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(54) Title: AGENTS FOR IMPROVED DELIVERY OF NUCLEIC ACIDS TO EUKARYOTIC CELLS

(57) Abstract: New cationic lipids are provided that are useful for delivering macromolecules, such as nucleic acids, into eukaryotic cells. The lipids can be used alone, in combination with other lipids and/or in combination with other transfection enhancing reagents to prepare transfection complexes.

AGENTS FOR IMPROVED DELIVERY OF NUCLEIC ACIDS TO EUKARYOTIC CELLS

This application claims priority to US application 61/476,240, filed April 15, 2011, the contents of which are incorporated herein by reference in their entirety.

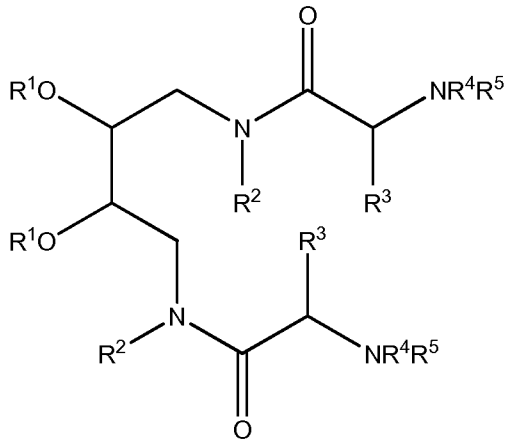
BACKGROUND

Transfection agents, such as lipid aggregates comprising cationic lipid components have been used to deliver large anionic molecules, such as nucleic acids, into certain types of cells. See Felgner et al., *Nature* 337:387-388 (1989); *Proc. Natl. Acad. Sci. USA* 84:7413 (1987). These agents are not, however, universally effective in all cell types. In many cases, cationic lipids alone are not effective or are only partially effective for transfection. Moreover, these methods do not work for all cell types, often require relatively complex protocols and are inconvenient. It is apparent, therefore, that new and improved methods for introducing macromolecules, and particularly nucleic acids, into cell, are greatly to be desired. In particular, improved methods for introducing nucleic acids into a wider variety of cells, and particularly into primary cells, are greatly to be desired.

SUMMARY OF THE INVENTION

New compounds, compositions and methods are provided that improve the efficiency of introducing macromolecules, such as nucleic acids, into cells. New cationic lipid molecules are provided, together with compositions containing those lipids and methods for using the new lipid molecules and compositions for transfection. The cationic lipids may be used alone for transfection, or they may be used in combination with additional reagents in transfection compositions. For example, the cationic lipids may be combined with one or more neutral lipids, additional cationic lipids, one or more cell surface ligands, one or more fusion enhancing agents, and/or one or more nuclear localization agents. The resulting compositions may be complexed with one or macromolecules, such as DNA or RNA and used to deliver the macromolecule into eukaryotic cells..

Specifically there is provided a compound having the structure (I)



(I)

wherein each R^1 independently is C_1-C_{23} alkyl, C_1-C_{23} alkenyl, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl;

each R^2 independently is C_1-C_6 alkyl, or C_1-C_6 alkenyl, optionally interrupted by O;

each R^3 independently is H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkyl-C_1-C_6 alkyl, C_5-C_7-cycloalkenyl, C_5-C_7-cycloalkenyl-C_1-C_6 alkyl, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2\text{-}6}NHR^7$, $-(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, $((CH_2)_mO_x)_y(CH_2)_zO_xR^8$, or $-(CH_2)_{0\text{-}3}$Het,;

each R^4 independently is H, C_1-C_6 alkyl, or C_1-C_6 alkenyl;

each R^5 independently is H, an amine protecting group, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2\text{-}6}NHR^7$, $-(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, –(CO)$C_1$-$C_{23}$ alkyl, –(CO)C_1-C_{23} alkenyl, or a peptide containing 1-20 amino acid residues;

each m independently is 2-5,

each n independently is 2-5,

each x independently is 0 or 1,

y is 0-2,

z is 1-6

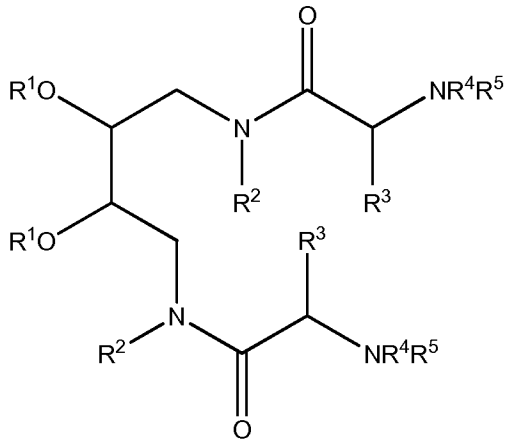
R^6 and R^7 independently are H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl;

R^8 is H, C_1-C_6 alkyl, or C_1-C_6 alkenyl

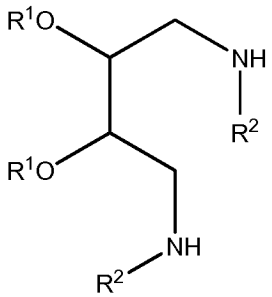
and

Het is a 5-7 membered monocyclic basic heterocycle, or an 8-11 membered bicyclic basic heterocycle.

A compound having the structure (I) or (II)



(I)



(II)

where each R^1 independently may be C_1-C_{23} alkyl, C_1-C_{23} alkenyl, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl;

each R^2 independently may be C_1-C_6 alkyl, or C_1-C_6 alkenyl, optionally interrupted by O;

or, in compound (II), each R^2 independently is $–CH_2-(CHR^9)_{2-7}-NHR^4$ or $–CH_2-(CHR^9)_{2-7}-OH$, wherein each R^9 independently is H, OH, or NH2,.

each R^3 independently may be H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkyl-C_1-C_6 alkyl, C_5-C_7-cycloalkenyl, C_5-C_7-cycloalkenyl-C_1-C_6 alkyl, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2-6}NHR^7$, $-(CH_2)_{3-6}NHC(=NH)NH_2$, $((CH_2)_mO_x)_y(CH_2)_zO_xR^8$, or $-(CH_2)_{0-3}$Het,;

each R^4 independently may be H, C_1-C_6 alkyl, or C_1-C_6 alkenyl;

each R^5 independently may be H, an amine protecting group, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2-6}NHR^7$, $-(CH_2)_{3-6}NHC(=NH)NH_2$, –(CO)$C_1$-$C_{23}$ alkyl, –(CO)C_1-C_{23} alkenyl, or a peptide containing 1-20 amino acid residues;

each m independently may be 2-5, each n independently may be 2-5, each x independently may be 0 or 1, y may be 0-2, z may be 1-6, R^6 and R^7 independently are H, an

amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl; R^8 may be H, C_1-C_6 alkyl, or C_1-C_6 alkenyl

and

Het may be a 5-7 membered monocyclic basic heterocycle, or an 8-11 membered bicyclic basic heterocycle.

R^1, R^2, R^3, R^4 and/or R^5 may be the same or different.

In any of these compounds R^2 may be C_1-C_3 alkyl and/or R^1 may be monounsaturated C_{12}-C_{20} alkenyl, for example *cis* alkenyl. R^1 advantageously may be $C_{14\text{-}20}$ alkyl or monounsaturated $C_{14\text{-}20}$ alkenyl.

In other embodiments, for example, R^3 may be $-(CH_2)_mNR^6(CH_2)_nNHR^7$ and/or R^5 may be $-(CH_2)_{2\text{-}6}NHR^7$. In another embodiment m may be 3, n may be 3, and R^5 may be $-(CH_2)_3NHR^7$

In any of these embodiments R^6 and each R^7 may be H.

In a particular embodiment, at least one R^6 or R^7 may be –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl and the remainder are H.

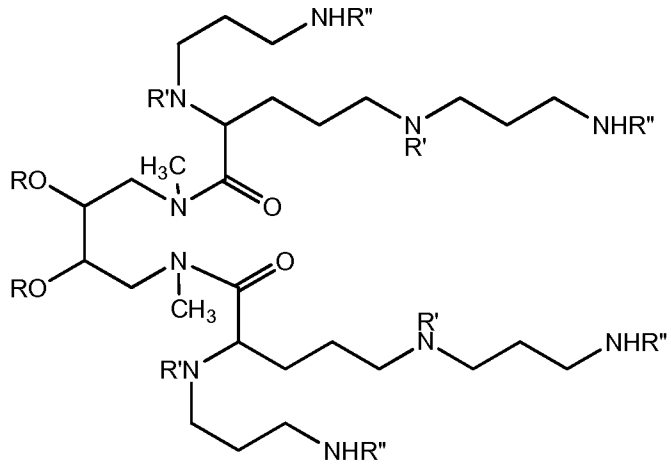
In further specific embodiments, each R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl; each R^2 may be C_1-C_3 alkyl; each R^3 may be $-(CH_2)_3NH(CH_2)_3NH_2$; each R^4 may be H; and each R^5 may be $-(CH_2)_3NH_2$.

In other embodiments, R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl; each R^2 may be C_1-C_3 alkyl; each R^3 may be $-(CH_2)_3NR^6(CH_2)_3NHR^7$; each R^4 may be H; and each R^5 may be $-(CH_2)_3NHR7$, where at least one R^6 or R^7 may be –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl and the remainder are H.

For compounds having structure (II), advantageously R^2 is $–CH_2-(CHR^3)_{2\text{-}7}-NHR^4$ and R^3 is H or OH. In certain embodiments, R^1 may be C_{14}-C_{20} alkyl or monounsaturated C_{14}-C_{20}-alkenyl. In other embodiments, no more than 3 R^3 groups are OH in each R^3 moiety. Advantageously, in compounds having structure (II) each R^1 is the same and/or each R^2 is the same. R^2 may be C_1-C_3 alkyl. In any of these compounds of structure (II) R^1 may advantageously be monounsaturated C_{12}-C_{20} alkenyl, which may be *cis* alkenyl.

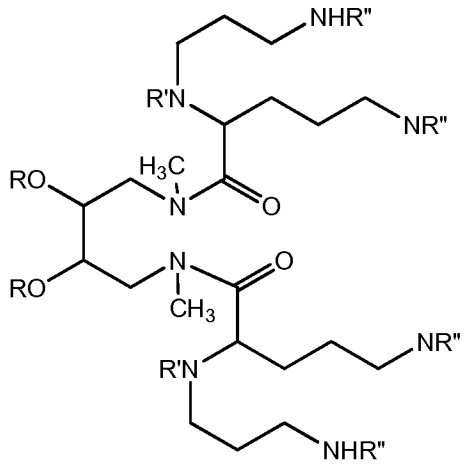
In other embodiments of compounds of structure (II), R^2 may be$–CH_2-CHOH-(CHR^3)_{1\text{-}6}-NHR^4$, and advantageously may be $–CH_2-CHOH-CH_2-NH_2$.

Specific compounds include:



where R may be C_{12-20} alkyl or C_{12-20} alkenyl; andeach R' and each R" independently may be H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl. For example, R may be C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl, and each R' and each R" independently may be H, C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl.

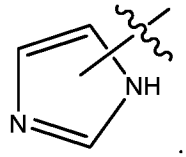
Another specific compound is



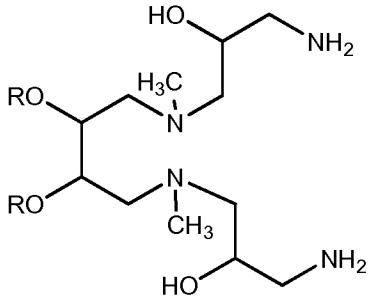
where R may be C_{12-20} alkenyl; and each R' and each R" independently may be H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl. For example, R may be oleyl and each R' and each R" independently may be H, or oleoyl. At least one R' or R" may be oleoyl and the remainder are H.

In specific compounds of Formula (I), each R^3 independently may be -$(CH_2)_{2-6}NHR^7$, -$(CH_2)_{3-6}NHC(=NH)NH_2$, or -$(CH_2)_{1-3}$Het, each R^4 may be H; and each R^5 independently may be H or a peptide containing 1-20 amino acid residues. In a specific compound, each R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} alkenyl; each R^2 may be C_1-C_3 alkyl; and and each R^5 may be H.

In compounds containing Het moieties, Het may be



A specific example of a compound of structure (II) is



where R advantageously is C_{12-20} alkyl or monounsaturated alkenyl.

Also provided are compositions comprising a compound of formula (I) or (II) and at least one cationic lipid and/or at least one neutral lipid. In these compositions, the cationic lipid when present may be selected from the group consisting of LipofectAmine™ 2000, LipofectAmine™, Lipofectin®, DMRIE-C, CellFectin®(Invitrogen), Oligofectamine®(Invitrogen), LipofectAce® (Invitrogen), Fugene® (Roche, Basel, Switzerland), Fugene® HD (Roche), Transfectam® (Tranfectam, Promega, Madison, WI), Tfx-10® (Promega), Tfx-20® (Promega), Tfx-50® (Promega), Transfectin™ (BioRad, Hercules, CA), SilentFect™(Bio-Rad), Effectene® (Qiagen, Valencia, CA), DC-chol (Avanti Polar Lipids), GenePorter® (Gene Therapy Systems, San Diego, CA), DharmaFect 1® (Dharmacon, Lafayette, CO), DharmaFect 2® (Dharmacon), DharmaFect 3® (Dharmacon), DharmaFect 4® (Dharmacon), Escort™ III (Sigma, St. Louis, MO), Escort™ IV (Sigma), DOTMA, DOTAP, DMRIE, DC-Chol, DDAB, DOSPA, DOSPER, DOGS, TMTPS, TMTOS, TMTLS, TMTMS, TMDOS, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, L-spermine-5-carboxyl-3-(DL-1,2-dipalmitoyl-dimethylaminopropyl-β-hyd roxyethylamine, 3,5-(N,N-di-lysyl)-diaminobenzoyl-glycyl-3-(DL-1,2-dipalmitoyl-dimethylami nopropyl-β-

hydroxyethylamine), L-Lysine-bis(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, L-Lysine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-alkylamino)-2-hydroxypropyl)piperazine, L-Lysine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxypropyl]piperazine, L-Ornithine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4,-bis[(3-amino-2-hydroxypropyl)-oleylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-palmitylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-myristylamino]-butane-2,3-diol, 1,4-bis[(3-oleylamino)propyl]piperazine, L-Arginine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, bis[(3-(3-aminopropyl)-myristylamino)2-hydroxypropyl]piperazine, L-Arginine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Serine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxypropyl]piperazine, Glycine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, Sarcosine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Histidine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, cholesteryl-3β-carboxyl-amidoethylenetrimethylammonium iodide, 1,4-bis[(3-myristylamino)propyl]piperazine, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine, cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysucc inate iodide, 2-[(2-trimethylammonio)-ethylmethylamino] ethyl-cholesteryl-3β-oxysuccinate iodide, 3β[N-(N', N'-dimethylaminoethane)carbamoyl]cholesterol, and 3β-[N-(polyethyleneimine)-carbamoyl] cholesterol,1,4-bis[(3-palmitylamino)propyl]piperazine, L-Ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide, N^2,N^5 -Bis(3-aminopropyl)-L-ornithylglycyl-N- (1-heptadecyloctadecyl)glycinamide, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-alkylamino)-2-hydroxypropyl]piperazine N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-glutamine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioctadecyl-L-glutaminyl]-L-glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-diolyl-L-glutamine, N^2-

[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-glutamine,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)-2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioleyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-oleylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dipalmityl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-asparagine,

N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dipalmityl-L-glutaminyl]-L-glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dimyristyl-L-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-asparagine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)-2-hydroxypropyl]piperazine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dimyristyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-myristylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dilaureyl-L-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dilaureyl-L-glutaminyl]-L-glutamic acid, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dioctadec-9-enylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dipalmitylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dimyristylpropionamide, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)propyl]piperazine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)propyl]piperazine, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-diolylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dipalmitylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dimyristylaminopropane, 1,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)propyl]piperazine, [(3-aminopropyl)-bis-(2-

tetradecyloxyethyl)]methyl ammonium bromide, [(3-aminopropyl)-bis-(2-oleyloxyethyl)]methyl ammonium bromide,

[(3-aminopropyl)-bis-(2-palmityloxyethyl)]methyl ammonium bromide, Oleoyl-2-hydroxy-3-N,N-dimethyamino propane, 2-didecanoyl-1-N,N-dimethylaminopropane, palmitoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dipalmitoyl-1-N,N-dimethylaminopropane,

myristoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dimyristoyl-1-N,N-dimethylaminopropane, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-tetradecylcarbamoyl-butylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino-4-carbamoylbutylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-(2-dimethylamino-ethylcarbamoy l)-butylcarbamic acid cholestryl ester, Spermine-5-carboxyglycine (N'-stearyl-N'-oleyl) amide tetratrifluoroacetic acid salt, Spermine-5-carboxyglycine (N'-stearyl-N'-elaidyl) amide tetratrifluoroacetic acid salt, Agmatinyl carboxycholesterol acetic acid salt, Spermine-5-carboxy-β-alanine cholesteryl ester tetratrifluoroacetic acid salt, 2,6-Diaminohexanoeyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, 2,4-Diaminobutyroyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, N,N-Bis (3-aminopropyl)-3-aminopropionyl β-alanine cholesteryl ester tristrifluoroacetic acid salt., [N,N-Bis(2-hydroxyethyl)-2-aminoethyl]aminocarboxy cholesteryl ester, Stearyl carnitine ester, Palmityl carnitine ester, Myristyl carnitine ester, Stearyl stearoyl carnitine ester chloride salt, L-Stearyl Stearoyl Carnitine Ester, Stearyl oleoyl carnitine ester chloride, Palmityl palmitoyl carnitine ester chloride, Myristyl myristoyl carnitine ester chloride, L-Myristyl myristoyl carnitine ester chloride, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)propyl]piperazine, N-(3-aminopropyl)-N,N'-bis-(dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(myristyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-myristyloxyethyl)-piperazinium bromide, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxy-propyl]piperazine, 1,4-bis[(3-(3-aminopropyl)-myristylamino)-2-hydroxy-propyl]piperazine, and 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxy-propyl]piperazine.

