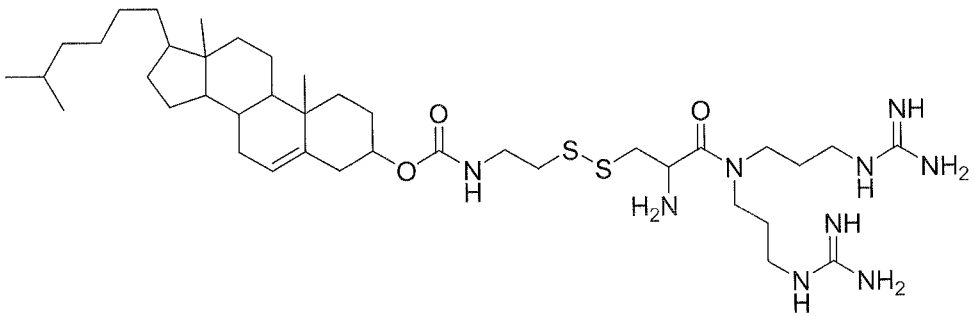
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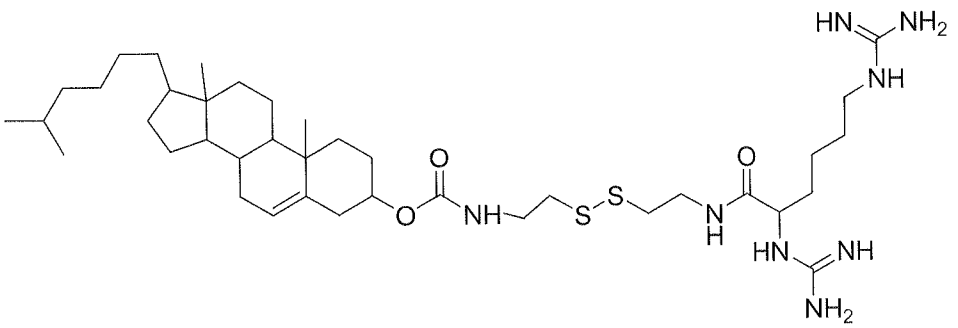
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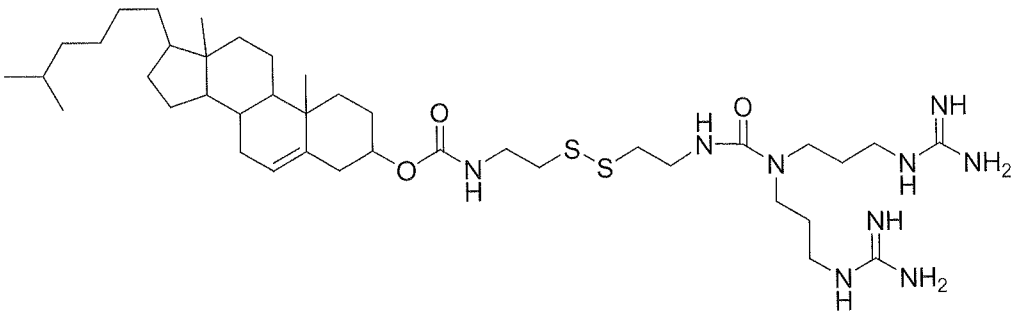
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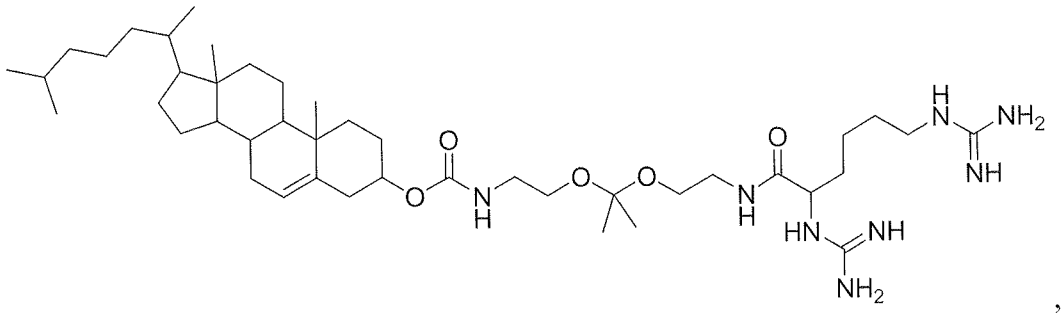

