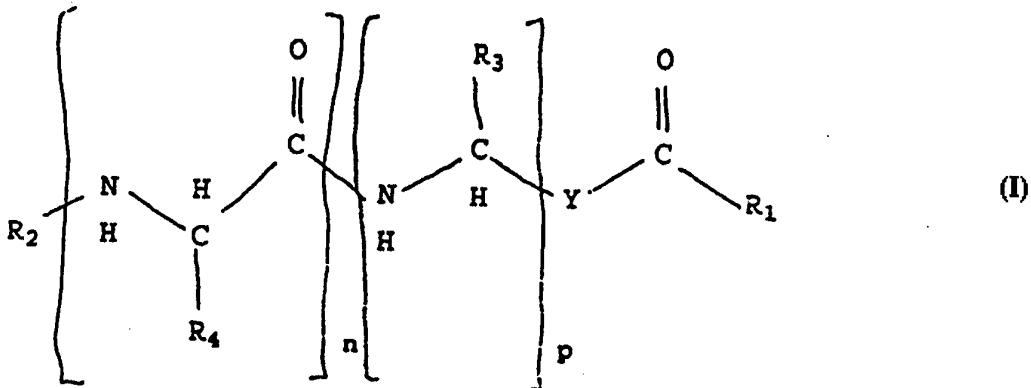




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07C 49/105, 59/00, 205/00, 207/00, 233/00		A1	(11) International Publication Number: WO 97/03939
			(43) International Publication Date: 6 February 1997 (06.02.97)
(21) International Application Number: PCT/US96/12087		(81) Designated States: AU, CA, IL, JP, KR, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 22 July 1996 (22.07.96)			
(30) Priority Data: 08/505,802 21 July 1995 (21.07.95) US		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): GENTA INCORPORATED [US/US]; 3550 General Atomics Court, San Diego, CA 92121 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): SCHWARTZ, David, Aaron [CA/US]; 1544 Valeda, Encinitas, CA 92024 (US). DAILY, William, S. [US/US]; 9940 Reagan Road #145, San Diego, CA 92126 (US). DWYER, Brian, Patrick [US/US]; 13639 Comuna Drive, Poway, CA 92064 (US). SRINIVASAN, Kumar [IN/US]; 8889 Caminito Plaza Centro #7302, San Diego, CA 92122 (US). BROWN, Bob, Dale [US/US]; 445 North Willow Spring Drive, Encinitas, CA 92024 (US).			
(74) Agents: BIGGS, Suzanne, L. et al.; Lyon & Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).			

(54) Title: NOVEL AMIDE-BASED CATIONIC LIPIDS


 $[X^-]_m$

(57) Abstract

The present invention provides novel amide-based cationic lipids of general structure (I) or a salt, or solvate, or enantiomers thereof wherein: (a) Y is a direct link or an alkylene of 1 to about 20 carbon atoms; (b) R₁ is H or a lipophilic moiety; (c) R₂, R₃, and R₄ are positively charged moieties, or at least one but not all of R₂, R₃, or R₄ is a positive moiety and the remaining are independently selected from H, an alkyl moiety of 1 to about 6 carbon atoms, or a heterocyclic moiety of about 5 to about 10 carbon atoms; (d) n and p are independently selected integers from 0 to 8, such that the sum of n and p is from 1 to 16; (e) X⁻ is an anion or polyanion and (f) m is an integer from 0 to a number equivalent to the positive charge(s) present on the lipid; provided that if Y is a direct link and the sum of n and p is 1 then one of either R₃ or R₄ must have an alkyl moiety of at least 10 carbon atoms. The present invention further provides compositions of these lipids with polyanionic macromolecules, methods for interfering with protein expression in a cell utilizing these compositions and a kit for preparing the same.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

NOVEL AMIDE-BASED CATIONIC LIPIDS

Technical Field

5 The present invention is directed to novel amide-based cationic lipid compounds useful in lipid aggregates for the delivery of macromolecules into cells.

Background of the Invention

10 Lipid aggregates, such as liposomes, have been previously reported to be useful as agents for the delivery of macromolecules such as DNA, RNA, oligonucleotides, proteins, and pharmaceutical compounds into cells. In particular, lipid aggregates which include charged as well as uncharged lipids have been especially effective for delivering polyanionic 15 molecules to cells. The reported effectiveness of cationic lipids may result from charge interactions with cells which are said to bear a net negative charge. It has also been postulated that the net positive charge on the cationic lipid aggregates may enable them to bind polyanions, such as nucleic 20 acids. Lipid aggregates containing DNA have been reported to be effective agents for efficient transfection of cells.

25 The structure of various types of lipid aggregates vary depending on factors which include composition and methods of forming the aggregate. Lipid aggregates include, for example, liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, and may have particle sizes in the nanometer to micrometer range. Various methods of making lipid aggregates have been reported in the art. One type of lipid aggregate comprises phospholipid containing liposomes. 30 An important drawback to the use of this type of aggregate as a cell delivery vehicle is that the liposome has a negative charge that reduces the efficiency of binding to a negatively

charged cell surface. It has been reported that positively charged liposomes that are able to bind DNA may be formed by combining cationic lipid compounds with phospholipids. These liposomes may then be utilized to transfer DNA into target

5 cells. (See, e.g. Felgner et al., *Proc. Nat. Acad. Sci.* 84:7413-7417, 1987; Eppstein et al. U.S. Patent No. 4,897,355; Felgner et al. U.S. Patent No. 5,264,618; and Gebeyehu et al. U.S. Patent No. 5,334,761).

Known cationic lipids include N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride ("DOTMA") and combinations of DOTMA with dioleoylphosphatidylethanolamine ("DOPE") are commercially available. Formulation of DOTMA, either by itself or in 1:1 combination with DOPE, into liposomes by conventional techniques has been reported. However,

10 compositions comprising DOTMA have been reported to show some

15 toxicity to cells.

Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether, linkages to the propylamine. However, DOTAP is reported to be more readily degraded by target cells. Other cationic lipids which represent structural modifications of DOTMA and DOTAP have also been reported.

Other reported cationic lipid compounds include those which have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamine ("DOGS") and dipalmitoyl-phosphatidylethanolamine 5-carboxyspermyl-amide ("DPPE") (See, e.g. Behr et al., U.S. Patent No. 5,171,678).

Another reported cationic lipid composition is a cationic cholesterol derivative ("DC-Chol") which has been formulated into liposomes in combination with DOPE. (See, Gao, X. and Huang, L., *Biochim. Biophys. Res. Commun.* 179:280, 35 1991). For certain cell lines, these liposomes were said to exhibit lower toxicity and provide more efficient transfection

than the DOTMA-containing compositions.

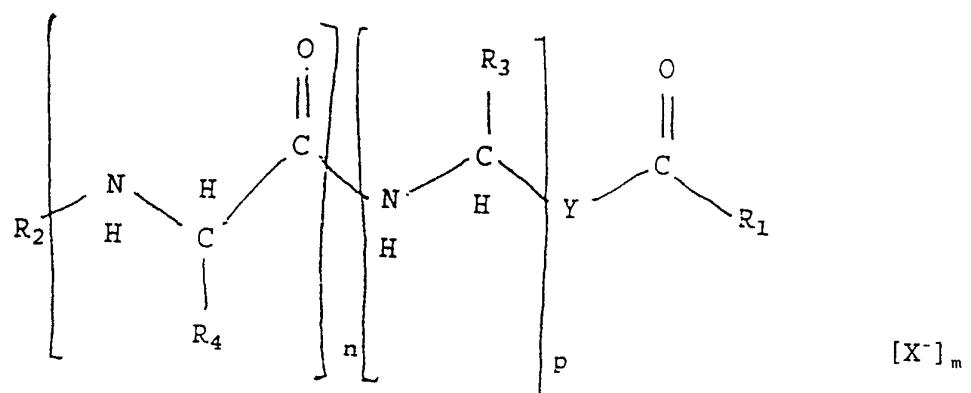
Lipopolylysine, made by conjugating polylysine to DOPE has been reported to be effective for transfection in the presence of serum. (Zhou, X. et al., *Biochim. Biophys. Acta* 1065:8, 1991).

5 1065:8, 1991).

However, of the cationic lipids which have been proposed
for use in delivering macromolecules to cells, no particular
cationic lipid has been reported to work well with a wide
variety of cell types. Since cell types differ from one
10 another in membrane composition, different cationic lipid
compositions and different types of lipid aggregates may be
effective for different cell types, either due to their
ability to contact and fuse with target cell membranes
directly or due to different interactions with intracellular
15 membranes or the intracellular environment. For these and
other reasons, design of effective cationic lipids has largely
been empirical. In addition, to content and transfer, other
factors believed important include, for example, ability to
form lipid aggregates suited to the intended purpose, toxicity
20 of the composition to the target cell, stability as a carrier
for the macromolecule to be delivered, and function in an *in vivo*
environment. Thus, there remains a need for improved
cationic lipids which are capable of delivering macromolecules
to a wide variety cell types with greater efficiency.

Summary of the Invention

In one aspect of the present invention novel amide-based cationic lipids having the structure:



or a salt, or solvate, or enantiomers thereof are provided wherein; (a) Y is a direct link or an alkylene of 1 to about 20 carbon atoms; (b) R₁ is H or a lipophilic moiety; (c) R₂, R₃, and R₄ are positively charged moieties, or at least one but not all of R₂, R₃, or R₄ is a positively charged moiety and the remaining are independently selected from H, an alkyl moiety of 1 to about 6 carbon atoms or a heterocyclic moiety; (d) n and p are independently selected integers from 0 to 8, such that the sum of n and o is from 1 to 16; (e) X⁻ is an anion or polyanion and (f) m is an integer from 0 to a number equivalent to the positive charge(s) present on the lipid; provided that if Y is a direct link and the sum of n and p is 1 then one of either R₃ or R₄ must have an alkyl moiety of at least 10 carbon atoms.

In one embodiment R₁ may be a variety of lipophilic moieties including a straight chain alkyl moiety of 1 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a steroidyl moiety, a amine derivative, a glyceryl derivative, or OCH(R₅R₆) or N(R₅R₆), wherein R₅ and R₆ are straight chain or branched alkyl moieties of about 10 to about 30 carbon.

In another embodiment when R₂, R₃, or R₄ are positively charged moieties it is preferable that the positively charged moiety be an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium moiety, an enamine moiety, a cyclic amine moiety, an amidine moiety, an isothiourea moiety, and a substituted heterocyclic amine moiety, a substituted heterocyclic moiety or a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with a substituent selected from the group consisting of NH₂, C(=O)NH₂, NHR₇, C(=O)NHR₇, NHR₇R₈, or C(=O)NHR₇R₈, wherein R₇ and R₈ are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to

about 20 carbon atoms, and an aralkyl moiety of about 6 to about 25 carbon atoms.

When any of R_2 , R_3 , or R_4 are not a positively charged moiety it is preferable that the at least one but not all of R_2 , R_3 , or R_4 are independently selected from a substituted heterocyclic moiety of 1 to about 6 carbon atoms, or a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with substituents selected from OH, thio, aryl of 1 to about 20 carbon atoms, or OR₇, wherein R₇ is an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl of 2 to about 24 carbon atoms, an aryl of about 5 to about 20 carbon atoms or an aralkyl of about 6 to about 25 carbon atoms.

It is particularly preferred that when R_2 , R_3 , or R_4 is an arylamine moiety that it be tryptophane, phenylalanine, or tyrosine.

In another preferred embodiment R_2 is an amino acid residue having a positively charged side chain wherein the amino group(s) may be optionally substituted with an alkyl of 1 to about 6 carbon atoms or substituted to form a secondary, tertiary, or quaternary amine with an alkyl moiety of 1 to about 6 carbon atoms optionally substituted with substituents selected from hydroxyl, amino, alkoxy moiety of 1 to about 6 carbon atoms, alkylamino moiety of 1 to about 6 carbon atoms, or dialkylamino moiety of 2 to about 12 carbon atoms.

Preferably when R_1 is steroidyl moiety it is a cholesteryl moiety.

It is preferable when R_2 is an amino acid that it be lysine, arginine, histidine, ornithine, or an amino acid analog. In particular, when R_2 is an amino acid analog it is preferable that it be 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine, or monoalkyl, dialkyl, or peralkyl substituted derivatives which are substituted on one or more amine nitrogens with an alkyl group of 1 to about 6 carbon atoms.

It is preferable that R_3 and R_4 independently be a lipophilic moiety of 1 to about 24 carbon atoms, a positively charged moiety, or a negatively charged moiety. In particular,

when both or either R_3 and R_4 are a lipophilic moiety it is preferable that it be a straight chain alkyl moiety of 1 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, or a steroidyl moiety.

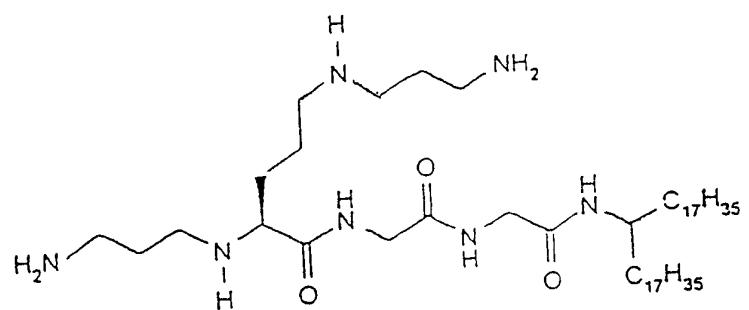
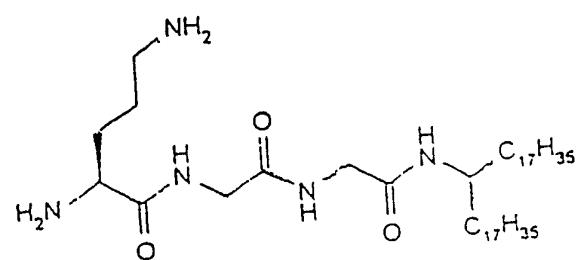
When both or either R_3 and R_4 are a positively charged moiety it is preferable that the moiety be an amino acid residue having a positively charged group on the side chain, an alkylaminoalkyl moiety, a fluoroalkylaminoalkyl moiety, a perfluoroalkylaminoalkyl moiety, a guanidiniumalkyl moiety, an enaminoalkyl moiety, a cyclic aminoalkyl moiety, an amidinoalkyl moiety, an isothiourea alkyl moiety, or a heterocyclic amine moiety.

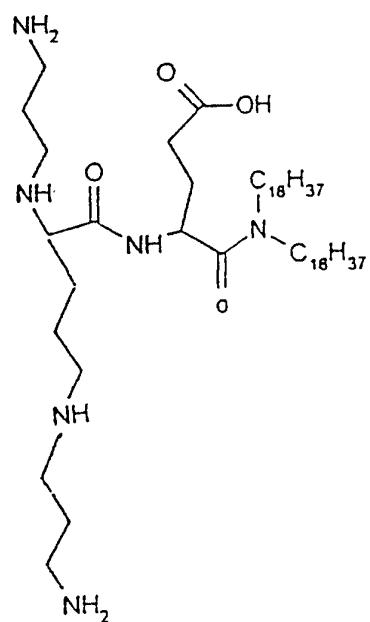
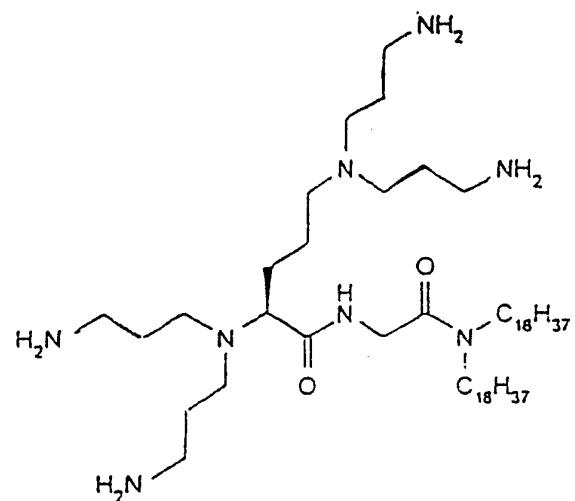
It is also preferable that when both or either R_3 and R_4 are a negatively charged moiety it be a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, or a phosphatidylalkyl moiety of 1 to about 24 carbon atoms.

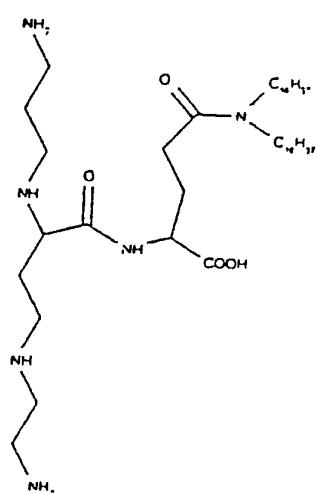
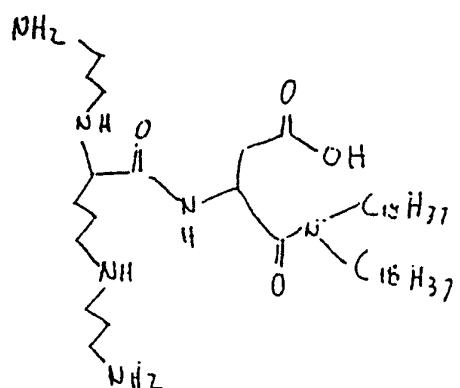
It is further preferred that the sum of the integers n and p be from 1 to 8, more preferably from 1 to 4 and most preferably from 1 to 2.

It is also preferable that X^- be a pharmaceutically acceptable anion or polyanion.