The neutral lipid may be selected from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

In some embodiments at least two neutral lipids are present, which may, for example, be selected from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

These compositions also may contain one or more polyamine transfection agent, such as dense star dendrimers, PAMAM dendrimers, NH_3 core dendrimers, ethylenediamine core dendrimers, dendrimers of generation 5 or higher, dendrimers with substituted groups, dendrimers comprising one or more amino acids, grafted dendrimers, activated dendrimers, polyethylenimine, and/or polyethylenimine conjugates.

The compositions may contain a fusion agent, a cell surface ligand. a nuclear localization peptide or protein, and/or a nuclear localization agent. A nucleic acid also may be present.

Also provided are methods of introducing a nucleic acid into a eukaryotic cell, comprising contacting the cell with a composition as described above, thereby introducing the nucleic acid, into the cell, for example a human cell or an animal cell. The nucleic acid may, for example, be an expression vector.

Also provided are methods of inhibiting expression of a protein in a cell, comprising contacting the cell with a double stranded RNAi molecule and a compound according to formula (I) or (II), or a composition containing that compound.

Also provided are kits containing a compound of formula (I) or (II) and a neutral lipid, a cationic lipid, a cell surface ligand, a fusion agent and/or a nuclear localization peptide or protein.

Also provided are methods of expressing a protein in a cell, comprising contacting the cell with an expression vector encoding the protein and a compound of formula (I) or (II), or a composition containing that compound.

Also provided is a method of increasing the transfection efficiency of a polycationic lipid containing *N* amine groups, comprising contacting said cationic lipid with an acylating reagent in an amount sufficient to acylate no more than *N-1* of the amine groups.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed

description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1(A) and **1(B)** show the results of transfection of a β-galactosidase gene into 293 GT and CHO K1 cells respectively, compared to the results obtained using commercial reagents.

Figures 2(A) and 2(B) show the the results of transfection of a β-galactosidase gene into HeLa and NIH 3T3 cells respectively, compared to the results obtained using commercial reagents.

Figures 3(A) and **3(B)** show the the results of transfection of a β-galactosidase gene into A549 and HMSC-Ad cells respectively, compared to the results obtained using commercial reagents.

Figures 4(A) and **4(B)** show the the results of transfection of a β-galactosidase gene into Nuff and UB Stem cells respectively, compared to the results obtained using commercial reagents.

DETAILED DESCRIPTION

Positively charged (cationic) moloecules are provided that are useful for improved methods of delivering macromolecules into eukaryotic cells. The compositions and methods are effective in a wide variety of cells, and provide a high efficiency of transfection. Specifically, it has been found that molecules based on a core of N,N'-disubstituted 2,3,-dihydroxy-1,4-butanediamine are useful for efficient delivery of macromolecules into cells. These molecules advantageously can be used with one or more neutral lipids and additional components such as fusogenic or fusion-enhancing molecules, additional cationic lipids, cell surface ligands, cell adhesion molecules, and nuclear localization agents, in a complex with the macromolecule. The complex is easily prepared by straightforward methods and can be used on a wide variety of cells.

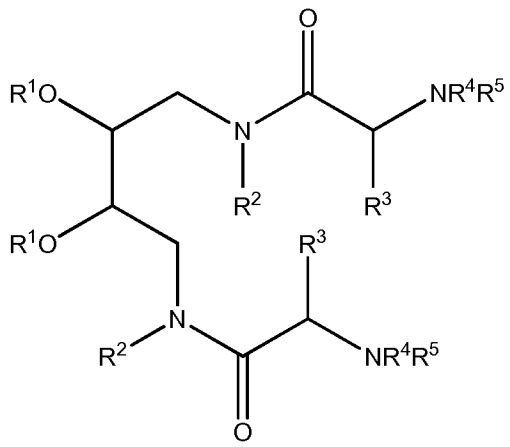
Surprisingly, it also has been found that the nucleic acid transfection efficiency of cationic lipids in general, and the new cationic lipids described herein in particular, can be

dramatically enhanced in many cases by reducing the net positive charge on the lipid by partial acylation of free primary and secondary amine functions on the lipid. Unexpectedly, this reduction in charge has been shown to greatly increase the ability of transfection complexes containing the modified lipid to efficiently transfect cells. Thus, for a lipid with *N* primary or secondary amines, it is possible to acylate up to *N*-1 of the amine groups. In most cases, the skilled artisan will recognize that the distribution of acyl groups in a lipid preparation with distinct amino groups will be statistical, because regiospecific acylation likely will not be possible unless the acylation is carried out as part of a more elaborate synthetic scheme. Thus the distribution of acyl groups will be affected not only by the stoichiometry of the acylation reagent with respect to the lipd, but will also be affected by the reactivity of the amine groups, both initially (in the non-acylated amine) but also during the reaction, as acylation activity at a free amine is potentially affected by acylation at another amine elsewhere in the molecule.

The enhancement of transfection is particularly marked for lipids containing 4 or more reactive amines, in addition to the possible presence of tertiary or quaternary amines, but is not necessarily limited to these lipids. This observed result is surprising in light of the prejudice in the art that a relatively high charge on a cationic lipid is desirable to enhance binding of negatively charged nucleic acids.

New cationic lipids

New lipid molecules are provided having the structure I:



(I)

In this structure, each R^1 independently may be C_1-C_{23} alkyl, C_1-C_{23} alkenyl, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl. Each R^2 independently may be C_1-C_6 alkyl, or C_1-C_6 alkenyl, optionally interrupted by up to 2 O atoms. Each R^3 independently may be H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkyl-C_1-C_6 alkyl,

C_5-C_7-cycloalkenyl, C_5-C_7-cycloalkenyl-C_1-C_6 alkyl, -$(CH_2)_mNR^6(CH_2)_nNHR^7$, -$(CH_2)_{2\text{-}6}NHR^7$, -$(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, $((CH_2)_mO_x)_y(CH_2)_zO_xR^8$, or -$(CH_2)_{0\text{-}3}$Het. Each R^4 independently may be H, C_1-C_6 alkyl, or C_1-C_6 alkenyl and each R^5 independently may be H, an amine protecting group, -$(CH_2)_mNR^6(CH_2)_nNHR^7$, -$(CH_2)_{2\text{-}6}NHR^7$, -$(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, –$(CO)C_1$-$C_{23}$ alkyl, –$(CO)C_1$-C_{23} alkenyl, or a peptide containing 1-20 amino acid residues. The peptide advantageously contains multiple positively charged amino acid side chains. Thus, for example, the peptide may contain one or more lysine, arginine, and/or histidine residues. Other positively charged amino acids also may be used, whether or not naturally occurring. Thus, for example, ornithine, homo-arginine and other amino acids containing amine, guanidine; imidazole and other basic heterocycles and the like can be used. Each m independently may be 2-5, and each n independently may be 2-5, while each x independently may be 0 or 1, each y may be 0-2, and each z may be 1-6. R^6 and R^7 independently may be H, an amine protecting group, –$(CO)C_1$-C_{23} alkyl, or –$(CO)C_1$-C_{23} alkenyl, R^8 may be H, C_1-C_6 alkyl, or C_1-C_6 alkenyl, and Het may be a 5-7 membered monocyclic basic heterocycle, or an 8-11 membered bicyclic basic heterocycle.

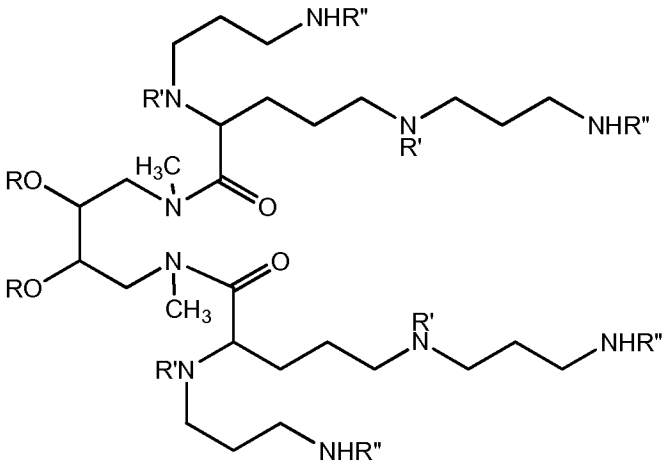
The molecule may be symmetrical or non-symmetrical with regard to each or all of the substituents R^1-R^5 independently; that is each R^1 may be the same or different, each R^2 may be the same or different, each R^3 may be the same or different, each R^4 may be the same or different, and/or each R^5 may be the same or different.

In specific embodiments R^2 may be C_1-C_3 alkyl and/or R^1 may be monounsaturated C_{12}-C_{20} alkenyl; for example one or both C_{12}-C_{20} alkenyl moieties in R^1 may be *cis* alkenyl.

In other embodiments, R^3 may be -$(CH_2)_mNR^6(CH_2)_nNHR^7$ and/or R^5 may be -$(CH_2)_{2\text{-}6}NHR^7$. In these or other embodiments, m may be 3, n may be 3, and/or R^5 may be -$(CH_2)_3NHR^7$ In these and other embodiments, each R^6 and each R^7 may be H.

In still other embodiments, at least one R^6 or R^7 may be -$(CO)C_1$-C_{23} alkyl, or -$(CO)C_1$-C_{23} alkenyl and the remainder are H. In a specific embodiment, each R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl, each R^2 may be C_1-C_3 alkyl, each R^3 may be -$(CH_2)_3NH(CH_2)_3NH_2$, each R^4 may be H; and each R^5 may be -$(CH_2)_3NH_2$. In another specific embodiment, each R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl, each R^2 may be C_1-C_3 alkyl, each R^3 may be -$(CH_2)_3NR^6(CH_2)_3NHR^7$, each R^4 may be H; each R^5 may be -$(CH_2)_3$NHR7, and at least one R^6 or R^7 may be –$(CO)C_1$-C_{23} alkyl, or –$(CO)C_1$-C_{23} alkenyl and the remainder are H.

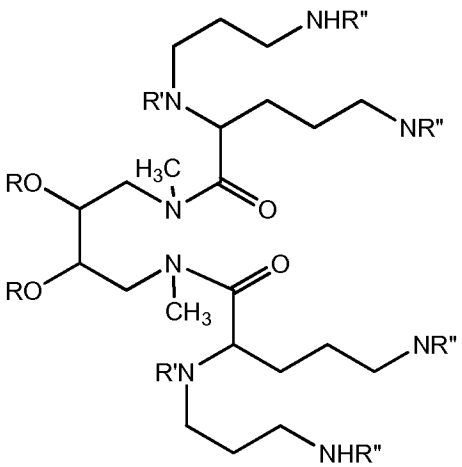
Specific examples of compounds include compounds having the structure :



where each R for example may be, but is not limited to, C_{12-20} alkyl or C_{12-20} alkenyl; and each R' and each R" independently may be, but is not limited to, H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl.

In these and other embodiments, R may be C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl, and/or each R' and each R" independently may be H, C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl.

Another specific example of the compounds is the set of compounds having the structure:

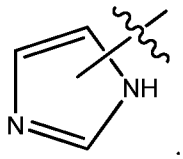


where, for example, R may be, but is not limited to, C_{12-20} alkenyl; and/or each R' and each R" independently may be, but is not limited to, H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl.

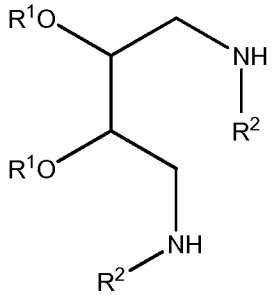
In these and other embodiments R may be oleyl and each R' and each R" independently may be H, or oleoyl. In still other embodiments at least one R' or R" may be oleoyl and the remainder are H.

In still other specific embodiments, each R^3 independently may be $-(CH_2)_{2-6}NHR^7$, $-(CH_2)_{3-6}NHC(=NH)NH_2$, or $-(CH_2)_{1-3}Het$, each R^4 is H and each R^5 independently is H or a peptide containing 1-20 amino acid residues.

In these and other embodiments, each R^1 may be C_{12}-C_{20} alkyl or C_{12}-C_{20} alkenyl, each R^2 may be C_1-C_3 alkyl, and/or and each R^5 may be H. In still other embodiments, each R^3 may be $-(CH_2)_{2-6}NH_2$, $-(CH_2)_{3-6}NHC(=NH)NH_2$ or each R^3 may be $-(CH_2)_{1-3}Het$. In one specific embodiment, Het may be

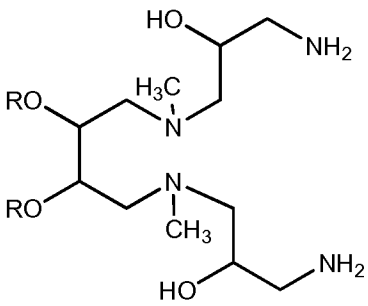


In another, the lipid may have the general formula (II):



(II)

where each R^1 independently may be C_1-C_{23} alkyl, C_1-C_{23} alkenyl, $-(CO)C_1$-C_{23} alkyl, or $-(CO)C_1$-C_{23} alkenyl; and each R^2 independently may be C_1-C_6 alkyl, or C_1-C_6 alkenyl, optionally interrupted by O. Alternatively, each R^2 independently is $-CH_2-(CHR^9)_{2-7}-NHR^4$ or $-CH_2-(CHR^9)_{2-7}-OH$, wherein each R^9 independently is H, OH, or NH2, and R^4 is H or CH_3. Advantageously, each R^2 is $-CH_2-(CHR^9)_{2-7}-NHR^4$ where R^9 is H or OH, and no more than 3 R^9 groups are OH in each R^9 moiety. In a specific embodiment, each R^2 independently is $-CH_2-CHOH-(CHR^9)1_{-6}-NHR^4$, and in one specific embodiment, the compound has the structure:



where R is R^1 as defined above. In this compound R advantageously is C_{14-18} alkyl or C_{14-18} monounsaturated alkenyl.

