

LIPIDS FOR USE IN LIPID NANOPARTICLE FORMULATIONS

BACKGROUND

Technical Field

The present disclosure generally relates to novel cationic lipids that can be used in
5 combination with other lipid components, such as neutral lipids, cholesterol, and polymer
conjugated lipids, to form lipid nanoparticles with oligonucleotides, to facilitate the intracellular
delivery of therapeutic nucleic acids (*e.g.*, oligonucleotides, messenger RNA) both *in vitro* and *in*
vivo.

Description of the Related Art

10 There are many challenges associated with the delivery of nucleic acids to affect a
desired response in a biological system. Nucleic acid-based therapeutics have enormous potential
but there remains a need for more effective delivery of nucleic acids to appropriate sites within a
cell or organism to realize this potential. Therapeutic nucleic acids include, *e.g.*, messenger RNA
(mRNA), antisense oligonucleotides, ribozymes, DNazymes, plasmids, immune stimulating
15 nucleic acids, antagomir, antimir, mimic, supermir, and aptamers. Some nucleic acids, such as
mRNA or plasmids, can be used to effect expression of specific cellular products as would be
useful in the treatment of, for example, diseases related to a deficiency of a protein or enzyme.
The therapeutic applications of translatable nucleotide delivery are extremely broad as constructs
can be synthesized to produce any chosen protein sequence, whether indigenous to the system.
20 The expression products of the nucleic acid can augment existing levels of protein, replace
missing or non-functional versions of a protein, or introduce new protein and associated
functionality in a cell or organism.

Some nucleic acids, such as miRNA inhibitors, can be used to effect expression of
specific cellular products that are regulated by miRNA as would be useful in the treatment of, for
25 example, diseases related to deficiency of protein or enzyme. The therapeutic applications of
miRNA inhibition are extremely broad as constructs can be synthesized to inhibit one or more
miRNA that would in turn regulate the expression of mRNA products. The inhibition of
endogenous miRNA can augment its downstream target endogenous protein expression and
restore proper function in a cell or organism as a means to treat disease associated to a specific
30 miRNA or a group of miRNA.

Other nucleic acids can down-regulate intracellular levels of specific mRNA and, as a result, down-regulate the synthesis of the corresponding proteins through processes such as RNA interference (RNAi) or complementary binding of antisense RNA. The therapeutic applications of antisense oligonucleotide and RNAi are also extremely broad, since oligonucleotide constructs
5 can be synthesized with any nucleotide sequence directed against a target mRNA. Targets may include mRNAs from normal cells, mRNAs associated with disease-states, such as cancer, and mRNAs of infectious agents, such as viruses. To date, antisense oligonucleotide constructs have shown the ability to specifically down-regulate target proteins through degradation of the cognate mRNA in both *in vitro* and *in vivo* models. In addition, antisense oligonucleotide
10 constructs are currently being evaluated in clinical studies.

However, two problems currently face using oligonucleotides in therapeutic contexts. First, free RNAs are susceptible to nuclease digestion in plasma. Second, free RNAs have limited ability to gain access to the intracellular compartment where the relevant translation machinery resides. Lipid nanoparticles formed from cationic lipids with other lipid components,
15 such as neutral lipids, cholesterol, PEG, PEGylated lipids, and oligonucleotides have been used to block degradation of the RNAs in plasma and facilitate the cellular uptake of the oligonucleotides.

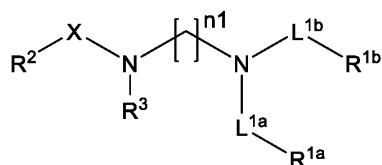
There remains a need for improved cationic lipids and lipid nanoparticles for the delivery of oligonucleotides. Preferably, these lipid nanoparticles would provide optimal drug to lipid
20 ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with unacceptable toxicity and/or risk to the patient. The present disclosure provides these and related advantages.

25 BRIEF SUMMARY

In brief, the present disclosure provides lipid compounds, including stereoisomers, pharmaceutically acceptable salts, or tautomers thereof, which can be used alone or in combination with other lipid components such as neutral lipids, charged lipids, steroids (including for example, all sterols) and/or their analogs, and/or polymer conjugated lipids to
30 form lipid nanoparticles for the delivery of therapeutic agents. In some instances, the lipid nanoparticles are used to deliver nucleic acids such as antisense and/or messenger RNA.

Methods for use of such lipid nanoparticles for treatment of various diseases or conditions, such as those caused by infectious entities and/or insufficiency of a protein, are also provided.

In one embodiment, compounds having the following Structure (I) are provided:



5

(I)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein R^{1a} , R^{1b} , R^2 , R^3 , L^{1a} , L^{1b} , $n1$, and X are as defined herein.

Pharmaceutical compositions comprising one or more of the foregoing compounds of Structure (I) and a therapeutic agent are also provided. In some embodiments, the pharmaceutical compositions further comprise one or more components selected from neutral lipids, charged lipids, steroids, and polymer conjugated lipids. Such compositions are useful for formation of lipid nanoparticles for the delivery of the therapeutic agent.

In other embodiments, the present disclosure provides a method for administering a therapeutic agent to a patient in need thereof, the method comprising preparing a composition of lipid nanoparticles comprising the compound of Structure (I) and a therapeutic agent and delivering the composition to the patient. Such methods are useful for inducing expression of a protein in a subject, for example for expressing an antigen for purposes of vaccination or a gene editing protein.

These and other aspects of the disclosure will be apparent upon reference to the following detailed description.

DETAILED DESCRIPTION

In the following description, certain specific details are set forth in order to provide a thorough understanding of various embodiments of the disclosure. However, one skilled in the art will understand that the embodiments of the disclosure may be practiced without these details.

The present disclosure is based, in part, upon the discovery of novel cationic (amino) lipids that provide advantages when used in lipid nanoparticles for the *in vivo* delivery of an active or therapeutic agent such as a nucleic acid into a cell of a mammal. In particular, embodiments of the present disclosure provide nucleic acid-lipid nanoparticle compositions

comprising one or more of the novel cationic lipids described herein that provide increased activity of the nucleic acid and improved tolerability of the compositions *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid nanoparticle compositions previously described.

5 In particular embodiments, the present disclosure provides novel cationic lipids that enable the formulation of improved compositions for the *in vitro* and *in vivo* delivery of mRNA and/or other oligonucleotides. In some embodiments, these improved lipid nanoparticle compositions are useful for expression of protein encoded by mRNA. In other embodiments, these improved lipid nanoparticles compositions are useful for upregulation of endogenous
10 protein expression by delivering miRNA inhibitors targeting one specific miRNA or a group of miRNA regulating one target mRNA or several mRNA. In other embodiments, these improved lipid nanoparticle compositions are useful for down-regulating (*e.g.*, silencing) the protein levels and/or mRNA levels of target genes. In some other embodiments, the lipid nanoparticles are also useful for delivery of mRNA and plasmids for expression of transgenes. In yet other
15 embodiments, the lipid nanoparticle compositions are useful for inducing a pharmacological effect resulting from expression of a protein, *e.g.*, increased production of red blood cells through the delivery of a suitable erythropoietin mRNA, or protection against infection through delivery of mRNA encoding for a suitable antibody.

The lipid nanoparticles and compositions of the present disclosure may be used for a
20 variety of purposes, including the delivery of encapsulated or associated (*e.g.*, complexed) therapeutic agents such as nucleic acids to cells, both *in vitro* and *in vivo*. Accordingly, embodiments of the present disclosure provide methods of treating or preventing diseases or disorders in a subject in need thereof by contacting the subject with a lipid nanoparticle that encapsulates or is associated with a suitable therapeutic agent, wherein the lipid nanoparticle
25 comprises one or more of the novel cationic lipids described herein.

As described herein, embodiments of the lipid nanoparticles of the present disclosure are particularly useful for the delivery of nucleic acids, including, *e.g.*, mRNA, antisense oligonucleotide, plasmid DNA, microRNA (miRNA), miRNA inhibitors (antagomirs/antimirs), messenger-RNA-interfering complementary RNA (micRNA), DNA, multivalent RNA, dicer
30 substrate RNA, complementary DNA (cDNA), etc. Therefore, the lipid nanoparticles and compositions of the present disclosure may be used to induce expression of a desired protein both *in vitro* and *in vivo* by contacting cells with a lipid nanoparticle comprising one or more novel cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is

associated with a nucleic acid that is expressed to produce the desired protein (*e.g.*, a messenger RNA or plasmid encoding the desired protein). Alternatively, the lipid nanoparticles and compositions of the present disclosure may be used to decrease the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a lipid nanoparticle comprising one or more novel cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that reduces target gene expression (*e.g.*, an antisense oligonucleotide or small interfering RNA (siRNA)). The lipid nanoparticles and compositions of the present disclosure may also be used for co-delivery of different nucleic acids (*e.g.*, mRNA and plasmid DNA) separately or in combination, such as may be useful to provide an effect requiring colocalization of different nucleic acids (*e.g.*, mRNA encoding for a suitable gene modifying enzyme and DNA segment(s) for incorporation into the host genome).