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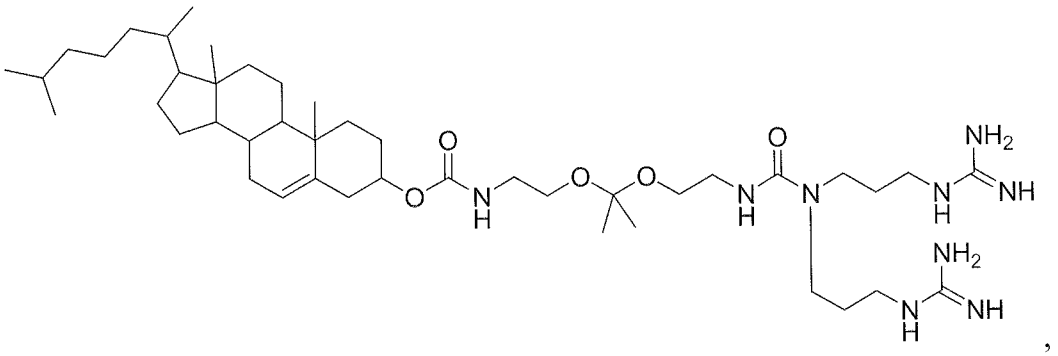

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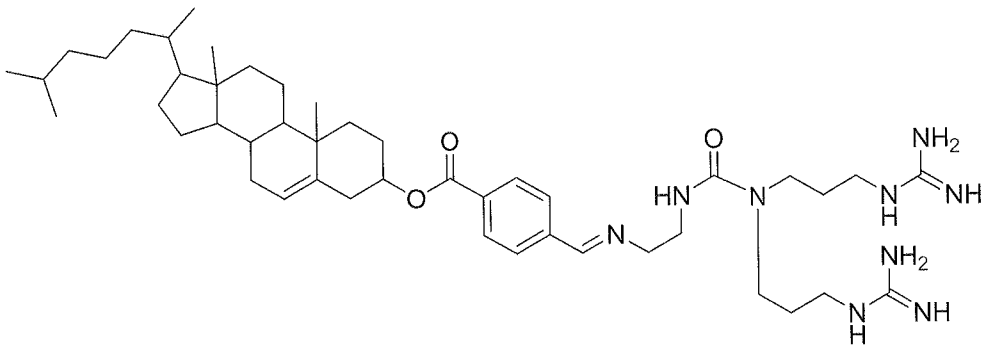
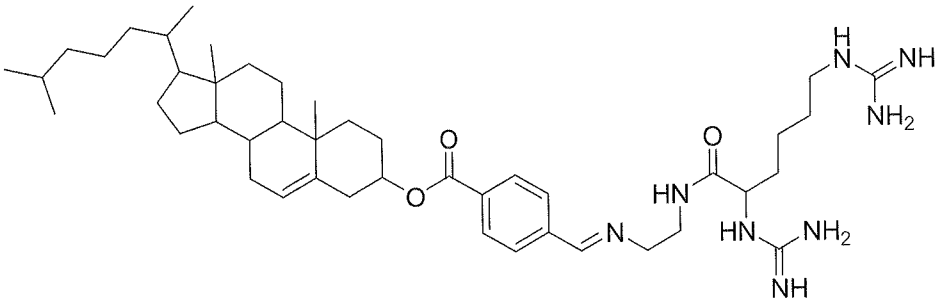


, and



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28. The nanoparticle composition of claim 26, wherein the fusogenic lipid is selected from the group consisting of DOPE, DOGP, POPC, DSPC, EPC, and combinations thereof.

29. The nanoparticle composition of claim 26, wherein the PEG lipid is selected from the group consisting of PEG-DSPE, PEG-dipalmitoylglycamide, C16mPEG-ceramide and combinations thereof.

30. The nanoparticle composition of claim 26, further comprising cholesterol.

31. The nanoparticle composition of claim 30, wherein the compound of Formula (I) has a molar ratio ranging from about 10% to about 99.9% of the total lipid present in the nanoparticle composition.

32. The nanoparticle composition of claim 30, wherein the compound of Formula (I) has a molar ratio ranging from about 15% to about 25% of the total lipid present in the nanoparticle composition.

33. The nanoparticle composition of claim 30, wherein a molar ratio of a cationic lipid including a compound of Formula (I), a non-cholesterol-based fusogenic lipid, a PEG lipid and cholesterol is about 15-25%: 20-78%: 0-50%: 2-10%: of the total lipid present in the nanoparticle composition.

34. The nanoparticle composition of claim 30 selected from the group consisting of:

a mixture of a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a mixture of a compound of Formula (I), a diacylphosphatidylcholine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a mixture of a compound of Formula (I), a diacylphosphatidylethanolamine, a diacylphosphatidyl-choline, a PEG conjugated to phosphatidylethanolamine (PEG-PE), and cholesterol;

a mixture of a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to ceramide (PEG-Cer), and cholesterol; and

a mixture of a compound of Formula (I), a diacylphosphatidylethanolamine, a PEG conjugated to phosphatidylethanolamine (PEG-PE), a PEG conjugated to ceramide (PEG-Cer), and cholesterol.