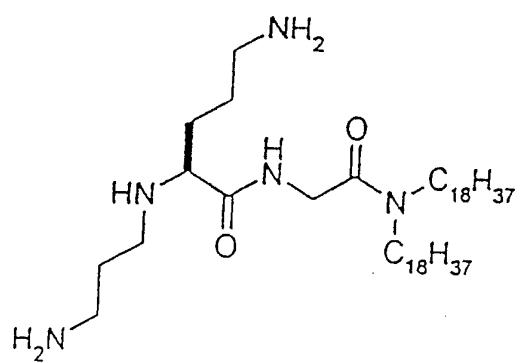
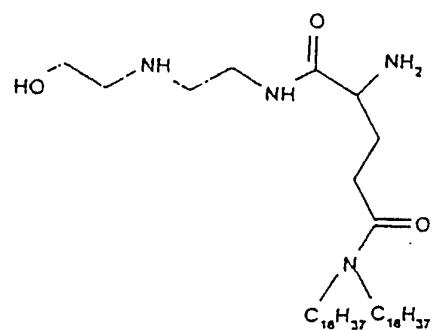
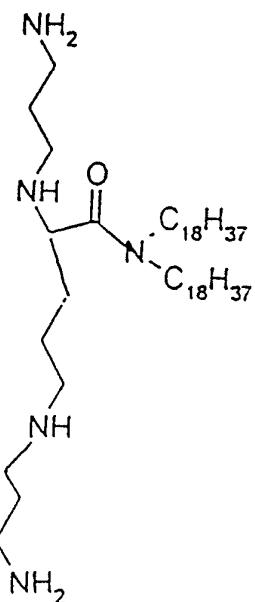
In a particularly preferred embodiment the amide-based cationic lipid has the structure

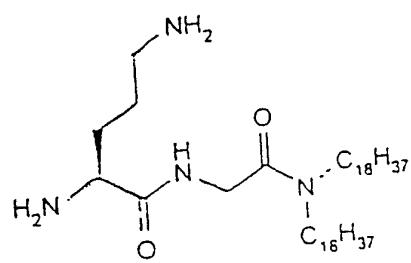
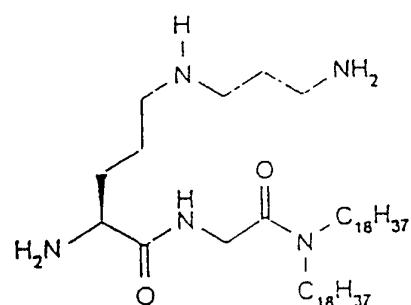
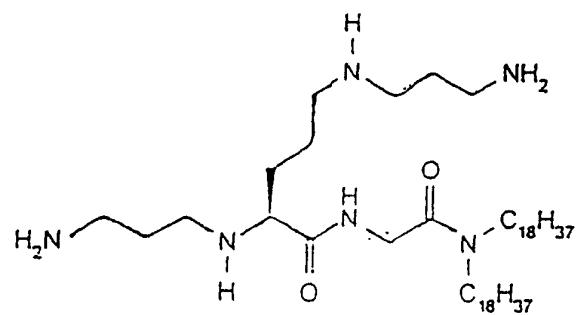


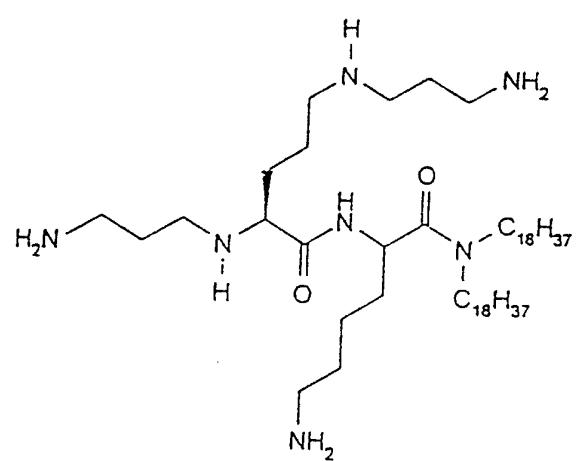
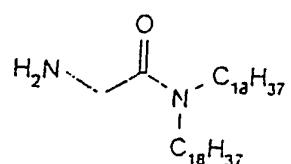
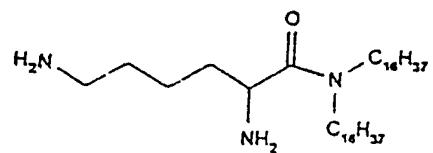




10







In another aspect of the present invention compositions comprising a polyanionic macromolecule and any of the lipids described above are provided. In particular, the polyanionic macromolecule may be a variety of molecules including an expression vector capable of expressing a polypeptide in a cell. In a preferred embodiment the polyanionic macromolecule is an oligonucleotide or an oligomer and most preferably DNA.

In still another aspect of the invention methods for the delivery of a polyanionic macromolecule into a cell by contacting any of the compositions above with the cell are provided. In particular, a method is provided to interfere with the expression of a protein in a cell by contacting any of the compositions described above with a cell wherein the composition comprises an oligomer having a base sequence that is substantially complementary to an RNA sequence in the cell that encodes the protein.

The present invention further provides a kit for delivering a polyanionic macromolecule into a cell comprising any of the compositions described above.

20

Brief Description of the Drawings

Figure 1 depicts synthetic schemes for the preparation of compounds 1-9 and 1-10. In this figure, i denotes hydroxylamine hydrochloride; ii denotes Raney nickel, H₂ at 45 psi, and 1:1 dichloromethane:methanol; iii denotes benzyl chloroformate, aqueous sodium hydroxide, water and toluene; iv denotes dicyclohexylcarbodiimide ("DCC"), 1-hydroxybenzotriazole ("HOBT" or "HOBt") and tetrahydrofuran ("THF"); v denotes palladium on carbon, H₂ gas at 50 psi and 9:1 dichloromethane:methanol; vi denotes N,N²-bis-[(1,1-dimethylethoxy)carbonyl]-L-ornithine, N-hydroxysuccinimidyl ester; vii denotes compound 1-6; and viii denotes trifluoroacetic acid and dichloromethane.

Figure 2 depicts a synthetic scheme for the preparation of the compound 2-5. In this figure, i denotes dioctadecylamine ("DODA"), DCC, and HOBT in dichloromethane;

ii denotes trifluoroacetic acid and 1,2-dichloroethane ("DCE") to give a quantitative (99%) yield of compound 2-2; iii denotes compound 1-6, N²,N⁵-bis[(1,1-dimethylethoxy)carbonyl]-N²,N⁵-bis[3-[(1,1-dimethylethoxy)carbonyl]amino-propyl]-L-ornithine, DCC and HOBT to give a 77% yield of compound 2-3; iv denotes 10% palladium on carbon and H₂ at 55 psi to give a 97% yield of compound 2-4; and v denotes 7.0 M HCl in dioxane to give a quantitative yield of compound 2-5.

Figure 3 depicts a synthetic scheme for the preparation of compound 3-5. In this figure, i denotes DODA, DCC and HOBT in dichloromethane to give a 85% yield of compound 3-1; ii denotes deprotection using 1:1 trifluoroacetic acid:dichloromethane to give a quantitative compound 3-2; iii denotes compound 1-6, triethylamine ("TEA") and DCM to give a 60% yield of compound 3-3; iv denotes catalytic hydrogenation using palladium on carbon and H₂ at 55 psi to give an 85% yield of compound 3-4; and v denotes Boc deprotection using 1:1 TFA:DCM to give a quantitative yield of compound 3-5.

Figure 4 depicts a reaction scheme for the preparation of compound 4-5. In this figure, i denotes DODA, DCC and HOBT in DCM to give a quantitative (95%) yield of compound 4-1; ii denotes TFA to give a quantitative yield of compound 4-2; iii denotes tetra-Boc-carboxy-spermidine, TEA and DCM to give a 27% yield of compound 4-3 (67% of unreacted lipid (compound 4-2) was isolated); iv denotes palladium on carbon and H₂ at 50 psi to give a 53% yield of compound 4-4; and v denotes Boc deprotection with TFA to give a 94% yield of compound 4-5.

Figure 5 depicts a reaction scheme for the preparation of compound 5-6. In this figure, i denotes DODA, DCC and HOBT in DCM to give an 85% yield of compound 2-1; ii denotes palladium on carbon with H₂ at 55 psi to give an 88% yield of compound 5-1; iii denotes L-glutamic acid bis(phenylmethyl)ester, toluene sulfonic acid salt, DCC, HOBT, TEA, and DCM to give a 85% yield of compound 5-2; iv denotes deprotection with TFA and DCE to give a quantitative yield of compound 5-3; v denotes compound 1-6 in DCM to give a

90% yield of compound 5-4; vi denotes palladium on carbon with H₂ at 55% psi to give 95% yield of compound 5-5; and vii denotes Boc deprotection with TFA and DCE to give a quantitative yield of compound 5-6.

5 Figure 6 depicts a reaction scheme for the preparation of compound 6-5. In this figure, i denotes DODA, DCC, and NHS in DCM to give a 70% yield of compound 6-2; ii denotes Pd/C in 2:1 methanol:DCM to give a 66% yield of compound 6-3; iii denotes TetraBoc carboxyspermine, DCC and HOBr in DCM to give 10 a 20% yield of compound 6-4; and iv denotes TFA to give a quantitative yield of compound 6-5. In connection with compounds 6-1 and 6-2 "Z" represents benzylloxycarbonyl.

15 Figure 7 depicts the structure of a cationic lipid, compound 7-1. This compound and its synthesis is described in the commonly assigned and co-pending United States Patent application, "Novel Methylphosphonate-Based Cationic Lipids", United States Serial No. 08/484,716, filed June 7, 1995, the disclosure of which is incorporated herein by reference.

20 Detailed Description of the Invention

25 Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used herein after. These terms will have the following meanings unless explicitly stated otherwise:

30 The term "lipophilic moiety" refers to a moiety which demonstrates one or more of the following characteristics: tend to be water insoluble, tend to be soluble in non-polar solvent, tend to favor octanol in octanol/water partition measurements, or tend to be compatible with lipid bilayers and may be bilayer forming.

35 The phrase "charged moiety" as used in the terms "positively charged moiety" and "negatively charged moiety" refers to a moiety, independent of the cationic lipid for which it is a substituent, having a net positive or negative charge respectively within the pH range of 2 to 12. The net

charge for the cationic lipid is the summation of all charged moieties occurring on the lipid, such that the net charge may be positive, neutral or negative.

The term "alkyl" refers to saturated aliphatic groups including straight-chain, branched-chain and cyclic groups. Suitable alkyl groups include, but are not limited to, cycloalkyl groups such as cyclohexyl and cyclohexylmethyl. "Lower alkyl" refers to alkyl groups of 1 to 6 carbon atoms. Fluoroalkyl or perfluoroalkyl refers to singly, partially, or fully fluorinated alkyl groups.

The term "alkenyl" refers to an unsaturated aliphatic group having at least one double bond.

The term "arylamine" refers to aromatic groups that have at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted.

The term "aralkylamine" refers to an alkyl group substituted with an aryl group. Suitable aralkyl groups include benzyl, picolyl, and the like, all of which may be optionally substituted.

The term "aralkyl" refers to an alkyl group substituted with an aryl group. Suitable aralkyl groups include benzyl, picolyl, and the like, all of which may be optionally substituted.

The term "oligonucleoside" or "oligomer" refers to a chain of nucleosides that are linked by internucleoside linkages that is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "oligomer" refers to a chain of oligonucleosides that have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs, alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides,

phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as phosphotriesters and other oligonucleoside analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The 5 term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a morpholino linkage, a sulfamate linkage, a silyl linkage, a carbamate linkage, an amide linkage, a guanidine linkage, a nitroxide linkage or a substituted hydrazine linkage. It also includes 10 nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also 15 includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone of the non-nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety 20 may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

"Lipid aggregate" is a term that includes liposomes of all types both unilamellar and multilamellar as well as 25 micelles and more amorphous aggregates of cationic lipid or lipid mixed with amphipathic lipids such as phospholipids.

"Target cell" refers to any cell to which a desired compound is delivered, using a lipid aggregate as carrier for the desired compound.

"Transfection" is used herein to mean the delivery of 30 expressible nucleic acid to a target cell, such that the target cell is rendered capable of expressing said nucleic acid. It will be understood that the term "nucleic acid" includes both DNA and RNA without regard to molecular weight, and the term "expression" means any manifestation of the 35 functional presence of the nucleic acid within the cell, including without limitation, both transient expression and

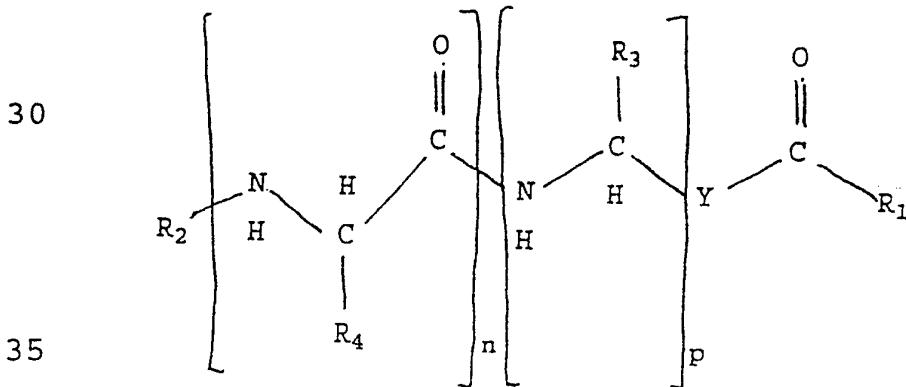
stable expression.

"Delivery" is used to denote a process by which a desired compound is transferred to a target cell such that the desired compound is ultimately located inside the target cell or in, 5 or on the target cell membrane. In many uses of the compounds of the invention, the desired compound is not readily taken up by the target cell and delivery via lipid aggregates is a means for getting the desired compound into the cell. In certain uses, especially under *in vivo* conditions, delivery to 10 a specific target cell type is preferable and can be facilitated by compounds of the invention.

All references which have been cited below are hereby incorporated by reference in their entirety.

The generic structure of functionally active cationic 15 lipids requires three contiguous moieties, e.g. cationic-head- group, a linker, and a lipid-tail group. While a wide range of structures can be envisioned for each of the three moieties, it has been demonstrated that there is no *a priori* means to predict which cationic lipid will successfully 20 transfect anionic macromolecules into a particular cell line. The property of a cationic lipid to be formulated with an anionic macromolecule which will then successfully transfect a cell line is empirical. We demonstrate the abilities of novel 25 cationic lipids which are chemically linked into multimeric constructions to enhance the uptake of macromolecules.

The novel amide-based cationic lipids of the present invention have the general structure:



comprising any salt, solvate, or enantiomers thereof. The symbols R_1 , R_2 , R_3 , R_4 , Y , X , n , and m are described as follows;

5 R_1 represents the lipid-tail group of the amide-based cationic lipid and may be hydrogen or a variety of lipophilic moieties, in particular, these include for example, a straight chain alkyl of 1 to about 24 carbon atoms, a straight chain alkenyl of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl of about 10 to about 50 carbon atoms, a unsymmetrical branched alkyl or alkenyl of about 10 to about 50 carbon atoms,
10 a amine derivative, a steroidyl moiety, a glyceryl derivative, and $OCH(R_5R_6)$ or $N(R_5R_6)$, wherein R_5 and R_6 are straight chain or branched alkyl moieties of about 10 to about 30 carbon atoms.

15 When R_1 is an amine derivative a variety of such derivatives may be utilized, for example, a straight chain alkylamine moiety of 1 to about 24 carbon atoms, a straight chain alkenylamine moiety of 2 to about 24 carbon atoms, a symmetrical branched alkylamine or alkenylamine moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkylamine or alkenylamine moiety of about 10 to about 50 carbon atoms, cyclic amine moiety of about 3 to about 10 carbon atoms, or a steroidyl moiety.

20 In the case where R_1 is a steriodal moiety a variety of such moieties may be utilized including for example pregnenolone, progesterone, cortisol, corticosterone, aldosterone, androstenedione, testosterone, or cholesterol or analogs thereof.

25 R_2 represents the cationic head group of the amide-based cationic lipid and may be a positively charged moiety independent of R_3 and R_4 . In such case, R_2 may be an amino acid residue having a unsubstituted or substituted positively charged side chain. Where the amino acid residue is substituted the substituent may be an alkyl of 1 to about 6 carbon atoms or a substituent which renders a secondary, tertiary, or
30 35 quaternary amine having an alkyl moiety of 1 to about 6 carbon atoms which is substituted with a hydroxyl, an amino, an alkoxy

of 1 to about 6 carbon atoms, an alkylamino of 1 to about 6 carbon atoms, or a dialkylamino of 2 to about 12 carbon atoms.

In particular, when R_2 is an amino acid residue it may be, for example, lysine, arginine, histidine, ornithine, or an amino acid analog. Specific examples of amino acid analogs include 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine and a monoalkyl, dialkyl, or peralkyl substituted derivative which is substituted on one or more amine nitrogens with an alkyl group of 1 to about 6 carbon atoms.

R_2 , R_3 , and R_4 may be positively charged moieties, or at least one but not all of R_2 , R_3 , or R_4 may be a positively charged moiety. In the latter case, the remaining R group(s) may independently be a hydrogen, a substituted or unsubstituted alkyl moiety of 1 to about 6 carbon atoms, or a substituted or unsubstituted heterocyclic moiety of about 5 to about 10 carbon atoms. More specifically, these groups may be substituted with a hydroxyl, a thio, an aryl of 1 to about 20 carbon atoms, or OR_7 , wherein R_7 is an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl of 2 to about 24 carbon atoms, an aryl of about 5 to about 20 carbon atoms or an aralkyl of about 6 to about 25 carbon atoms.

When R_2 , R_3 , and R_4 are positively charged moieties they may independently be, for example, an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium moiety, an enamine moiety, a cyclic amine moiety, an amidine moiety, an isothiourea moiety, a heterocyclic amine moiety, or a substituted heterocyclic moiety or a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with a substituent selected from the group consisting of NH_2 , $C(=O)NH_2$, NHR_7 , $C(=O)NHR_7$, NHR_7R_8 , and $C(=O)NHR_7R_8$, wherein R_7 and R_8 are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, and an aralkyl moiety of about 6 to about 25 carbon atoms. In

particular, when at least R_2 , R_3 , or R_4 is an arylamine moiety a variety of such moieties may be utilized including, for example, tryptophane, phenylalanine, and tyrosine.

5 R_3 and R_4 may also be independently a lipophilic moiety or a negatively charged moiety. In particular, when R_3 and/or R_4 is a lipophilic moiety it may be a straight chain alkyl moiety of about 3 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a 10 unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, or a steroidyl moiety.

15 In the case when R_3 and/or R_4 is a negatively charged moiety it may be a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, or a phosphatidylalkyl moiety of 1 to about 24 carbon atoms.

20 The linker comprises the structure joining the head group, R_1 to the lipid-tail group, R_2 . This structure includes Y may be a direct link from $-(C=O)-$ to $-(CHR_3)-$ or an alkylene of 1 to about 20 carbon atoms.

25 n and p are integers indicating the number of repeating units enclosed by the brackets and having magnitudes independent from each other ranging from 0 to 8, such that the sum of n and p is from 1 to 16, in particular cases the sum of the integers range from 1 to 4 and in specific instances from 1 to 2.