Nucleic acids for use with this disclosure may be prepared according to any available technique. For mRNA, the primary methodology of preparation is, but not limited to, enzymatic synthesis (also termed *in vitro* transcription) which currently represents the most efficient method to produce long sequence-specific mRNA. *In vitro* transcription describes a process of template-directed synthesis of RNA molecules from an engineered DNA template comprised of an upstream bacteriophage promoter sequence (*e.g.*, including but not limited to that from the T7, T3, and SP6 coliphage) linked to a downstream sequence encoding the gene of interest. Template DNA can be prepared for *in vitro* transcription from a number of sources with appropriate techniques which are well known in the art including, but not limited to, plasmid DNA and polymerase chain reaction amplification (see Linpinsel, J.L. and Conn, G.L., General protocols for preparation of plasmid DNA template and Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012)

Transcription of the RNA occurs *in vitro* using the linearized DNA template in the presence of the corresponding RNA polymerase and adenosine, guanosine, uridine and cytidine ribonucleoside triphosphates (rNTPs) under conditions that support polymerase activity while minimizing potential degradation of the resultant mRNA transcripts. *In vitro* transcription can be performed using a variety of commercially available kits including, but not limited to RiboMax Large Scale RNA Production System (Promega), MegaScript Transcription kits (Life Technologies) as well as with commercially available reagents including RNA polymerases and rNTPs. The methodology for *in vitro* transcription of mRNA is well known in the art. (*see, e.g.*,

Losick, R., 1972, *In vitro* transcription, Ann Rev Biochem v.41 409-46; Kamakaka, R. T. and Kraus, W. L. 2001. *In Vitro* Transcription. Current Protocols in Cell Biology. 2:11.6:11.6.1–11.6.17; Beckert, B. And Masquida, B., (2010) Synthesis of RNA by *In Vitro* Transcription in RNA in Methods in Molecular Biology v. 703 (Neilson, H. Ed), New York, N.Y. Humana Press, 5 2010; Brunelle, J.L. and Green, R., 2013, Chapter Five – *In vitro* transcription from plasmid or PCR-amplified DNA, Methods in Enzymology v. 530, 101-114; all of which are incorporated herein by reference).

The desired *in vitro* transcribed mRNA is then purified from the undesired components of the transcription or associated reactions (including unincorporated rNTPs, protein enzyme, salts, 10 short RNA oligos *etc.*). Techniques for the isolation of the mRNA transcripts are well known in the art. Well known procedures include phenol/chloroform extraction or precipitation with either alcohol (ethanol, isopropanol) in the presence of monovalent cations or lithium chloride. Additional, non-limiting examples of purification procedures which can be used include size exclusion chromatography (Lukavsky, P.J. and Puglisi, J.D., 2004, Large-scale preparation and 15 purification of polyacrylamide-free RNA oligonucleotides, RNA v.10, 889-893), silica-based affinity chromatography and polyacrylamide gel electrophoresis (Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012). Purification can be performed using a variety of 20 commercially available kits including, but not limited to SV Total Isolation System (Promega) and *In Vitro* Transcription Cleanup and Concentration Kit (Norgen Biotek).

Furthermore, while reverse transcription can yield large quantities of mRNA, the products can contain a number of aberrant RNA impurities associated with undesired polymerase activity which may need to be removed from the full-length mRNA preparation. These include 25 short RNAs that result from abortive transcription initiation as well as double-stranded RNA (dsRNA) generated by RNA-dependent RNA polymerase activity, RNA-primed transcription from RNA templates and self-complementary 3' extension. It has been demonstrated that these contaminants with dsRNA structures can lead to undesired immunostimulatory activity through interaction with various innate immune sensors in eukaryotic cells that function to recognize 30 specific nucleic acid structures and induce potent immune responses. This in turn, can dramatically reduce mRNA translation since protein synthesis is reduced during the innate cellular immune response. Therefore, additional techniques to remove these dsRNA contaminants have been developed and are known in the art including but not limited to scalable

HPLC purification (see, *e.g.*, Kariko, K., Muramatsu, H., Ludwig, J. And Weissman, D., 2011, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, Nucl Acid Res, v. 39 e142; Weissman, D., Pardi, N., Muramatsu, H., and Kariko, K., HPLC Purification of *in vitro* transcribed long RNA in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013). HPLC purified mRNA has been reported to be translated at much greater levels, particularly in primary cells and *in vivo*.

A significant variety of modifications have been described in the art which are used to alter specific properties of *in vitro* transcribed mRNA and improve its utility. These include but are not limited to modifications to the 5' and 3' termini of the mRNA. Endogenous eukaryotic mRNA typically contains a cap structure on the 5'-end of a mature molecule which plays an important role in mediating binding of the mRNA Cap Binding Protein (CBP), which is in turn responsible for enhancing mRNA stability in the cell and efficiency of mRNA translation. Therefore, highest levels of protein expression are achieved with capped mRNA transcripts. The 5'-cap contains a 5'-5'-triphosphate linkage between the 5'-most nucleotide and guanine nucleotide. The conjugated guanine nucleotide is methylated at the N7 position. Additional modifications include methylation of the ultimate and penultimate most 5'-nucleotides on the 2'-hydroxyl group.

Multiple distinct cap structures can be used to generate the 5'-cap of *in vitro* transcribed synthetic mRNA. 5'-capping of synthetic mRNA can be performed co-transcriptionally with chemical cap analogs (*i.e.*, capping during *in vitro* transcription). For example, the Anti-Reverse Cap Analog (ARCA) cap contains a 5'-5'-triphosphate guanine-guanine linkage where one guanine contains an N7 methyl group as well as a 3'-O-methyl group. However, up to 20% of transcripts remain uncapped during this co-transcriptional process and the synthetic cap analog is not identical to the 5'-cap structure of an authentic cellular mRNA, potentially reducing translatability and cellular stability. Alternatively, synthetic mRNA molecules may also be enzymatically capped post-transcriptionally. These may generate a more authentic 5'-cap structure that more closely mimics, either structurally or functionally, the endogenous 5'-cap which have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5' endonucleases and/or reduced 5' de-capping. Numerous synthetic 5'-cap analogs have been developed and are known in the art to enhance mRNA stability and translatability (see, *e.g.*, Grudzien-Nogalska, E., Kowalska, J., Su, W., Kuhn, A.N., Slepenev, S.V., Darynkiewicz, E., Sahin, U., Jemielity, J., and Rhoads, R.E., Synthetic mRNAs with superior translation and

stability properties in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013).

On the 3'-terminus, a long chain of adenine nucleotides (poly-A tail) is normally added to mRNA molecules during RNA processing. Immediately after transcription, the 3' end of the transcript is cleaved to free a 3' hydroxyl to which poly-A polymerase adds a chain of adenine nucleotides to the RNA in a process called polyadenylation. The poly-A tail has been extensively shown to enhance both translational efficiency and stability of mRNA (see Bernstein, P. and Ross, J., 1989, Poly (A), poly (A) binding protein and the regulation of mRNA stability, Trends Bio Sci v. 14 373-377; Guhaniyogi, J. And Brewer, G., 2001, Regulation of mRNA stability in mammalian cells, Gene, v. 265, 11-23; Dreyfus, M. And Regnier, P., 2002, The poly (A) tail of mRNAs: Bodyguard in eukaryotes, scavenger in bacteria, Cell, v.111, 611-613).

Poly (A) tailing of *in vitro* transcribed mRNA can be achieved using various approaches including, but not limited to, cloning of a poly (T) tract into the DNA template or by post-transcriptional addition using Poly (A) polymerase. The first case allows *in vitro* transcription of mRNA with poly (A) tails of defined length, depending on the size of the poly (T) tract, but requires additional manipulation of the template. The latter case involves the enzymatic addition of a poly (A) tail to *in vitro* transcribed mRNA using poly (A) polymerase which catalyzes the incorporation of adenine residues onto the 3' termini of RNA, requiring no additional manipulation of the DNA template, but results in mRNA with poly(A) tails of heterogeneous length. 5'-capping and 3'-poly (A) tailing can be performed using a variety of commercially available kits including, but not limited to Poly (A) Polymerase Tailing kit (EpiCenter), mMESSAGE mMACHINE T7 Ultra kit and Poly (A) Tailing kit (Life Technologies) as well as with commercially available reagents, various ARCA caps, Poly (A) polymerase, etc.

In addition to 5' cap and 3' poly adenylation, other modifications of the *in vitro* transcripts have been reported to provide benefits as related to efficiency of translation and stability. It is well known in the art that pathogenic DNA and RNA can be recognized by a variety of sensors within eukaryotes and trigger potent innate immune responses. The ability to discriminate between pathogenic and self DNA and RNA has been shown to be based, at least in part, on structure and nucleoside modifications since most nucleic acids from natural sources contain modified nucleosides. In contrast, *in vitro* synthesized RNA lacks these modifications, thus rendering it immunostimulatory which in turn can inhibit effective mRNA translation as outlined above. The introduction of modified nucleosides into *in vitro* transcribed mRNA can be used to prevent recognition and activation of RNA sensors, thus mitigating this undesired

immunostimulatory activity and enhancing translation capacity (see, *e.g.*, Kariko, K. And Weissman, D. 2007, Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development, *Curr Opin Drug Discov Devel*, v.10 523-532; Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in *Methods in Molecular Biology* v.969 (Rabinovich, P.H. Ed), 2013); Kariko, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., Weissman, D., 2008, Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability, *Mol Ther* v.16, 1833-1840. The modified nucleosides and nucleotides used in the synthesis of modified RNAs can be prepared monitored and utilized using general methods and procedures known in the art. A large variety of nucleoside modifications are available that may be incorporated alone or in combination with other modified nucleosides to some extent into the *in vitro* transcribed mRNA (see, *e.g.*, US Publication No. 2012/0251618). *In vitro* synthesis of nucleoside-modified mRNA has been reported to have reduced ability to activate immune sensors with a concomitant enhanced translational capacity.

Other components of mRNA which can be modified to provide benefit in terms of translatability and stability include the 5' and 3' untranslated regions (UTR). Optimization of the UTRs (favorable 5' and 3' UTRs can be obtained from cellular or viral RNAs), either both or independently, have been shown to increase mRNA stability and translational efficiency of *in vitro* transcribed mRNA (see, *e.g.*, Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in *Methods in Molecular Biology* v.969 (Rabinovich, P.H. Ed), 2013).