35. The nanoparticle composition of claim 30, wherein a compound of Formula (I), DOPE, cholesterol, and C16mPEG-Ceramide are included in a molar ratio of about 17%: 60%: 20%: 3% of the total lipid present in the nanoparticle composition.

36. The nanoparticle composition of claim 30, wherein a compound of Formula (I), DOPE, cholesterol, PEG-DSPE, and C16mPEG-Ceramide are included in a molar ratio of about 18%: 60%: 20%: 1%: 1% of the total lipid present in the nanoparticle composition.

37. A nanoparticle comprising nucleic acids encapsulated within the nanoparticle composition of claim 30.

38. The nanoparticle of claim 37, wherein the nucleic acids are a single stranded or double stranded oligonucleotide.

39. The nanoparticle of claim 37, wherein the nucleic acids are selected from the group consisting of deoxynucleotide, ribonucleotide, locked nucleic acids (LNA), short interfering RNA (siRNA), microRNA (miRNA), aptamers, peptide nucleic acid (PNA), phosphorodiamidate morpholino oligonucleotides (PMO), tricyclo-DNA, double stranded oligonucleotide (decoy ODN), catalytic RNA (RNAi), aptamers, spiegelmers, CpG oligomers and combinations thereof.

40. The nanoparticle of claim 38, wherein the oligonucleotide is an antisense oligonucleotide.

41. The nanoparticle of claim 38, wherein the oligonucleotide has phosphodiester or phosphorothioate linkages, and combinations thereof.

42. The nanoparticle of claim 38, wherein the oligonucleotide includes LNA.

43. The nanoparticle of claim 38, wherein the oligonucleotide has from about 8 to 50 nucleotides.

44. The nanoparticle of claim 38, wherein the oligonucleotide inhibits expression of oncogenes, pro-angiogenesis pathway genes, pro-cell proliferation pathway genes, viral infectious agent genes, and pro-inflammatory pathway genes.

45. The nanoparticle of claim 38, wherein the oligonucleotide is selected from the group consisting of antisense bcl-2 oligonucleotides, antisense HIF-1α oligonucleotides, antisense survivin oligonucleotides, antisense ErbB3 oligonucleotides, antisense PIK3CA oligonucleotides, antisense HSP27 oligonucleotides, antisense androgen receptor oligonucleotides, antisense Gli2 oligonucleotides, and antisense beta-catenin oligonucleotides.

46. The nanoparticle of claim 38, wherein the oligonucleotide comprises eight or more consecutive nucleotides set forth in SEQ ID NO: 1, SEQ ID NOs 2 and 3, SEQ ID NO:4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, and SEQ ID NO: 16, and each nucleic acid is a naturally occurring or modified nucleic acid.

47. The nanoparticle of claim 37, wherein the charge ratio of the nucleic acids and the compound of Formula (I) ranges from about 1:20 to about 20: 1.

48. The nanoparticle of claim 37, wherein the nanoparticle has a size ranging from about 50 nm to about 150 nm.

49. A method of treating disease in a mammal comprising administering a nanoparticle of claim 37 to a mammal in need thereof.

50. A method of introducing an oligonucleotide into a cell comprising:
contacting a cell with a nanoparticle of claim 37.

51. A method of inhibiting a gene expression in human cells or tissues, comprising: contacting human cells or tissues with a nanoparticle of claim 37.

52. The method of claim 51, wherein the cells or tissues are cancer cells or tissues.

53. A method of downregulating a gene expression in a mammal, comprising: administering an effective amount of a nanoparticle of claim 37 to a mammal in need thereof.

54. A method of inhibiting the growth or proliferation of cancer cells comprising: contacting a cancer cell with a nanoparticle of claim 37.