30 The counterion represented by X^- is an anion or a polyanion that binds to the positively charged groups present on the phosphonic acid-based cationic lipid via charge-charge interactions. When these cationic lipids are to be used *in vivo* the anion or polyanion should be pharmaceutically acceptable.

35 m is an integer indicating the number of anions or polyanions associated with the cationic lipid. In particular this integer ranges in magnitude from 0 to a number equivalent to the positive charge(s) present on the lipid.

In particular, when Y is a direct link and the sum of n

and p is 1, then one or either R₃ or R₄ must comprise an alkyl moiety of at least 10 carbon atoms.

The cationic lipids of the present invention include salts, solvates, or enantiomeric isomers resulting from any or 5 all asymmetric atoms present in the lipid. Included in the scope of the invention are racemic mixtures, diastereomeric mixtures, optical isomers or synthetic optical isomers which are isolated or substantially free of their enantiomeric or diasteriomic partners. The racemic mixtures may be separated 10 into their individual, substantially optically pure isomers by techniques known in the art, such as, for example, the separation of diastereomeric salts formed with optically active acid or base adjuncts followed by conversion back to the optically active substances. In most instances, the 15 desired optical isomer is synthesized by means of stereospecific reactions, beginning with the appropriate stereoisomer of the desired starting material. Methods and theories used to obtain enriched and resolved isomers have been described (Jacques et al., "Enantiomers, Racemates and 20 Resolutions." Kreiger, Malabar, FL, 1991).

The salts include pharmaceutically or physiologically acceptable non-toxic salts of these compounds. Such salts may include those derived by combination of appropriate cations such as alkali and alkaline earth metal ions or ammonium and 25 quaternary amino ions with the acid anion moiety of the phosphate or phosphorothioate acid group present in polynucleotides. Suitable salts include for example, acid addition salts such as HCl, HBr, HF, HI, H₂SO₄, and trifluoroacetate. The salts may be formed from acid addition 30 of certain organic and inorganic acids, e.g., HCl, HBr, H₂SO₄, amino acids or organic sulfonic acids, with basic centers, (e.g. amines), or with acidic groups. The composition herein also comprise compounds of the invention in their un-ionized, as well as zwitterionic forms.

35 Exemplary invention cationic lipids have the structures shown in the Summary of the Invention above.

The cationic lipids form aggregates with polyanionic macromolecules such as oligonucleotides, oligomers, peptides, or polypeptides through attraction between the positively charged lipid and the negatively charged polyanionic 5 macromolecule. The aggregates may comprise multilamellar or unilamellar liposomes or other particles. Hydrophobic interactions between the cationic lipids and the hydrophobic substituents in the polyanionic macromolecule such as aromatic and alkyl moieties may also facilitate aggregate formation.

10 Cationic lipids have been shown to efficiently deliver nucleic acids and peptides into cells and are suitable for use *in vivo* or *ex vivo*.

Cationic lipid-polyanionic macromolecule aggregates may be formed by a variety of methods known in the art.

15 Representative methods are disclosed by Felgner *et al.*, *supra*; Eppstein *et al.* *supra*; Behr *et al.* *supra*; Bangham, *et al.* *M. Mol. Biol.* 23:238, 1965; Olson, *et al.* *Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al.* *Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al.* *Biochim. Biophys. Acta* 775:169, 1984;

20 Kim, *et al.* *Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al.* *Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, e.g., Mayer, *et al.* *Biochim.* 25 *Biophys. Acta* 858:161, 1986). Microfluidization is used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, E., *supra*). In general aggregates may be formed by preparing lipid particles consisting of either (1) a cationic lipid of the invention or

30 (2) a cationic lipid mixed with a colipid, followed by adding a polyanionic macromolecule to the lipid particles at about room temperature (about 18 to 26° C). In general, conditions are chosen that are not conducive to deprotection of protected groups. The mixture is then allowed to form an aggregate over 35 a period of about 10 minutes to about 20 hours, with about 15

to 60 minutes most conveniently used. The complexes may be formed over a longer period, but additional enhancement of transfection efficiency will not usually be gained by a longer period of complexing. Colipids may be neutral or synthetic 5 lipids having no net charge or a positive or a negative charge. In particular, natural colipids that are suitable for preparing lipid aggregates with the cationic lipids of the present invention are dimyristoylphosphatidylethanolamine, dipalmitoyl-phosphatidylethanolamine, palmitoyloleolphosphatidyl-ethanolamine, cholesterol, 10 distearoylphosphatidyl-ethanolamine, phosphatidylethanolamine covalently linked to polyethylene glycol and mixtures of these colipids.

The optimal cationic lipid:colipid ratios for a given 15 cationic lipid is determined by mixing experiments to prepare lipid mixtures for aggregation with a polyanionic macromolecule using cationic lipid:colipid ratios between about 1:0.1 and 1:10. Methods to determine optimal cationic lipid:colipid ratios have been described (see, Felgner, 20 *infra*). Each lipid mixture is optionally tested using more than one oligonucleotide-lipid mixture having different nucleic acid:lipid molar ratios to optimize the oligonucleotide:lipid ratio.

Suitable molar ratios of cationic lipid:colipid are 25 about 0.1:1 to 1:0.1, 0.2:1 to 1:0.2, 0.4:1 to 1:0.4, or 0.6:1 to 1:0.6. Lipid particle preparation containing increasing molar proportions of colipid have been found to enhance oligonucleotide transfection into cells with increasing colipid concentrations.

30 In addition, the cationic lipids can be used together in admixture, or different concentrations of two or more cationic lipids in admixture, with or without colipid.

Liposomes or aggregates maybe conveniently prepared by first 35 drying the lipids in solvent (such as chloroform) under reduced pressure. The lipids may then be hydrated and converted to liposomes or aggregates by adding water or low

ionic strength buffer (usually less than about 200 mM total ion concentration) followed by agitating (such as vortexing and/or sonication) and/or freeze/thaw treatments. The size of the aggregates or liposomes formed range from about 40 nm to 5 600 nm in diameter.

The amount of an oligonucleotide delivered to a representative cell by at least some of the cationic lipids was found to be significantly greater than the amount delivered by commercially available transfection lipids. The 10 amount of oligonucleotide delivered into cells was estimated to be about 2- to 100-fold greater for the cationic lipids of the invention based on the observed fluorescence intensity of transfected cells after transfection using a fluorescently labeled oligonucleotide. The cationic lipids described herein 15 also transfect some cell types that are not detectably transfected by commercial lipids. Functionality of cationic lipid-DNA aggregates was demonstrated by assaying for the gene product of the exogenous DNA. Similarly, the functionality of cationic lipid-oligonucleotide aggregates were demonstrated by 20 antisense inhibition of a gene product.

The cationic lipids described herein also differed from commercially available lipids by efficiently delivering an oligonucleotide into cells in tissue culture over a range of cell confluency from about 50 to 100%. Most commercially 25 available lipids require cells that are at a relatively narrow confluency range for optimal transfection efficiency. For example, Lipofectin™ requires cells that are 70-80% confluent for transfecting the highest proportion of cells in a population. The cationic lipids described herein may be used 30 to transfect cells that are about 10-50% confluent, but toxicity of the lipids was more pronounced, relative to that seen using cells that are about 50-100% confluent. In general, the cationic lipids transfected cells that were about 35 60-100% confluent with minimal toxicity and optimal efficiency. Confluency ranges of 60-95% or 60-90% are thus convenient for transfection protocols with most cell lines in

tissue culture.

The cationic lipid aggregates were used to transfect cells in tissue culture and the RNA and the DNA encoded gene products were expressed in the transfected cells.

5 The cationic lipid aggregates may be formed with a variety of macromolecules such as oligonucleotides and oligomers. Oligonucleotides used in aggregate formation may be single stranded or double stranded DNA or RNA, oligonucleotide analogs, and plasmids.

10 In general, relatively large oligonucleotides such as plasmids or mRNAs will carry one or more genes that are to be expressed in a transfected cell, while comparatively small oligonucleotides will comprise (1) a base sequence that is complementary (via Watson Crick or Hoogsteen binding) to a DNA 15 or RNA sequence present in the cell or (2) a base sequence that permits oligonucleotide binding to a molecule inside a cell such as a peptide, protein, or glycoprotein. Exemplary RNAs include ribozymes and antisense RNA sequences that are complementary to a target RNA sequence in a cell.

20 An oligonucleotide may be a single stranded unmodified DNA or RNA comprising (a) the purine or pyrimidine bases guanine, adenine, cytosine, thymine and/or uracil: (b) ribose or deoxyribose; and (c) a phosphodiester group that linkage adjacent nucleoside moieties. Oligonucleotides typically 25 comprise 2 to about 100 linked nucleosides. Typical oligonucleotides range in size from 2-10, 2-15, 2-20, 2-25, 2-30, 2-50, 8-20, 8-30 or 2-100 linked nucleotides.

30 Oligonucleotides are usually linear with uniform polarity and, when regions of inverted polarity are present, such regions comprise no more than one polarity inversion per 10 nucleotides. One inversion per 20 nucleotides is typical. Oligonucleotides can also be circular, branched or double-stranded. Antisense oligonucleotides generally will comprise a sequence of about from 8-30 bases or about 8-50 bases that 35 is substantially complementary to a DNA or RNA base sequence present in the cell. The size of oligonucleotide that is

delivered into a cell is limited only by the size of polyanionic macromolecules that can reasonably be prepared and thus DNA or RNA that is 0.1 to 1 Kilobase (Kb), 1 to 20 Kb, 20 Kb to 40 Kb or 40 Kb to 1,000 Kb in length may be delivered

5 into cells.

Oligonucleotides also include DNA or RNA comprising one or more covalent modifications. Covalent modifications include (a) substitution of an oxygen atom in the phosphodiester linkage of an polynucleotide with a sulfur atom, a methyl group or the like, (b) replacement of the phosphodiester group with a nonphosphorus moiety such as -O-CH₂O-, -S-CH₂O- or -O-CH₂O-S, and (c) replacement of the phosphodiester group with a phosphate analog such as -O-P(S)(O)-O-, -O-P(S)(S)-O-, -O-P(CH₃)(O)-O or -O-P(NHR¹⁰)(O)-O- where R¹⁰ is alkyl of 1 to about 6 carbon atoms, or an alkyl ether of 1 to about 6 carbon atoms. Such substitutions may constitute from about 10% to 100% or about 20% to about 80% of the phosphodiester groups in unmodified DNA or RNA. Other modifications include substitutions of or on sugar moiety such as morpholino, arabinose 2'-fluororibose, 2'-fluoroarabinose, 2'-O-methylribose, or 2'-O-allylribose. Oligonucleotides and methods to synthesize them have been described (for example see PCT/US90/03138, PCT/US90/06128, PCT/US90/06090, PCT/US90/06110, PCT/US92/03385, PCT/US91/08811, PCT/US91/03680, PCT/US91/06855, PCT/US91/01141, PCT/US92/10115, PCT/US92/10793, PCT/US93/05110, PCT/US93/05202, PCT/US92/04294, WO 86/05518, WO 89/12060, WO 91/08213, WO 90/15065, WO 91/15500, WO 92/02258, WO 92/20702, WO 92/20822, WO 92/20823, U.S. Patent No.: 5,214,136 and

30 Uhlmann *Chem Rev.* 90:543, 1990).

The linkage between the nucleotides of the oligonucleotide may be a variety of moieties including both phosphorus-containing moieties and non phosphorus-containing moieties such as formacetal, thioformacetal, riboacetal and the like. A linkage usually comprises 2 or 3 atoms between the 5' position of a nucleotide and the 2' or 3' position of

an adjacent nucleotide. However, other synthetic linkers may contain greater than 3 atoms.

The bases contained in the oligonucleotide may be unmodified or modified or natural or unnatural purine or 5 pyrimidine bases and may be in the α or β anomer form. Such bases may be selected to enhance the affinity of oligonucleotide binding to its complementary sequence relative to bases found in native DNA or RNA. However, it is preferable that modified bases are not incorporated into an 10 oligonucleotide to an extent that it is unable to bind to complementary sequences to produce a detectably stable duplex or triplex.

Exemplary bases include adenine, cytosine, guanine, hypoxanthine, inosine, thymine, uracil, xanthine, 2- 15 aminopurine, 2,6-diaminopurine, 5-(4-methylthiazol-2-yl)uracil, 5-(5-methylthiazol-2-yl)uracil, 5-(4-methylthiazol-2-yl)cytosine, 5-(5-methylthiazol-2-yl)cytosine and the like. Other exemplary bases include alkylated or alkynylated bases having substitutions at, for example, the 5 position of 20 pyrimidines that results in a pyrimidine base other than uracil, thymine or cytosine, (i.e., 5-methylcytosine, 5-(1-propynyl)cytosine, 5-(1-butynyl)cytosine, 5-(1-butynyl)uracil, 5-(1-propynyl)uracil and the like). The use of modified bases or base analogs in oligonucleotides have been previously 25 described (see PCT/US92/10115; PCT/US91/08811; PCT/US92/09195; WO 92/09705; WO 92/02258; Nikiforov, et al., *Tet. Lett.* 33:2379, 1992; Clivio, et al., *Tet. Lett.* 33:65, 1992; Nikiforov, et al., *Tet. Lett.* 32:2505, 1991; Xu, et al., *Tet. Lett.* 32:2817, 1991; Clivio, et al., *Tet. Lett.* 33:69, 1992; 30 and Connolly, et al., *Nucl. Acids Res.* 17:4957, 1989).

Aggregates may comprise oligonucleotides or oligomers encoding a therapeutic or diagnostic polypeptide. Examples of such polypeptides include histocompatibility antigens, cell adhesion molecules, cytokines, antibodies, antibody fragments, 35 cell receptor subunits, cell receptors, intracellular enzymes and extracellular enzymes or a fragment of any of these. The

oligonucleotides also may optionally comprise expression control sequences and generally will comprise a transcriptional unit comprising a transcriptional promoter, an enhancer, a transcriptional terminator, an operator or other expression control sequences.

Oligonucleotides used to form aggregates for transfecting a cell may be present as more than one expression vector. Thus, 1, 2, 3, or more different expression vectors may be delivered into a cell as desired. Expression vectors

will typically express 1, 2, or 3 genes when transfected into a cell, although many genes may be present such as when a herpes virus vector or a artificial yeast chromosome is delivered into a cell. Expression vectors may further encode selectable markers (e.g. neomycin phosphotransferase,

thymidine kinase, xanthine-guanine phosphoribosyl-transferase, and the like) or biologically active proteins such as metabolic enzymes or functional proteins (e.g. immunoglobulin genes, cell receptor genes, cytokines (e.g. IL-2, IL-4, GM-CSF, γ -INF and the like), or genes that encode enzymes that mediate purine or pyrimidine metabolism and the like)).

The nucleic acid sequence of the oligonucleotide coding for specific genes of interest may be retrieved, without undue experimentation, from the GenBank or EMBL DNA libraries. Such sequences may include coding sequences, for example, the coding sequences for structural proteins, hormones, receptors and the like, and the DNA sequences for other DNAs of interest, for example, transcriptional and translational regulatory elements (promoters, enhancers, terminators, signal sequences and the like), vectors (integrating or autonomous) and the like. Non-limiting examples of DNA sequences which may be introduced into cells include those sequences coding for fibroblast growth factor (see WO 87/01728); ciliary neurotrophic factor (Lin et al., *Science*, 246:1023, 1989); human interferon- α receptor (Uze, et al., *Cell*, 60:225, 1990); the interleukins and their receptors (reviewed in Mizal, *FASEB J.*, 3:2379, 1989); hybrid interferons (see EPO 051,873); the

RNA genome of human rhinovirus (Callahan, *Proc. Natl. Acad. Sci.*, 82:732, 1985); antibodies including chimeric antibodies (see U.S. Pat. No.: 4,816,567); reverse transcriptase (see Moelling, et al., *J. Virol.*, 32:370, 1979); human CD4 and soluble forms thereof (Maddon et al., *Cell*, 47:333, 1986), WO 88/01304 and WO 89/01940); and EPO 330,191, which discloses a rapid immunoselection cloning method useful for the cloning of a large number of desired proteins.

Aggregates can be used in antisense inhibition of gene expression in a cell by delivering an antisense oligonucleotide into the cell (see Wagner, *Science* 260:1510, 1993 and WO 93/10820). Such oligonucleotides will generally comprise a base sequence that is complementary to a target RNA sequence that is expressed by the cell. However, the oligonucleotide may regulate intracellular gene expression by binding to an intracellular nucleic acid binding protein (see Clusel, *Nucl. Acids Res.* 21:3405, 1993) or by binding to an intracellular protein or organelle that is not known to bind to nucleic acids (see WO 92/14843). A cell that is blocked for expression of a specific gene(s) is useful for manufacturing and therapeutic applications. Exemplary manufacturing uses include inhibiting protease synthesis in a cell to increase production of a protein for a therapeutic or diagnostic application (e.g., reduce target protein degradation caused by the protease). Exemplary therapeutic applications include inhibiting synthesis of cell surface antigens to reduce rejection and/or to induce immunologic tolerance of the cell either after it is implanted into a subject or when the cell is transfected *in vivo* (e.g. histocompatibility antigens, such as MHC class II genes, and the like).

Methods to introduce aggregates into cells *in vitro* and *in vivo* have been previously described (see U.S. Patent Nos.: 5,283,185 and 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.*

3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

Entry of liposomes or aggregates into cells may be by endocytosis or by fusion of the liposome or aggregate with the cell membrane. When fusion takes place, the liposomal membrane is integrated into the cell membrane and the aqueous contents of the liposome merge with the fluid in the cell.

Endocytosis of liposomes occurs in a limited class of cells; those that are phagocytic, or able to ingest foreign particles. When phagocytic cells take up liposomes or aggregates, the cells move the spheres into subcellular organelles known as lysosomes, where the liposomal membranes are thought to be degraded. From the lysosome, the liposomal lipid components probably migrate outward to become part of cell's membranes and other liposomal components that resist lysosomal degradation (such as modified oligonucleotides or oligomers) may enter the cytoplasm.

Lipid fusion involves the transfer of individual lipid molecules from the liposome or aggregate into the plasma membrane (and vice versa); the aqueous contents of the liposome may then enter the cell. For lipid exchange to take place, the liposomal lipid must have a particular chemistry in relation to the target cell. Once a liposomal lipid joins the cell membrane it can either remain in the membrane for a period of time or be redistributed to a variety of intracellular membranes. The cationic lipids of the present invention can be used to deliver an expression vector into a cell for manufacturing or therapeutic use. The expression vectors can be used in gene therapy protocols to deliver a therapeutically useful protein to a cell or for delivering nucleic acids encoding molecules that encode therapeutically useful proteins or proteins that can generate an immune response in a host for vaccine or other immunomodulatory purposes according to known methods (see U.S. Patent Nos.: 5,399,346 and 5,336,615, WO 94/21807 and WO 94/12629). The vector-transformed cell can be used to produce commercially

useful cell lines, such as a cell line for producing therapeutic proteins or enzymes (e.g. erythropoietin, and the like), growth factors (e.g. human growth hormone, and the like) or other proteins. The aggregates may be utilized to 5 develop cell lines for gene therapy applications in humans or other species including murine, feline, bovine, equine, ovine or non-human primate species. The aggregates may be used to deliver polyanionic macromolecules into cells in tissue culture medium *in vitro* or in an animal *in vivo*.