In addition to mRNA, other nucleic acid payloads may be used for this disclosure. For oligonucleotides, methods of preparation include but are not limited to chemical synthesis and enzymatic, chemical cleavage of a longer precursor, *in vitro* transcription as described above, etc. Methods of synthesizing DNA and RNA nucleotides are widely used and well known in the art (see, *e.g.*, Gait, M. J. (ed.) *Oligonucleotide synthesis: a practical approach*, Oxford [Oxfordshire], Washington, D.C.: IRL Press, 1984; and Herdewijn, P. (ed.) *Oligonucleotide synthesis: methods and applications*, *Methods in Molecular Biology*, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

For plasmid DNA, preparation for use with this disclosure commonly utilizes but is not limited to expansion and isolation of the plasmid DNA *in vitro* in a liquid culture of bacteria containing the plasmid of interest. The presence of a gene in the plasmid of interest that encodes resistance to a particular antibiotic (penicillin, kanamycin, *etc.*) allows those bacteria containing the plasmid of interest to selective grow in antibiotic-containing cultures. Methods of isolating plasmid DNA are widely used and well known in the art (see, *e.g.*, Heilig, J., Elbing, K. L. and Brent, R (2001) Large-Scale Preparation of Plasmid DNA. Current Protocols in Molecular Biology. 41:II:1.7:1.7.1–1.7.16; Rozkov, A., Larsson, B., Gillström, S., Björnstedt, R. and Schmidt, S. R. (2008), Large-scale production of endotoxin-free plasmids for transient expression in mammalian cell culture. Biotechnol. Bioeng., 99: 557–566; and US6197553B1). Plasmid isolation can be performed using a variety of commercially available kits including, but not limited to Plasmid Plus (Qiagen), GenJET plasmid MaxiPrep (Thermo) and PureYield MaxiPrep (Promega) kits as well as with commercially available reagents.

Various exemplary embodiments of the cationic lipids of the present disclosure, lipid nanoparticles and compositions comprising the same, and their use to deliver active or therapeutic agents such as nucleic acids to modulate gene and protein expression, are described in further detail below.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open and inclusive sense, that is, as "including, but not limited to".

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this disclosure belongs. As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

The phrase "induce expression of a desired protein" refers to the ability of a nucleic acid to increase expression of the desired protein. To examine the extent of protein expression, a test sample (*e.g.*, a sample of cells in culture expressing the desired protein) or a test mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or a non-human primate (*e.g.*, monkey) model) is contacted with a nucleic acid (*e.g.*, nucleic acid in combination with a lipid of the present disclosure). Expression of the desired protein in the test sample or test animal is compared to expression of the desired protein in a control sample (*e.g.*, a sample of cells in culture expressing the desired protein) or a control mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or non-human primate (*e.g.*, monkey) model) that is not contacted with or administered the nucleic acid. When the desired protein is present in a control sample or a control mammal, the expression of a desired protein in a control sample or a control mammal may be assigned a value of 1.0. In particular embodiments, inducing expression of a desired protein is achieved when the ratio of desired protein expression in the test sample or the test mammal to the level of desired protein expression in the control sample or the control mammal is greater than 1, for example, about 1.1, 1.5, 2.0, 5.0 or 10.0. When a desired protein is not present in a control sample or a control mammal, inducing expression of a desired protein is achieved when any measurable level of the desired protein in the test sample or the test mammal is detected. One of ordinary skill in the art will understand appropriate assays to determine the level of protein expression in a sample, for example dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, and phenotypic assays, or assays based on reporter proteins that can produce fluorescence or luminescence under appropriate conditions.

The phrase "inhibiting expression of a target gene" refers to the ability of a nucleic acid to silence, reduce, or inhibit the expression of a target gene. To examine the extent of gene silencing, a test sample (*e.g.*, a sample of cells in culture expressing the target gene) or a test mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or a non-human primate (*e.g.*, monkey) model) is contacted with a nucleic acid that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (*e.g.*, a sample of cells in culture expressing the target gene) or a control mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or non-human primate (*e.g.*, monkey) model) that is not contacted with or administered the nucleic acid. The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%. In particular

embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of target gene expression in the control sample or the control mammal is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the nucleic acids are capable of silencing, reducing, or inhibiting the expression of a target gene by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the nucleic acid. Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.*, dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as a therapeutic nucleic acid is an amount sufficient to produce the desired effect, *e.g.*, an increase or inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the nucleic acid. An increase in expression of a target sequence is achieved when any measurable level is detected in the case of an expression product that is not present in the absence of the nucleic acid. In the case where the expression product is present at some level prior to contact with the nucleic acid, an increase in expression is achieved when the fold increase in value obtained with a nucleic acid such as mRNA relative to control is about 1.05, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 5000, 10000 or greater. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with a nucleic acid such as antisense oligonucleotide relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, fluorescence or luminescence of suitable reporter proteins, as well as phenotypic assays known to those of skill in the art.

The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes

DNA, RNA, and hybrids thereof. DNA may be in the form of antisense molecules, plasmid DNA, cDNA, PCR products, or vectors. RNA may be in the form of small hairpin RNA (shRNA), messenger RNA (mRNA), antisense RNA, miRNA, micRNA, multivalent RNA, dicer substrate RNA or viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res., 19:5081 (1991); Ohtsuka et al., J. Biol. Chem., 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

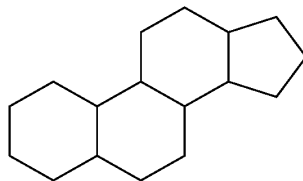
The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple

lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

A "steroid" is a compound comprising the following carbon skeleton:



5 Non-limiting examples of steroids include cholesterol, and the like.

A "cationic lipid" refers to a lipid capable of being positively charged. Exemplary cationic lipids include one or more amine group(s) which bear the positive charge. Preferred cationic lipids are ionizable such that they can exist in a positively charged or neutral form depending on pH. The ionization of the cationic lipid affects the surface charge of the lipid
10 nanoparticle under different pH conditions. This charge state can influence plasma protein absorption, blood clearance and tissue distribution (Semple, S.C., et al., Adv. Drug Deliv Rev 32:3-17 (1998)) as well as the ability to form endosomolytic non-bilayer structures (Hafez, I.M., et al., Gene Ther. 8:1188-1196 (2001)) critical to the intracellular delivery of nucleic acids.

The term "lipid nanoparticle" refers to particles having at least one dimension on the
15 order of nanometers (*e.g.*, 1-1,000 nm) which include one or more of the compounds of formula (I) or other specified cationic lipids. In some embodiments, lipid nanoparticles are included in a formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA) to a target site of interest (*e.g.*, cell, tissue, organ, tumor, and the like). In some embodiments, the lipid nanoparticles of the disclosure comprise a nucleic acid. Such lipid
20 nanoparticles typically comprise a compound of Structure (I) and one or more excipient selected from neutral lipids, charged lipids, steroids and polymer conjugated lipids. In some embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other
25 undesirable effects induced by the mechanisms of the host organism or cells *e.g.*, an adverse immune response.

In various embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from
30 about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from

about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In certain embodiments, nucleic acids, when present in the lipid nanoparticles, are resistant in aqueous solution to degradation with a nuclease. Lipid nanoparticles comprising nucleic acids and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 2004/0142025, 2007/0042031 and PCT Pub. Nos. WO 2013/016058 and WO 2013/086373, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

As used herein, "lipid encapsulated" refers to a lipid nanoparticle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA), with full encapsulation, partial encapsulation, or both. In an embodiment, the nucleic acid (*e.g.*, mRNA) is fully encapsulated in the lipid nanoparticle.

The term "polymer conjugated lipid" refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid. The term "pegylated lipid" refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art and include 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) and the like.

The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, but are not limited to, phosphatidylcholines such as 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), phosphatidylethanolamines such as 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), sphingomyelins (SM), ceramides, steroids such as sterols and their derivatives. Neutral lipids may be synthetic or naturally derived.

The term "charged lipid" refers to any of a number of lipid species that exist in either a positively charged or negatively charged form independent of the pH within a useful physiological range *e.g.*, pH ~3 to pH ~9. Charged lipids may be synthetic or naturally derived. Examples of charged lipids include phosphatidylserines, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, sterol hemisuccinates, dialkyl trimethylammonium-propanes, (*e.g.*, DOTAP, DOTMA), dialkyl dimethylaminopropanes, ethyl phosphocholines, dimethylaminoethane carbamoyl sterols (*e.g.*, DC-Chol).

As used herein, the term "aqueous solution" refers to a composition comprising water.

"Serum-stable" in relation to nucleic acid-lipid nanoparticles means that the nucleotide is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a
5 DNase assay, or an RNase assay.

"Systemic delivery," as used herein, refers to delivery of a therapeutic product that can result in a broad exposure of an active agent within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts
10 of the body. Systemic delivery of lipid nanoparticles can be by any means known in the art including, for example, intravenous, intraarterial, subcutaneous, and intraperitoneal delivery. In some embodiments, systemic delivery of lipid nanoparticles is by intravenous delivery.

"Local delivery," as used herein, refers to delivery of an active agent directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a
15 disease site such as a tumor, other target site such as a site of inflammation, or a target organ such as the liver, heart, pancreas, kidney, and the like. Local delivery can also include topical applications or localized injection techniques such as intramuscular, subcutaneous, or intradermal injection. Local delivery does not preclude a systemic pharmacological effect.

"Alkyl" refers to a straight or branched hydrocarbon chain radical consisting solely of
20 carbon and hydrogen atoms that is saturated (*i.e.*, contains no double and/or triple bonds), having from one to twenty-four carbon atoms (C₁-C₂₄ alkyl), one to twelve carbon atoms (C₁-C₁₂ alkyl), one to eight carbon atoms (C₁-C₈ alkyl) or one to six carbon atoms (C₁-C₆ alkyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, methyl, ethyl, n-propyl, 1-methylethyl (iso propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), 3-methylhexyl, 2-methylhexyl, and
25 the like. Unless stated otherwise specifically in the specification, an alkyl group is optionally substituted.