55. The method of claim 54, further comprising administering an anticancer agent.

FIG. 1

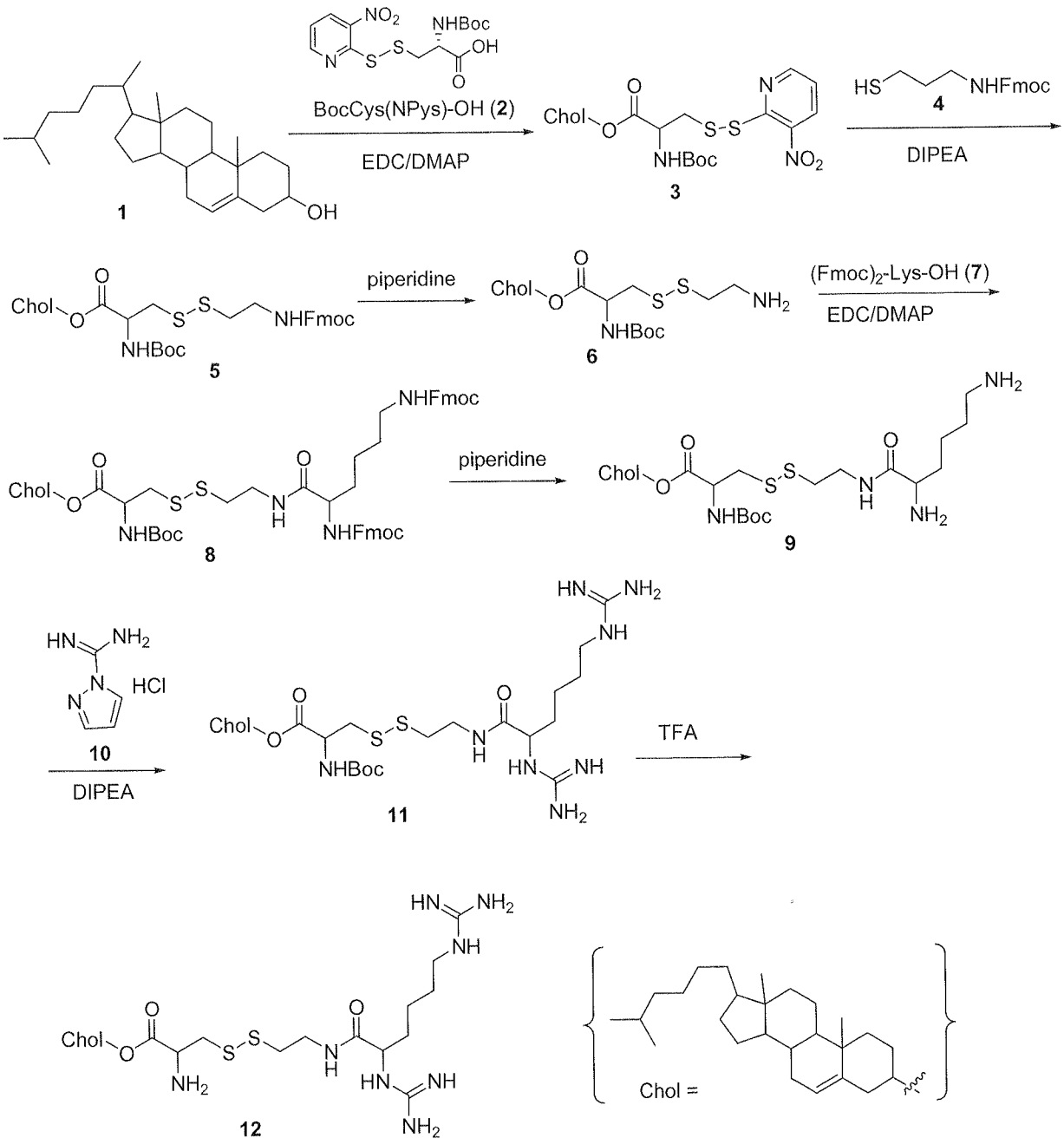

BocCys(NPys)-OH (2)
EDC/DMAP
DIPEA
piperidine
(Fmoc)2-Lys-OH (7)
EDC/DMAP
piperidine
HCl
10
DIPEA
TFA
Chol =