10 The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

General Methods

5

All reactions were run under a positive pressure of dry argon. Reactions requiring anhydrous conditions were performed in flame-dried glassware which was cooled under argon.

Tetrahydrofuran (THF, Aldrich Milwaukee, WI) was distilled from potassium/benzophenone ketyl immediately prior to use.

Methylene chloride, pyridine, toluene, heptane, methanol, and ethanol were obtained as anhydrous reagent (<0.005% water) or reagent grade and were used without further purification. TLC was performed on 0.2 mm E. Merck precoated silica gel 60 F₂₅₄

15 TLC plates (20 x 20 cm aluminum sheets, Fisher, Pittsburgh, PA). Flash chromatography was performed using E. Merck 230-400 mesh silica gel. All ¹H, ¹³C and ³¹P NMR spectra were recorded on a 300 MHz Bruker ARX Spectrometer (Bruker, Boston, MA) and were obtained in CDCl₃, unless otherwise indicated. Mass spectra were provided by The Scripps Research Institute Mass Spectrometry Facility of La Jolla, CA. FAB mass spectra were obtained on a Fisons VG ZAB-VSE double focusing mass spectrometer equipped with a cesium ion gun (Fisons, Altrincham, UK). ESI mass spectra were obtained on an API III

25 PE Sciex triple-quadrupole mass spectrometer (Sciex, Toronto, CA).

Example 1

30 Synthesis of L-Ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide, Dihydrotrifluoroacetate (1-9) and N²,N⁵-Bis(3-aminopropyl)-L-ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide, Tetrahydrotrifluoroacetate (1-10)

35

Synthesis of Stearone oxime (1-1) was prepared as

follows and used without further purification. A 500 mL, round-bottomed reaction flask equipped with a magnetic stirrer, reflux condenser, and an argon inlet tube was charged with stearone (12.3 g, (24.3 mmol)), hydroxylamine hydrochloride (8.5 g, (121.6 mmol)), pyridine (12.0 mL, (148.4 mmol)), and ethanol (125 mL). After the mixture was refluxed for 2 hours it was cooled to room temperature and left standing overnight. The resulting white solid was collected, washed with water and then with ethanol, air-dried for 30 minutes, and then dried under vacuum (0.5 mm Hg) at room temperature for 15 hours to afford 11.59 g (91% yield) of compound 1-1 as a white solid: mp 68-69°C (lit. (Grun et al., *Angew. Chem.* 39, 421, 1926) mp 66-67°C); R_f 0.49 (9:1 heptane:ethyl acetate); 1H NMR δ 7.40 (br s, 1 H), 2.32 (t, J = 7.8 Hz, 2 H), 2.15 (t, J = 7.8 Hz, 2 H), 1.49 (m, 4 H), 1.25 (br s, 56 H), 0.88 (t, J = 6.5 Hz, 6 H).

Synthesis of 18-Pentatriacontanamine (1-2) was prepared as follows and used without further purification. A Parr bottle was charged with stearone oxime (5.0 g, (9.6 mmol)), 50 mL of a 1:1 mixture of methylene chloride:methanol, and 1 g of wet Raney nickel (washed with 95% ethanol to remove water). The reaction bottle was placed in a Parr shaker apparatus, filled with H_2 gas and evacuated by vacuum to remove air, and then subjected to 45 psi H_2 gas at room temperature with shaking for 15 hours. The reaction mixture was then filtered and washed with 1:1 methylene chloride:methanol. The filtrate was concentrated by rotary evaporation and the crude product was purified by flash chromatography on silica gel (100 g) using 95:5 methylene chloride:methanol to afford 1.99 g (41% yield) of 1-2 as a white solid and 1.22 g of a mixture (ratio not determined) of 1-2 and stearone. Compound 1-2 gave the following spectral data: R_f 0.58 (4:1 methylene chloride:methanol); 1H NMR δ 7.50-6.70 (br s, 2 H), 3.03 (apparent quintet, J = 6.3 Hz, 1 H), 1.62-1.05 (m, 64 H), 0.88 (t, J = 6.6 Hz, 6 H); MS (ESI) m/z 509 (MH^+).

Synthesis N-Carbobenzyloxyglycylglycine (1-3) was prepared as follows and used without further purification. A 250 mL round-bottomed reaction flask equipped with a magnetic stirrer was charged with glycylglycine (1.0 g, (7.6 mmol)), aqueous sodium hydroxide (8.3 mL of a 2 M solution, (16.6 mmol)), water (2.0 mL) and toluene (5.0 mL). The resulting biphasic mixture was cooled in an ice-water bath and then benzyl chloroformate (1.2 mL, (8.3 mmol)) was added to the reaction mixture dropwise via syringe. The reaction mixture was allowed to warm to room temperature and stirred for 3 hours. The reaction mixture was then transferred to a separatory funnel and extracted with ethyl acetate. The phases were separated and the aqueous phase was acidified to pH 2-3 by dropwise addition of a 6 N aqueous HCl solution. The acidified aqueous phase was placed in a refrigerator for 30 minutes. The resulting white solid was collected, air-dried and then vacuum-dried to afford 1.21 (60% yield) of compound 1-3 as a white solid. R_f 0.44 (1:1 methylene chloride:methanol); 1H NMR (CD₃OD) δ 7.39-7.27 (m, 5 H), 5.10 (s, 2 H), 3.97 (s, 2 H), 3.82 (s, 2 H).

Synthesis of (Carbobenzyloxy)glycyl-N-(1heptadecyl-octadecyl)glycinamide (1-4) was prepared as follows and used without further purification. A 100 mL round bottomed reaction flask equipped with a magnetic stirrer was charged with N-carbobenzyloxyglycylglycine (0.21 g, (0.78 mmol)), 18-pentatriacontanamine (1-2) (0.40 g, (0.78 mmol)), dicyclohexylcarbodiimide (0.18 g, (0.86 mmol)), 1-hydroxybenzotriazole hydrate (0.12 g, (0.86 mmol)) and THF (10 mL). The reaction mixture was stirred at room temperature under argon for 15 hours. The reaction mixture was filtered and washed with ethyl acetate. The filtrate was concentrated by rotary evaporation and the crude product was purified by flash chromatography on silica gel using 1:1 ethyl acetate-heptane to afford 0.39 g (66% yield) of compound 1-4 as a white solid. R_f 0.22 (1:1 ethyl acetate-heptane); 1H NMR δ 7.15 (t, J = 4.5 Hz, 1 H), 6.18 (d, J = 9.6 Hz, 1 H), 5.74 (t, J = 4.5 Hz, 1 H), 5.10 (s,

2 H), 3.90 (m, 5 H), 2.20-1.00 (m, 64 H), 0.88 (t, J = 6.6 Hz, 6 H); MS (FAB) m/z 757 (MH⁺).

Synthesis of Glycyl-N- (1-heptadecylocta decyl)-
5 glycinamide (1-5) was prepared as follows and used without
further purification. A Parr bottle was charged with N-
(carbobenzyloxy)glycyl-N- (1-heptadecylocta decyl)glycinamide
(1-4) (2.95 g, (3.9 mmol)), palladium on carbon (0.5 g, 5% Pd
content), and 50 mL of a 9:1 mixture of methylene
10 chloride:methanol. The reaction bottle was secured in a Parr
shaker apparatus, filled with H₂ gas, evacuated by vacuum twice
to remove oxygen, and then subjected to 50 psi H₂ gas, with
shaking, at room temperature for 10 hours. The reaction
mixture was filtered through a filter agent and washed with
15 1:1 methylene chloride:methanol to remove catalyst. The
filtrate was concentrated by rotary evaporation to afford 1.75
g (72% yield) of compound 1-5 as a white solid. R_f 0.42 (4:1
methylene chloride:methanol); ¹H NMR δ 7.88 (br s, 1 H), 5.85
(br d, J = 10.8 Hz, 1 H), 4.02-3.79 (m, 3 H), 3.40 (br s, 2
20 H), 1.69 (br s, 3 H), 1.58-0.98 (m, 63 H), 0.88 (t, J = 6.5
Hz, 6 H).

Synthesis of N²,N⁵-Bis[(1,1-dimethylethoxy)carbonyl]-
25 N²,N⁵-bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-
ornithine, N-hydroxysuccinimydyl ester (1-6) was prepared as
follows and used without further purification. A 100 mL round-
bottomed reaction flask was charged with N²,N⁵-Bis[(1,1-
dimethylethoxy)carbonyl]-N²,N⁵-bis[3-[(1,1-
dimethylethoxy)carbonyl]aminopropyl]-L-ornithine (Behr, Acc.
30 Chem. Res. 26:274, 1993; (2.08 g, (3.2 mmol)),
dicyclohexylcarbodiimide (0.73 g, (3.5 mmol)), N-
hydroxysuccinimide (0.41 g, (3.5 mmol)), and
methylene:chloride (20 mL). The reaction mixture was stirred
35 for 5 hours and then placed in a refrigerator (0-5°C)
overnight. The mixture was filtered and washed with
methylene chloride. The filtrate was concentrated by rotary

vaporation and the crude product was purified by flash chromatography on silica gel using 1:1 ethyl acetate-heptane to provide 1.2 g (50% yield) of compound 1-6 as a white solid. ^1H NMR δ 5.26 (br s, 1 H), 4.77 (br s, 1 H), 4.28 (br s, 1 H), 3.22-3.09 (m, 10 H), 2.84 (s, 4 H), 2.05-1.61 (m, 8 H), 1.48 and 1.46 and 1.44 (3 s, 36 H); MS (ESI) m/z 744 (MH^+).

Synthesis of N^2,N^5 -Bis-[(1,1-dimethyethoxy)carbonyl]-L-ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide (1-7) was prepared as follows and used without further purification. A 50-mL round-bottomed reaction flask was charged with glycylglycinamide 1-5 (0.05 g, (0.08 mmol)), N,N^2 -bis-[(1,1-dimethylethoxy)carbonyl]-L-ornithine, N -hydroxysuccinimidyl ester (0.04 g, (0.09 mmol)), and methylene chloride (3 mL). The reaction was stirred at room temperature for 1 hour. The reaction mixture was concentrated by rotary evaporation and then purified by flash chromatography on silica gel (10 g) using ethyl acetate to afford 0.06 g (80% yield) of compound 1-7 as a white waxy semi-solid. R_f 0.52 (9:1 methylene chloride:methanol); 1H NMR δ 7.66 (br s, 1 H), 7.43 (br s, 1 H), 6.43 (br s, 1 H), 5.55 (br s, 1 H), 4.87 (br s, 1 H), 4.21 (br s, 1 H), 4.07-3.77 (m, 4 H), 3.31-3.02 (m, 2 H), 2.35 (br s, 1 H), 1.93-1.00 (m, 68 H) 1.43 (s, 18 H), 0.88 (t, J = 6.6 Hz, 6 H).

25

Synthesis of N^2,N^5 -Bis[(1,1-dimethylethoxy)carbonyl]-
 N^2,N^5 -Bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-
30 ornithylglycyl-N-(1-heptadecyloctadecyl)glycinamide (1-8) was
prepared as follows and used without further purification. A
50-mL round-bottomed reaction flask was charged with
glycylglycinamide 1-5 (0.05 g, (0.08 mmol)), N^2,N^5 -bis[(1,1-
dimethylethoxy)carbonyl]- N^2,N^5 -bis[3-[(1,1-
35 dimethylethoxy)carbonyl]aminopropyl]-L-ornithine, N-
hydroxysuccinimydyl ester (1-6) (0.07 g, (0.09 mmol)), and

methylene chloride (3 mL). The reaction was stirred at room temperature for 1 hour. The reaction mixture was concentrated by rotary evaporation and then purified by flash chromatography on silica gel (10 g) using ethyl acetate to afford 0.055 g (55% yield) of compound 1-8 as a colorless thick oil: R_f 0.57 (9:1 methylene chloride:methanol); 1H NMR δ 7.87-7.57 (m, 2 H), 6.48 (m, 1 H), 5.06 (br s, 1 H), 4.45-3.38 (m, 6 H), 3.37-2.92 (m, 10 H), 2.38-0.98 (m, 72 H), 1.45 (s, 36 H), 0.88 (t, J = 6.6 Hz, 6 H); MS (ESI) m/z 1251 (MH^+).

Compound 1-9 was synthesized according to the following procedure. To a 50-mL round-bottomed reaction flask containing glycylglycinamide 1-7 (0.07 g, (0.08 mmol)) was added methylene chloride (2.0 mL) and to this suspension was added trifluoroacetic acid (0.5 mL). The reaction mixture became a homogeneous yellow solution and was left standing for 20 minutes. The reaction mixture was concentrated by rotary evaporation and then coevaporated with heptane (3 x 10 mL). The resulting residue was subjected to high vacuum (0.1 mm Hg) at room temperature for 15 hours to afford 0.07 g (97% yield) of compound 1-9 as a pale yellow waxy solid: 1H NMR (CD_3OD) δ 7.72 (d, J = 9.0 Hz, 1 H), 4.13 and 4.07 and 3.94 and 3.88 (4 s, amide rotamers, 4 H), 3.95-3.82 (m, 2 H), 2.97 (m, 2 H), 1.95-1.76 (m, 4 H), 1.48-1.27 (m, 64 H), 0.89 (t, J = 6.6 Hz, 6 H); MS (ESI) m/z 694 (MH^+).

Compound 1-10 was synthesized according to the following procedure. To a 50-mL round-bottomed reaction flask containing glycylglycinamide 1-8 (0.06 g, (0.04 mmol)) was added methylene chloride (2.0 mL) and to this suspension was added trifluoroacetic acid (0.5 mL). The reaction mixture became a homogeneous yellow solution and was left standing for 20 minutes. The reaction mixture was concentrated by rotary evaporation and then coevaporated with heptane (3 x 10 mL). The resulting residue was subjected to high vacuum (0.1 mm Hg) at room temperature for 15 hours to afford 0.06 g (99% yield) of compound 1-10 as a pale yellow waxy solid: 1H NMR (CD_3OD) δ 7.72 (d, J = 9.0 Hz, 1 H), 4.03 (s, 2 H), 3.88 (s, 2 H), 4.03-

3.79 (m, 2 H), 3.16-3.02 (m, 10 H), 2.20-1.05 (m, 72 H), 0.89 (t, J = 6.6 Hz, 6 H); MS (ESI) m/z 851 (MH⁺).

Example 2

5

Synthesis N²-[N²,N⁵-Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-glutamine, tetrahydrochloride (2-5)

Synthesis of N²-[(1,1-Dimethylethoxy)carbonyl]-N,N-dioctadecyl-L-glutamine, phenylmethyl ester (2-1) was prepared as follows

10 and used without further purification. To a solution of 5.0 g (14.8 mmol) of Boc-Glu-a-OBn in 75 mL of dry DCM was added 8.5 g (16.3 mmol, 1.1 equivalents) of dioctadecylamine (DODA), 3.4 g (4.6 mmol, 1.1 equivalents) of DCC and 2.0 g of HOBT (14.8 mmol, 1.0 equivalents). The reaction mixture was allowed to

15 stir under argon atmosphere overnight. The precipitated dicyclohexylurea was filtered, the residue was washed with 15 mL of DCM, and the combined filtrate was concentrated under reduced pressure to give a colorless oil. The crude product was purified by chromatography on silica gel (9:1

20 heptanes/ethyl acetate) to afford 12.5 g (quantitative yield) of compound 2-1 as a colorless oil. Rf 0.28 (4:1 heptanes:ethyl acetate) ¹HNMR δ: 7.35-7.30 (m, 5H), 5.44-5.42 (broad d, J = 6 Hz, 1H), 5.16 (AB q, J = 12.3 Hz, 2H), 4.31-4.29 (m, 1H), 3.31-3.21 (m, 2H), 3.13-3.08 (dd, J = 8.1, 7.6 Hz, 2H) 2.38-2.01 (m, 4H), 1.50- 1.45 (m overlaps s, 13H) 1.26 (br s, 60H), 0.88 (t, J = 6 Hz, 3H).

30 Synthesis of N,N-Dioctadecyl-L-glutamine, phenylmethyl ester, hydrotrifluoroacetate (2-2) was prepared as follows and used without further purification. To a solution of 12.5 (14.8 mmol) N²-[(1,1-dimethylethoxy)carbonyl]-N,N-dioctadecyl-L-glutamine, phenylmethyl ester 2-1 in 70 mL of 1,2-dichloroethane was added 50 mL of TFA. The reaction mixture was allowed to stir at room temperature for 30 minutes and 35 concentrated under reduced pressure. The crude oil obtained was further coevaporated with heptane (4 x 50 mL) and left to

stand under high vacuum overnight to afford 12.7 g (quantitative) of compound 2-2 as a waxy solid. ^1H NMR (CD_3OD) δ : 7.23-7.14 (m, 5H), 5.07 (AB q, J = 12.0 Hz, 2H), 3.94 (t, J = 6.6 Hz, 1H), 3.11-2.92 (m, 4H), 2.33 (t, J = 7.5 Hz, 2H), 5 1.97 (dd, J = 6.6, 12.0 Hz, 2H), 1.35-1.31 (m, 4H), 1.08 (m, 60H), 0.69 (t, J = 6.6 Hz, 3H).

Synthesis of 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-glutamine, phenylmethyl ether (2-3) was prepared as follows and used without further purification. To a solution of N,N-Dioctadecyl-L-glutamine, phenylmethyl ester, hydrotrifluoroacetate, compound 2-2, (3.75 g, 4.4 mmol) in 40 mL of dry DCM, was added triethylamine (10 mL). The reaction mixture was allowed to stir at room temperature for 5 minutes and N^2 , N^5 -Bis[(1,1-dimethylethoxy)carbonyl]- N^2 , N^5 -bis[3-[(1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithine (1-6A) (3.1 g, 4.8 mmol, 1.1 equivalents) was added in one portion followed by DCC (992 mg 4.8 mmol, 1.1 equivalents) and HOBT (300 mg, 2.2 mmol, 0.5 equivalents). The progress of the reaction was followed by TLC (7:3 heptane:ethyl acetate), and was considered was complete after 3 hours. The reaction mixture was concentrated under reduced pressure to give a crude oil that was purified by chromatography on silica gel (7:3 heptane:ethyl acetate) to afford 4.6 g (77% yield) of compound 2-3 as a colorless oil. ^1H NMR δ : 7.33-7.29 (m, 5H), 5.25 (m, 1H), 5.14 (AB q, J = 12.2 Hz), 4.47 (m, 1H), 3.23-3.06 (m, 14H), 1.44-1.31 (br, 40H), 1.29-1.24 (br, 60H), 0.87 (t, J = 6.6 Hz, 3H). Mass spec(ESI+) calcd for : 1369, 20 30 found 1370 (MH^+).