Each R^1 and/or each R^2 may be the same or different. In particular embodiments, R^2 may be C_1-C_3 alkyl and/or R^1 is monounsaturated C_{12}-C_{20} alkenyl. One or both C_{12}-C_{20} alkenyl moieties in R^1, when present, may be *cis* alkenyl.

The skilled artisan will recognize that, although the molecules of the invention are shown here for convenience in their neutral (unprotonated) forms, these molecules will exist in a partially or fully protonated form in solutions of appropriate pH, and that the present invention encompasses the molecules in all their protonated, unprotonated, ionized and non-ionized forms without limitation, unless specifically indicated otherwise.

Definitions

The term "alkyl", alone or in combination with any other term, refers to a straight-chain or branch-chain saturated aliphatic hydrocarbon radical containing the specified number of carbon atoms, or where no number is specified, in one embodiment from 1 to about 15 (i.e. (C_{1-15})alkyl), in another embodiment from 1 to about 10 carbon atoms (i.e. (C_{1-10})alkyl), and in another embodiment from 1 to about 6 carbon atoms (i.e. (C_{1-6})alkyl). Examples of alkyl radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isoamyl, n-hexyl and the like.

The term "alkenyl", alone or in combination with any other term, refers to a straight-chain or branched-chain mono- or poly-unsaturated aliphatic hydrocarbon radical containing the specified number of carbon atoms, or where no number is specified, in one embodiment from 2-10 carbon atoms (i.e. (C_{2-10})alkenyl) and in another embodiment, from 2-6 carbon atoms (i.e. (C_{2-6})alkenyl). Examples of alkenyl radicals include, but are not limited to, ethenyl, E- and Z-propenyl, isopropenyl, E- and Z-butenyl, E- and Z-isobutenyl, E- and Z-pentenyl, E- and Z-hexenyl, E,E-, E,Z-, Z,E- and Z,Z-hexadienyl and the like.

The term "alkynyl," alone or in combination with any other term, refers to a straight-chain or branched-chain hydrocarbon radical having one or more triple bonds containing the specified number of carbon atoms, or where no number is specified, in one embodiment from 2 to about 10 carbon atoms. Examples of alkynyl radicals include, but are not limited to, ethynyl, propynyl, propargyl, butynyl, pentynyl and the like.

The term "alkoxy" refers to an alkyl ether radical, wherein the term "alkyl" is defined above. Examples of suitable alkyl ether radicals include, but are not limited to, methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy and the like.

The term "aryl," alone or in combination with any other term, refers to a carbocyclic aromatic radical (such as phenyl or naphthyl) containing the specified number of carbon

atoms, in one embodiment from 6-15 carbon atoms (i.e. (C_{6-15})aryl), and in another embodiment from 6-10 carbon atoms (i.e. (C_{6-10})aryl), optionally substituted with one or more substituents selected from alkyl, alkoxy, (for example methoxy), nitro, halogen, (for example chloro), amino, carboxylate and hydroxy. Examples of aryl radicals include, but are not limited to phenyl, p-tolyl, 4-hydroxyphenyl, 1-naphthyl, 2-naphthyl, indenyl, indanyl, azulenyl, fluorenyl, anthracenyl and the like.

The term "aralkyl", alone or in combination, means an alkyl radical as defined above in which one hydrogen atom is phenyl, benzyl, 2-phenylethyl and the like.

The term "aralkoxycarbonyl", alone or in combination, means a radical of the formula -C(O)-O-aralkyl in which the term "aralkyl" has the significance given above. An example of an aralkoxycarbonyl radical is benzyloxycarbonyl.

The term "aryloxy", alone or in combination, means a radical of the formula aryl-O- in which the term "aryl" has the significance given above.

The term "alkanoyl", alone or in combination, means an acyl radical derived from an alkanecarboxylic acid, examples of which include acetyl, propionyl, butyryl, valeryl, 4-methylvaleryl, and the like.

The term "aryloxyalkanoyl" means an acyl radical of the formula aryl-O-alkanoyl wherein aryl and alkanoyl have the significance given above.

The term "aralkanoyl" means an acyl radical derived from an aryl-substituted alkanecarboxylic acid such as phenylacetyl, 3-phenylpropionyl (hydrocinnamoyl), 4-phenylbutyryl, (2-naphthyl)acetyl, 4-chlorohydrocinnamoyl, 4-aminohydrocinnamoyl, 4-phenylbutyryl, (1-naphthyl)acetyl, 4-chlorohydrocinnamoyl, 4-aminohydrocinnamoyl, 4-methoxyhydrocinnamoyl, and the like.

The term "aroyl" means an acyl radical derived from an aromatic carboxylic acid. Examples of such radicals include aromatic carboxylic acids, an optionally substituted benzoic or naphthoic acid such as benzoyl, 4-chlorobenzoyl, 4-carboxybenzoyl, 4-benzyloxycarbonyl)benzoyl, 1-naphthoyl, 2-naphthoyl, 6-carboxy-2-naphthoyl, 6-(benzyloxycarbonyl)-2-naphthoyl, 3-benzyloxy-2-naphthoyl, 3-hydroxy-2-naphthoyl, 3-(benzyloxyformamido)-2-naphthoyl, and the like.

The term "aminocarbonyl" alone or in combination, means an amino-substituted carbonyl (carbamoyl) group derived from an amino-substituted carboxylic acid wherein the amino group can be a primary, secondary or tertiary amino group continuing substituents selected from hydrogen, alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl radicals and the like.

The term "aminoalkanoyl" means an acyl radical derived from an amino substituted alkanecarboxylic acid wherein the amino group can be a primary, secondary or tertiary amino group containing substituents selected from the group consisting of hydrogen, cycloalkyl, cycloalkylalkyl radicals and the like, examples of which include N,N-dimethylaminoacetyl and N-benzylaminoacetyl.

The term "carbocycle" refers to a non-aromatic stable 3- to 8-membered carbon ring which may be saturated, mono-unsaturated or poly-unsaturated. The carbocycle may be attached at any endocyclic carbon atom which results in a stable structure. Carbocycles in one embodiment have 5-7 carbons.

The term "cycloalkyl", alone or in combination, means an alkyl radical which contains from about 3 to about 8 carbon atoms and is cyclic. Examples of such cycloalkyl radicals include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and the like.

The term "cycloalkylalkyl" means an alkyl radical as defined above which is substituted by a cycloalkyl radical containing from about 3 to about 8, in one embodiment from about 3 to about 6, carbon atoms.

The term "cycloalkylcarbonyl" means an acyl group derived from a monocyclic or bridged cycloalkanecarboxylic acid such as cyclopropanecarbonyl, cyclohexanecarbonyl, adamantanecarbonyl, and the like, or from a benz-fused monocyclic cycloalkanecarboxylic acid which is optionally substituted by, for example, alkanoylamino, such as 1,2,3,4-tetrahydro-2-naphthoyl, 2-acetamido-1,2,3,4-tetrahydro-2-naphthoyl.

The term "cycloalkylalkoxycarbonyl" means an acyl group derived from a cycloalkylalkoxycarboxylic acid of the formula cycloalkylalkyl-O-COOH wherein cycloalkylalkyl has the significance given above.

The term "basic heterocycle" refers to a stable 5-7 membered monocyclic heterocyclic ring or 8-11 membered bicyclic heterocyclic ring which is either saturated or partially unsaturated, and which may be optionally benzofused if monocyclic and which is optionally substituted on one or more carbon atoms by halogen, alkyl, alkoxy, oxo, and the like, and/or on a secondary nitrogen atom (i.e., -NH-) by alkyl, aralkoxycarbonyl, alkanoyl, phenyl or phenylalkyl or on a tertiary nitrogen atom (i.e., +N-) by oxido and which is attached via a carbon atom. Each heterocycle consists of one or more carbon atoms and from one to four heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, provided that at least one heteroatom is a basic nitrogen atom. A heterocyclyl radical may be attached at any endocyclic carbon or heteroatom which results in the creation of a stable structure. Heterocycles include 5-7 membered monocyclic heterocycles and 8-10 membered bicyclic

heterocycles. Examples of basic heterocycles include imidazolinoyl, imidazolidinyl, indazolinolyl, perhydropyridazyl, pyrrolinyl, pyrrolidinyl, piperidinyl, pyrazolinyl, piperazinyl, morpholinyl, thiamorpholinyl, thiazolidinyl, thiamorpholinyl sulfone, oxopiperidinyl, oxopyrrolidinyl, and oxoazepinyl.

The term "halogen" means fluorine, chlorine, bromine or iodine.

The term "surface ligand" or "cell surface ligand" refers to a chemical compound or structure which will bind to a surface receptor of a cell. The term "cell surface receptor" as used herein refers to a specific chemical grouping on the surface of a cell to which the ligand can attach. Cell surface receptors can be specific for a particular cell, i.e., found predominantly in one cell rather than in another type of cell (e.g., LDL and asialoglycoprotein receptors are specific for hepatocytes). The receptor facilitates the internalization of the ligand and attached molecules. A cell surface receptor includes but is not limited to a folate receptor, biotin receptor, lipoic acid receptor, low-density lipoprotein receptor, asialoglycoprotein receptor, insulin-like growth factor type II/cation-independent mannose-6-phosphate receptor, calcitonin gene-related peptide receptor, insulin-like growth factor I receptor, nicotinic acetylcholine receptor, hepatocyte growth factor receptor, endothelin receptor, bile acid receptor, bone morphogenetic protein receptor, cartilage induction factor receptor or glycosylphosphatidylinositol (GPI)-anchored proteins (e.g., .beta. andrenargic receptor, T-cell activating protein, Thy-1 protein, GPI-anchored 5' nucleotidase). These are nonlimiting examples.

A receptor is a molecule to which a ligand binds specifically and with relatively high affinity. It is usually a protein or a glycoprotein, but may also be a glycolipid, a lipidpolysaccharide, a glycosaminoglycan or a glycocalyx. For purposes of this disclosure, epitopes to which an antibody or its fragments binds is construed as a receptor since the antigen:antibody complex undergoes endocytosis. Furthermore, surface ligand includes anything which is capable of entering the cell through cytosis (e.g. endocytosis, potocytosis, pinocytosis).

As used herein, the term "ligand" refers to a chemical compound or structure which will bind to a receptor. This includes but is not limited to ligands such as asialoorosomucoid, asialoglycoprotein, lipoic acid, biotin, apolipoprotein E sequence, insulin-like growth factor II, calcitonin gene-related peptide, thymopoietin, hepatocyte growth factor, endothelin-1, atrial natriuretic factor, RGD-containing cell adhesion peptides and the like.

One skilled in the art will readily recognize that the ligand chosen will depend on which receptor is being bound. Since different types of cells have different receptors, this

provides a method of targeting nucleic acid to specific cell types, depending on which cell surface ligand is used. Thus, the preferred cell surface ligand may depend on the targeted cell type.

The term "nuclear localization agent," "nuclear localization signal," or "nuclear ligand" as used herein refers to a ligand, such as a peptide, which will cause an agent covalently or non-covalently linked to it to localize at the cell nucleus, typically by binding a nuclear receptor. The term "nuclear receptor" as used herein refers to a chemical grouping on the nuclear membrane which will bind a specific ligand and help transport the ligand, and accompanying linked moieties, through the nuclear membrane. Nuclear receptors can be but are not limited to those receptors which bind nuclear localization sequences. Nonlimiting examples of nuclear ligands include GYSTPPKKKRKVEDP, GYSTPPKTRRRP, GYSTPGRKKR, GYSTPRRNRRRRW, PDEVKRKKKPPTSYG, PRRRTKPPTSYG, RKKRGPTSYG, WRRRRNRRPTSYG, and GYGPPKKKRKVEAPYKA$(K)_{20-40}$K, may be used to transport nucleic acid to the nucleus.

The term "lysis agent" as used herein refers to a molecule, compound, protein or peptide which is capable of breaking down an endosomal membrane and freeing the DNA transporter into the cytoplasm of the cell. This term includes but is not limited to viruses, synthetic compounds, lytic peptides, or derivatives thereof. The term "lytic peptide" refers to a chemical grouping which penetrates a membrane such that the structural organization and integrity of the membrane is lost. As a result of the presence of the lysis agent, the membrane undergoes lysis, fusion or both.

The term "nucleic acid," when not applied to a specific type of molecule such as unmodified DNA or RNA, refers to any type of nucleic acid that presently is known or that may be prepared or identified in the future, provided that the nucleic acid is sufficiently negatively charged to form a lipid aggregate, liposome, or liposome-like complex when admixed with any lipid of Formula (I) or (II). Nucleic acid, as used herein, refers to deoxyribonucleotides or ribonucleotides and mixtures and polymers thereof in single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as a reference nucleic acid, and which are metabolized in a manner similar to a reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs). The nucleic acid may be in the form of an antisense molecule, for example a “gap-

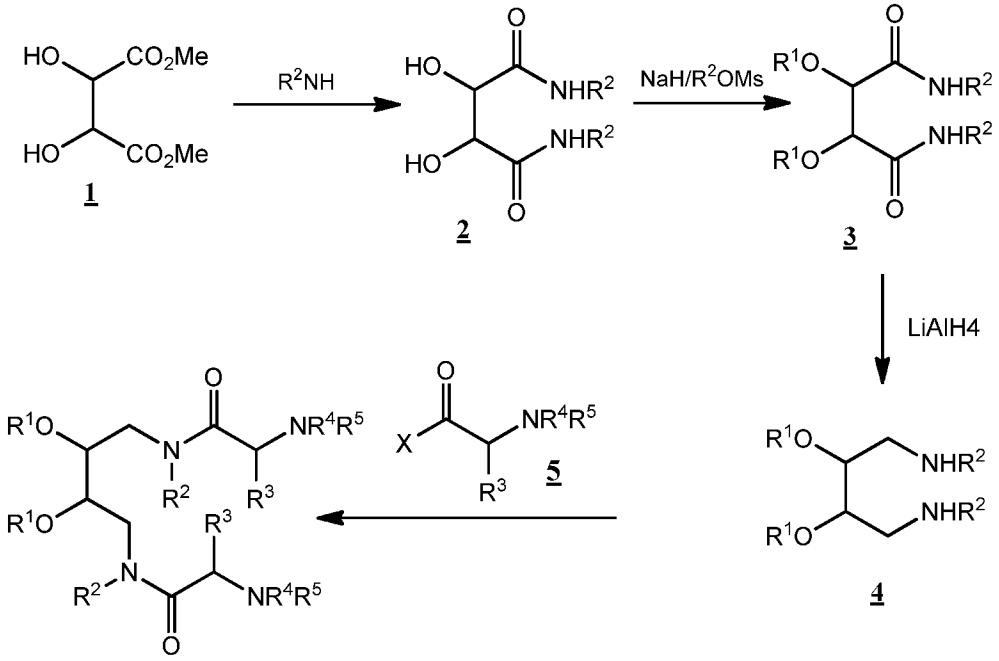
mer" containing an RNA-DNA-RNA structure that activates RNAseH. The nucleic acid can be, for example, DNA or RNA, or RNA-DNA hybrid, and can be an oligonucleotide, plasmid, parts of a plasmid DNA, pre-condensed DNA, product of a polymerase chain reaction (PCR), vectors, expression cassettes, chimeric sequences, chromosomal DNA, or derivatives of these groups or other form of nucleic acid molecule. The nucleic acid may be a double-stranded RNA molecule of the type used for inhibiting gene expression by RNA interference. The nucleic acid may be a short interfering double stranded RNA molecule (siRNA). The nucleic acid molecule can also be a Stealth™RNAi molecule (Invitrogen Corporation/Life Technologies Corporation, Carlsbad, CA).