FIG. 2

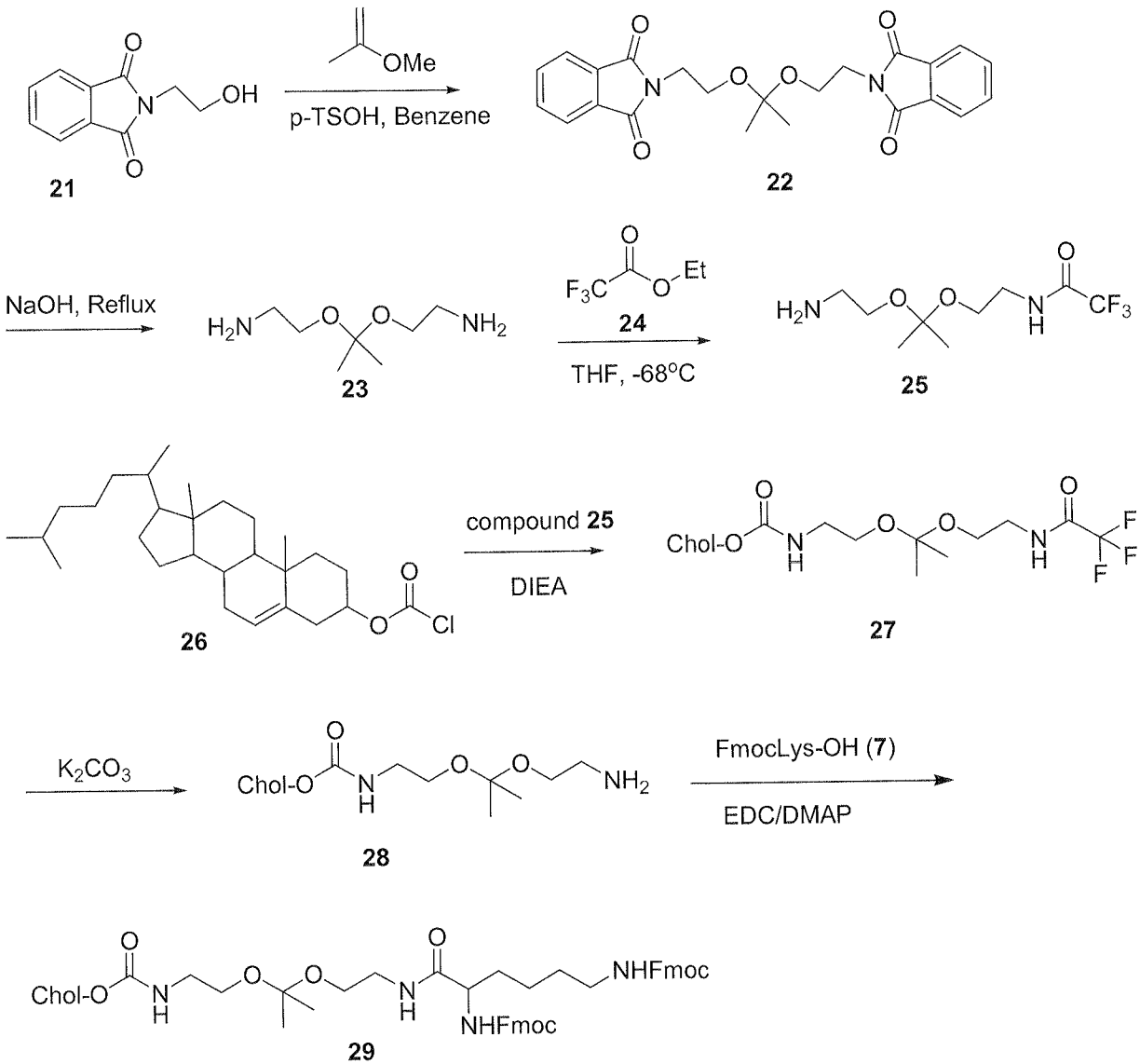

OMe
p-TSOH, Benzene
21
22
NaOH, Reflux
23
24
THF, -68°C
25
compound 25
DIEA
26
27
K2CO3
28
FmocLys-OH (7)
EDC/DMAP
29