"Cycloalkyl" or "carbocyclic ring" refers to a stable non-aromatic monocyclic or polycyclic hydrocarbon radical consisting solely of carbon and hydrogen atoms, which may include fused or bridged ring systems, having from three to fifteen carbon atoms, preferably
30 having from three to ten carbon atoms, and which is saturated or unsaturated and attached to the rest of the molecule by a single bond. Monocyclic radicals include, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. Polycyclic radicals include, for example, adamantyl, norbornyl, decalyl, 7,7-dimethyl bicyclo[2.2.1]heptanyl, and the like.

Unless otherwise stated specifically in the specification, a cycloalkyl group is optionally substituted.

"Alkylene" or "alkylene chain" refers to a straight or branched divalent saturated hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon and hydrogen. In some embodiments, an alkylene chain has from one to twenty-four carbon atoms (C₁-C₂₄ alkylene), one to fifteen carbon atoms (C₁-C₁₅ alkylene), one to twelve carbon atoms (C₁-C₁₂ alkylene), one to eight carbon atoms (C₁-C₈ alkylene), one to six carbon atoms (C₁-C₆ alkylene), four to six carbon atoms (C₄-C₆ alkylene), two to four carbon atoms (C₂-C₄ alkylene), one to two carbon atoms (C₁-C₂ alkylene), *e.g.*, methylene, ethylene, propylene, *n*-butylene, and the like. The alkylene chain is attached to the rest of the molecule through a single bond and to the radical group through a single bond. The points of attachment of the alkylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkylene chain is optionally substituted.

"Alkylene oxide" refers to an alkylene group as defined herein, wherein at least one carbon-carbon bond is replaced with a carbon-oxygen-carbon bond. Examples of alkylene oxides include, ethylene oxide, methylene oxide, propylene oxide and the like. Multiple repeats of alkylene oxide groups are included within the definition of alkylene oxide. For example, polyethylene oxide and ethylene oxides with fewer repeating units, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10 repeating ethylene oxide units are included within alkylene oxide. Unless stated otherwise specifically in the specification, an alkylene oxide is optionally substituted.

"Aryl" refers to a carbocyclic ring system radical comprising hydrogen, 6 to 18 carbon atoms and at least one aromatic ring. For purposes of this disclosure, the aryl radical is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include fused or bridged ring systems. Aryl radicals include, but are not limited to, aryl radicals derived from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene, chrysene, fluoranthene, fluorene, *as*-indacene, *s*-indacene, indane, indene, naphthalene, phenalene, phenanthrene, pleiadene, pyrene, and triphenylene.

"Arylalkyl" refers to a radical of the formula -R_b-R_c where R_b is an alkylene or alkenylene as defined above and R_c is one or more aryl radicals as defined above, for example, benzyl, diphenylmethyl and the like. Unless stated otherwise specifically in the specification, an arylalkyl group is optionally substituted.

The term "substituted" used herein means any of the above groups (*e.g.*, alkyl, cycloalkyl or heterocyclyl) wherein at least one hydrogen atom is replaced by a bond to a non-hydrogen atoms such as, but not limited to: a halogen atom such as F, Cl, Br, and I; oxo groups (=O); hydroxyl groups (-OH); alkoxy groups (-OR^a, where R^a is C₁-C₁₂ alkyl or cycloalkyl); carboxyl groups (-OC(=O)R^a or -C(=O)OR^a, where R^a is H, C₁-C₁₂ alkyl or cycloalkyl); amine groups (-NR^aR^b, where R^a and R^b are each independently H, C₁-C₁₂ alkyl or cycloalkyl); C₁-C₁₂ alkyl groups; and cycloalkyl groups. In some embodiments the substituent is a C₁-C₁₂ alkyl group. In other embodiments, the substituent is a cycloalkyl group. In other embodiments, the substituent is a halo group, such as fluoro. In other embodiments, the substituent is an oxo group. In other
10 embodiments, the substituent is a hydroxyl group. In other embodiments, the substituent is an alkoxy group. In other embodiments, the substituent is a carboxyl group. In other embodiments, the substituent is an amine group.

"Optional" or "optionally" (*e.g.*, optionally substituted) means that the subsequently described event or circumstances may or may not occur, and that the description includes
15 instances where said event or circumstance occurs and instances in which it does not. For example, "optionally substituted alkyl" means that the alkyl radical may or may not be substituted and that the description includes both substituted alkyl radicals and alkyl radicals having no substitution. In some embodiments, "optionally substituted" means a particular radical is substituted with one or more substituents selected from the group consisting of halo (*e.g.*, F, Cl, Br, and I), oxo (=O), hydroxyl (-OH), alkoxy (-OR^a, where R^a is C₁-C₁₂ alkyl), cycloalkoxy (-OR^a, where R^a is C₃-C₈ cycloalkyl), carboxyl (-OC(=O)R^a or -C(=O)OR^a, where R^a is H, C₁-C₁₂ alkyl, or C₃-C₈ cycloalkyl), amine (-NR^aR^b, where R^a and R^b are each independently H, C₁-C₁₂ alkyl, or C₃-C₈ cycloalkyl), C₁-C₁₂ alkyl, and C₃-C₈ cycloalkyl.
20

In some embodiments, "optionally substituted" means substituted with one or more halo substituents. In some embodiments, "optionally substituted" means substituted with one or more oxo substituents. In some embodiments, "optionally substituted" means substituted with one or more hydroxyl substituents. In certain embodiments, "optionally substituted" means substituted with one or more alkoxy substituents. In some embodiments, "optionally substituted" means substituted with one or more cycloalkoxy substituents. In certain embodiments, "optionally
30 substituted" means substituted with one or more carboxy substituents. In some embodiments, "optionally substituted" means substituted with one or more amine substituents. In certain embodiments, "optionally substituted" means substituted with one or more C₁-C₁₂ alkyl

substituents. In some embodiments, "optionally substituted" means substituted with one or more C₃-C₈ cycloalkyl substituents.

When a functional group is described as "optionally substituted," and in turn, substituents on the functional group are also "optionally substituted" and so on, for the purposes of this disclosure, such iterations are limited to five, preferably such iterations are limited to two. In some embodiments, such iterations are limited to one. In some embodiments, such iterations are limited to zero.

This disclosure is also meant to encompass all pharmaceutically acceptable compounds of the compound of Structure (I) being isotopically labelled by having one or more atoms replaced by an atom having a different atomic mass or mass number. Examples of isotopes that can be incorporated into the disclosed compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine, and iodine, such as ²H, ³H, ¹¹C, ¹³C, ¹⁴C, ¹⁵N, ¹⁵N, ¹⁵O, ¹⁷O, ¹⁸O, ³¹P, ³²P, ³⁵S, ¹⁸F, ³⁶Cl, ¹²³I, and ¹²⁵I, respectively. These radio labelled compounds could be useful to help determine or measure the effectiveness of the compounds, by characterizing, for example, the site or mode of action, or binding affinity to pharmacologically important site of action. Certain isotopically labelled compounds of Structure (I), for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, *i.e.*, ³H, and carbon-14, *i.e.*, ¹⁴C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection.

Substitution with heavier isotopes such as deuterium, *i.e.*, ²H, may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as ¹¹C, ¹⁸F, ¹⁵O and ¹³N, can be useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy.

Isotopically labeled compounds of Structure (I) can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the Preparations and Examples as set out below using an appropriate isotopically labeled reagent in place of the non-labeled reagent previously employed.

This disclosure is also meant to encompass the *in vivo* metabolic products of the disclosed compounds. Such products may result from, for example, the oxidation, reduction, hydrolysis, amidation, esterification, and the like of the administered compound, primarily due to enzymatic processes. Accordingly, the disclosure includes compounds produced by a process comprising administering a compound of this disclosure to a mammal for a period sufficient to

yield a metabolic product thereof. Such products are typically identified by administering a radio labelled compound of the disclosure in a detectable dose to an animal, such as rat, mouse, guinea pig, monkey, or to human, allowing sufficient time for metabolism to occur, and isolating its conversion products from the urine, blood, or other biological samples.

- 5 "Stable compound" and "stable structure" are meant to indicate a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into an efficacious therapeutic agent.

"Mammal" includes humans and both domestic animals such as laboratory animals and household pets (*e.g.*, cats, dogs, swine, cattle, sheep, goats, horses, rabbits), and non-domestic
10 animals such as wildlife and the like.

"Pharmaceutically acceptable carrier, diluent or excipient" includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug
15 Administration as being acceptable for use in humans or domestic animals.

"Pharmaceutically acceptable salt" includes both acid and base addition salts.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases, which are not biologically or otherwise undesirable, and which are formed with inorganic acids such as, but are not limited to,
20 hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as, but not limited to, acetic acid, 2,2-dichloroacetic acid, adipic acid, alginic acid, ascorbic acid, aspartic acid, benzenesulfonic acid, benzoic acid, 4-acetamidobenzoic acid, camphoric acid, camphor-10-sulfonic acid, capric acid, caproic acid, caprylic acid, carbonic acid, cinnamic acid, citric acid, cyclamic acid, dodecylsulfuric acid, ethane-1,2-disulfonic acid,
25 ethanesulfonic acid, 2-hydroxyethanesulfonic acid, formic acid, fumaric acid, galactaric acid, gentisic acid, glucoheptonic acid, gluconic acid, glucuronic acid, glutamic acid, glutaric acid, 2-oxo-glutaric acid, glycerophosphoric acid, glycolic acid, hippuric acid, isobutyric acid, lactic acid, lactobionic acid, lauric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, mucic acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid,
30 1-hydroxy-2-naphthoic acid, nicotinic acid, oleic acid, orotic acid, oxalic acid, palmitic acid, pamoic acid, propionic acid, pyroglutamic acid, pyruvic acid, salicylic acid, 4-aminosalicylic acid, sebacic acid, stearic acid, succinic acid, tartaric acid, thiocyanic acid, *p*-toluenesulfonic acid, trifluoroacetic acid, undecylenic acid, and the like.