Synthesis of 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-glutamine (2-4) was prepared as follows and used without further purification. A

solution of N^2 -[N^2, N^5 -bis(1,1-dimethylethoxy)carbonyl]- N^2, N^5 -bis[3-[(1,1-dimethyl-ethoxy)carbonyl]aminopropyl]-L-ornithyl]-N,N-dioctadecyl-L-glutamine, phenylmethyl ether, compound 2-3, (4.5 g, 3.2 mmol) in 75 mL of ethyl acetate was hydrogenated
5 in presence 10% Pd/C (450 mg) under 55 psi hydrogen in a Parr hydrogenator. After 12 hours the reaction mixture was filtered to remove the catalyst and concentrated to give compound 2-4 as a colorless oil (4.1 g, 97% yield). 1 H NMR δ : 9.49 (m, 1H), 4.29 (m, 3H), 3.31-3.23 (m, 16H), 1.28 (m, 10 60H), 0.89 (t, J = 6.6 Hz, 6H).

Compound 2-5 was synthesized according to the following procedure. To the acid, compound 2-4 (3.7 g), was added warm dioxane (20 mL). This solution was cooled to room temperature
15 and then was added 7.0 M solution of HCl in dioxane (20 mL). The reaction mixture was then allowed to stir at room temperature for 5 hours. It was then concentrated at reduced pressure to give a white solid which was coevaporated with heptane (4 x 10 mL) to give compound 2-5 a white solid
20 (quantitative yield). 1 H NMR (CD₃OD) δ : 4.50 (t, J = 6.0 Hz, 1H), 4.15 (m, 1H), 3.38-3.11 (m, 14H), 2.59-2.55 (m, 2H), 1.61-1.56 (m, 4H), 1.54 (br, 60H), 0.90 (t J = 6.0 Hz, 6H). Mass spec (ESI+) calcd for 879, found 880 (MH⁺) (ESI-) found : 878.

25

Example 3

30 Synthesis of N^2 -[N^2, N^5 -Bis(aminopropyl)-L-ornithyl]-N-N-dioctadecyl-L-a-glutamine, tetrahydrotrifluoroacetate (3-5)

Synthesis of 4-[(1,1-dimethylethoxy)carbonyl]amino-5-(dioctadecyl)amino-5-oxopentanoic acid, phenylmethyl-ester (3-35 1) was prepared as follows and used without further purification. To a solution (338 mg, 1.0 mmol) of Boc-Glu-g-

benzyl ester in DCM (10 mL) was added dioctadecylamine (574 mg 1.1 mmol), DCC (227 mg, 1.1 mmol) and HOBT (14 mg, 0.1 mmol). The reaction mixture was allowed to stir at room temperature for 3 days and then filtered. The filtrate was concentrated under reduced pressure to give a colorless oil that was purified by silica gel chromatography (4:1 heptanes:ethyl acetate) to afford 717 mg (85% yield) of compound 3-1. ¹H NMR δ: 7.36-7.29 (m, 5H), 5.39-5.36 (m 1H), 5.13 (AB q, J = 12.0 Hz), 4.66, 4.61 (m, 1H), 3.52-3.45 (m, 2H), 3.09-3.07 (m, 2H), 1.41 (s, 9H), 1.41 (br, 60H), 0.88 (t, J = 6.0 Hz, 6H).

Synthesis of 5-(Dioctadecyl)amino-5-oxopentanoic acid, phenylmethyl ester, hydrotrifluoroacetate (3-2) was prepared using standard Boc deprotecting procedures (see, e.g., Example 15 2 using TFA:DCM (1:1) (quantitative yield). ¹H NMR δ: 7.56-7.36 (m, 5H), 5.18, (AB q, J = 12.2 Hz), 4.35 (br dd, J = 4.2, 3.3 Hz. 1H), 3.57 (dd overlapping dd, 2H), 3.22- 3.12 (dd over laps dd, 2H), 2.60 (dd over laps dd 2H), 2.07-2.05 9m, 2H), 1.59-1.57(m, 4H), 1.27 9(br s, 60H), 0.88 (t, J = 6.3 Hz, 6H).

Synthesis of 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-a-glutamine, phenylmethyl ester (3-3) was prepared as follows and used without further 5 purification. (4S)-5-(Dioctadecyl)amino-5-oxopentanoic acid, phenylmethyl ester, hydrotrifluoroacetate (3-2) (285 mg 0.33 mmol) was coupled with compound 1-6 (261 mg, 0.35 mmol) using protocol such as described in Example 1. The crude product was purified by silica gel chromatography gave 200 mg (60% 10 yield) of compound 3-3. (Also recovered was 150 mg of starting amine). 1H NMR δ : 7.34-7.29 (m, 5H), 5.10 (br 2H), 3.16-3.10 (m, 14 H), 1.47 (m overlaps singlet, 40 H), 1.24 (m, 60H), 0.87 (t, J = 6.3 Hz, 6H).

15 Synthesis of 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-a-glutamine (3-4) was prepared by catalytic hydrogenation of 3-3 in EtOAc under 55 psi using 10% Pd/C (85% yield) (see Example 2).

20 Compound 3-5 was synthesized according to the following procedure. Compound 3-4 was subjected to standard Boc deprotection using TFA:DCM (1:1) to give compound 3-5 in quantitative yield (see Example 1). 1H NMR (CD₃OD/CDCl₃) δ : 4.01-3.98 (m, 1H), 3.59-3.57 (m 2H), 3.16-3.01 (m, 12H), 3.01 (m, 2H), 1.21(br m, 60H), 0.89 (t, J = 6.6 Hz). Mass spec: calcd 879. found 880 (MH⁺).

Example 4

30 Synthesis of N^2 -[N^2, N^5 -Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-a-asparagine, tetrahydrotrifluoroacetate (4-5)

35 Synthesis of 3-Amino-4-(dioctadecyl)amino-4-oxobutanoic acid, phenylmethyl ester (4-1) was prepared as follows and used without further purification. Boc-Asp-b-benzyl ester was

coupled with dioctadecylamine using DCC coupling procedures such as those described in Examples 2. (quantitative yield of compound 4-1). ^1H NMR δ : 7.39-7.27 (m, 5H), 5.28 (br d, J = 12 Hz, 1H), 5.10, (AB q, J = 12.0 Hz, 2H), 4.94, (dd, J = 5.4, 6.0 Hz, 1H), 3.40- 3.15 (m, 4H), 2.82 (dd, J = 6.0, 15.6 Hz, 1H), 2.63 (dd, J = 5.9, 15.6 Hz, 1H), 1.47 (m overlaps s, 13 H), 1.25 (br m, 60H, 0.87 (t, J = 6.3 Hz, 6H).

Synthesis of 3-Amino-4-(dioctadecyl)amino-4-oxobutanoic acid, phenylmethyl ester, hydrotrifluoroacetate (4-2) was prepared as follows and used without further purification.

(3S)-3-Amino-4-(dioctadecyl)amino-4-oxobutanoic acid, phenylmethyl ester, compound 4-1, was exposed to TFA to remove Boc protecting group. A quantitative yield of compound 4-2 was obtained. ^1H NMR δ : 7.39 (m, 5H), 5.19 (AB q, J = 12.3 Hz, 2H), 4.55 (t, J = 6.3, Hz, 1H), 3.54-3.10 (m, 4H), 2.90 (d, J = 6.1 Hz, 2H), 1.60-1.50 (m, 4H), 1.38 (m, 60H), 0.89 (t, J = 6.6 Hz, 6H).

Synthesis of N^2 -[N^2, N^5 -Bis[(1,1-dimethylethoxy) carbonyl]- N^2 - N^5 -bis[3-[(1,1-dimethyl-ethoxy) carbonyl]aminopropyl]-L-ornithyl]-N-N-dioctadecyl-L-a-asparagine (4-4) was prepared as follows and used without further purification. N^2 -[N^2, N^5 -Bis[(1,1-dimethylethoxy) carbonyl]- N^2 - N^5 -bis[(1,1-dimethylethoxy) carbonyl]aminopropyl]-L-ornithyl]-N,N-dioctadecyl-L-a-asparagine, phenylmethyl ester (4-3) (186 mg, 0.13 mmol) was hydrogenated using standard procedures (see Example 2) to afford 92 mg of product (53 % yield).

10

Compound 4-5 was synthesized according to the following procedure. 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-a-asparagine (4-4) was subjected to standard 15 Boc deprotection conditions (see Example 1). to give the product as a white waxy solid (94% yield). 1 H NMR δ : 3.84 (t, J + 4.5 Hz, 1H), 3.49-3.44 (m, 1H), 3.33-3.26 (m, 2H), 3.07-2.93 (m, 12H), 2.68-2.59 (m, 2H), 2.04-1.68 (m, 12 H), 1.19 (br m, 60H), 0.80 9t J = 5.4 Hz). Mass spec calcd: 865 found: 20 866 (MH $^+$)

Example 5

Synthesis 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 (5-6)

Synthesis of N^2 -(1,1-Dimethylethoxy) carbonyl-N-N-dioctadecyl-L-glutamine (5-1) was prepared as follows and used 30 without further purification. N^2 -[1,1-Dimethylethoxy) carbonyl]-N,N-dioctadecyl-L-glutamine, phenylmethyl ester (2-1) (2.4 g, 2.85 mmol) was dissolved in 30 mL of ethyl acetate and 500 mg of 10% Pd/C was added to this solution. The resulting mixture was hydrogenated in a Parr hydrogenation apparatus at 55 psi 35 for 12 hours. The catalyst was filtered and the filtrate was

concentrated to give the product, compound 5-1, as colorless oil.

Synthesis of N-[N²-(1,1-Dimethylethoxy)carbonyl-N-N-dioctadecyl-L-glutaminyl]-L-glutamic acid, bis(phenylmethyl)ester (5-2) was prepared as follows and used without further purification. N²-(1,1-Dimethylethoxy)carbonyl-N-N-dioctadecyl-L-glutamine 5-1, (680 mg, 0.9 mmol) was coupled with L-glutamic acid, bis(phenylmethyl)ester, toluene sulfonic acid salt (450 mg, 0.9 mmol) using standard DCC, HOBT mediated coupling (see Examples 2 and 3) to afford 812 mg of product (85% yield). ¹H NMR δ: 7.88 (m, 1H), 7.38-7.32 (m, 10H), 5.83 (br, 1H), 5.17, 5.12 (2 apparent s, 4H), 4.68 (m, 1H), 4.15 (dd, J = 4.6, 6 Hz, 1H), 3.30-3.18 (m, 4H), 2.48-2.42 (m, 5H), 2.1-1.09, (m, 4H), 1.55-1.53 (m, 4H), 1.20 (br, 60H), 0.91 (t, J = 6.6 Hz).

Synthesis of N-[N,N-Dioctadecyl-L-glutaminyl]-L-glutamic acid, bis(phenylmethyl) ester, hydrotrifluoro-acetate (5-3) was prepared as follows and used without further purification. N-[N²-(1,1-Dimethylethoxy)carbonyl-N-N-dioctadecyl-L-glutaminyl]-L-glutamic acid, bis(phenylmethyl)ester (5-2) was subjected to standard TFA deprotection (see, e.g., Example 2). This product was used in Example 5 with out further characterization.

Synthesis of N-[N²-[N²,N⁵-Bis[(1,1-dimethylethoxy)carbonyl]-N²-N⁵-bis[3-[1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithyl]-N-N-dioctadecyl]-L-glutaminyl]-L-glutamic acid, bis(phenylmethyl)ester (compound 5-4) was prepared as follows and used without further purification. Compound 5-3 (200 mg, 0.19 mmol) was coupled with N²,N⁵-Bis[(1,1-dimethylethoxy)carbonyl]-N²,N⁵-bis[3-[1,1-dimethylethoxy)carbonyl]aminopropyl]-L-ornithine, N-hydroxysuccinimydyl ester (1-6) (156 mg, 0.21 mmol) in DCM (5 mL). After 6 hours the reaction mixture was concentrated under reduced pressure and the crude product thus obtained was purified by silica gel chromatography (10% MeOH in DCM) to afford 270 mg of product as a white foamy solid. (90% yield) ¹H NMR δ: 8.71 (m, 1H), 8.32 (m, 1H), 7.35-7.29 (m, 10H), 5.35 (m, 2H), 5.20-5.11 (m, 4H), 4.39-4.37 (q, J = 6.1 Hz), 3.36-3.10 (m, 14H), 1.45 (br s, 36H), 1.27 (br, 60H), 0.89 (t, J = 6.5 Hz, 6H).

Synthesis of N-[N²-[N²,N⁵-Bis(3-aminopropyl)-L-ornithyl]-N,N-dioctadecyl-L-glutaminyl]-L-glutamic acid, Tetrahydrotrifluoroacetate (5-5) was prepared as follows and used without further purification. Compound 5-4 (200 mg, 0.12 mmol) was hydrogenated with catalytic Pd/C at 55 psi (95% yield). This compound was carried to next stage with out further characterization and/or isolation.

Compound 5-6 was synthesized according to the following procedure. Compound 5-5 (100 mg) was subjected to standard Boc deprotection using TFA (see Example 1). Obtained was the product as dirty white waxy solid (quantitative yield). ¹H NMR (CDCl₃/CD₃OD) δ: 7.36 (d, J = 3.0 Hz, 1H), 4.54 (dd, J = 4.7, 9.0 Hz, 1H), 4.41 (t, J = 7 Hz, 1H), 4.00 (m, 1H), 3.37-3.30 (m, 14H), 1.57 (m, 4H), 1.29 (br m, 60H), 0.88 (t, J = 6.6Hz, 6H). Mass spec(ESI \pm): calcd : 1008, found 1009 (MH⁺) and 1007 (M-H⁺)

Example 6

Synthesis of Compound 6-5

5 Synthesis of compound 6-2 was prepared as follows and used without further purification. To a solution of 6-benzyloxycarbonyl amino caprioc acid, (6-1), (300 mg, 1.1 mmol) in 10 mL of DCM, was added dioctadecylamine (DODA) (590 mg, 1.1 mmol), DCC (233 mg, 1.1 mmol) and N-hydroxysuccinimide ("NHS") (143 mg, 1.1 mmol). The reaction mixture was warmed to enable the starting material to completely dissolve. It was then allowed to stir under argon atmosphere for 2 days; then the precipitated DC urea was filtered off. The filtrate was washed with 1N HCl, and then saturated NaHCO₃ solution, dried over MgSO₄, filtered and concentrated to afford 620 mg (71% yield) of a viscous oil. ¹H NMR δ:7.36-7.30 (m, 5H), 5.43 (m, 2H), 4.84 (br m 1H), 3.49-319, (m, 6H), 2.25 (t, J = 7.5 Hz, 1H), 1.67-1.50 (m, 6H), 1.26 (br s, 60H), 0.88 (t, J = 6.0 Hz, 6H).

20

 Synthesis of Compound 6-3 was prepared as follows and used without further purification. Compound 6-2 (620 mg, 0.62 mmol) was dissolved in 15 mL of a 2:1 mixture of methanol:DCM and 100 mg 10% Pd/C was added. This mixture was subjected to 25 hydrogenation at 35 psi for 12 hours. The reaction mixture was then filtered to remove the catalyst and concentrated to afford 420 mg of Compound 6-3 as a waxy solid. ¹H NMR δ:3.25-3.01 (overlapping m, 6H), 2.28 (t, J = 7.2 Hz, 2H), 1.81 (m, 2H), 1.64-1.59 (m, 10H), 1.23 (br s, 60H), 0.83 (t, J = 6.6 Hz, 6H).

30 Synthesis of Compound 6-4 was prepared as follows and used without further purification. To a solution of amine, Compound 6-3, (195 mg, 0.3 mmol) in 5 mL of DCM, was added tetraboc carboxyspermine (220 mg, 0.34 mmol) and HOEt (50 mg, 0.37 mmol). To this solution was then added a solution of DDC

(70 mg, 0.34 mmol) in 3 mL of DCM. The reaction mixture was allowed to stir overnight. After usual DCC coupling workup, a crude mixture was obtained that was then purified by chromatography on silica gel (13 g) with hexane:ethyl acetate (4:6) as eluent to afford 75 mg of product, Compound 6-4, as a glassy solid. ¹H NMR δ:4.38 (br, 1H), 3.24-3.11 (m, 16 H), 2.26 (t, J = 7.2 Hz, 2H), 1.57 (overlapping s and m, 50H), 1.24 (br s, 60H), 0.86 (t J = 6.6 Hz, 6H). Mass spectrometry calculated 1263, observed 1264 (mH⁺), 1266 (m+Na⁺).

10

Compound 6-5 was synthesized according to the following procedure. Compound 6-4 (4 mg) was subjected to standard Boc deprotecting conditions and workup conditions using trifluoroacetic acid (see Example 1) to give a quantitative yield of the product compound 6-5 as a pale yellow waxy solid. ¹H NMR δ:3.3-2.9 (m, 12 H), 2.4-2.2 (m, 20H), 1.25 (br s, 60H), 0.85 (t, 6H).

15

Example 7

20

Preparation and transfection protocols for COS-7, SNB-19, RD and C8161 cells with mixtures of cationic lipids and CAT plasmid

25 A. Culturing and Transfection of Cells

Cell lines were plated at 1.5 x 10⁵ cells/well in a 12 well plate format on the day before transfection. Cultures were maintained at 37°C in 5% CO₂. On the next day, when the cells reached approximately 80% confluence, the transfection mixes were prepared as follows: 126 μg of the target CAT plasmid pG1035 (described below) was added to 36.0 mL of Opti-MEM® (Gibco/BRL, Gaithersburg, MD) to make a plasmid stock solution. 63 μg of each lipid mix (from a high concentration stock in 100% ethanol) was added to individual 1.5 mL aliquots Opti-MEM® and mixed thoroughly. Then, 2 mL of the DNA stock

(containing 7 μ g of plasmid) were added to each 1.5 mL aliquot of lipid/Opti-MEM® and gently vortexed. This procedure yielded 3.5 mL of plasmid/lipid mixture at 2 μ g/mL plasmid and 18 μ g/mL lipid for a 9 to 1 lipid to DNA ratio. The quantity 5 of ethanol in the final cell cultures was 2% or less. This small quantity of ethanol was confirmed to have no adverse effect on any of the cell lines.