FIG. 3

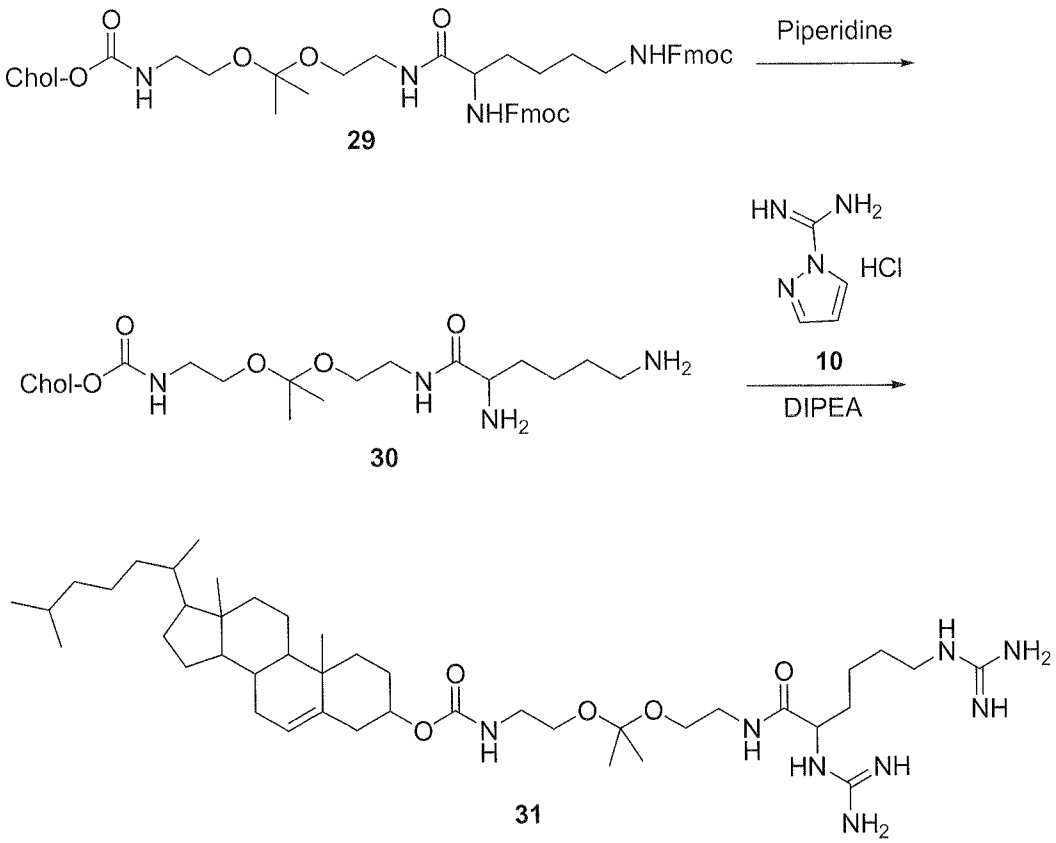

Chol-O
NHFmoc
NHFmoc
29
Piperidine
HN
NH2
HCl
10
DIPEA
NH2
30
31
Chol =

FIG. 4

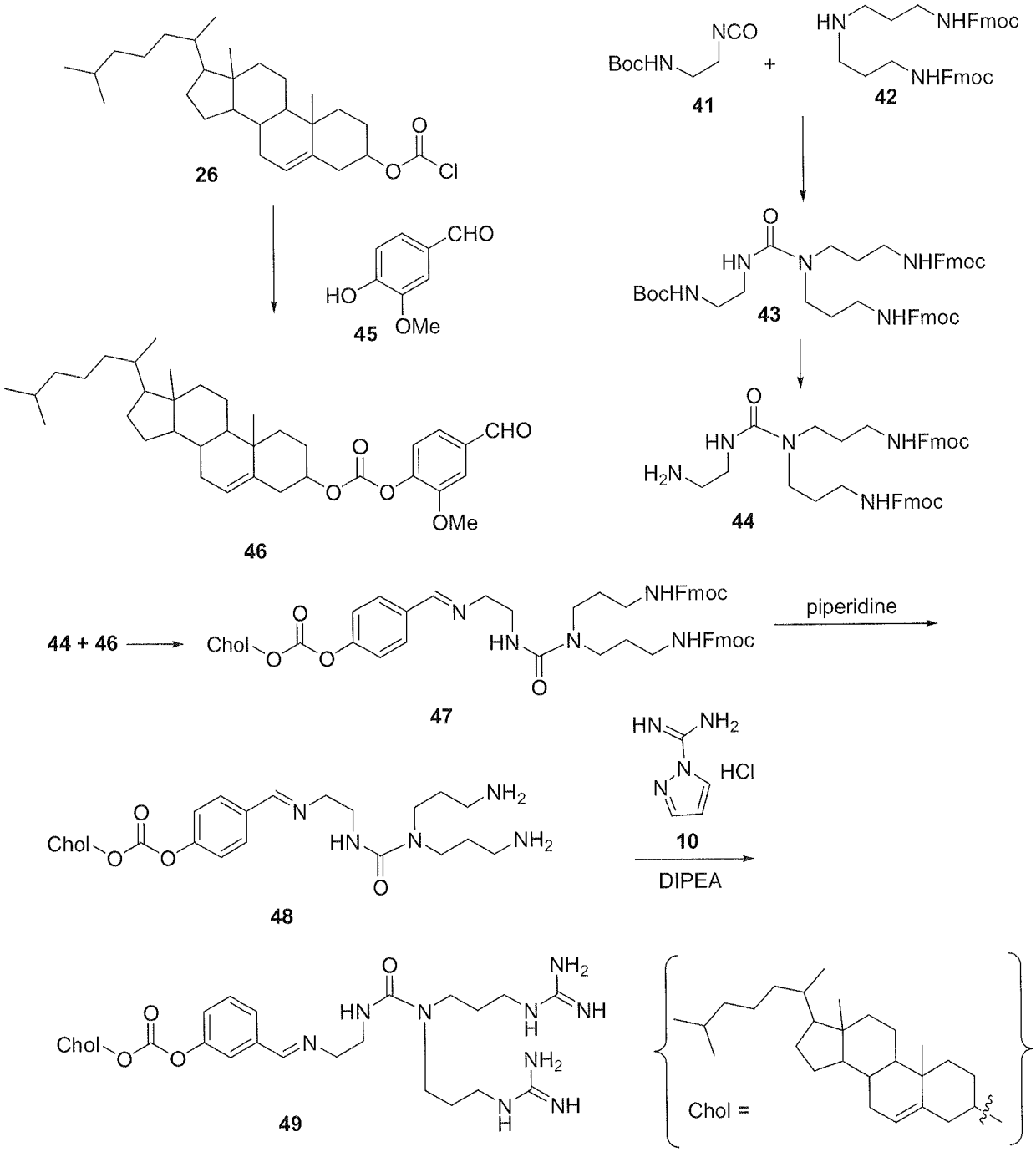