"Pharmaceutically acceptable base addition salt" refers to those salts which retain the biological effectiveness and properties of the free acids, which are not biologically or otherwise undesirable. These salts are prepared from addition of an inorganic base or an organic base to the free acid. Salts derived from inorganic bases include, but are not limited to, the sodium, 5 potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Preferred inorganic salts are the ammonium, sodium, potassium, calcium, and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as ammonia, 10 isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, diethanolamine, ethanolamine, deanol, 2-dimethylaminoethanol, 2-diethylaminoethanol, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, benethamine, benzathine, ethylenediamine, glucosamine, methylglucamine, theobromine, triethanolamine, tromethamine, purines, piperazine, piperidine, *N*-ethylpiperidine, polyamine resins and the like. 15 Particularly preferred organic bases are isopropylamine, diethylamine, ethanolamine, trimethylamine, dicyclohexylamine, choline and caffeine.

Often crystallizations produce a solvate of the compound of the disclosure. As used herein, the term "solvate" refers to an aggregate that comprises one or more molecules of a compound of the disclosure with one or more molecules of solvent. The solvent may be water, in 20 which case the solvate may be a hydrate. Alternatively, the solvent may be an organic solvent. Thus, the compounds of the present disclosure may exist as a hydrate, including a monohydrate, dihydrate, hemihydrate, sesquihydrate, trihydrate, tetrahydrate and the like, as well as the corresponding solvated forms. The compound of the disclosure may be true solvates, while in other cases, the compound of the disclosure may merely retain adventitious water or be a mixture 25 of water plus some adventitious solvent.

A "pharmaceutical composition" refers to a formulation of a compound of the disclosure and a medium generally accepted in the art for the delivery of the biologically active compound to mammals, *e.g.*, humans. Such a medium includes all pharmaceutically acceptable carriers, diluents, or excipients therefor.

30 "Effective amount" or "therapeutically effective amount" refers to that amount of a compound of the disclosure which, when administered to a mammal, preferably a human, is sufficient to effect treatment in the mammal, preferably a human. The amount of a lipid nanoparticle of the disclosure which constitutes a "therapeutically effective amount" will vary

depending on the compound, the condition and its severity, the manner of administration, and the age of the mammal to be treated, but can be determined routinely by one of ordinary skill in the art having regard to his own knowledge and to this disclosure.

"Treating" or "treatment" as used herein covers the treatment of the disease or condition of interest in a mammal, preferably a human, having the disease or condition of interest, and includes:

- (i) preventing the disease or condition from occurring in a mammal, in particular, when such mammal is predisposed to the condition but has not yet been diagnosed as having it;
- (ii) inhibiting the disease or condition, *i.e.*, arresting its development;
- 10 (iii) relieving the disease or condition, *i.e.*, causing regression of the disease or condition; or
- (iv) relieving the symptoms resulting from the disease or condition, *i.e.*, relieving pain without addressing the underlying disease or condition. As used herein, the terms "disease" and "condition" may be used interchangeably or may be different in that the particular malady or
- 15 condition may not have a known causative agent (so that etiology has not yet been worked out) and it is therefore not yet recognized as a disease but only as an undesirable condition or syndrome, wherein a more or less specific set of symptoms have been identified by clinicians.

The compounds of the disclosure, or their pharmaceutically acceptable salts may contain one or more asymmetric centers and may thus give rise to enantiomers, diastereomers, and other

20 stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (*R*)- or (*S*)- or, as (*D*)- or (*L*)- for amino acids. The present disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (-), (*R*)- and (*S*)-, or (*D*)- and (*L*)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques, for example, chromatography and fractional

25 crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high-pressure liquid chromatography (HPLC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the

30 compounds include both *E* and *Z* geometric isomers. Likewise, all tautomeric forms are also intended to be included.

A "stereoisomer" refers to a compound made up of the same atoms bonded by the same bonds but having different three-dimensional structures, which are not interchangeable. The

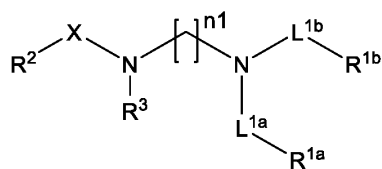
present disclosure contemplates various stereoisomers and mixtures thereof and includes "enantiomers", which refers to two stereoisomers whose molecules are non-superimposable mirror images of one another.

A "tautomer" refers to a proton shift from one atom of a molecule to another atom of the same molecule. The present disclosure includes tautomers of any said compounds.

Compounds

In an aspect, the disclosure provides novel lipid compounds which are capable of combining with other lipid components such as neutral lipids, charged lipids, steroids, and/or polymer conjugated lipids to form lipid nanoparticles with oligonucleotides. Without wishing to be bound by theory, it is thought that these lipid nanoparticles shield oligonucleotides from degradation in the serum and provide for effective delivery of oligonucleotides to cells *in vitro* and *in vivo*.

One embodiment provides a compound having the following Structure (I):



(I)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein:

L^{1a} and L^{1b} are each independently optionally substituted C₃-C₁₂ alkyl;

R^{1a} is -C(=O)OR^{4a} or -O(C=O)R^{4a};

R^{1b} is -C(=O)OR^{4b} or -O(C=O)R^{4b};

R² is -NR⁶(C=O)R⁵, -(C=O)N(R⁶)R⁵ or -(C=O)OR⁷;

R³ and R⁶ are each independently hydrogen or optionally substituted C₁-C₆ alkyl;

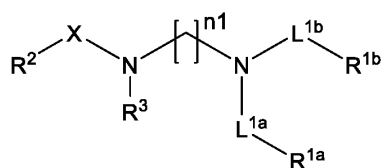
R^{4a}, R^{4b}, and R⁵ are each independently optionally substituted alkyl;

R⁷ is optionally substituted arylalkyl;

n₁ is 2, 3, 4, 5, or 6; and

X is C₂-C₆ alkylene or C₄-C₂₀ alkyleneoxide.

One embodiment provides a compound having the following Structure (I):



(I)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein:

L^{1a} and L^{1b} are each independently optionally substituted C₃-C₁₂ alkyl;

$$R^{1a} \text{ is } -C(=O)OR^{4a} \text{ or } -O(C=O)R^{4a},$$

5 R^{1b} is -C(=O)OR^{4b} or -O(C=O)R^{4b}.

R² is -NR⁶(C=O)R⁵, -(C=O)N(R⁶)R⁵ or -(C=O)OR⁷;

R³ and R⁶ are each independently hydrogen or optionally substituted C₁-C₁₂ alkyl;

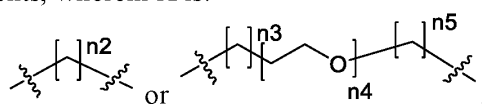
R^{4a}, R^{4b}, and R⁵ are each independently optionally substituted alkyl;

R⁷ is optionally substituted C₁-C₆ alkyl or optionally substituted arylalkyl;

10 n_1 is 2, 3, 4, 5, or 6; and

X is C₂-C₆ alkylene or C₄-C₂₀ alkyleneoxide.

In some embodiments, wherein X is:



wherein:

15 n2 is 2, 3, 4, 5, or 6;

n3 is 0, 1, 2, 3, or 4;

n_4 is 2, 3, or 4; and

n_5 is 2, 3, 4, or 5.

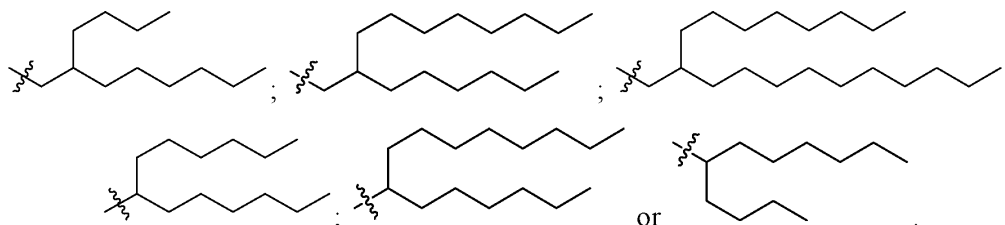
In some embodiments, L^{1a} is C₅-C₉ alkyl. In certain embodiments, L^{1b} is C₅-C₉ alkyl. In some embodiments, L^{1a} is C₅-, C₆-, C₇-, or C₉-alkyl. In certain embodiments, L^{1b} is C₅-, C₆-, C₇-, or C₉-alkyl. In some embodiments, L^{1a} is C₅-alkyl. In certain embodiments, L^{1a} is C₆-alkyl. In some embodiments, L^{1a} is C₇-alkyl. In certain embodiments, L^{1a} is C₉-alkyl. In some embodiments, L^{1b} is C₅-alkyl. In certain embodiments, L^{1b} is C₆-alkyl. In some embodiments, L^{1b} is C₇-alkyl. In certain embodiments, L^{1b} is C₉-alkyl. In some embodiments, L^{1a} is unsubstituted. In certain embodiments, L^{1b} is unsubstituted. In some embodiments, L^{1a} is unbranched. In certain embodiments, L^{1b} is unbranched.

In some embodiments, one of R^{1a} is -O(C=O)R^{4a}. In certain embodiments, one of R^{1a} is -(C=O)OR^{4a}. In some embodiments, R^{1b} is -O(C=O)R^{4b}. In certain embodiments, one of R^{1b} is -(C=O)OR^{4b}.