In order to prepare cells for transfection, the culture medium was then aspirated out of the wells and the cells were 10 rinsed twice in 1 mL Opti-MEM® per well. The transfection experiments were performed in triplicate; thus, 1 mL of each transfection mix was then added to each of three wells. The cells were cultured in the transfection mix for 5 to 6 hours. The transfection mix was then removed and replaced with 1 mL 15 of complete culture medium (DMEM or DMEM/F12 plus 10% fetal bovine serum and 1:100 dilution of penicillin:streptomycin stock, all from Gibco/BRL, (Gaithersburg, MD) and the cells were allowed to recover overnight before expression the CAT gene was measured.

20 Cell lysates were prepared by rinsing twice in PBS and then were treated with 0.5 mL of 1X Reporter Lysis Buffer (Promega, Madison, WI). The lysed cells were pipetted into 1.5 mL tubes and frozen in CO₂/EtOH once and thawed. The crude lysate was then clarified by microcentrifugation at 14,000 rpm 25 for 10 minutes to pellet cell debris. The clear supernatant was recovered and assayed directly or stored at -20°C for assay later.

The cell lysates were then assayed for CAT activity and the total protein concentration was determined as described 30 below in Example 7. The CAT activity was normalized to total protein and plotted as shown.

B. Chloramphenicol Acetyltransferase Assay

35 This assay was performed generally as follows. First, the following reaction mixture was prepared for each sample:

65 mL 0.23 M Tris, pH 8/0.5% BSA (Sigma, St. Louis, MO),
4 mL ¹⁴C-chloramphenicol, 50 nCi/mL (Dupont, Boston, MA),
and

5 5 mL mg/mL n-butyryl coenzyme A (Pharmacia, Piscataway,
NJ).

10 A CAT activity standard curve was prepared by serially
diluting CAT stock (Promega, Madison, WI) 1:1000, 1:10,000 and
1:90,000 in 0.25 M Tris, pH 8:0.5% BSA. The original stock
CAT was at 7000 Units/mL. CAT lysate was then added in a
labeled tube with Tris/BSA buffer for final volume of 50 mL.

15 Approximately 74 mL of reaction mixture was then added
to each sample tube, which was then typically incubated for
approximately 1 hour in a 37°C oven. The reaction was
terminated by adding 500 mL pristane:mixed xylenes (2:1)
(Sigma, St. Louis, MO) to each tube. The tubes were then
vortexed for 2 minutes and spun for 5 minutes. Approximately
400 mL of the upper phase was transferred to a scintillation
vial with 5 mL Scintiverse (Fisher, Pittsburgh, PA). The
sample was then counted in a scintillation counter (Packard).

20

C. Preparation of Plasmid pG1035

25 The plasmid pG1035 was used for transient transfections
of COS-7 (ATCC # CRL-1651), SNB-19, C8161 and RD (ATCC # CCL-
136) cells. pG1035 consists of a modified CAT (chloramphenicol
acetyl transferase) gene (named SplicerCAT) inserted into the
eukaryotic vector pRc/CMV (Invitrogen, San Diego, CA).
SplicerCAT was created by inserting an artificial intron into
the wild type CAT gene sequence in a plasmid named pG1036
30 (also based on pRc/CMV) using the polymerase chain reaction.
Transfection results are set forth in Tables IA to ID.

35 (i) Description of inserted CAT sequences of plasmids
pG1035 and pG1036. The sequences of the synthetic splice
sites of pG1035 (SplicerCAT) and pG1036 (wild-type CAT) are
set forth below:

(a) Partial sequence of the wild type CAT gene used to create plasmid pG1036:

+409 +410

1

5 GCC UAU UUC CCU AUU UCC CUA AAG GGU UUA UUG AGA AUA
 [SEQ. ID. NO. 1]

(b) Full sequence of the intron inserted within the CAT coding sequence of pG1036 to create the SplicerCAT gene and plasmid pG1035:

10

+409 1

1

... ACC UGG CCU AUU UCC CUA *AAG[^]gug agu gac uaa cua aqu*

39

15

cga cug cag acu agu cau ug(a) uug agu gua aca aga ccg

87 +410

1

gau auc uuc gaa ccu cuc ucu cuc ucu cag[^]GGU UUA UUG
AGA...

20

[SEQ. ID. NO. 2]

(ii) Preparation of Plasmids

The region of the CAT gene into which the intron was inserted is shown as the mRNA in sequence (a) above. The wild type CAT gene (Pharmacia) was inserted into pRc/CMV (Invitrogen, San Diego, CA) via Hind III restriction sites to create plasmid pG1036. Bases +409 and +410 are labeled on SEQ. ID. NOS. 1 and 2 for comparison of sequences only to pG1035. A synthetic intron, shown in sequence (b) above, was inserted into the CAT DNA to create plasmid pG1035. Mature mRNA sequences are shown uppercase, intronic sequences are lower case. The canonical guanosine of the splice donor is labeled +409, which corresponds to base +409 of the CAT open reading frame. The first base of the intron is labeled 1. The canonical branchpoint adenosine is base 39 and the

canonical intronic splice acceptor guanosine is base 87 of the intron. Base +410 marks the resumption of the CAT open reading frame. The sequences against which the oligomers are targeted are underlined. The consensus splice site bases are given in bold face italics (Smith *et al.*, *Ann. Rev. Genet.* 23: 5 527, 1989; and Green, *Ann. Rev. Genet.* 20: 671, 1986).

The clone pG1035 was assembled from two fragments created using phosphorylated synthetic DNA PCR primers and plasmid pG1036 as the PCR template in two separate PCR reactions. One reaction produced a Hind III-Spe I 5'-fragment containing the first 2/3 of the open reading frame and half of the synthetic intron. The second PCR reaction produced an Spe I-Not I fragment containing the second half of the intron and the last 1/3 of the open reading frame. These PCR products were combined with Hind III-Not I cut pRc/CMV in a 3-way ligation to yield the final plasmid. The artificial CAT gene containing the intron is named SplicerCAT. References applicable to the foregoing include Smith *et al.*, *supra* and Green *supra*.

20 D. Coomassie Protein Assay

The total protein content of the clarified cell lysates was determined by mixing 6 μ L of each cell lysate to 300 mL of 25 Coomassie protein assay reagent (Pierce, Rockford, MD) in the wells of an untreated microtiter assay plate. Concentration curve standards were prepared using 6 μ L of 0, 75, 100, 200, 250, 400, 500, 1000, and 1500 mg/mL BSA stock solutions and 300 mL of the Coomassie reagent. The assay samples were allowed to sit for approximately 30 minutes before reading the optical absorbance at 570 nm in a microplate reader (Molecular Probes).

35 The cells were assayed for CAT protein as described above. The results of the transfection efficiency of the cationic lipids are tabulated in Tables IA to ID.

Example 8

FITC-Oligonucleotide Uptake Assay

5 A. Oligomers Used

The oligonucleotides used for the determination of cationic lipid mediated oligonucleotide uptake in all cell lines tested were:

10

#3498-PS: 5' FITC-ggt-ata-tcc-agt-gat-ctt-ctt-ctc [SEQ. ID NO. 1],

Oligomer 3498-PS has an all-phosphorothioate backbone. This oligonucleotide has 23 negative charges on the backbone 15 and is considered to be 100% negatively charged.

#3498: 5' FITC-ggt-ata-tcc-agt-gat-ctt-ctt-ctc [SEQ. ID NO. 2],

Oligomer 3498 is a chimeric oligonucleoside. The underlined bases were linked by a phosphorothioate backbone, while the 20 other linkages in the oligomer consisted of alternating methylphosphonates and phosphodiesters. The oligomer had 11 methylphonate, 7 diester, and 5 phosphorothioates linkages. The total charge density was 57% of 3498-PS.

25

#3793-2: 5' FITC-ggu-aua-ucc-agu-gau-cuu-cut
[SEQ. ID NO. 3],

Oligomer 3293-2 has an alternating methylphosphonate and 30 diester backbone with all 2'-O-methyl groups on each ribose in the oligonucleotide. The total charge density was 50% of 3498-PS.

Stocks of oligomers 3498-PS and 3498 were prepared at 300 micromolar, while the oligomer 3793-2 stock was prepared at 440 micromolar.

35

B. Reagents and Cells

The commercially available lipids used in the assays were:

Lipofectin® ("LFN") Lot#EF3101 1 mg/mL, Gibco/BRL (Gaithersburg, MD)

5 LipofectAMINE® ("LFA") Lot#EFN101 2 mg/mL, Gibco/BRL (Gaithersburg, MD)

Transfectam® ("TFM") Lot#437121 1 mg dry, Promega, (Madison, WI) and resuspended in 100% ethanol.

10 The novel lipids of the present invention used in these evaluations, as listed in the data tables (**Tables II A to C**), were at 1 mg/mL in 100% ethanol.

15 The tissue culture cell stocks, SNB-19 (human glioblastoma), C8161 (a human amelanotic melanoma), RD (human rhabdomyosarcoma, ATCC # CCL-136) and COS-7 (African green monkey kidney cells, ATCC # CRL-1651) were maintained in standard cell culture media: DMEM:F12 (1:1) mix from Mediatech, Lot#150901126, 10% fetal bovine serum from Gemini Bioproducts, Lot#A1089K, 100 units/mL penicillin and 100 20 micrograms/mL streptomycin, from Mediatech, Lot#30001044 and 365 micrograms/mL L-glutamine. The cells were maintained under standard conditions (37°C, 5% CO₂ atmosphere) at all times prior to fixation and microscopic examination.

25 C. Preparation of Cells and Transfection Mixes

30 For each FITC labeled oligomer delivery determination, the appropriate cells were plated into 16 well slides (Nunc #178599, glass microscope slide with 16 removable plastic wells attached to the slide surface with a silicone gasket) according to standard tissue culture methods. Each cell line was plated at a starting density (approximately 20,000 35 cells/well) that allowed them to be healthy and 60-80% confluent one to two days after plating. The cells were allowed to adhere to the glass and recover from the plating procedure in normal growth medium for 24 to 48 hours before beginning the transfection procedure.

Oligonucleotide transfection mixes were made up in Opti-MEM® without antibiotics as follows: 500 μ L aliquots of Opti-MEM® containing a 0.25 micromolar solution of either oligomer 3498-PS, 3498, or 3793-2 (2 micrograms of oligomer per sample) 5 were pipetted into 1.5 mL Eppendorf tubes. Cationic lipid or lipid mixture was then added to the oligomer solution to give a final 9:1 or 6:1 ratio (18 or 12 μ g of lipid total) of cationic lipid to oligomer by weight, as listed in **Tables IIIA to IIC**. The tubes were mixed by vortexing immediately after 10 the addition of lipid.

Prior to beginning the transfection reactions the cells were rinsed in 200 μ L Opti-MEM®; then, the cells were rinsed with Dulbecco's phosphate buffered saline (PBS) solution, and 15 200 μ L of oligomer transfection mix was added directly to a well to begin each transfection reaction. Transfection reactions were allowed to continue for four to six hours.

At that time, the cells were then rinsed in PBS from Mediatech and fixed for ten minutes in 200 μ L of 3.7% formaldehyde (Sigma, St. Louis, MO) to terminate the 20 transfection reaction. Then the wells were rinsed again in PBS. The formaldehyde was quenched with 200 μ L of 50 mM glycine (Sigma, St. Louis, MO) for ten minutes. Finally, the wells were then emptied by shaking out the glycine solution. At that time, the plastic chambers and silicone gasket were 25 removed and the cells were covered with Fluoromount-G mounting medium (from Fisher, Pittsburgh, PA, with photobleaching inhibitors) and a cover slip.

Intracellular fluorescence was evaluated under 200X magnification with a Nikon Labophot-2 microscope with an 30 episcopic-fluorescence attachment. Using this equipment we could distinguish extracellular from nuclear and endosomal fluorescence.

The cells were scored for uptake of FITC labelled oligomer as follows: No nuclear fluorescence, 0; up to 20% 35 fluorescent nuclei, 1; up to 40% fluorescent nuclei, 2; up to 60% fluorescent nuclei, 3; up to 80% fluorescent nuclei, 4;

and up to 100% fluorescent nuclei, 5.

The results of the transfections in COS-7, SB-19, C-8161 and RD cells are tabulated in **Tables IIA to IIC**.

TABLE I

Transient transfection efficiency of cationic lipids in COS-7,
SNB-19, RD and C8161 cells

5

<u>A. Cell line COS-7</u>			
<u>Lipid</u>	<u>Mean</u>	<u>SDV</u>	<u>REL</u>
None	894	23	0
Transfectam	89437	14746	0.52
Lipofectamine	119902	7350	0.7
Lipofectin	57084	4288	0.33
2-5	55598	4643	0.33
2-5/DOPE	159419	18564	0.93
2-5/7-1	162560	9944	0.95
1-9/DOPE	170801	5457	1
1-10	148040	3932	0.87
1-10/DOPE	138397	11512	0.81

<u>B. Cell line SNB-19</u>			
<u>Lipid</u>	<u>Mean</u>	<u>SDV</u>	<u>REL</u>
None	807	24	0
Transfectam	106060	17596	0.56
Lipofectamine	143064	12699	0.76
Lipofectin	177312	3487	0.94
2-5	103280	12908	0.55

10

15

20

25

2-5/DOPE	134725	13224	0.71
2-5/7-1	172245	99236	0.91
1-9/DOPE	187651	5480	1
1-10	166179	18702	0.88
1-10/DOPE	188468	2650	1

<u>C. Cell line RD</u>				
	<u>Lipid</u>	<u>Mean</u>	<u>SDV</u>	
	<u>REL</u>			
5	None	743	32	0
	Transfectam	51255	1490	0.29
	Lipofectamine	85689	9618	0.48
	Lipofectin	128481	8972	0.72
	2-5	73921	3839	0.41
10	2-5/DOPE	104283	6701	0.58
	2-5/7-1	178331	4630	1
	1-9/DOPE	123060	5312	0.69
	1-10	124232	5248	0.7
	1-10/DOPE	42824	2629	0.24

<u>D. Cell line C8161</u>				
	<u>Lipid</u>	<u>Mean</u>	<u>SDV</u>	
	<u>REL</u>			
15	None	851	32	0
	Transfectam	141138	2049	0.71
	Lipofectamine	133571	5823	0.67
20	Lipofectin	144780	11981	0.73
	2-5	137710	16610	0.69
	2-5/DOPE	199253	5307	1
	2-5/7-1	153079	13322	0.77

1-9/DOPE	61088	8087	0.31
1-10	159578	6067	0.8
1-10/DOPE	84229	7287	0.42

Table II

5 Demonstration of nuclear delivery of oligonucleotides
 of varying charge densities by novel cationic lipids

<u>A. 3498-PS (phosphorothioate)</u>					
<u>Lipids:</u>		<u>Cell lines:</u>			
		<u>COS7</u>	<u>SNB19</u>	<u>C8161</u>	<u>RD</u>
10	TFM	4	3	5	4
	LFA	4	3	4	4
	LFN	5	3	3	4
	2-5	2	3	3	3
	2-5/DOPE	4	3	2	3
	2-5/7-1	4	3	2	4
	1-9/DOPE	5	3	2	3
	1-10	4	4	5	5
15	1-10/DOPE	5	4	3	3

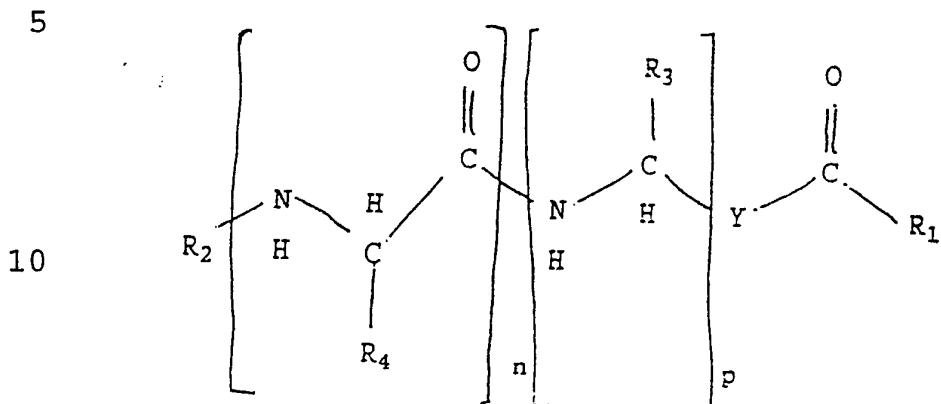
<u>B. 3498 Chimera</u>				
	<u>Lipids:</u>	<u>Cell lines:</u>		
		<u>COS7</u>	<u>SNB19</u>	<u>C8161</u>
5	TFM	5	5	5
	LFA	5	4	5
	LFN	1	2	3
	2-5	2	3	4
	2-5/DOPE	4	2	3
	2-5/7-1	4	2	4
	1-9/DOPE	5	3	2
	1-10	3	4	3
10		3	3	0
1-10/DOPE				1

<u>C. 3793-2 alternating</u>				
	<u>Lipids:</u>	<u>Cell lines:</u>		
		<u>COS7</u>	<u>SNB19</u>	<u>C8161</u>
5	TFM	5	5	5
	LFA	5	4	5
	LFN	0	0	2
	2-5	1	3	3
	2-5/DOPE	3	1	1
	2-5/7-1	4	3	3
	1-9/DOPE	4	2	1
	1-10	0	0	0
	1-10/DOPE	0	0	0

Claims

We claim:

1. A lipid having the structure:



[X⁻]_m

15

or a salt, or solvate, or enantiomers thereof wherein; (a) Y is a direct link or an alkylene of 1 to about 20 carbon atoms; (b) R₁ is H or a lipophilic moiety; (c) R₂, R₃, and R₄ are positively charged moieties, or at least one but not all of R₂, R₃, or R₄ is a positive moiety and the remaining are independently selected from H, an alkyl moiety of 1 to about 6 carbon atoms, or a heterocyclic moiety of about 5 to about 10 carbon atoms; (d) n and p are independently selected integers from 0 to 8, such that the sum of n and p is from 1 to 16; (e) X⁻ is an anion or polyanion and (f) m is an integer from 0 to a number equivalent to the positive charge(s) present on the lipid; provided that if Y is a direct link and the sum of n and p is 1 then one of either R₃ or R₄ must have an alkyl moiety of at least 10 carbon atoms.