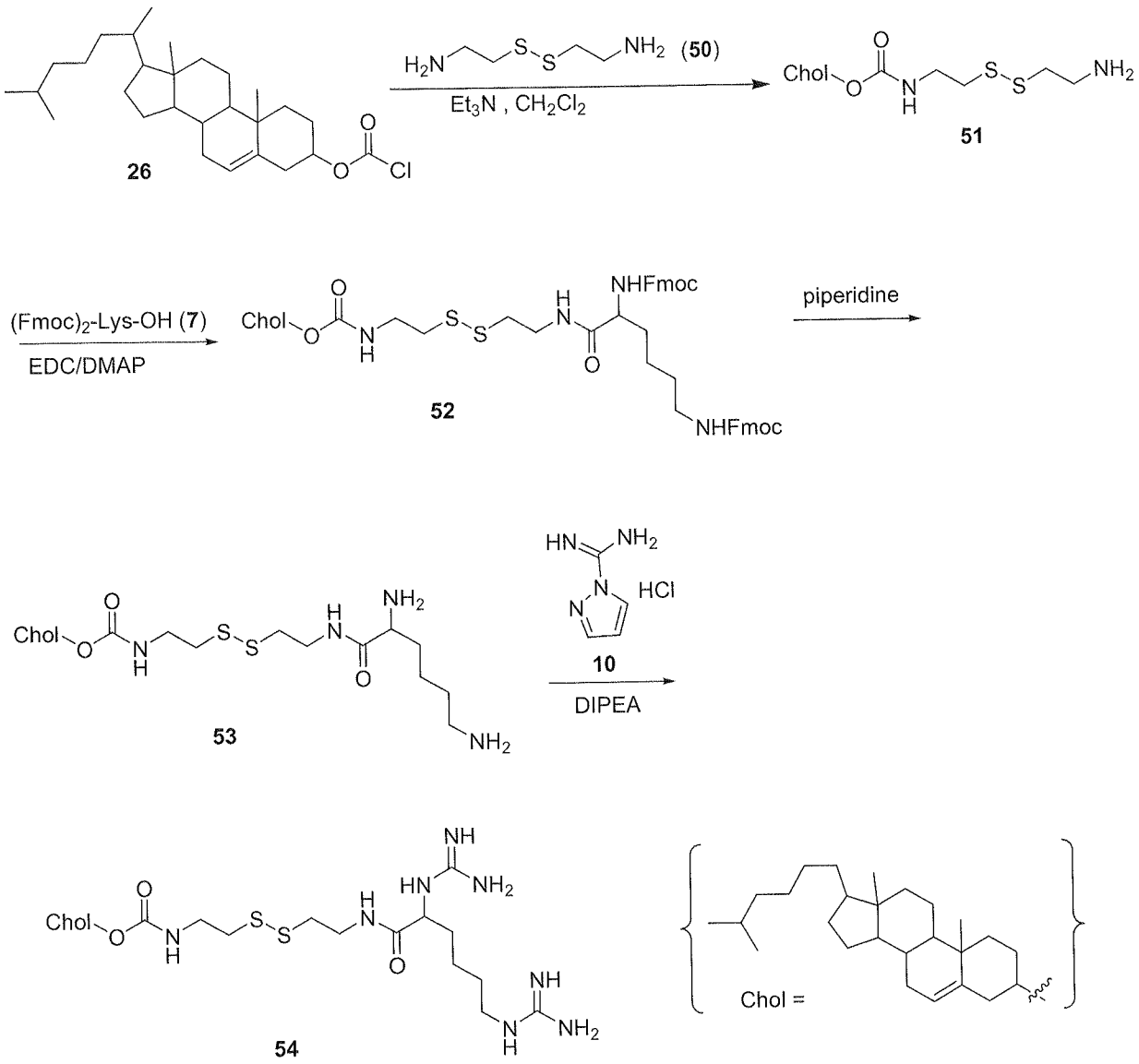


FIG. 5


26
(50)
Et3N , CH2Cl2
Chol
51
(Fmoc)2-Lys-OH (7)
EDC/DMAP
52
NHFmoc
piperidine
53
10
DIPEA
54
Chol =