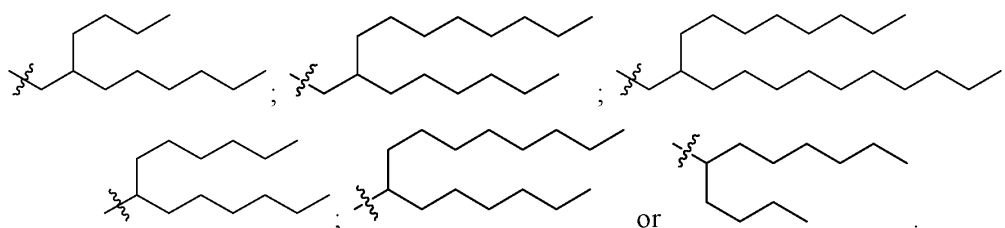
30 In some embodiments, R^{4a} is C₈-C₂₄-alkyl. In certain embodiments, R^{4a} is C₁₀-C₁₈-alkyl. In certain embodiments, R^{4a} is C₁₁-C₁₆-alkyl. In some embodiments, R^{4a} is C₁₁-alkyl. In certain embodiments, R^{4a} is C₁₅-alkyl. In some embodiments, R^{4a} is C₁₆-alkyl. In certain embodiments, R^{4b} is C₈-C₂₄-alkyl. In some embodiments, R^{4b} is C₁₀-C₁₈-alkyl. In certain embodiments, R^{4b} is

C₁₁-C₁₆-alkyl. In some embodiments, R^{4b} is C₁₁-alkyl. In certain embodiments, R^{4b} is C₁₅-alkyl. In some embodiments, R^{4b} is C₁₆-alkyl.

In certain embodiments, R^{4a} is branched. In some embodiments, R^{4b} is branched. In certain embodiments, R^{4a} is unsubstituted. In some embodiments, R^{4b} is unsubstituted. In certain
5 embodiments, R^{4a} has one of the following structures:

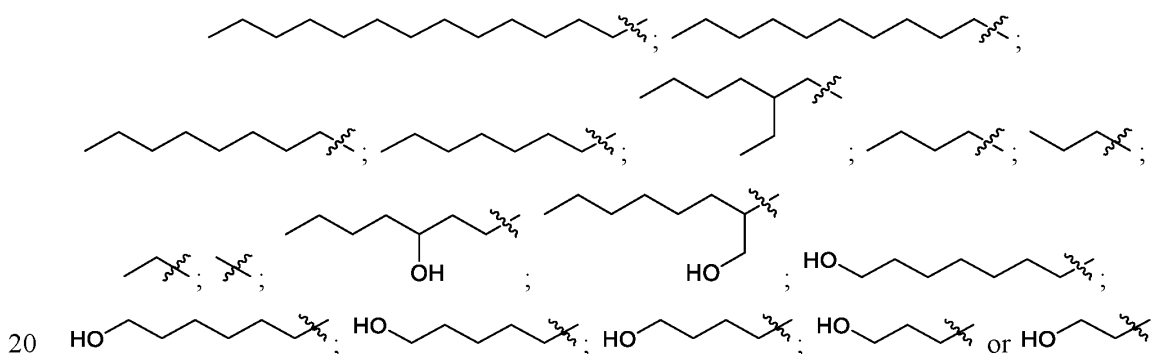


In some embodiments, R^{4b} has one of the following structures:



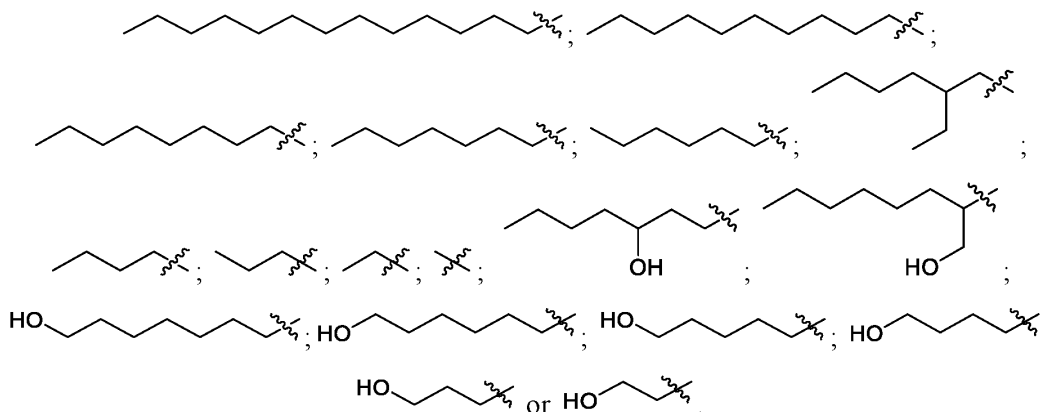
10

In some embodiments, R² is -NR⁶(C=O)R⁵. In certain embodiments, R² is -
(C=O)N(R⁶)R⁵. In some embodiments, R⁵ is C₂-C₁₆-alkyl. In certain embodiments, R⁵ is C₄-C₁₃-
alkyl. In some embodiments, R⁵ is C₄-, C₇-, C₈-, C₁₀-, or C₁₃-alkyl. In certain embodiments, R⁵ is
unsubstituted. In some embodiments, R⁵ is substituted with hydroxyl. In some embodiments, R⁵ is branched. In certain embodiments, R⁵ is unbranched. In some embodiments, R⁵ has one of the
15 following structures:



20

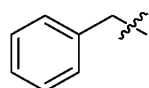
In certain embodiments, R⁵ is unbranched. In some embodiments, R⁵ has one of the following structures:



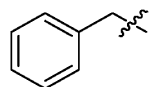
In some embodiments, R⁶ is C₁-C₆ alkyl. In some embodiments, R⁶ is C₁-C₁₀ alkyl. In certain embodiments, R⁶ is C₁-C₄-alkyl. In some embodiments, R⁶ is C₁-, C₂-, C₃-, C₆-, C₈-, or C₁₀-alkyl. In certain embodiments, methyl, ethyl, n-butyl, n-hexyl, n-octyl, or n-decyl. In some embodiments, R⁶ is unbranched. In certain embodiments, R⁶ is methyl or n-butyl. In some
10 embodiments, R⁶ is unsubstituted. In some embodiments, R⁶ is substituted. In some embodiments, R⁶ is C₁-C₆ alkyl substituted with one or more hydroxyl. In some embodiments, R⁶ is C₂-, C₃-, C₄-, or C₆- alkyl substituted with one or more hydroxyl. In certain embodiments, R⁶ is hydrogen. In some embodiments, R² is -(C=O)OR⁷.

In some embodiments, R⁷ is C₁-C₃ alkyl or C₇-C₁₆ arylalkyl. In certain embodiments, R⁷ is C₇-C₁₆ arylalkyl. In some embodiments, R⁷ is C₁-C₃ alkyl. In some embodiments, R⁷ is unsubstituted.

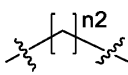
In certain embodiments, R⁷ is -CH₃ or has the following structure:

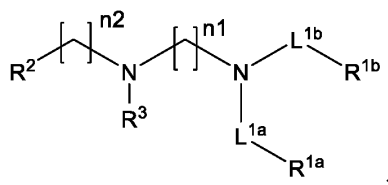


In certain embodiments, $\mathbf{R}^7$ has the following structure:



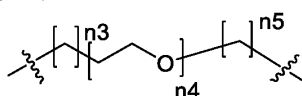
In some embodiments, R³ is optionally substituted C₁-C₆ alkyl. In certain embodiments, R³ is optionally substituted methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl. In some embodiments, R³ is optionally substituted methyl. In some embodiments, R³ is C₁-C₆ alkyl substituted with one or more hydroxyl. In some embodiments, R³ is C₂- or C₄-alkyl substituted with one or more hydroxyl. In certain embodiments, R³ is unsubstituted. In some embodiments, R³ is hydrogen.

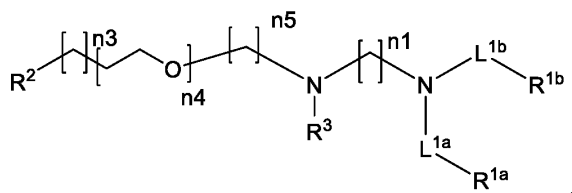
In some embodiments, X is . For example, in certain aspects the compound has the following structure (II):



(II)

- 5 or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof. For example, in some of these embodiments n2 is 3, 4, or 5.

In other embodiments X is . In some such embodiments, the compound has the following structure (III):



(III)

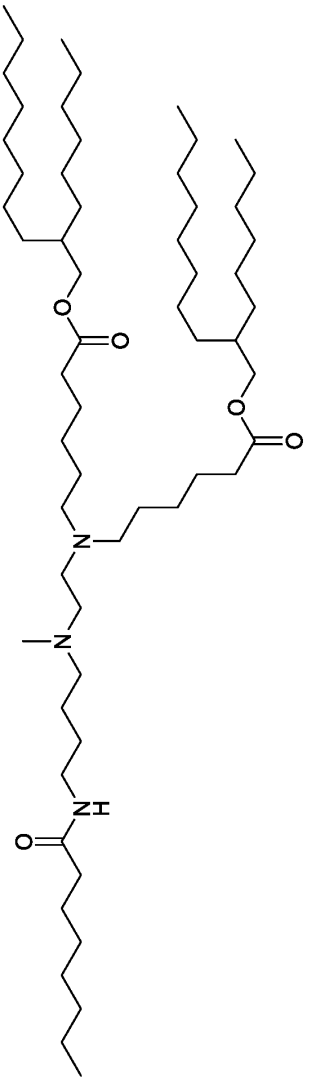
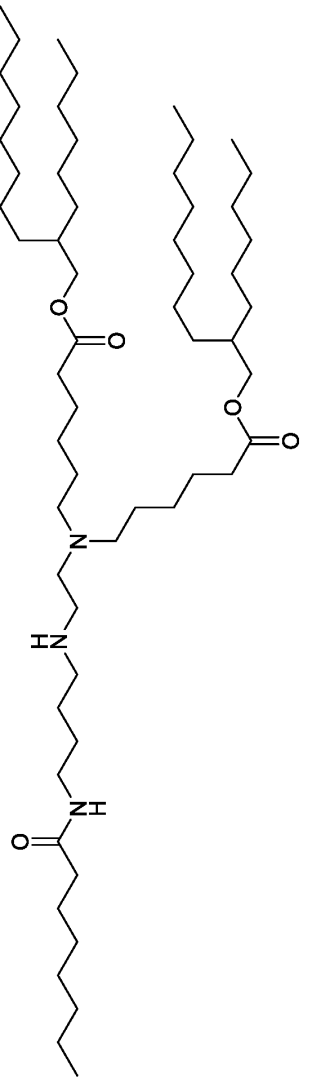
10