Preparation of the lipids

Symmetric and asymmetric cationic lipids of general structure (I) may be synthesized using methods that are well known in the art, as shown, for example in Scheme 1. Dimethyl tartrate (1) can be treated with an alkylamine at elevated temperature (e.g. 70°C) in a sealed pressure reactor to obtain compound **2**. This compound may be alkylated with an alkyl mesylate to obtain compound **3,** which is then reduced using lithium aluminum hydride to produce the bis-amine **4**. Compound **4** may be acylated with a suitably protected amino acid **5** using, for example, a carbodiimide as a coupling agent to obtain the protected precursor of compound **6.** This precursor is then deprotected to produce the desired symmetric compound **6**. Specific examples of suitable amino acids for the acylation include Boc-protected carboxyspermine, histidine or lysine, which generate compounds **7**, **8**, and **9** respectively, after deprotection.

Alternatively, asymmetric cationic lipids (*i.e.* compounds lacking a plane of symmetry) may be prepared using a tartaric acid monoester, readily prepared from diacetyl tartaric anhydride (see *Organic Syntheses, Coll. Vol. 4, p.242 (1963); Vol. 35, p.49 (1955))* or from an erythronolactone such as **10**. See Scheme 2. Protection of the diol, followed by DIBAL reduction, reductive amination and amine protection produce compound **11**. Mild oxidation, reductive amination and protection produce compound **12**. Diol deprotection and alkylation produce compound **13**. Stepwise selective amine deprotection and coupling reactions then produces the asymmetric compound **14**.. .

Scheme 1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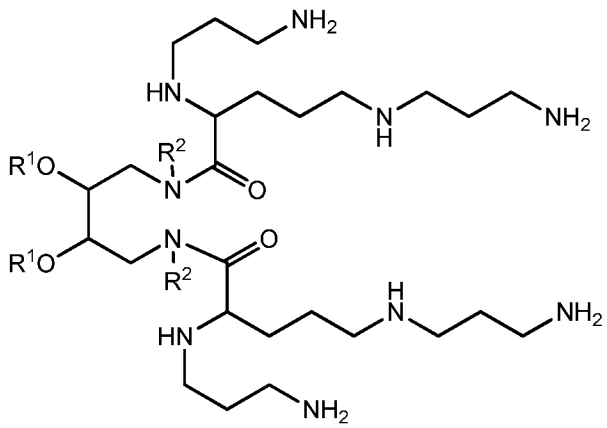
CO2Me
HO
CO2Me
1
R2NH
HO
NHR2
HO
NHR2
2
NaH/R2OMs
R1O
NHR2
R1O
NHR2
3
LiAlH4
R1O
NHR2
R1O
NHR2
4
X
NR4R5
R3
5
R1O
N
R2
NR4R5
R3
R1O
R2
N
NR4R5
O
6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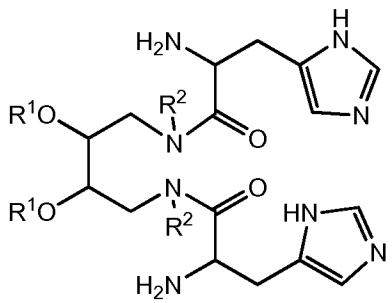
N
H
NH2
R1O
N
R2
O
R1O
N
R2
O
H
N
NH2
HN
NH2
7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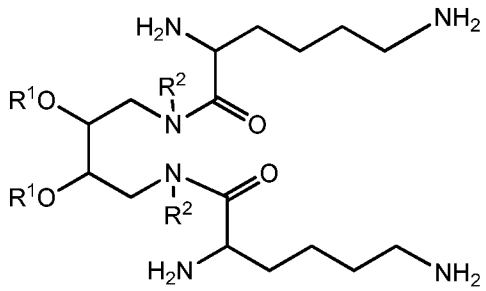
H
N
R1O
R2
N
O
R1O
N
R2
O
HN
N
H2N
8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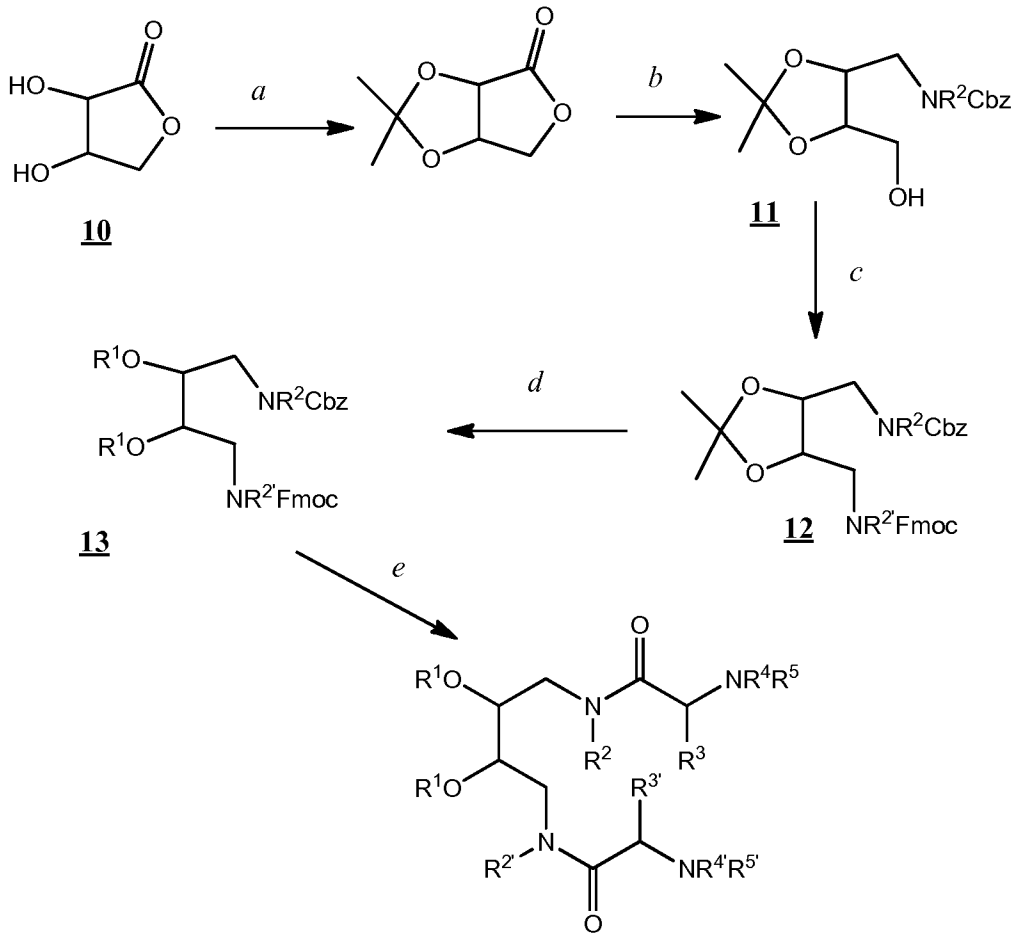
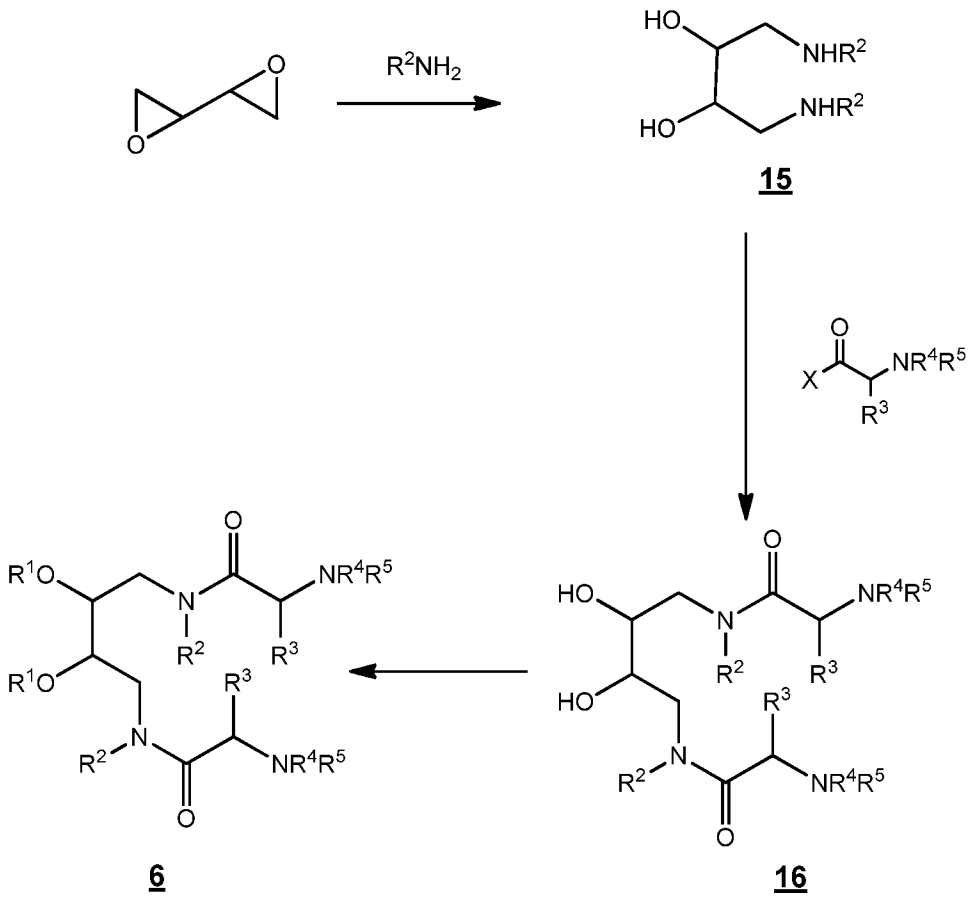
NH2
R1O
R2
N
O
R1O
N
R2
O
H2N
NH2
9

Scheme 2

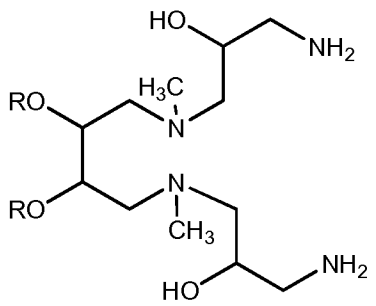


In an alternative synthesis, a bis-epoxide is reacted with an alkylamine to produce the amino alcohol **15**. This can be acylated to produce **16** followed by alkylation and deprotection, if necessary, to produce **6**. See Scheme 3.

Scheme 3



Compounds of formula (II) may be prepared from intermediates of structure **4** above by methods that are well known in the art. For example, compound **4** where R^2 is alkyl may be reacted with a suitable protected epoxyamine, for example, an epoxyphthalimide, followed by deprotection to provide β-hydroxyamines such as



Other methods of preparing compounds of formula (II) will be apparent to the skilled artisan. For example, reductive amination of a protected carbohydrate molecule can be used to provide compounds of formula (II) that contain multiple hydroxyl groups in R^2.

Formulation and use of the lipids for transfection

The lipids described above may be formulated by various methods to be used in transfection. One of the simplest methods for formulation is reverse evaporation. In this procedure the required amount of the cationic lipid and a co-lipid (if used, e.g. a neutral lipid) are transferred into a round bottom flask. An amount of chloroform that is enough to dissolve the lipids is added, followed with enough molecular biology grade water to make the desired concentration of total lipids/volume (e.g. 2 mg/ml). The solution is evapoted *in vacuo* (e.g. on a rotary evaporator) and the chloroform removed under vacuum. As the chloroform is removed liposomes are formed in the aqueous medium. Other methods for formulation that can be used are sonication and microfluidization. In both cases the required amount of the cationic lipid and the co-lipid are transferred into a flask and an amount of chloroform that will dissolve the lipids to homogenous solution is added. The chloroform is evaporated to leave a thin film of lipid mixture in the flask. The lipid film is then hydrated with molecular biology grade water to make the desired concentration and sonicated or microfludized.

Advantageously, the new lipids are formulated with one or more colipids, most advantageously neutral colipids, although the skilled artisan will recognize that other lipids, including cationic lipids, may be used. For example, formulations where the molar ratio of cationic lipid : DOPE was varied from 2:1 to 1:16 were prepared using the above methods. Compounds having the structure **7** above were prepared where the hydrocarbon chain was varied from C_{10}–C_{20} and the histidine and lysine analogs **8** and **9** where the hydrocarbon chain was varied from C_{10} – C_{20} were formulated in the same manner.

The new lipids may be formulated with one ore more cationic lipids and/or one or more neutral lipids. The neutral lipid(s) may be, for example, DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and/or 3-alkyloxy-2-hydroxy-1-acetamidopropane.

The cationic lipid may be selected from the group consisting of DOTMA, DOTAP, DMRIE, DC-Chol, DDAB, DOSPA, DOSPER, DOGS, TMTPS, TMTOS, TMTLS, TMTMS, TMDOS, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, L-spermine-5-carboxyl-3-(DL-1,2-dipalmitoyl-

dimethylaminopropyl-β-hyd roxyethylamine, 3,5-(N,N-di-lysyl)-diaminobenzoyl-glycyl-3-(DL-1,2-dipalmitoyl-dimethylami nopropyl-β-hydroxyethylamine), L-Lysine-bis(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, L-Lysine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-alkylamino)-2-hydroxypropyl)piperazine, L-Lysine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxypropyl]piperazine, L-Ornithine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4,-bis[(3-amino-2-hydroxypropyl)-oleylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-palmitylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-myristylamino]-butane-2,3-diol, 1,4-bis[(3-oleylamino)propyl]piperazine, L-Arginine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, bis[(3-(3-aminopropyl)-myristylamino)2-hydroxypropyl]piperazine, L-Arginine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Serine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxypropyl]piperazine, Glycine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, Sarcosine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Histidine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, cholesteryl-3β-carboxyl-amidoethylenetrimethylammonium iodide, 1,4-bis[(3-myristylamino)propyl]piperazine, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine, cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysucc inate iodide, 2-[(2-trimethylammonio)-ethylmethylamino] ethyl-cholesteryl-3β-oxysuccinate iodide, 3β[N-(N', N'-dimethylaminoethane)carbamoyl]cholesterol, and 3β-[N-(polyethyleneimine)-carbamoyl] cholesterol,1,4-bis[(3-palmitylamino)propyl]piperazine, L-Ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide, N^2,N^5 -Bis(3-aminopropyl)-L-ornithylglycyl-N- (1-heptadecyloctadecyl)glycinamide, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-alkylamino)-2-hydroxypropyl]piperazine N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-glutamine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioctadecyl-L-glutaminyl]-L-

glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-diolyl-L-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-glutamine,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)-2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioleyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-oleylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dipalmityl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-asparagine,

N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dipalmityl-L-glutaminyl]-L-glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dimyristyl-L-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-asparagine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)-2-hydroxypropyl]piperazine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dimyristyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-myristylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dilaureyl-L-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dilaureyl-L-glutaminyl]-L-glutamic acid, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dioctadec-9-enylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dipalmitylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dimyristylpropionamide, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)propyl]piperazine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)propyl]piperazine, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-diolylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dipalmitylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dimyristylaminopropane, 1,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)propyl]piperazine, [(3-aminopropyl)-bis-(2-

tetradecyloxyethyl)]methyl ammonium bromide, [(3-aminopropyl)-bis-(2-oleyloxyethyl)]methyl ammonium bromide, [(3-aminopropyl)-bis-(2-palmityloxyethyl)]methyl ammonium bromide, Oleoyl-2-hydroxy-3-N,N-dimethyamino propane, 2-didecanoyl-1-N,N-dimethylaminopropane, palmitoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dipalmitoyl-1-N,N-dimethylaminopropane, myristoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dimyristoyl-1-N,N-dimethylaminopropane, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-tetradecylcarbamoyl-butylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino-4-carbamoylbutylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-(2-dimethylamino-ethylcarbamoy l)-butylcarbamic acid cholestryl ester, Spermine-5-carboxyglycine (N'-stearyl-N'-oleyl) amide tetratrifluoroacetic acid salt, Spermine-5-carboxyglycine (N'-stearyl-N'-elaidyl) amide tetratrifluoroacetic acid salt, Agmatinyl carboxycholesterol acetic acid salt, Spermine-5-carboxy-β-alanine cholesteryl ester tetratrifluoroacetic acid salt, 2,6-Diaminohexanoeyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, 2,4-Diaminobutyroyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, N,N-Bis (3-aminopropyl)-3-aminopropionyl β-alanine cholesteryl ester tristrifluoroacetic acid salt., [N,N-Bis(2-hydroxyethyl)-2-aminoethyl]aminocarboxy cholesteryl ester, Stearyl carnitine ester, Palmityl carnitine ester, Myristyl carnitine ester, Stearyl stearoyl carnitine ester chloride salt, L-Stearyl Stearoyl Carnitine Ester, Stearyl oleoyl carnitine ester chloride, Palmityl palmitoyl carnitine ester chloride, Myristyl myristoyl carnitine ester chloride, L-Myristyl myristoyl carnitine ester chloride, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)propyl]piperazine, N-(3-aminopropyl)-N,N'-bis-(dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(myristyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-myristyloxyethyl)-piperazinium bromide, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxy-propyl]piperazine, 1,4-bis[(3-(3-aminopropyl)-myristylamino)-2-hydroxy-propyl]piperazine, and 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxy-propyl]piperazine.