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/64719

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 43/04 (2010.01)
USPC - 514/44R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC- 514/44R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 514/1; 435/5; 435/6; 536/23.1; 424/450 (text search-see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents: cationic lipid, releasable linker, disulfide, nucleic acid, guanidine, cholesterol, nanoparticle, bifunctional linker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,153,434 A (Hughes et al.) 28 November 2000 (28.11.2000) col 2, ln 54-67, Fig. 2	1-3, 7, 9, 12-21, 24
Y	US 6,383,814 B1 (Lee et al.) 07 May 2002 (07.05.2002) col 3, ln 62-67, col 5, ln 54 to col 6, ln 8, col 6, ln 25-45, col 8, ln 66 to col 9, ln 12	1-3, 7, 9, 12-21, 24

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2010 (22.03.2010)	29 MAR 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/64719

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Group I+: claims 1-24, drawn to a compound of Formula (I). The first invention encompasses the first claimed compound of claim 24. Should an additional fee(s) be paid, Applicant is invited to elect an additional compound(s) to be searched . The exact claims searched will depend on the specifically elected compound(s).
[NOTE: Claims 4-6, 8, 10-11, 22-23 were excluded from Group I, because they are drawn to a non-elected subject matter.]

Group II+, claims 25-55, drawn to a nanoparticle composition comprising a compound of Formula (I), a method of using and a method of preparing said nanoparticle composition. Should an additional fee(s) be paid, Applicant is invited to elect a specific compound of Formula (I) and a specific oligonucleotide to be searched . The exact claims searched will depend on the specifically elected SEQ ID NO(s): and binding moieties.

---------- Please see extra sheet for continuation ----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 7, 9, 12-21, 24 restricted to the first compound of claim 24

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/64719

Continuation of:

Box NO III. Observations where unity of invention is lacking

The inventions listed as Groups I+ through II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I+ does not include the inventive concept of a nanoparticle composition, as required by Group II+.

The inventions of Group I+ share the technical feature of a compound of Formula (I). However, this shared technical feature does not represent a contribution over the prior art of US 6,153,434 A to Hughes et al. (hereinafter 'Hughes') that discloses a compound of Formula (I) (Fig. 2, CHDTAEA); wherein
R1 is cholesterol (Fig. 2, CHDTAEA);
Y1 is O (Fig. 2, CHDTAEA);
Y2 i s O (Fig. 2, CHDTAEA);
L1 is a bifunctional linkers (Fig. 2, CHDTAEA, -CH2-, see claim 15 of the instant invention wherein L1 is selected from -CH2-);
L2 is a bifunctional linkers (Fig. 2, CHDTAEA, -CH2-C(O)-NH-, see claim 16 of the instant invention wherein L2 is selected from (CR'21R'22)t'1(Y'14)a'2[C(=Y'16)]a'3Y'15(CR'23R'24)t'2; t'1 is 1, a'2 is 0, a'3 is 1 and t'2 is 0, Y'16 is O, Y'15 is NR'29, or represents -CH -C(O)-NH-);
M is an acid labile linker (Fig. 2, CHDTAEA, -S-S-);
(a), (d) and (f) are independently 0 (Fig. 2, CHDTAEA);
(b), (c) and (e) are independently positive integers (Fig. 2, CHDTAEA);
X is N (Fig. 2, CHDTAEA);
Q1 is -(L11)d1-R11 (Fig. 2, CHDTAEA);
Q2 is -(L12)d2-R12 (Fig. 2, CHDTAEA);
Q3 is a lone electron pair, provided that (ii) when X is N, Q3 is a lone electron pair (Fig. 2, CHDTAEA); wherein
L11 and L12 and L13 are independently selected bifunctional spacers (Fig. 2, CHDTAEA, -CH2-CH2-);
(dl), (d2) and (d3) are independently 0 or positive integers (Fig. 2, CHDTAEA);
R11 and R12 are independently NH2 (Fig. 2, CHDTAEA);
R2-3 are independently selected from hydrogen (Fig. 2, CHDTAEA);
As said compound was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

The inventions of Group II+ share the technical feature of a nanoparticle composition comprising a compound of Formula (I). However, this shared technical feature does not represent a contribution over the prior art, because Hughes discloses a compound of Formula (I) (Fig. 2, CHDTAEA), as set forth in the immediately preceding paragraph, and 2) Hughes further discloses a nanoparticle composition comprising a compound of Formula (I) (col 16, ln 12-24, Example 3, liposomes). As said nanoparticle composition was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another technical feature of the inventions listed as Group II+ is the specific oligonucleotide sequence recited therein. The inventions do not share a special technical feature, because US 2005/0014712 A1 to HANSEN, et al., in the context of MODULATION OF SURVIVIN EXPRESSION by OLIGOMERIC COMPOUNDS (title) discloses 16-nucleotide-long sequence comprising the claimed SEQ ID NO:1 (HANSEN, et al., nucleotides 1-16 of SEQ ID NO 130). Without a shared special technical feature, the inventions lack unity with one another.

The inventions of Groups I+ and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.