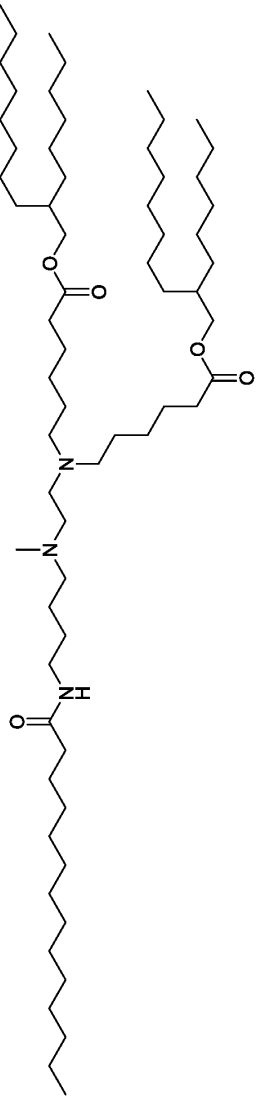
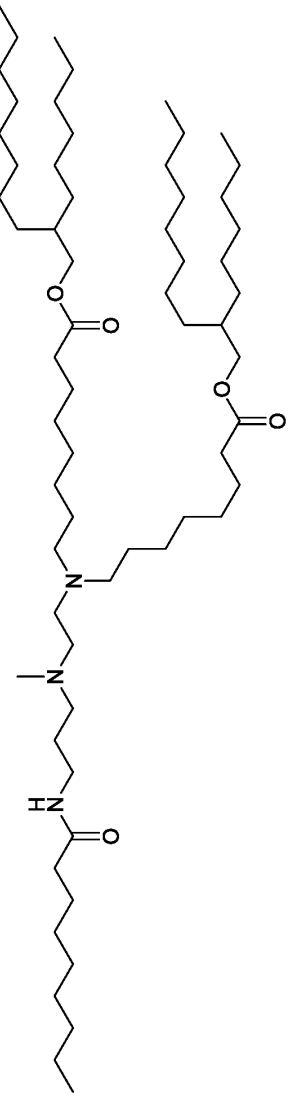
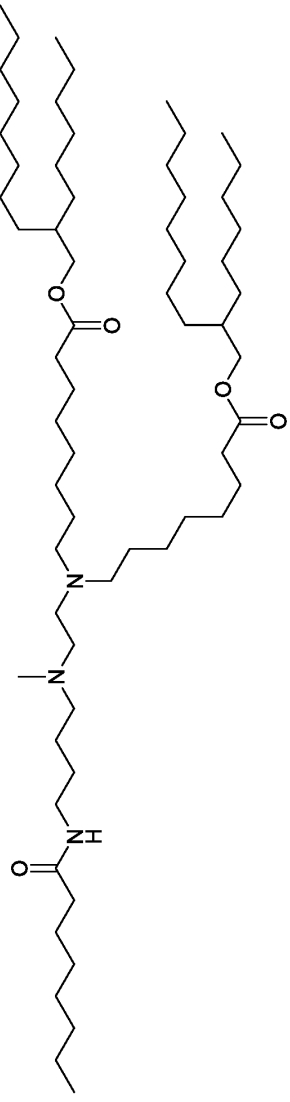
or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof. In different of these embodiments, n3 is 0 or 1. In other embodiments, n4 is 2 or 3. In some other different embodiments, n5 is 3.

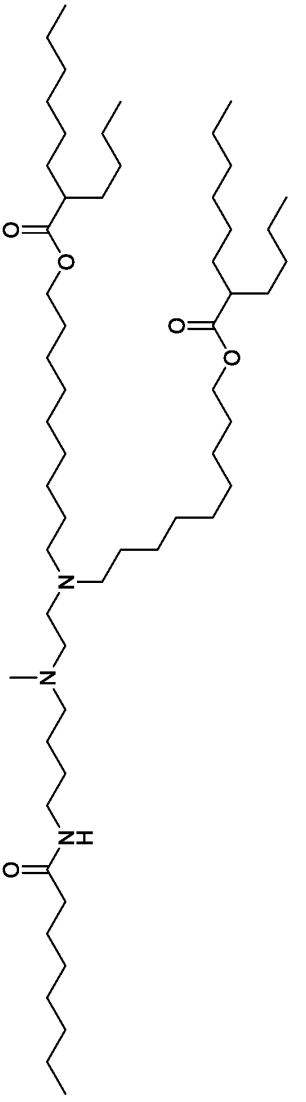
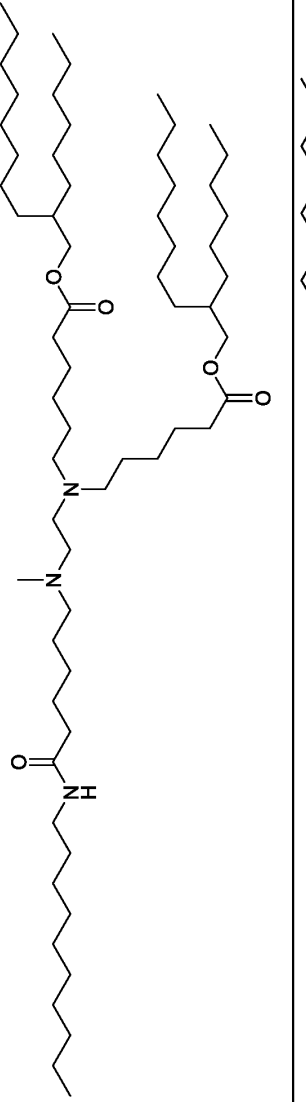
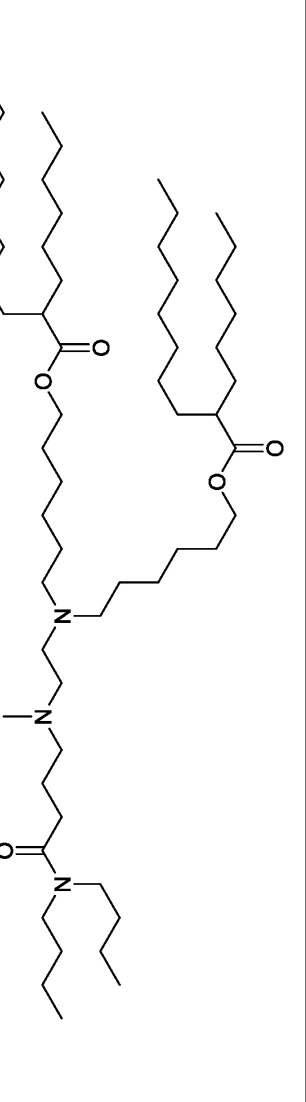
In some embodiments, n1 is 3, 4, or 5. In certain embodiments, n1 is 2.

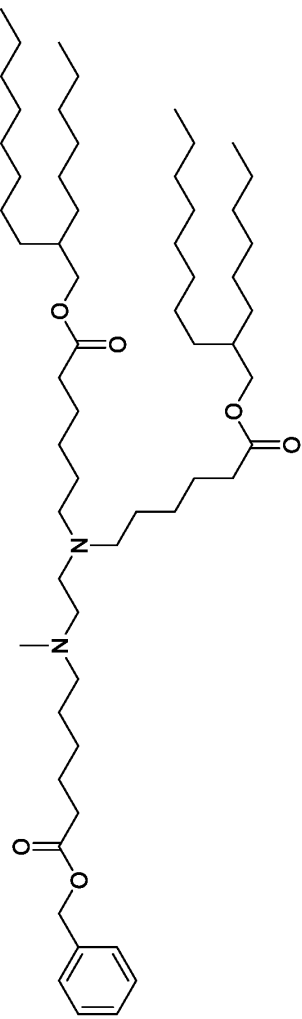
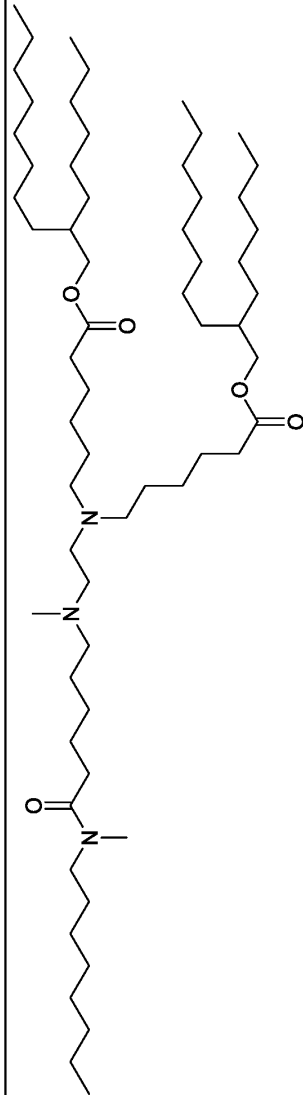
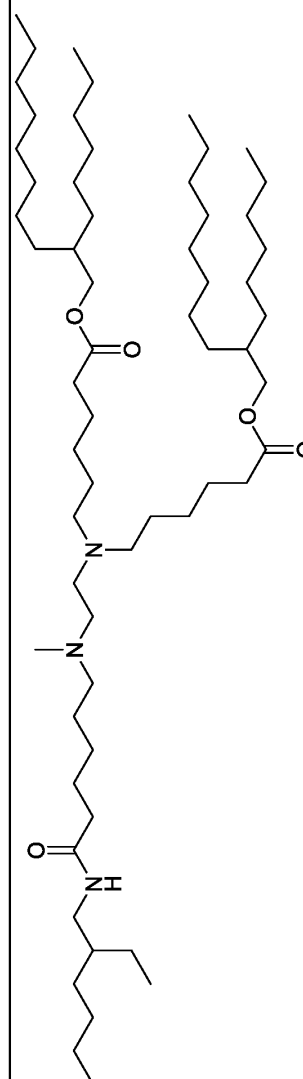
- 15 In some embodiments, the compound has one of the structures set forth in Table 1 below or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof.