Other formulations may also include one or more polyamine transfection agents, such as dense star dendrimers, PAMAM dendrimers, NH_3 core dendrimers, ethylenediamine core

dendrimers, dendrimers of generation 5 or higher, dendrimers with substituted groups, dendrimers comprising one or more amino acids, grafted dendrimers, activated dendrimers, polyethylenimine, and/or polyethylenimine conjugates.

Still other formulations may include transfection enhancing agents such as a fusion agent, a cell surface ligand and/or a nuclear localization agent such as a nuclear receptor ligand peptide, Examples of transfection enhancing agents include, but are not limited to, reovirus-related fusogenic peptides (see WO07/130073, which is hereby incorporated by reference in its entirety), insulin, a transferrin, epidermal growth factor, fibroblast growth factor, a cell targeting antibody, a lactoferrin, a fibronectin, an adenovirus penton base, Knob, a hexon protein, a vesicular stomatitis virus glycoprotein, a Semliki Forest Virus core protein, a influenza hemagglutinin, a hepatitis B core protein, an HIV Tat protein, a herpes simplex virus VP22 protein, a histone protein, a arginine rich cell permeability protein, a high mobility group protein, and invasin protein, and internalin protein, an endotoxin, a diptheria toxin, a shigella toxin, a melittin, a magainin, a gramicidin, a cecrophin, a defensin, a protegrin, a tachyplesin, a thionin, a indolicidin, a bactenecin, a drosomycin, an apidaecin, a cathelicidin, a bacteriacidal-permability-increasing protein, a nisin, a buforin, and fragments thereof.

Use of these compositions in transfection can be carried out by methods that are known in the art. See for example, WO07/130073, at pages 54-60 which describes "before" and "after" protocols for transfection where the components of a transfection complex are mixed in differing orders prior to addition to a cell culture. Typically, a liposomal preparation of the lipid, with or without colipid is prepared, and is then mixed with a macromolecule, such as a DNA molecule or RNAi molecule to form a transfection complex. The complex is then added to a cell culture and transfection is monitored using well known methods. Additional components such as cell surface ligands, fusion agents, nuclear localization agents and the like may be added to the nucleic acid prior to admixture with the liposome, or may be added to the liposome prior to addition of nucleic acid.

Cells which can be transfected according to these methods include, but are not limited to, virtually any eukaryotic cell including primary cells, cells in culture, a passaged cell culture or a cell line, and cells in cultured tissue. Suitable cells include human cell lines and animal cell lines. The cell may be a fibroblast. The cells can be attached cells or cells in suspension (suspension cells). In certain illustrative aspects, the cells are suspension CHO-S cells and suspension 293-F cells. Other cells that may be used include, without limitation, 293, 293-S, CHO, Cos, 3T3, Hela, primary fibroblasts, A549, Be2C, SW480, CHOK1,

Griptite 293, HepG2, Jurkat, LNCap, MCF-7, NIH-3T3, PC12, C6, Caco-2, COS-7, HL60, HT-1080, IMR-90, K-562, SK-BR3, PHP1, HUVEC, MJ90, NHFF, NDFF and primary neurons.

In another embodiment is a method for producing a protein which includes contacting a cell with a lipid-nucleic acid complex as described above, where the nucleic acid encodes the protein. The cells are incubated to produce the protein and the protein is collected. Cells which can be used for protein production are described above. In addition, any composition which includes a lipid of Formula (I) or (II) can be used for transfection of cells. Such compositions are further discussed herein, and include, but are not limited to compositions comprising lipids of Formula (I) or (II), a co-lipid and an optional transfection enhancing agent such as a fusogenic peptide or protein.

In another embodiment is a method for inhibiting production of a protein in a cell, comprising contacting the cell with a lipid-nucleic acid complex as described above, where the nucleic acid is a double stranded RNA molecule, such as an RNAi or siRNA molecule designed to inhibit expression of the protein. Methods of designing such RNA molecules are well known in the art. Lipids of Formula (II) are particularly suitable for deliver of RNAi molecules in this fashion. The cells are incubated and the phenotypic consequence of inhibiting production of the selected protein is observed. The lipids formulated in this manner were used in transfection. The transfection of 293, CHO-K1, NIH3T3, Hela,, A549, Umbilical Cord Mesenchymal Stem Cells (UB Stem), Adipose Mesenchymal Stem Cells(HMSC-Ad) and Neonatal Human Fibroblast (Nuff) with β-galactosidase reporter plasmid pCMV●SPORT-β-gal was carried out using the formulation **VII-97-G** which contains the histidine analog of Compound **6**. The result is shown in the figure below (Figure 1)

Reagent kits

Components of the transfection compositions described above can be provided in a reagent kit. The kits contain the lipid of formula I or II above, together with additional components, such as a neutral lipid, a cationic lipid, cell surface ligands, fusion agents, and/or nuclear localization agents and the like. The kit components may be separate or may be premixed in any manner. For example, the lipid of formula I or II may be admixed with one or more neutral lipid. Additional components may also be present in the same container or may be present in one or more separate containers. The kits typically include vessels, such as vials and/or tubes, that are packaged together, for example in a cardboard box. The kits can

be shipped from a supplier to a customer. For example, in one example provided herein is a kit that includes a vial that includes a liposomal formulation as described above and, optionally, a transfection agent and a transfection enhancing peptide. The kit can also include, for example, a separate vessel that includes a transfection enhancing agent, such as a transfection enhancing peptide, for example Plus Reagent™ (Invitrogen Corp., Carlsbad, CA). The kit can also include in separate containers, cells, cell culture medium, and a reporter nucleic acid sequence, such as a plasmid that expresses a reporter gene. In certain examples, the culture medium can be reduced-serum medium and/or protein expression medium.

In one embodiment, a kit comprises individual portions of, or a mixture of, cationic lipid, such as a lipid of Formula I, and peptide, protein or fragment thereof or modified peptide, protein or fragment thereof. In another embodiment, a kit comprises individual portions of, or a mixture of, polycationic polymers and peptide, protein or fragments thereof or modified peptide, protein or fragments thereof. Cationic lipid transfection kits can optionally include neutral lipid as well as other transfection-enhancing agents or other additives, and the relative amounts of components in the kit may be adjusted to facilitate preparation of transfection compositions. Kit components can include appropriate medium or solvents for other kit components.

Nucleic acids that can be transfected by the methods of this invention include DNA and RNA (including RNAi/siRNA) of any size from any source comprising natural bases or non-natural bases, and include those encoding and capable of expressing therapeutic or otherwise useful proteins in cells, those which inhibit undesired expression of nucleic acids in cells, those which inhibit undesired enzymatic activity or activate desired enzymes, those which catalyze reactions (ribozymes), and those which function in diagnostic assays (e.g., diagnostic nucleic acids). Thereapeutic nucleic acids include those nucleic acids that encode or can express therapeutically useful proteins, peptides or polypeptides in cells, those which inhibit undesired expression of nucleic acids in cells, those which inhibit undesired enzymatic activity or activate desired enzymes in cells.

The compositions and methods provided herein can also be readily adapted in view of the disclosure herein to introduce biologically-active macromolecules other than nucleic acids including, among others, polyamines, polyamine acids, polypeptides and proteins into eukaryotic cells. Other materials useful, for example as therapeutic agents, diagnostic materials, research reagents, which can be bound to the peptides and modified peptides and introduced into eukaryotic cells by the methods of this invention.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1: Synthesis of Compounds 7-9

Dimethyl tartrate **(1)**(7.12 g) was combined with 50 ml of methylamine solution in THF (2M) in a high pressure reactor tube. The tube was heated at 70°C overnight (22 hrs). The reaction mix was cooled to room temperature and kept at 4°C for about 1 hour. The precipitate was filtered and washed twice with 50 ml of THF to obtain compound **(2, R^2 = Me)** as a white solid material.

NaH (1g; 60% oil dispersion) was triturated twice with hexane (10 ml). Dimethylaminotartaramide (2)(1.78 g) was added followed by THF (500 ml). The reaction mix was heated under reflux overnight, after which oleyl mesylate was dissolved in 50 ml THF and added to the reaction mix. The reaction mix was was heated under reflux for 3–5 days. The reaction was stopped when TLC analysis (silica gel, ethyl acetate hexane 1:1) indicated that there was no change in the pattern of reaction products. The reaction mixture was cooled and water (400 ml) was added. The organic (top) layer was separated and concentrated *in vacuo* on a rotary evaporator. The resulting material was dissolved in 5 ml ethyl acetate and applied to a flash silica column (100 g) that was equilibrated with 1% ethyl acetate in hexane. The column was eluted with 1 L each of 1% ethyl acetate in hexane, ethyl acetate/hexane (3:7), ethyl acetate/hexane (4:6), ethyl acetate hexane (1:1) and 50 ml fractions were collected. The fractions that contained the desired material, Compound **(3, R^1 = oleyl)**, were combined and concentrated. The material was characterized by TLC and ESMS (MH^+ 677)

Compound **(3)** (0.7 g) was suspended in 100 ml anhydrous THF and 10 ml of 1 M lithium aluminum hydride solution in THF was added drop-wise.. After the addition was completed, the reaction mix was refluxed overnight. The mixture was cooled to room temperature and 0.5 ml of water was added very carefully followed by 0.5 ml 15% NaOH and 1.5 ml of water. The reaction mixture was stirred magnetically and the THF was decanted while the mixture was still hot. The THF was removed *in vacuo* on a rotary evaporator and the residue was dissolved in 400 ml chloroform and extracted with water (2 x 300 ml). The

solution was concentrated and the resulting gum was dissolved in 5 ml chloroform and applied to a flash silica column (100g) that was equilibrated with 1% methanol in chloroform. The column was eluted with 1L each of 1%, 5%, 10%, 20% and 30% methanol in chloroform. The fractions that contained the desired material, Compound (**4, R^1 = oleyl, R^2 = methyl**), were combined. It was characterized by TLC and ESMS (MH^+ 649).

BOC-protected carboxy-spermine (210 mg) was dissolved in 2 ml DMF/CH_2Cl_2. N-hydroxysuccinimide (36 mg) and diisopropylcarbodiimide (0.041 ml) were added to the solution and the mix was stirred for 2 hrs. Compound (**4**) in 1 ml CH_2Cl_2 was added to the reaction mix and stirring continued at room temperature overnight. The reaction mix was diluted with 100 ml CH_2Cl_2 and the solution was extracted with water (2 x 100 ml). The organic layer was separated and concentrated and taken up into 5 ml chloroform and loaded on a silica flash column (50 g) that previously was equilibrated with 1% methanol in chloroform. The column was eluted with 800 ml each of 1% and 3% methanol in chloroform. The fractions that contain the desired material, BOC-protected Compound 5, were combined and concentrated. The material was taken up into 2 ml methylene chloride and treated with 2 ml of trfluoroacetic acid for 1 hr. The mix was concentrated and co evaporated with ethanol (2 x50 ml) to obtain Compound (**7, R^1 = oleyl, R^2 = methyl**) as a gum. It was characterized by TLC and ESMS (MH^{2+} 553.8)

To prepare compound (**8, R^1 = oleyl, R^2 = methyl**), BOC –His(BOC)-NHS (332 mg) and diisopropylethylamine were dissolved in 10 ml CH_2Cl_2. The diamine, Compound (**4, R^1 = oleyl, R^2 = methyl**), in 2 ml CH_2Cl_2 was added in one portion to the NHS ester solution and stirred overnight. TLC analysis on silica showed the disappearance of starting material ($CHCl_3$: MeOH 90:10) and formation of product ($CHCl_3$: MeOH 95:5). The reaction mix was diluted with 200 ml CH_2Cl_2 and extracted with 200 ml of water followed by 200 ml of saturated $NaHCO_3$ solution. The organic layer was separated and concentrated and the residue dissolved in 5 ml chloroform and loaded on a flash column (50 g) equilibrated with chloroform (0.8% ethanol). The column was eluted with 1 L each of chloroform and 1% MeOH in chloroform. Fractions that contain the desired material, BOC protected Compound (**8**), were combined and concentrated. The gummy intermediate was characterized by TLC and ESMS (MH^+ 1323). The material was dissolved in 4 ml CH_2Cl_2 and 2 ml of trifluoroacetic acid was added and mixture incubated at room temperature for 2 hrs. The reaction mix was concentrated and co-vaporated with methylene chloride (2 x 50 ml) and methanol (1 x 50 ml) to obtain the trifluroacetat salt of Compound (**8, R^1 = oleyl, R^2 =**

methyl). It was characterized to be Compound **8** by TLC (Silica, $CHCl_3$: MeOH: H2O: NH_4OH 60:20:1:1) and ESMS (MH^+ 923).

Example 2 :Formulation of cationic lipids into liposomes:

The liposome was formulated using reverse evaporation. Thus, 6.32 mg of the TFA salt of Compound (**8, R^1 = oleyl, R^2 = methyl**) and 13.67 mg of DOPE (1:4 molar ration of TFA salt of Compound 8: DOPE) were combined and placed in a round bottom flask. The lipid mixture was dissolved in 2 ml of chloroform. 10 ml of water was added to the chloroform solution. The chloroform was removed under vacuum on a rotary evaporator to obtain a liposome solution. The solution was adjusted to 10 ml to obtain a 2 mg/ml liposome solution. It was designated as VII-97-G. Formulations where the molar ratios varied from 2:1 to 1:16 (TFA salt of Compound (**8, R^1 = oleyl, R^2 = methyl**): DOPE were prepared in this manner.

Example 3: Transfection protocol

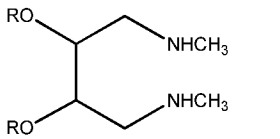
Transfection of 8 different cell types with β-galactosidase reporter plasmid pCMV●SPORT-β-gal was carried out as follows:

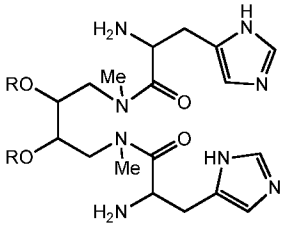
Cells were plated in a 96-well plates with 100µl of media containing 5- 10% fetal calf serum the day prior to transfection such that a desired confluency (70% - 95%) was achieved. The following day a transfection agent that included a liposomal composition of the lipid VII-97-G and DNA were mixed in Opti-MEM to form DNA/ lipid complexes. Complexes were formed by adding various amounts of lipids (1 µl to 6 µl to 50µl of Opti-MEM) DNA (100 ng) was added to 50µl Opti-MEM. The DNA and lipid solutions were then mixed to form DNA lipid complexes. The complexes were incubated for at least 20 minutes after which 10 µl DNA/ lipid complexes were added to cells. Lipofectin and Lipofectamine (Invitrogen, Carlsbad ,CA) were used as described by the manufacturer.

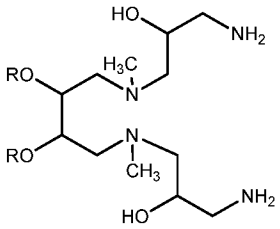
Cells were incubated for an additional 24 hours to allow expression of the plasmid. Medium was removed and the cells were lysed in 100-200 µl of lysis buffer. The lysates (20 µl) were assayed for β-gal activity using the enzymatic substrate ONPG. Total activity was determined by reading the OD at 405 using Bio-Rad Benchmark Microplate Spectrophotometer.