20

2. A lipid according to claim 1 wherein R₁ is a lipophilic moiety selected from the group consisting of a straight chain alkyl moiety of 1 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkyl or

25

alkenyl moiety of about 10 to about 50 carbon atoms, a steroidyl moiety, an amine derivative, a glyceryl moiety, or $OCH(R_5R_6)$ or $N(R_5R_6)$, wherein R_5 and R_6 are straight chain or branched alkyl moieties of about 10 to about 30 carbon atoms.

5

3. A lipid according to claim 2 wherein R_1 is an amine derivative selected from the group consisting of a straight chain alkylamine moiety of 1 to about 24 carbon atoms, a straight chain alkenylamine moiety of 2 to about 24 carbon atoms, a symmetrical branched alkylamine or alkenylamine moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkylamine or alkenylamine moiety of about 10 to about 50 carbon atoms, cyclic amine moiety of about 3 to about 10 carbon atoms, or a steroidyl moiety.

10

4. A lipid according to claim 3 wherein the steroidyl moiety is cholesteryl.

15

5. A lipid according to claim 1 wherein at least one but not all of R_2 , R_3 , and R_4 are independently selected from the group consisting of a substituted heterocyclic moiety of 1 to about 6 carbon atoms, or a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with substituents selected from the group consisting of OH, thio, aryl of 1 to about 20 carbon atoms, and OR_7 , wherein R_7 is an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl of 2 to about 24 carbon atoms, an aryl of about 5 to about 20 carbon atoms or an aralkyl of about 6 to about 25 carbon atoms.

20

25

30

6. A lipid according to claim 1 wherein at least one but not all R_2 , R_3 , and R_4 are independently selected from a group consisting of an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium moiety, an enamine moiety, a cyclic amine moiety, an amidine moiety, an isothiourea moiety,

a heterocyclic amine moiety, and a substituted heterocyclic moiety and a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with a substituent selected from the group consisting of NH_2 , $\text{C}(=\text{O})\text{NH}_2$, NHR_7 , $\text{C}(=\text{O})\text{NHR}_7$, NHR_7R_8 , and 5 $\text{C}(=\text{O})\text{NHR}_7\text{R}_8$, wherein R_7 and R_8 are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, and an aralkyl moiety of about 6 to about 25 carbon atoms.

10

7. A lipid according to claim 6 wherein the arylamine moiety is selected from the group consisting of tryptophane, phenylalanine, and tyrosine.

15

8. A lipid according to claim 1 wherein R_2 is an amino acid residue having a positively charged side chain, and wherein the amino group(s) is optionally substituted with an alkyl of 1 to about 6 carbon atoms or wherein the amino group(s) is substituted to form a secondary, tertiary, or 20 quaternary amine with an alkyl moiety of 1 to about 6 carbon atoms optionally substituted with a group selected from; hydroxyl, amino, alkoxy of 1 to about 6 carbon atoms, alkylamino of 1 to about 6 carbon atoms, or dialkylamino of 2 to about 12 carbon atoms.

25

9. A lipid according to claim 8 wherein R_2 is an amino acid residue selected from the group consisting of lysine, arginine, histidine, ornithine, and an amino acid analog.

30

10. A lipid according to claim 9 wherein the amino acid analog is selected from the group consisting of 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine and monoalkyl, dialkyl or peralkyl substituted derivatives which 35 are substituted on one or more amine nitrogens with an alkyl group of 1 to about 6 carbon atoms.

11. A lipid according to claim 1 wherein R_3 and R_4 are independently a lipophilic moiety of 1 to about 24 carbon atoms, a positively charged moiety, or a negatively charged moiety.

5

12. A lipid according to claim 11 wherein the lipophilic moiety is selected from the group consisting of a straight chain alkyl moiety of 1 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, and a steroidyl moiety.

10

13. A lipid according to claim 11 wherein positively charged moiety is selected from the group consisting of amino acid residues having a positively charged group on the side chain, an alkylaminoalkyl moiety, a fluoroalkylaminoalkyl moiety, a perfluoroalkylaminoalkyl moiety, a guanidiniumalkyl moiety, an enaminoalkyl moiety, a cyclic aminoalkyl moiety, an amidinoalkyl moiety, an isothiourea alkyl moiety, and a heterocyclic amine moiety.

15

14. A lipid according to claim 11 wherein the negatively charged moiety is selected from the group consisting of a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, and a phosphatidylalkyl moiety of 1 to about 24 carbon atoms.

20

15. A lipid according to claim 1 wherein the sum of n and p is from 1 to 8.

25

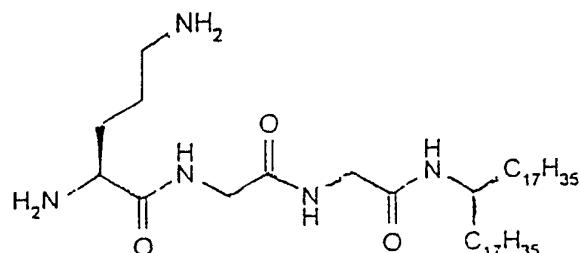
16. A lipid according to claim 1 wherein the sum of n and p is from 1 to 4.

17. A lipid according to claim 1 wherein the sum of n and p is from 1 to 2.

5 18. A lipid according to claim 1 wherein X⁻ is a pharmaceutically acceptable anion or polyanion.

19. An amide-based lipid having the structure:

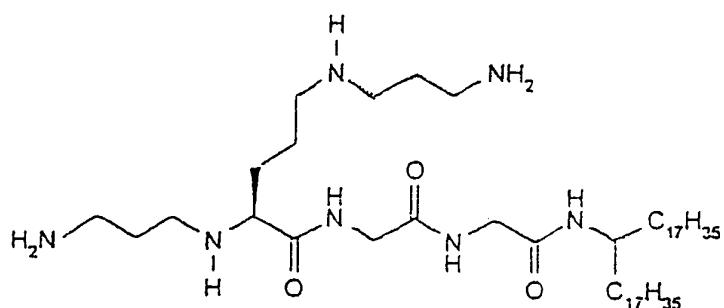
10



20

20. An amide-based lipid having the structure:

25



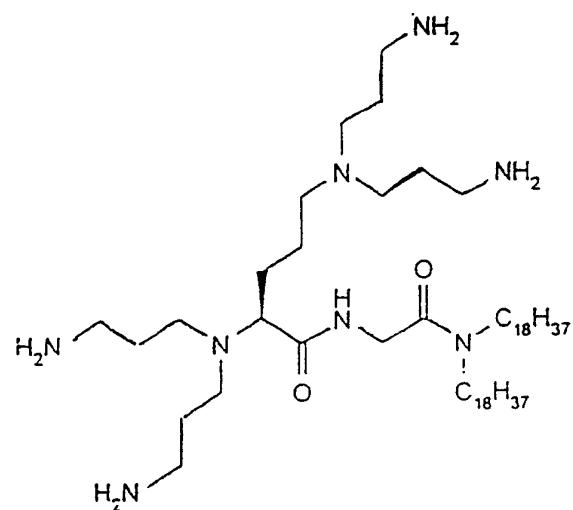
30

35

70

21. An amide-based lipid having the structure:

5



10

15

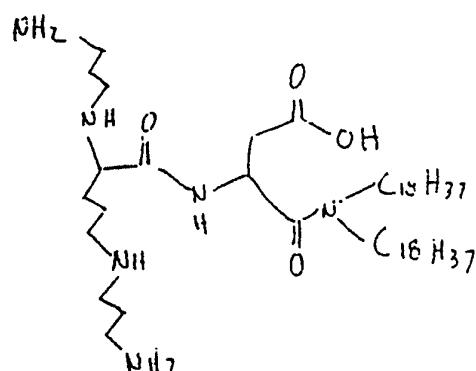
20

22. An amide-based lipid having the structure:

25

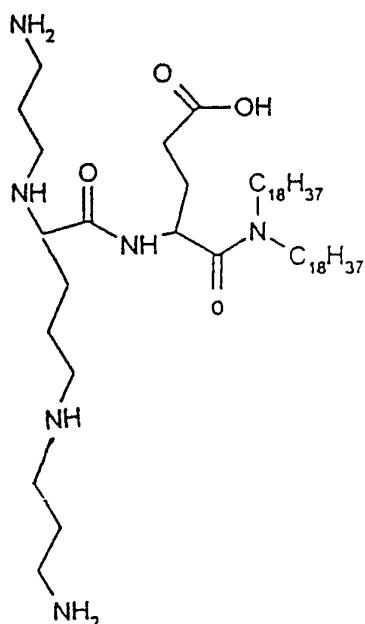
30

35



23. An amide-based lipid having the structure:

5



10

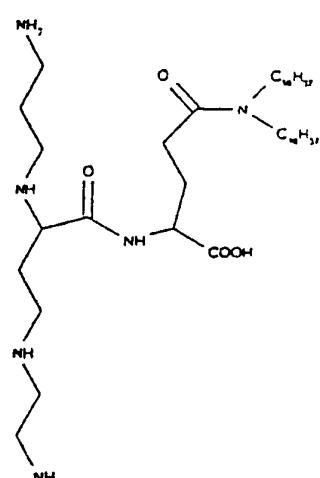
15

20

25

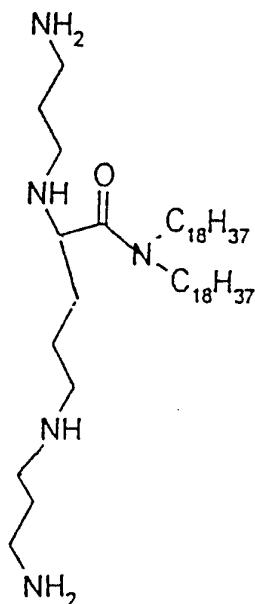
30

24. An amide-based lipid having the structure:



25. An amide-based lipid having the structure:

5



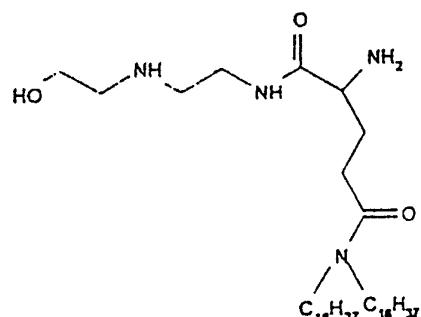
10

15

26. An amide-based lipid having the structure:

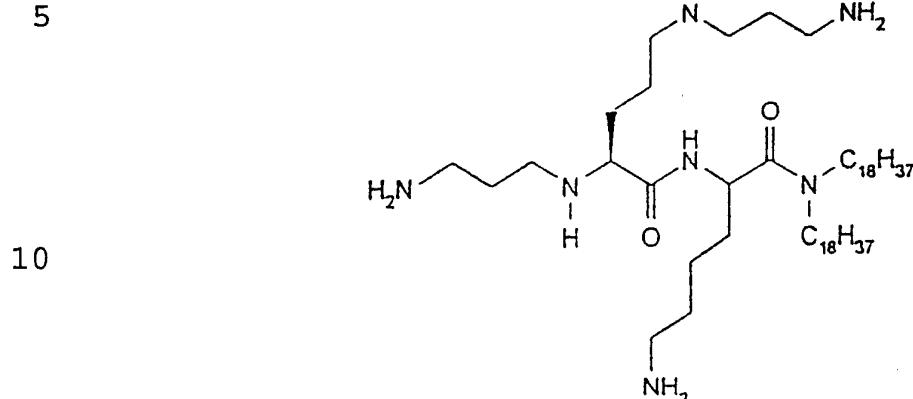
20

25

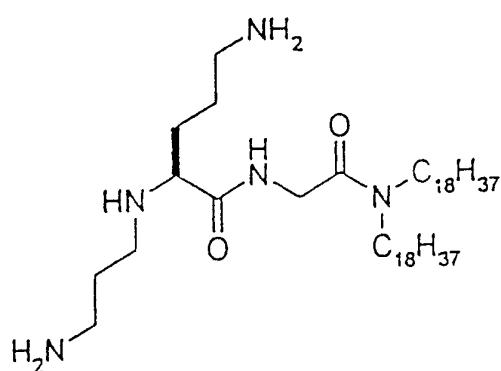


30

27. An amide-based lipid having the structure:



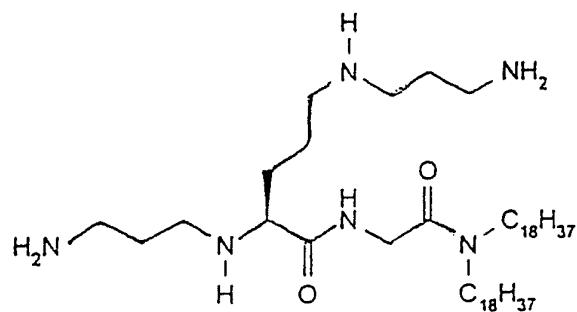
28. An amide-based lipid having the structure:



29. An amide-based lipid having the structure:

5

10



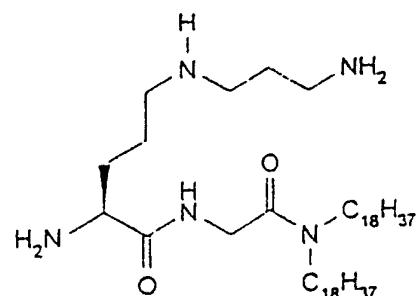
15

30. An amide-based lipid having the structure:

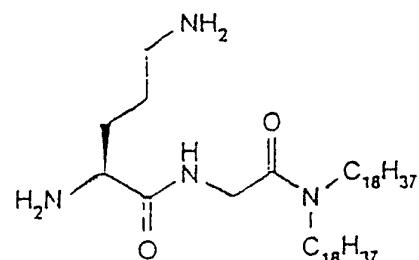
20

25

30

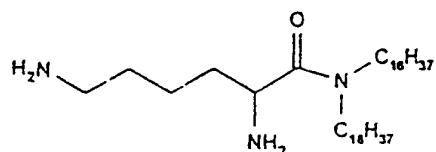


35



32. An amide-based lipid having the structure:

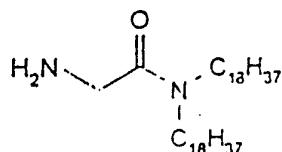
5



10

33. An amide-based lipid having the structure:

15



20

34. A composition comprising a polyanionic macromolecule and a lipid according to claim 1.

25

35. A composition according to claim 34 wherein the polyanionic macromolecule comprises an expression vector capable of expressing a polypeptide in a cell.

30

36. A composition according to claim 34 wherein the polyanionic macromolecule is an oligonucleotide or an oligomer.

35

37. A composition according to claim 34 wherein the polyanionic macromolecule is DNA.

38. A method of delivering a polyanionic macromolecule into a cell comprising contacting a composition of claim 34 with the cell.

5 39. A method to interfere with the expression of a protein in a cell comprising contacting a composition of claim 36 with the cell wherein the oligomer has a base sequence that is substantially complimentary to an RNA sequence in the cell that encodes the protein.

10

40. A kit for delivering a polyanionic macromolecule into a cell comprising a composition of claim 34.

Figure 1: Synthesis of 1-9 and 1-10

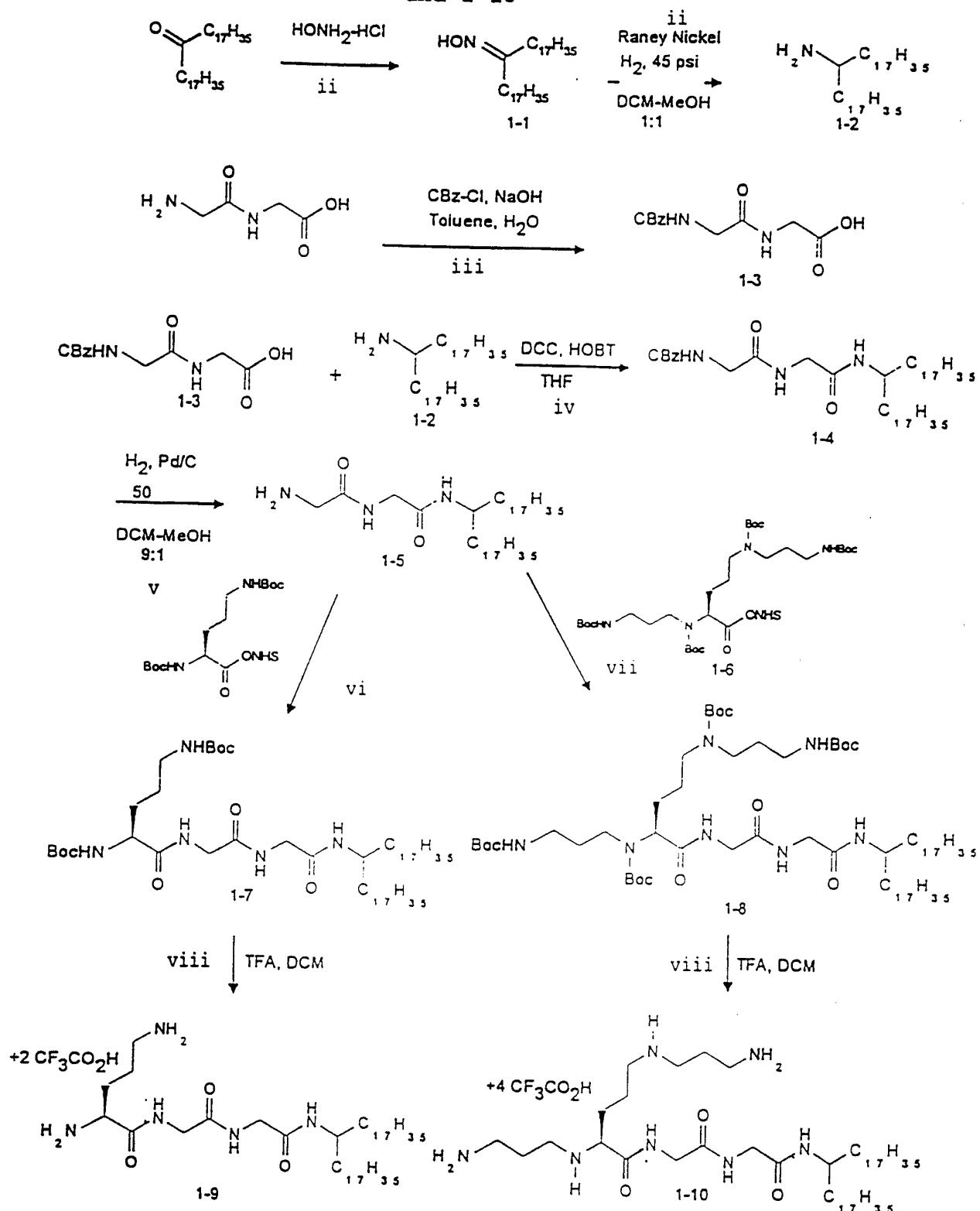


Figure 2: Synthesis of 2-5

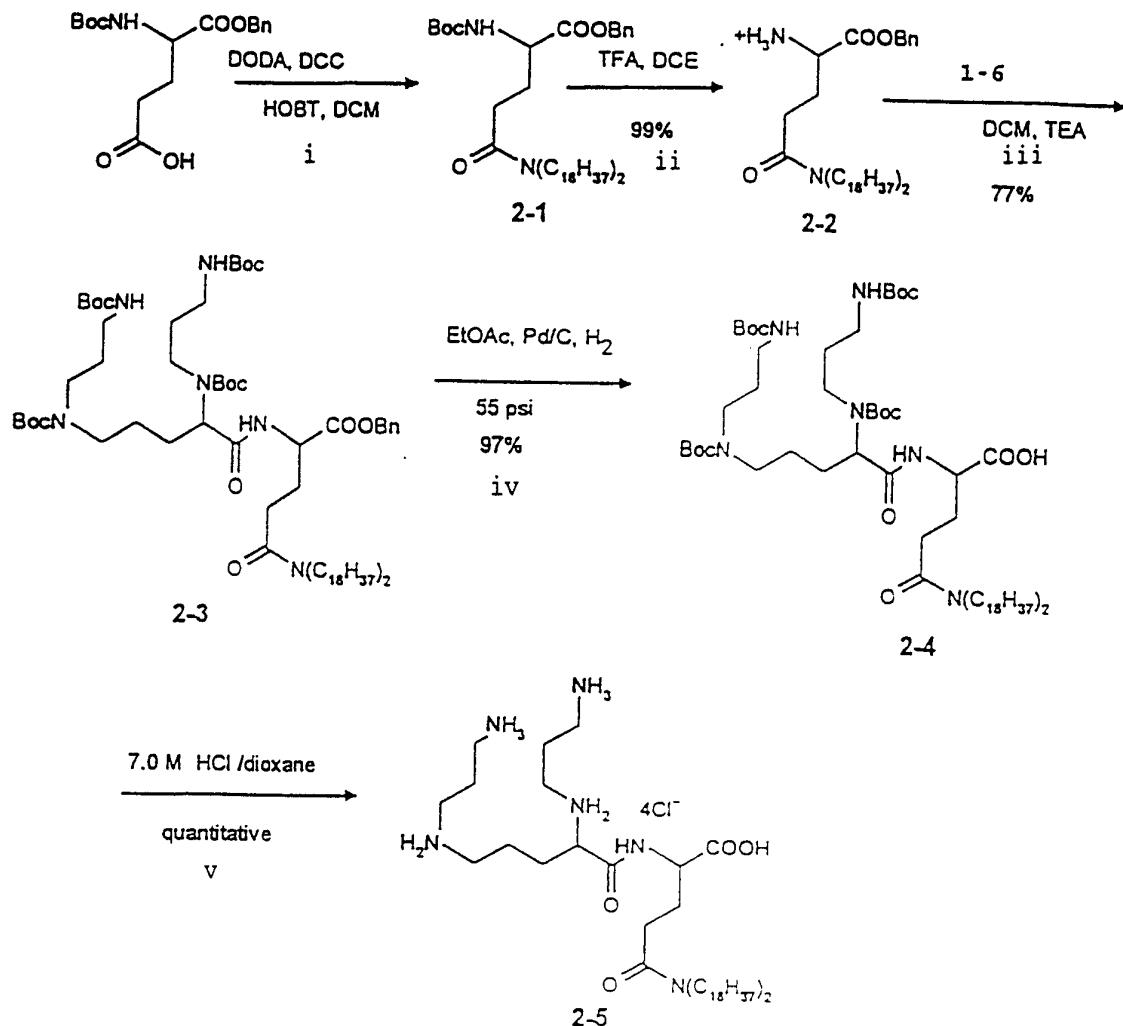


Figure 3: Synthesis of 3-5

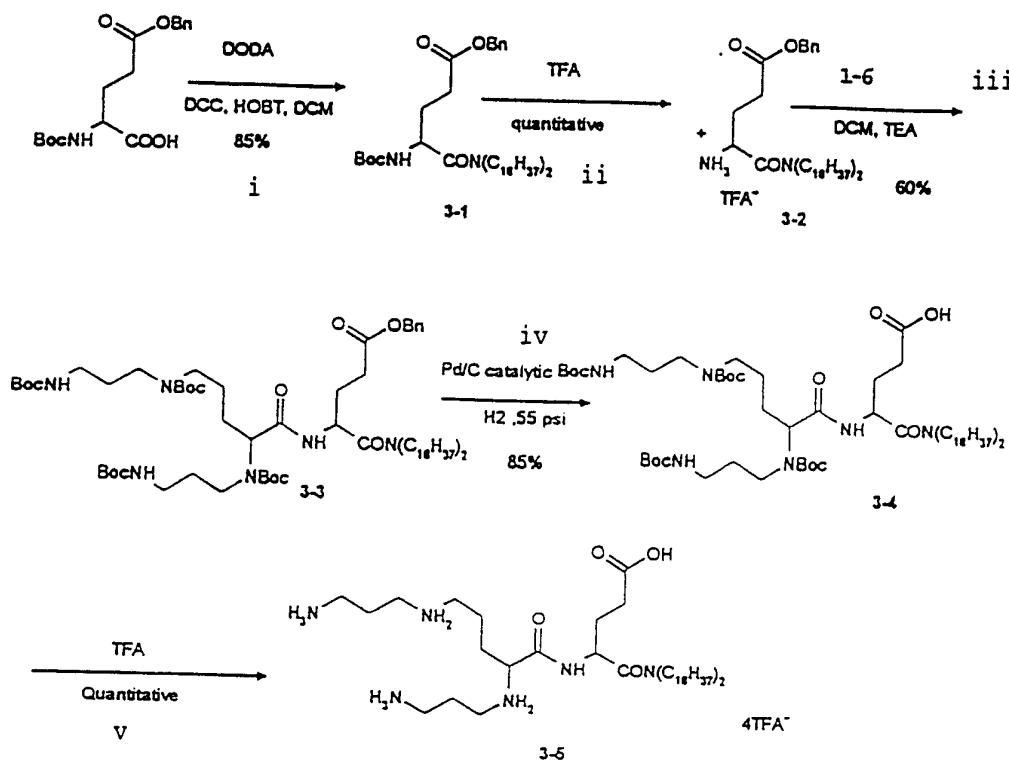
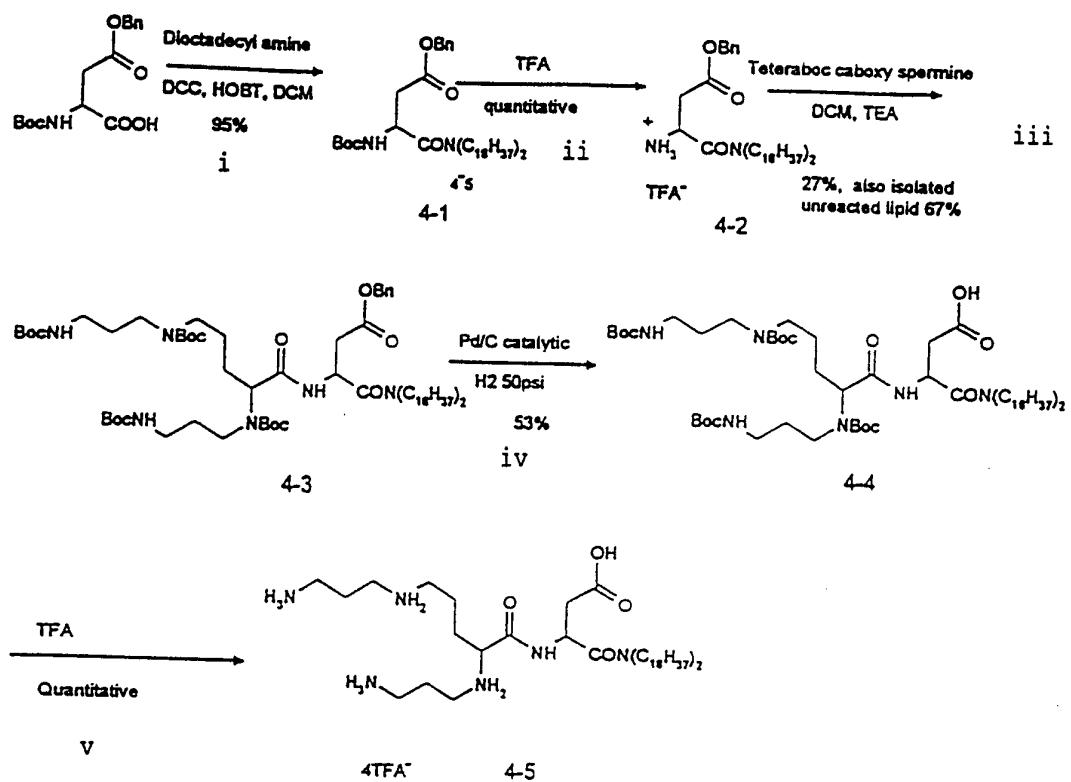


Figure 4 : Synthesis of 4-5



10

15

20

25

Figure 5: Synthetic route for the synthesis of 5-6

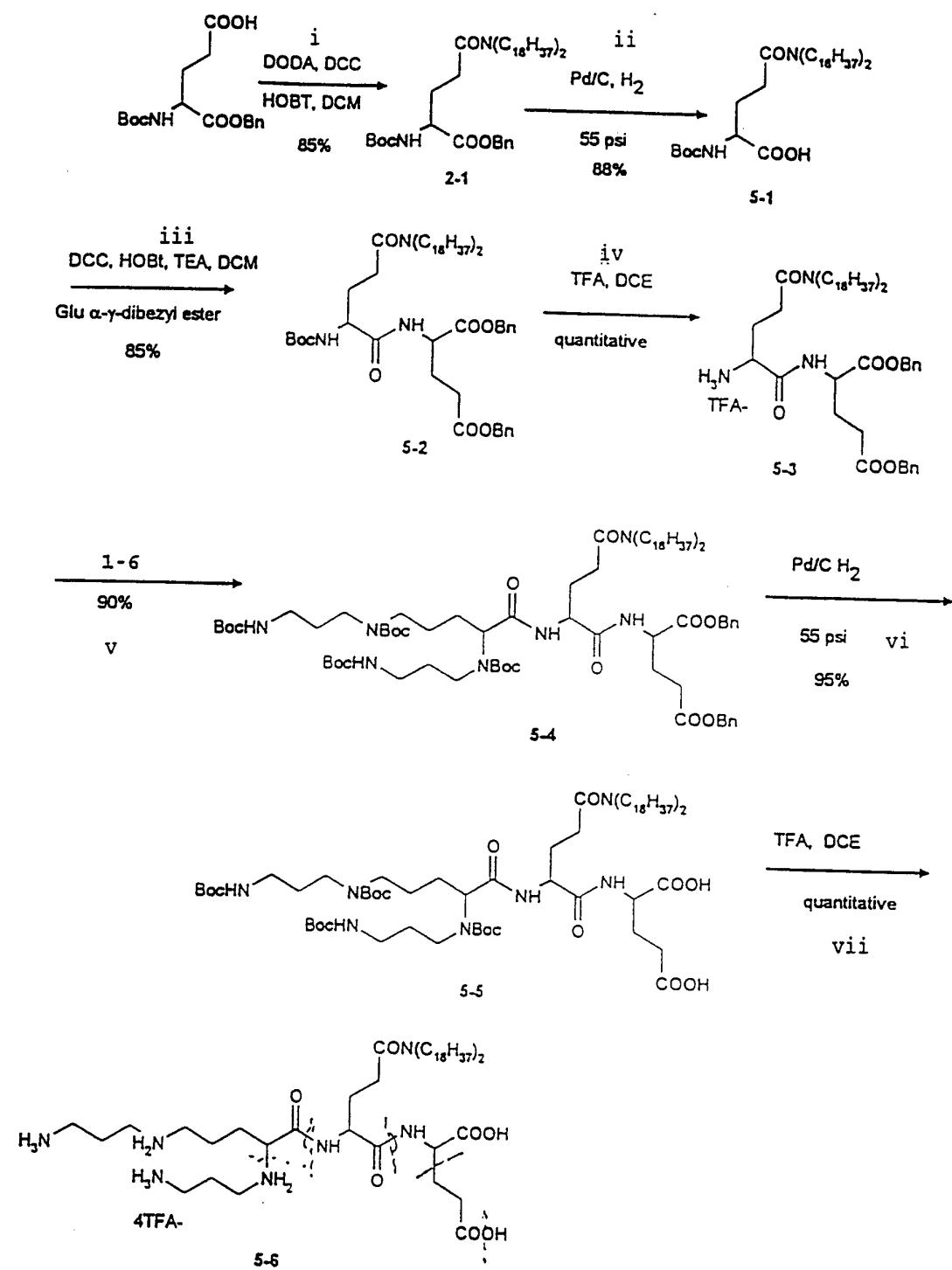


Figure 6: Synthesis of 6-5

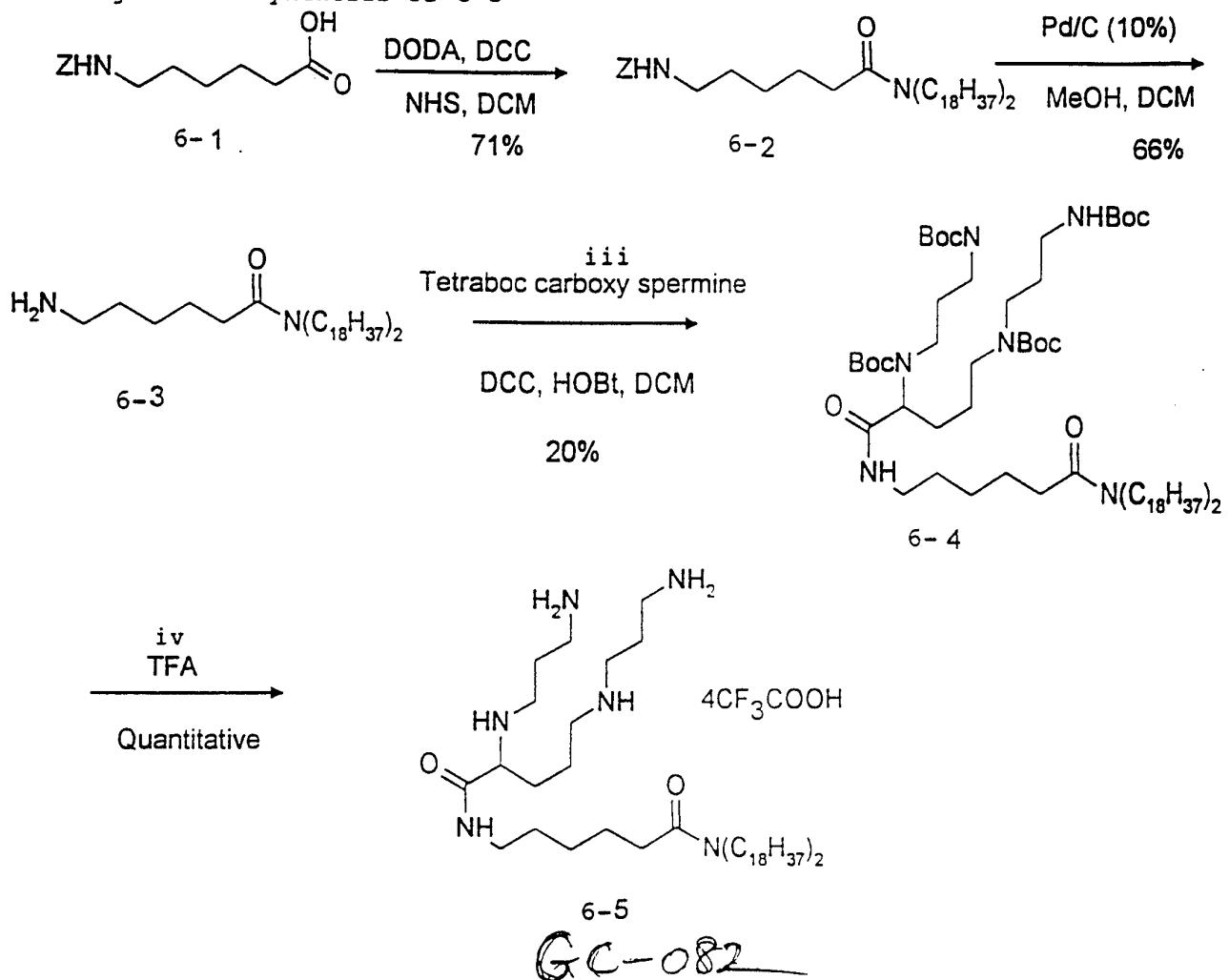
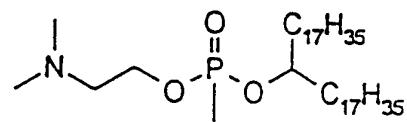


Figure 7: Structure of cationic lipid 7-1: (3b)Chole-5-en-3-ol, 2-(dimethylamino)ethyl methylphosphonate.

5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12087

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07C 49/105, 59/00, 205/00, 207/00, 233/00

US CL :554/56, 58, 104, 122; 564/152, 153, 159; 568/307, 369, 423

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)

U.S. : 554/56, 58, 104, 122; 564/152, 153, 159; 568/307, 369, 423

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,171,678 A (BEHR ET AL.) 15 December 1992 (15/12/92), see entire document.	1-3, 5-6, 11, 13-18, 28-31
X	US 2,426,891 A (K. LYNCH) 02 September 1947 (02/09/47), see entire document.	1-3, 5-6, 11, 13-18, 33
X	US 4,548,955 A (OKAHATA ET AL.) 22 October 1985 (22/10/85), see entire document, especially column 6, lines 34-36.	1-3, 5-6, 11, 13-18, 33

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"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
A document defining the general state of the art which is not considered to be of particular relevance	"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
E earlier document published on or after the international filing date	"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
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)	"&"	document member of the same patent family
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		

Date of the actual completion of the international search

19 OCTOBER 1996

Date of mailing of the international search report

03 DEC 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DEBORAH D. CARR

Telephone No. (703) 308-1235

gab for