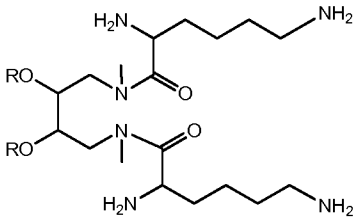
Figures 1-4 show the results obtained, and compare those results to two widely used commercial reagents. The results show that the lipids described above provided significantly enhanced transfection efficiency, as measure by β-gal expression, across all cell types.

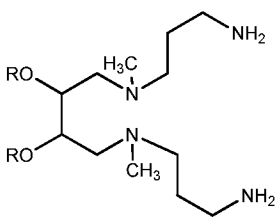
Compounds having the structures shown below, where R is C_{14}, C_{16}, or C_{18} alkyl, or C_{14}, C_{16}, or C_{18} monounsaturated alkenyl, were prepared and shown to be effective for transfection of nucleic acids as described above.

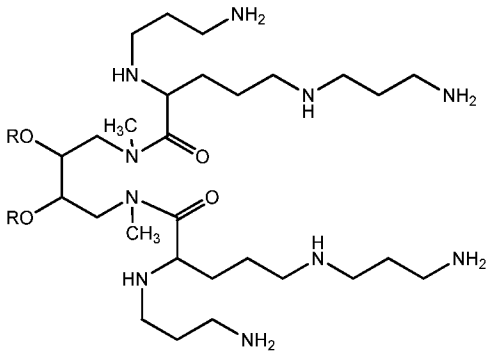






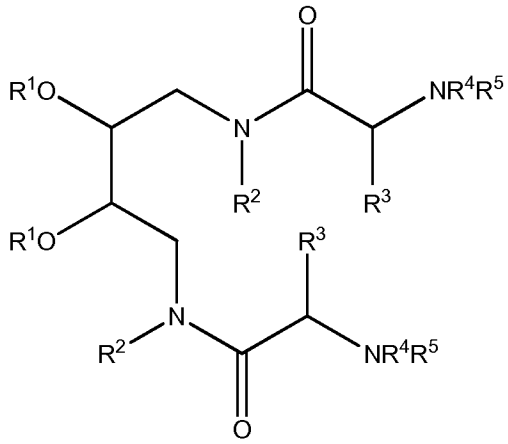






What is claimed is:

1. A compound having the structure



wherein each R^1 independently is C_1-C_{23} alkyl, C_1-C_{23} alkenyl, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl;

each R^2 independently is C_1-C_6 alkyl, or C_1-C_6 alkenyl, optionally interrupted by O;

each R^3 independently is H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkyl-C_1-C_6 alkyl, C_5-C_7-cycloalkenyl, C_5-C_7-cycloalkenyl-C_1-C_6 alkyl, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2\text{-}6}NHR^7$, $-(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, $((CH_2)_mO_x)_y(CH_2)_zO_xR^8$, or $-(CH_2)_{0\text{-}3}$Het,;

each R^4 independently is H, C_1-C_6 alkyl, or C_1-C_6 alkenyl;

each R^5 independently is H, an amine protecting group, $-(CH_2)_mNR^6(CH_2)_nNHR^7$, $-(CH_2)_{2\text{-}6}NHR^7$, $-(CH_2)_{3\text{-}6}NHC(=NH)NH_2$, –(CO)$C_1$-$C_{23}$ alkyl, –(CO)C_1-C_{23} alkenyl, or a peptide containing 1-20 amino acid residues;

each m independently is 2-5,

each n independently is 2-5,

each x independently is 0 or 1,

y is 0-2,

z is 1-6

R^6 and R^7 independently are H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl;

R^8 is H, C_1-C_6 alkyl, or C_1-C_6 alkenyl

and

Het is a 5-7 membered monocyclic basic heterocycle, or an 8-11 membered bicyclic basic heterocycle.

2. The compound of claim 1 wherein each R^1 is the same.

3. The compound of claim 1 or 2, wherein each R^2 is the same.

4. The compound of any preceding claim wherein each R^3 is the same.

5. The compound of any preceding claim wherein each R^4 is the same.

6. The compound of any preceding claim wherein each R^5 is the same.

7. The compound according to any preceding claim wherein R^2 is C_1-C_3 alkyl.

8. The compound according to any preceding claim wherein R^1 is monounsaturated C_{12}-C_{20} alkenyl.

9. The compound according to claim 8, where one or both C_{12}-C_{20} alkenyl moieties in R^1 are *cis* alkenyl.

10. The compound according to claim 4 wherein R^3 is $-(CH_2)_mNR^6(CH_2)_nNHR^7$.

11. The compound according to claim 10 wherein R^5 is $-(CH_2)_{2\text{-}6}NHR^7$.

12. The compound according to claim 11, wherein m is 3, n is 3, and R^5 is $-(CH_2)_3NHR^7$

13. The compound according to claim 12, wherein each R^6 and each R^7 is H.

14. The compound according to claim 12, wherein at least one R^6 or R^7 is $-(CO)C_1$-C_{23} alkyl, or $-(CO)C_1$-C_{23} alkenyl and the remainder are H.

15. The compound according to claim 1, wherein

each R^1 is C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl;

each R^2 is C_1-C_3 alkyl;

each R^3 is $-(CH_2)_3NH(CH_2)_3NH_2$;

each R^4 is H; and

each R^5 is $-(CH_2)_3NH_2$.

16. The compound according to claim 1, wherein

each R^1 is C_{12}-C_{20} alkyl or C_{12}-C_{20} monounsaturated alkenyl;

each R^2 is C_1-C_3 alkyl;

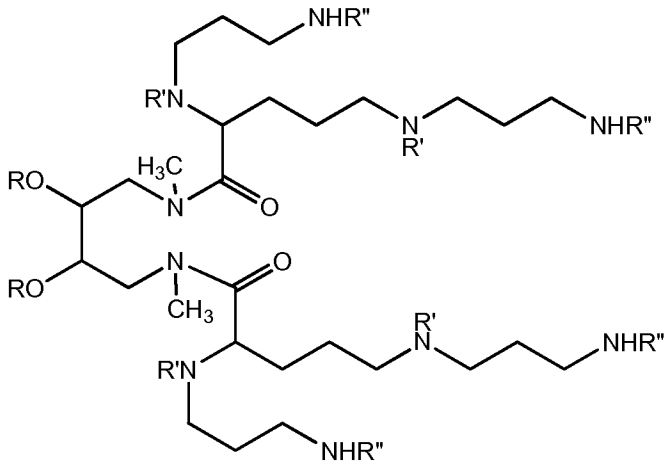
each R^3 is $-(CH_2)_3NR^6(CH_2)_3NHR^7$;

each R^4 is H;

each R^5 is $-(CH_2)_3NHR7$, and

wherein at least one R^6 or R^7 is $-(CO)C_1$-C_{23} alkyl, or $-(CO)C_1$-C_{23} alkenyl and the remainder are H.

17. The compound according to claim 1, having the structure:

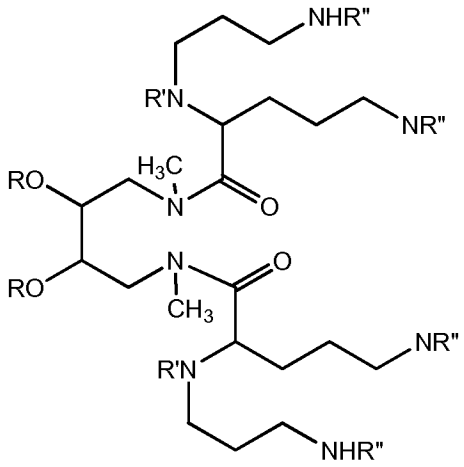


wherein R is C_{12-20} alkyl or C_{12-20} alkenyl; and

each R' and each R" independently is H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl.

18. The compound according to claim 17, wherein R is C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl, and each R' and each R" independently is H, C_{14}-C_{18} alkyl or C_{14}-C_{18} alkenyl.

19. The compound according to claim 1, having the structure:



wherein R is C_{12-20} alkenyl; and

each R' and each R" independently is H, an amine protecting group, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl.

20. The compound according to claim 19 wherein R is oleyl and each R' and each R" independently is H, or oleoyl.

21. The compound according to claim 18, wherein at least one R' or R" is oleoyl and the remainder are H.

22. The compound according to claim 20, wherein at least one R' or R" is oleoyl and the remainder are H.

23. The compound according to claim 1, wherein each R^3 independently is -$(CH_2)_{2-6}NHR^7$, -$(CH_2)_{3-6}NHC(=NH)NH_2$, or -$(CH_2)_{1-3}$Het,

each R^4 is H;

each R^5 independently is H or a peptide containing 1-20 amino acid residues.

24. The compound according to claim 23, wherein

each R^1 is C_{12}-C_{20} alkyl or C_{12}-C_{20} alkenyl;

each R^2 is C_1-C_3 alkyl;

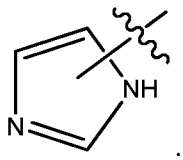
and each R^5 is H.

25. The compound according to claim 23 or 24, wherein each R^3 is -$(CH_2)_{2-6}NH_2$.

26. The compound according to claim 23 or 24, wherein each R^3 is -$(CH_2)_{3-6}NHC(=NH)NH_2$.

27. The compound according to claim 23 or 24 wherein each R^3 is -$(CH_2)_{1-3}$Het.

28. The compound according to claim 27, wherein Het is



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29. A composition comprising a compound according to claim 1 and at least one cationic lipid.

30. A composition comprising a compound according to claim 1 and at least one neutral lipid.

31.. The composition according to claim 29, wherein said cationic lipid is selected from the group consisting of DOTMA, DOTAP, DMRIE, DC-Chol, DDAB, DOSPA, DOSPER, DOGS, TMTPS, TMTOS, TMTLS, TMTMS, TMDOS, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-hydroxypropane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diaoleoyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diamyristyloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, N-1-dimethyl-N-1-(2,3-diapalmityloxypropyl)-2-(3-amino-2-hydroxypropyloxy)propane-1,3-diamine, L-spermine-5-carboxyl-3-(DL-1,2-dipalmitoyl-dimethylaminopropyl-β-hyd roxyethylamine, 3,5-(N,N-di-lysyl)-diaminobenzoyl-glycyl-3-(DL-1,2-dipalmitoyl-

dimethylami nopropyl-β-hydroxyethylamine), L-Lysine-bis(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, L-Lysine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-alkylamino)-2-hydroxypropyl)piperazine, L-Lysine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-myristoyl-β-hydroxyethyl)amide dihydrochloride, L-Ornithine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxypropyl]piperazine, L-Ornithine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, 1,4,-bis[(3-amino-2-hydroxypropyl)-oleylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-palmitylamino]-butane-2,3-diol, 1,4,-bis[(3-amino-2-hydroxypropyl)-myristylamino]-butane-2,3-diol, 1,4-bis[(3-oleylamino)propyl]piperazine, L-Arginine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, bis[(3-(3-aminopropyl)-myristylamino)2-hydroxypropyl]piperazine, L-Arginine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Serine-bis-(O,O'-oleoyl-β-hydroxyethyl)amide dihydrochloride, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxypropyl]piperazine, Glycine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, Sarcosine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, L-Histidine-bis-(O,O'-palmitoyl-β-hydroxyethyl)amide dihydrochloride, cholesteryl-3β-carboxyl-amidoethylenetrimethylammonium iodide, 1,4-bis[(3-myristylamino)propyl]piperazine, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl carboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine, cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propyl-cholesteryl-3β-oxysucc inate iodide, 2-[(2-trimethylammonio)-ethylmethylamino] ethyl-cholesteryl-3β-oxysuccinate iodide, 3β[N-(N', N'-dimethylaminoethane)carbamoyl]cholesterol, and 3β-[N-(polyethyleneimine)-carbamoyl] cholesterol,1,4-bis[(3-palmitylamino)propyl]piperazine, L-Ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide, N^2,N^5 -Bis(3-aminopropyl)-L-ornithylglycyl-N- (1-heptadecyloctadecyl)glycinamide, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-alkylamino)-2-hydroxypropyl]piperazine N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-glutamine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioctadecyl-L-glutaminyl]-L-glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-diolyl-L-glutamine, N^2-

[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-glutamine,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)-2-hydroxypropyl]piperazine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioleyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dioleyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-oleylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dipalmityl-L-glutamine,N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dipalmityl-L-α-asparagine,

N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dipalmityl-L-glutaminyl]-L-

glutamic acid, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dimyristyl-L-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dimyristyl-L-α-asparagine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)-2-hydroxypropyl]piperazine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dimyristyl-L-glutaminyl]-L-glutamic acid, 1,4-bis[(3-(3-aminopropyl)-myristylamino)propyl]piperazine, N^2-[N^2,N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dilaureyl-L-glutamine, N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-glutamine,

N^2-[N^2,N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dilaureyl-L-α-asparagine, N-[N^2-[N^2,N^5-Bis[(1,1-dimethylethoxy)carbonyl]- N^2,N^5-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl-N-N-dilaureyl-L-glutaminyl]-L-glutamic acid, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dioctadec-9-enylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dipalmitylpropionamide, 3-[N',N"-bis(2-tertbutyloxycarbonylaminoethyl)guanidino]-N,N-dimyristylpropionamide, 1,4-bis[(3-(3-aminopropyl)-palmitylamino)propyl]piperazine, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-oleylamino)propyl]piperazine, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-diolylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dipalmitylaminopropane, N,N-(2-hydroxy-3-aminopropyl)-N-2-hydroxypropyl-3-N,N-dimyristylaminopropane, 1,4-bis[(3-(3-amino-2-hydoxypropyl)-myristylamino)propyl]piperazine, [(3-aminopropyl)-bis-(2-

tetradecyloxyethyl)]methyl ammonium bromide, [(3-aminopropyl)-bis-(2-oleyloxyethyl)]methyl ammonium bromide,

[(3-aminopropyl)-bis-(2-palmityloxyethyl)]methyl ammonium bromide, Oleoyl-2-hydroxy-3-N,N-dimethyamino propane, 2-didecanoyl-1-N,N-dimethylaminopropane, palmitoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dipalmitoyl-1-N,N-dimethylaminopropane,

myristoyl-2-hydroxy-3-N,N-dimethyamino propane, 1,2-dimyristoyl-1-N,N-dimethylaminopropane, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-tetradecylcarbamoyl-butylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino-4-carbamoylbutylcarbamic acid cholestryl ester, (3-Amino-propyl)-›4-(3-amino-propylamino)-4-(2-dimethylamino-ethylcarbamoy l)-butylcarbamic acid cholestryl ester, Spermine-5-carboxyglycine (N'-stearyl-N'-oleyl) amide tetratrifluoroacetic acid salt, Spermine-5-carboxyglycine (N'-stearyl-N'-elaidyl) amide tetratrifluoroacetic acid salt, Agmatinyl carboxycholesterol acetic acid salt, Spermine-5-carboxy-β-alanine cholesteryl ester tetratrifluoroacetic acid salt, 2,6-Diaminohexanoeyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, 2,4-Diaminobutyroyl β-alanine cholesteryl ester bistrifluoroacetic acid salt, N,N-Bis (3-aminopropyl)-3-aminopropionyl β-alanine cholesteryl ester tristrifluoroacetic acid salt., [N,N-Bis(2-hydroxyethyl)-2-aminoethyl]aminocarboxy cholesteryl ester, Stearyl carnitine ester, Palmityl carnitine ester, Myristyl carnitine ester, Stearyl stearoyl carnitine ester chloride salt, L-Stearyl Stearoyl Carnitine Ester, Stearyl oleoyl carnitine ester chloride, Palmityl palmitoyl carnitine ester chloride, Myristyl myristoyl carnitine ester chloride, L-Myristyl myristoyl carnitine ester chloride, 1,4-bis[(3-(3-amino-2-hydroxypropyl)-palmitylamino)propyl]piperazine, N-(3-aminopropyl)-N,N'-bis-(dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N,N'-bis-(myristyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-dodecyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-oleyloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-palmityloxyethyl)-piperazinium bromide, N-(3-aminopropyl)-N'-methyl-N,N'-(bis-2-myristyloxyethyl)-piperazinium bromide, 1,4-bis[(3-(3-aminopropyl)-oleylamino)-2-hydroxy-propyl]piperazine, 1,4-bis[(3-(3-aminopropyl)-myristylamino)-2-hydroxy-propyl]piperazine, and 1,4-bis[(3-(3-aminopropyl)-palmitylamino)-2-hydroxy-propyl]piperazine.

32. The composition according to claim 30, wherein said neutral lipid is selected from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

33. The composition according to claim 30 further comprising at least one additional neutral lipid.

34. The composition according to claim 33 wherein said neutral lipids are selected from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

33. A composition comprising a compound according to claim 1 and a polyamine transfection agent.

34. The composition according to claim 33, wherein said polyamine transfection agent is selected from the group consisting of dense star dendrimers, PAMAM dendrimers, NH_3 core dendrimers, ethylenediamine core dendrimers, dendrimers of generation 5 or higher, dendrimers with substituted groups, dendrimers comprising one or more amino acids, grafted dendrimers, activated dendrimers, polyethylenimine, and polyethylenimine conjugates.

35. A composition comprising a compound according to claim 1 and a fusion agent.

36. A composition according to any of claims 29-34 and a fusion agent.

37. A composition comprising a compound according to claim 1 and a cell surface ligand.

38. A composition according to any of claims 29-37, further comprising a cell surface ligand.

39. A composition comprising a compound according to claim 1 and a nuclear localization peptide or protein.

40. A composition according to any of claims 29-38, further comprising a nuclear localization agent.

41. A composition according to any of claims 29-40, further comprising a nucleic acid.

42. A method of introducing a nucleic acid into a eukaryotic cell, comprising contacting the cell with a composition according to any of claims 29-40, thereby introducing the nucleic acid, protein, or peptide into the cell.

43. The method of claim 42, wherein said cell is a human cell.

44. The method of claim 42, wherein said cell is an animal cell.

45. A kit comprising a compound according to claim 1 and a neutral lipid.

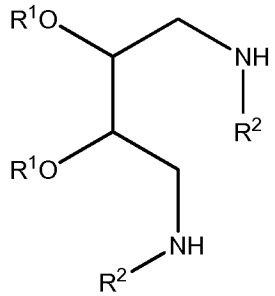
46. A kit comprising a compound according to claim 1 and a reagent selected from the group consisting of a cationic lipid, a cell surface ligand, a fusion agent and a nuclear localization peptide or protein.

47. A method of expressing a protein in a cell, comprising contacting the cell with an expression vector encoding the protein and a compound according to claim 1.

48. A method of expressing a protein in a cell, comprising contacting the cell with an expression vector encoding the protein and a composition according to claim 30. a compound according to claim 1.

49. A method of increasing the transfection efficiency of a polycationic lipid containing *N* amine groups, comprising contacting said cationic lipid with an acylating reagent in an amount sufficient to acylate no more than *N-1* of said amine groups.

50. A compound having the structure



wherein each R^1 independently is C_1-C_{23} alkyl, C_1-C_{23} alkenyl, –(CO)C_1-C_{23} alkyl, or –(CO)C_1-C_{23} alkenyl; and

each R^2 independently is C_1-C_6 alkyl or C_1-C_6 alkenyl, each optionally interrupted by O,

or each R^2 independently is $–CH_2-(CHR^3)_{2-7}-NHR^4$ or $–CH_2-(CHR^3)_{2-7}-OH$, wherein each R^3 independently is H, OH, or NH2, and

R^4 is H or CH_3.

51. The compound according to claim 50 wherein R^1 is C_{14}-C_{20} alkyl or monounsaturated C_{14}-C_{20}-alkenyl.

52. The compound according to claim 50, wherein R^2 is $–CH_2-(CHR^3)_{2-7}-NHR^4$ and R^3 is H or OH.

53. The compound according to claim 52, wherein no more than 3 R^3 groups are OH in each R^3 moiety.

54. The compound according to any of claims 50-53, wherein each R^1 is the same.

55. The compound according to any of claims 50-54 wherein each R^2 is the same.

56. The compound according to any of claims 50-55 wherein R^2 is C_1-C_3 alkyl.

57. The compound according to any of claims 50-56, wherein R^1 is monounsaturated C_{12}-C_{20} alkenyl.

58. The compound according to claim 57, wherein one or both C_{12}-C_{20} alkenyl moieties in R^1 are *cis* alkenyl.

59. The compound according to claim 50 or 52, wherein R^2 is $-CH_2$-CHOH-$(CHR^3)_{1\text{-}6}$-NHR^4.

60. The compound according to claim 50 or 52 wherein R^2 is $-CH_2$-CHOH-CH_2-NH_2.

61. A composition comprising a compound according to any of claims 50-60 and at least one cationic lipid.

62. A composition comprising a compound according to any of claims 50-60 and at least one neutral lipid.

63. A composition according to claim 62 wherein said at least one neutral lipid is selected from from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

64. A composition comprising a compound according to any of claims 50-60 and at least two neutral lipids.

65. The composition according to claim 64 wherein said at least two neutral lipids are selected from the group consisting of DOPE, DPhPE, cholesterol, DOPC, Lyso-PE (1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), Lyso-PC (1-acyl-3-hydroxy-sn-glycero-3-phosphocholine), and 3-alkyloxy-2-hydroxy-1-acetamidopropane.

66. A composition comprising a compound according to any of claims 50-60 and a polyamine transfection agent.

67. The composition according to claim 66, wherein said polyamine transfection agent is selected from the group consisting of dense star dendrimers, PAMAM dendrimers, NH_3 core dendrimers, ethylenediamine core dendrimers, dendrimers of generation 5 or higher, dendrimers with substituted groups, dendrimers comprising one or more amino acids,

grafted dendrimers, activated dendrimers, polyethylenimine, and polyethylenimine conjugates.

68. A composition comprising a compound according to any of claims 50-60 and a fusion agent.

69. A composition according to any of claims 61-67 further comprising a fusion agent.

70. A composition comprising a compound according to any of claims 50-60 and a cell surface ligand.

71. A composition according to any of claims 29-37, further comprising a cell surface ligand.

72. A composition comprising a compound according to any of claims 50-60 and a nuclear localization peptide or protein.

73. A composition according to any of claims 61-70, further comprising a nuclear localization agent.

74. A composition according to any of claims 60-73, further comprising a nucleic acid.

75. The composition according to claim 74, wherein said nucleic acid is an RNA molecule.

76. A method of introducing a nucleic acid into a eukaryotic cell, comprising contacting the cell with a composition according to claim 74, thereby introducing the nucleic acid into the cell.

77. The method of claim 76, wherein said cell is a human cell.

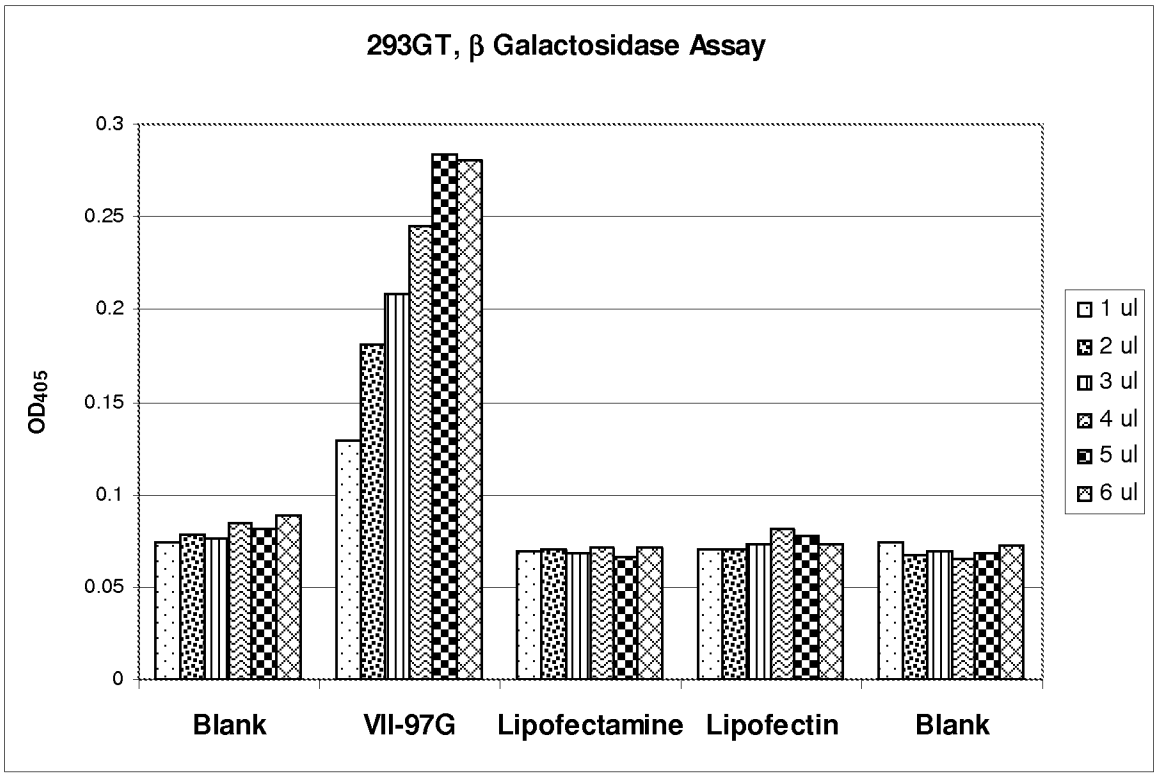
78. The method of claim 76, wherein said cell is an animal cell.

79. A kit comprising a compound according to claim 50 and a neutral lipid.

80. A kit comprising a compound according to claim 50 and a reagent selected from the group consisting of a cationic lipid, a cell surface ligand, a fusion agent and a nuclear localization peptide or protein.

81. A method of inhibiting expression of a protein in a cell, comprising contacting the cell with an RNAi molecule and a compound according to claim 50.

Figure 1(A)(upper) and 1(B)(lower)



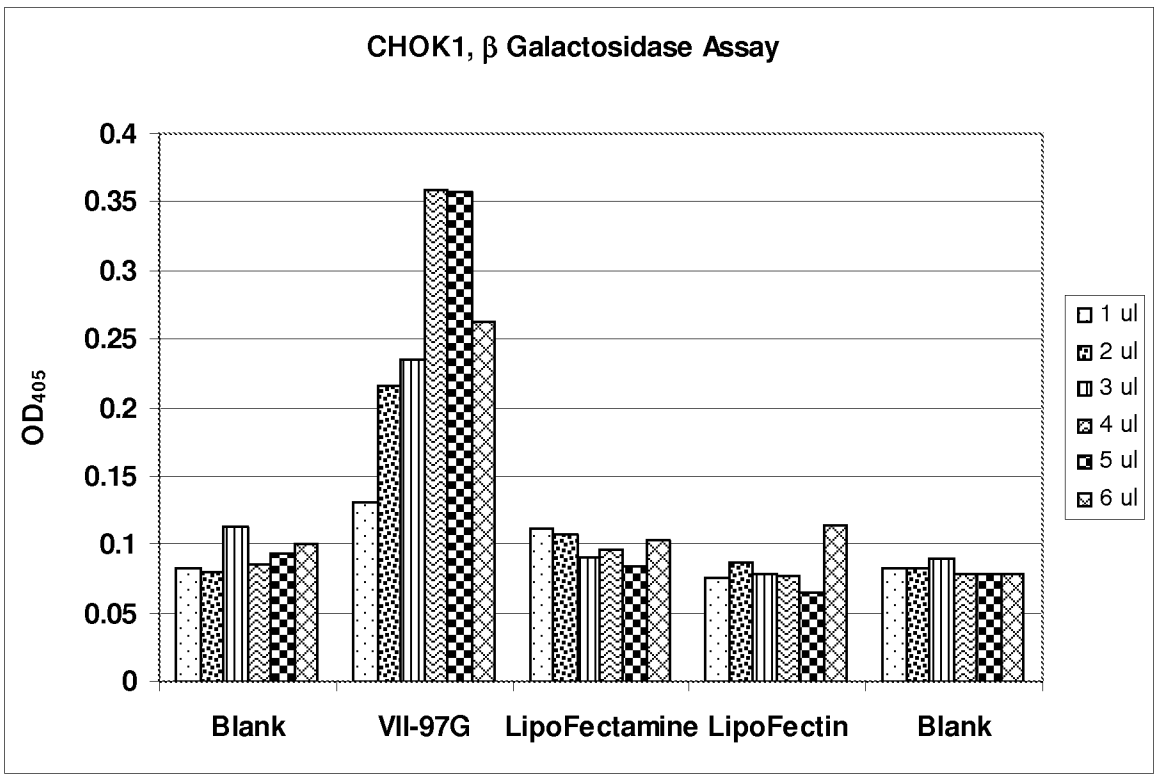
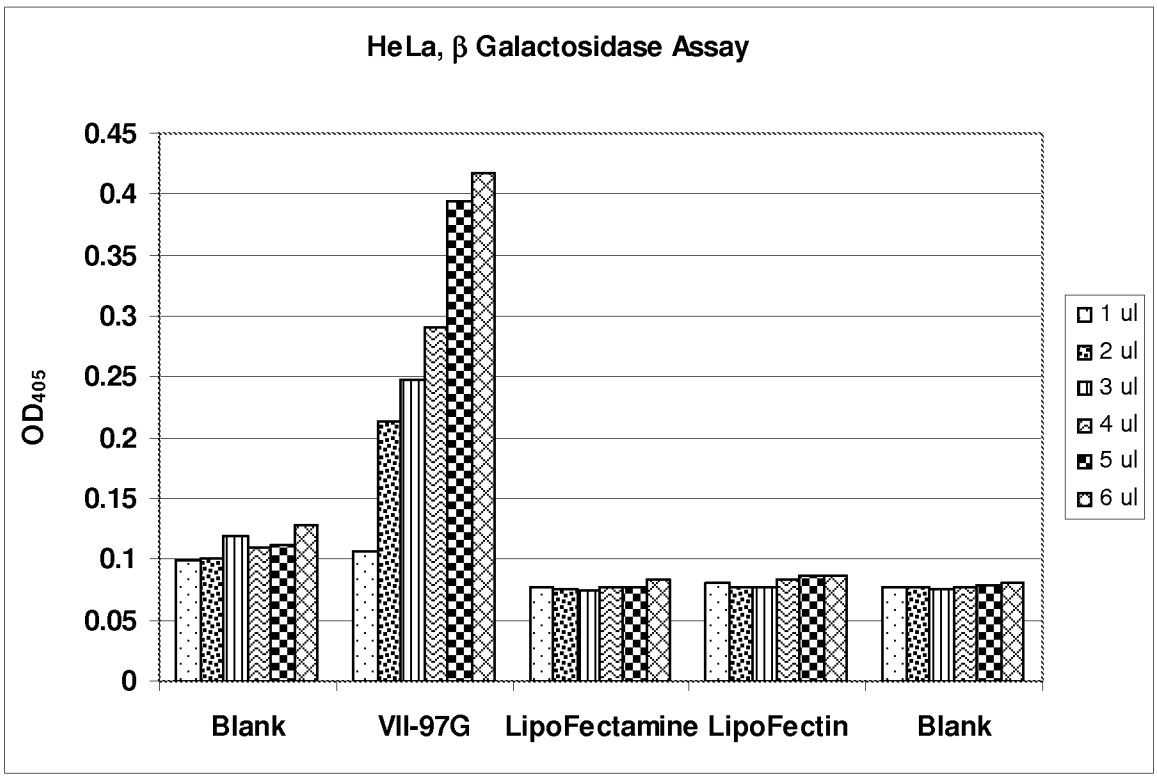


Figure 2(A)(upper) and 2(B)(lower)



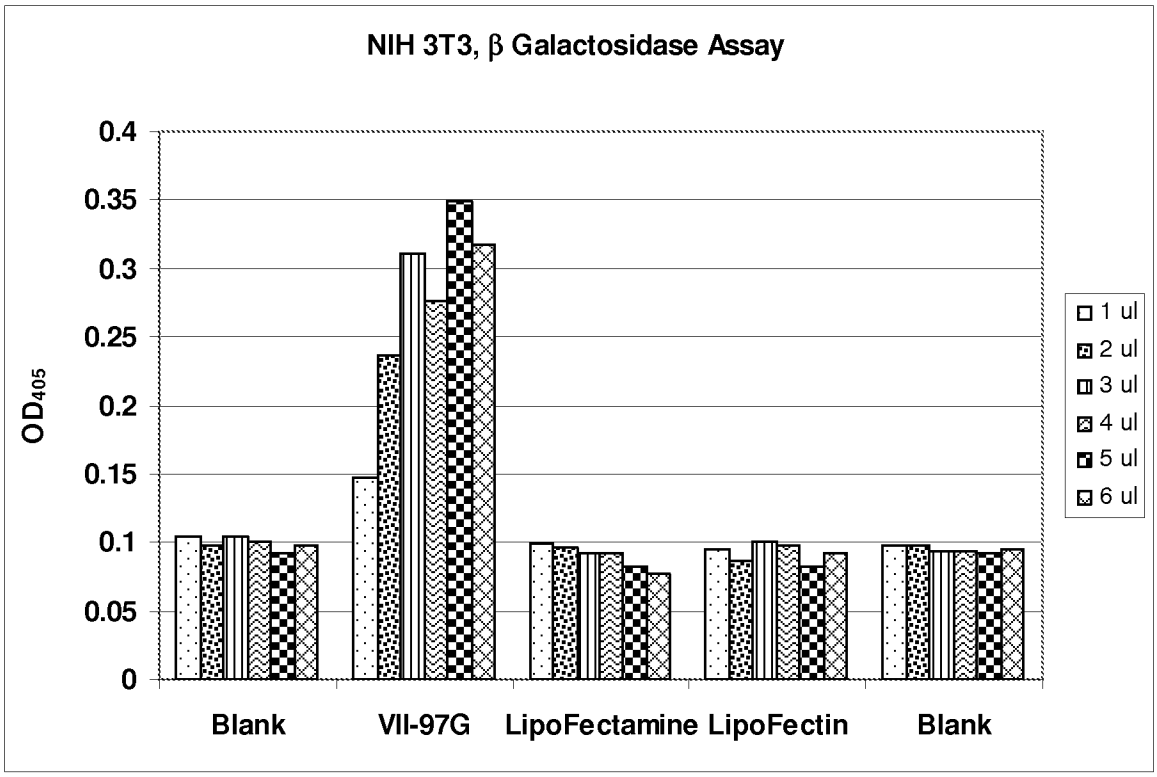
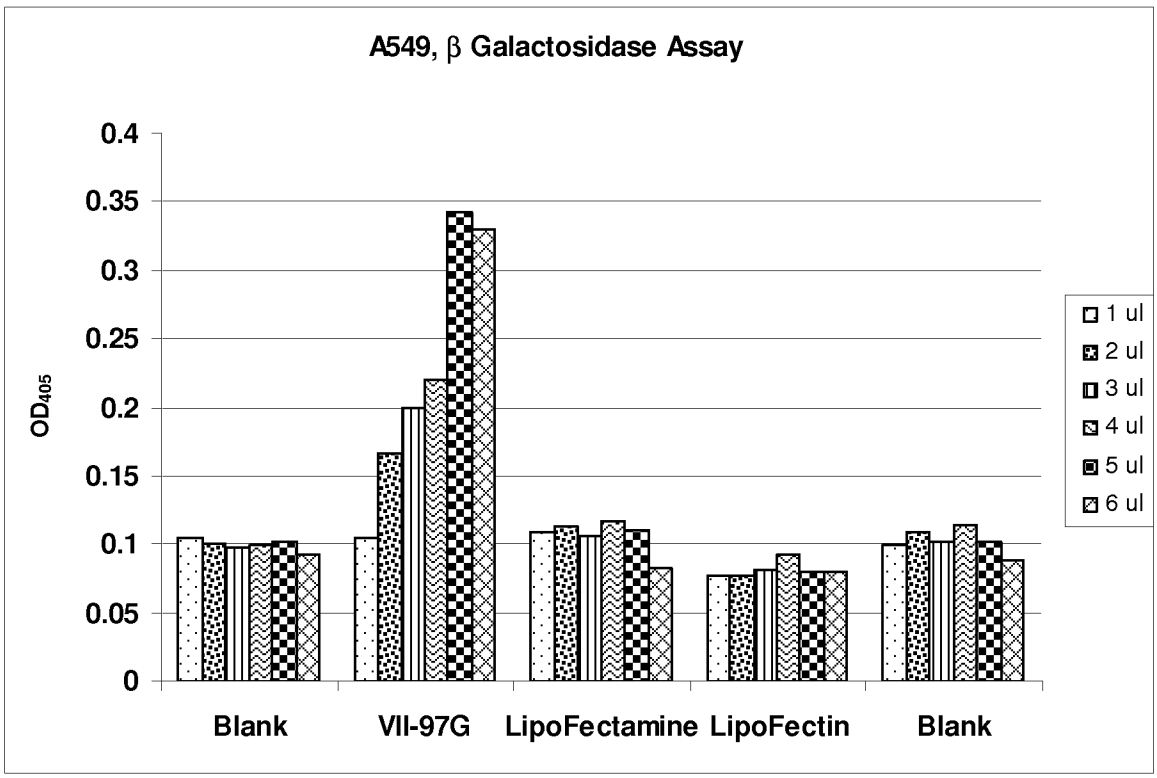


Figure 3(A)(upper) and 3(B)(lower)



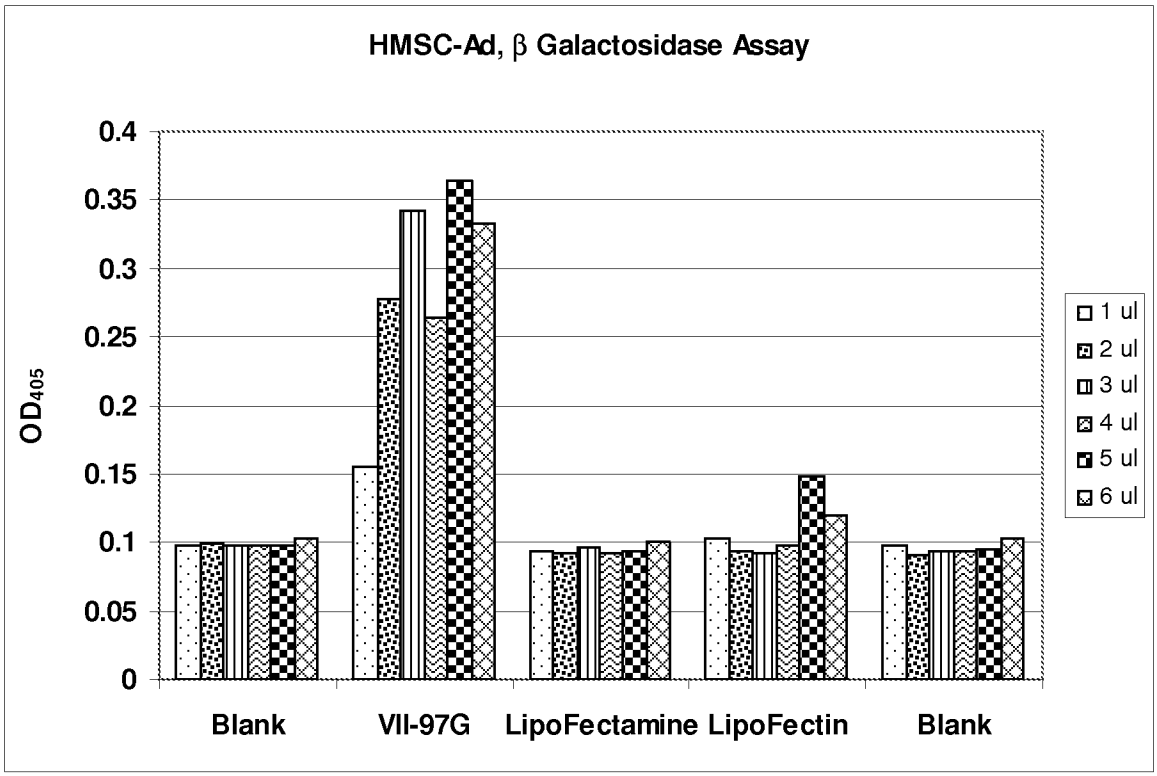
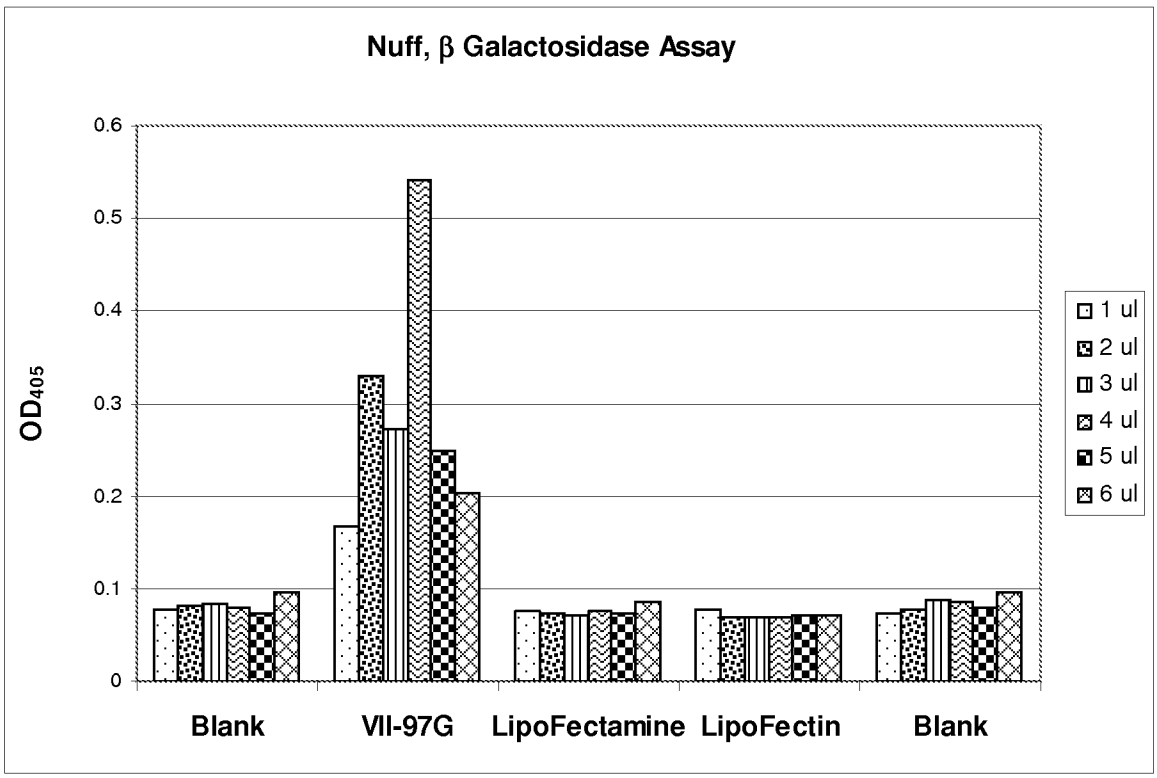
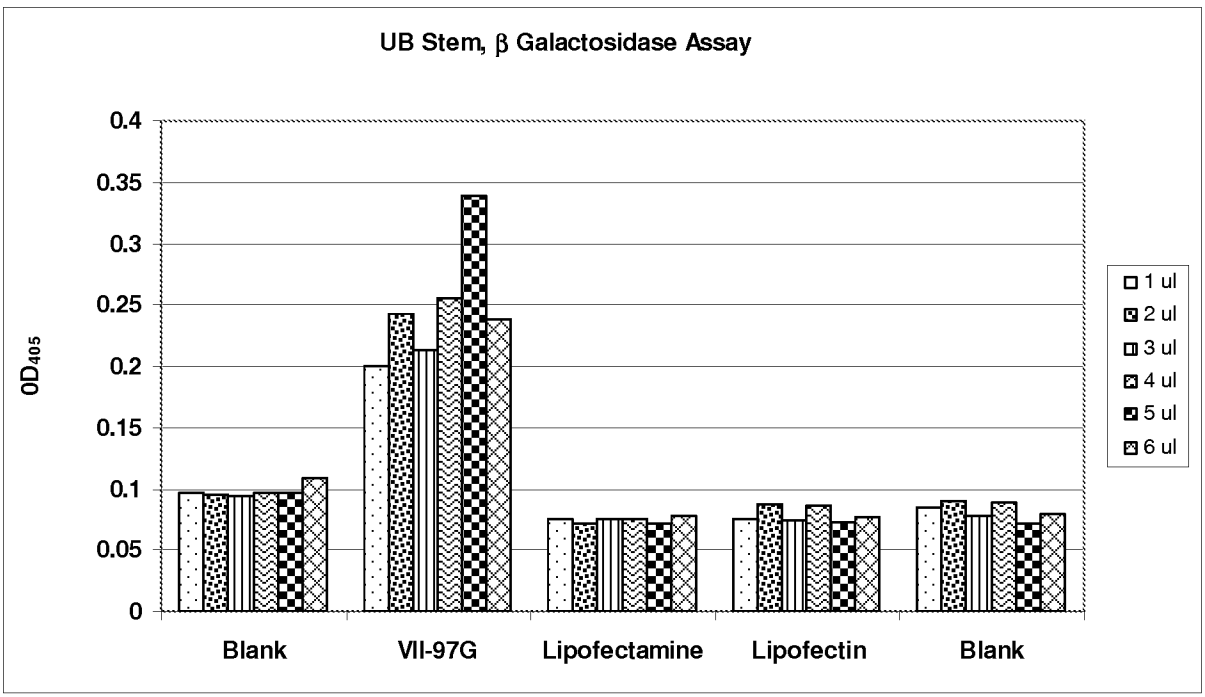


Figure 4(A)(upper) and 4(B)(lower)





INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/33847

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 37/12, 37/44; A61K 31/195 (2012.01)
USPC - 514/563
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: 514/563

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/613 (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/Scholar
Search Terms Used: Cationic lipid, polyamine, spermidine, acyl, DOTMA, DOPC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0260817 A1 (Slobodkin et al.) 14 October 2010 (14.10.2010) para [0017], [0019], [0362]	49
Y	US 5,824,812 A (Nantz et al.) 20 Octbober 1998 (20.10.1998) col 12, ln 1-19; col 19, ln 45-46; col 20, ln 1-23; col 23, ln 1-20; Table 2	1-3, 15-23, 29-32, 33A, 34A, 33B, 34B, 35-37, 39, 45-47, 50-54, 79-81
Y	US 2009/0143583 A1 (Chu et al.) 04 June 2009 (04.06.2009) para [0085], [0114]-[0117], [0240], [0293], [0299]	1-3, 15-23, 29-32, 33A, 34A, 33B, 34B, 35-37, 39, 45-47
Y	US 7,910,363 B1 (Phansitel, IV et al.) 22 March 2011 (22.03.2011) col 2, ln 41-45; col 7, ln 40-52; col 20, ln 8-35	50-54, 79-81
Y	US 2010/0298403 A1 (Tack et al.) 25 November 2010 (25.11.2010) para [0009], [0032]; Fig 2	33B, 34B
Y	US 5,736,392 A (Hawley-Nelson et al.) 07 April 1998 (07.04.1998) col 3, ln 66 to col 4, ln 13	35-37, 39, 46, 80

☐ 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
07 August 2012 (07.08.2012)	23 AUG 2012
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 12/33847

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.: 4-14, 24-28, 38, 40-44, 48 and 55-78
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:

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.:

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.