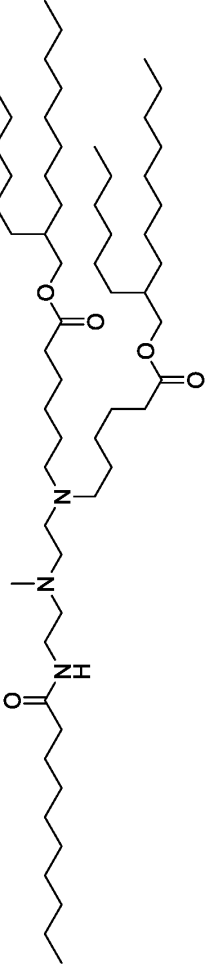
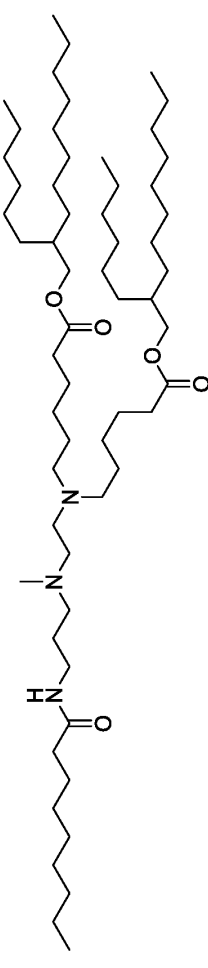
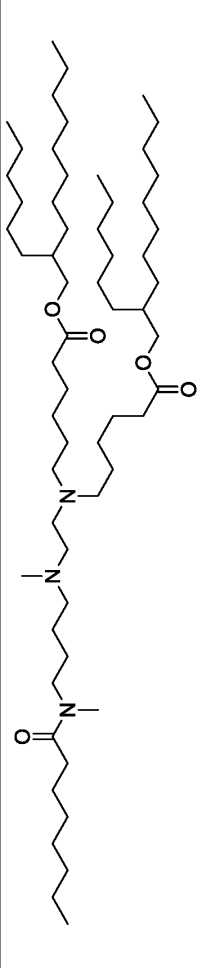
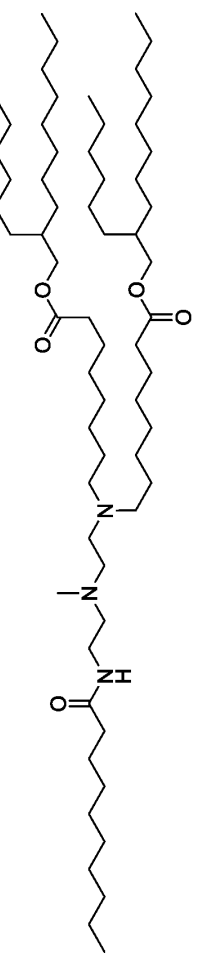
Table 1. Representative Compounds of Structure (I)

No.	Structure
I-1	 Chemical structure of compound I-1: A central tertiary amine nitrogen atom is bonded to a methyl group, a 1-octan-1-yl group, and a 1-octan-1-yl group. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge.
I-2	 Chemical structure of compound I-2: A central tertiary amine nitrogen atom is bonded to a methyl group, a 1-octan-1-yl group, and a 1-octan-1-yl group. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge. The nitrogen atom is also bonded to a 1-octan-1-yl group via a methylene bridge.

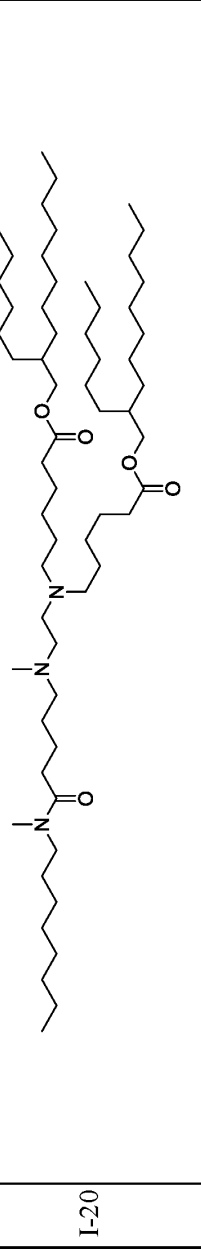
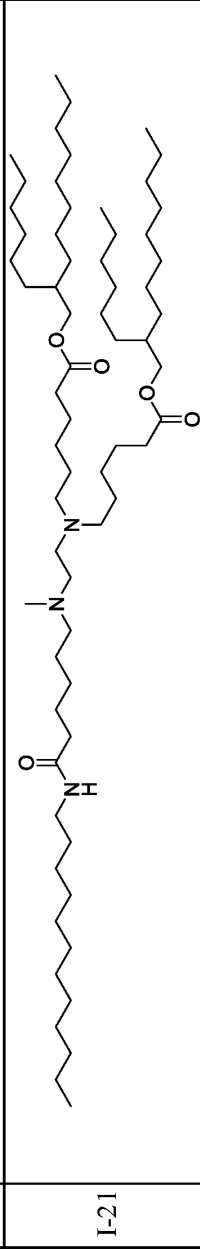
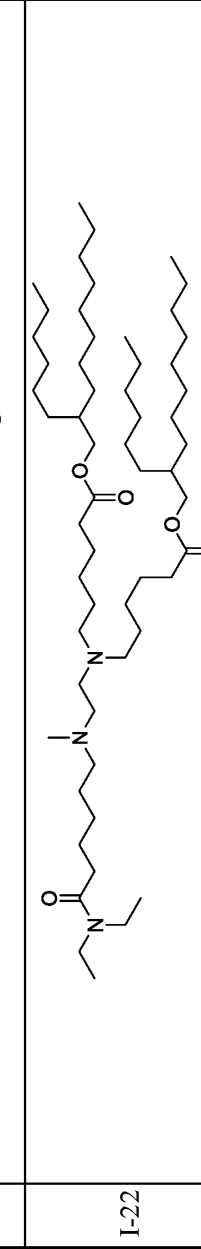
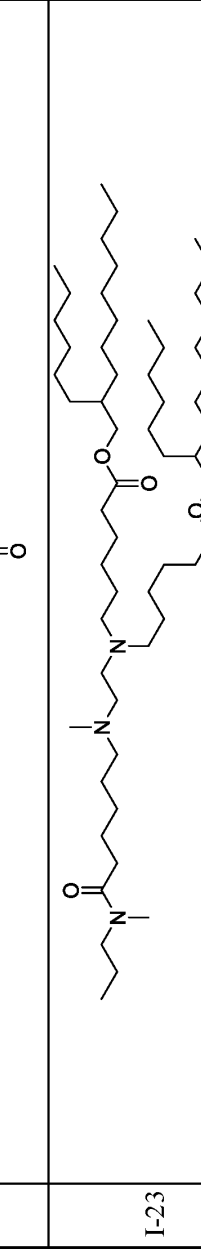
No.	Structure
I-3	
I-4	
I-5	

No.	Structure
I-6	
I-7	
I-8	

No.	Structure
I-9	
I-10	
I-11	

Structure	
I-12	
I-13	
I-14	
I-15	

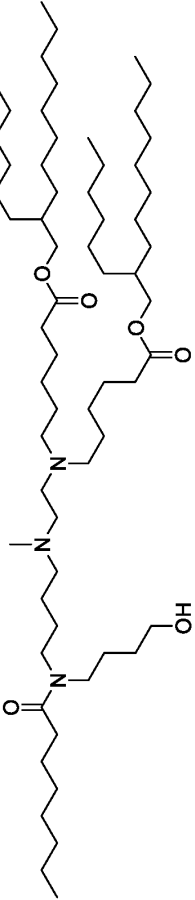
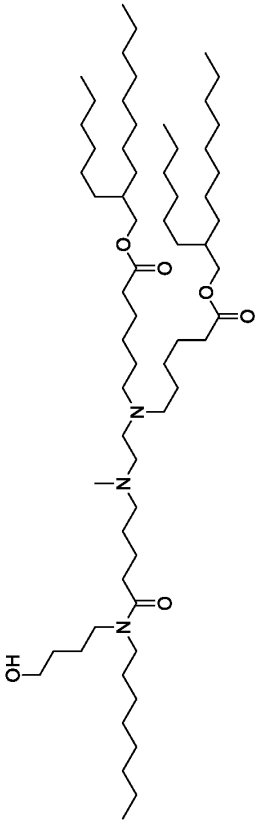
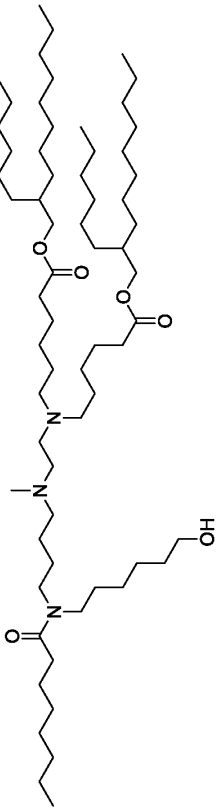
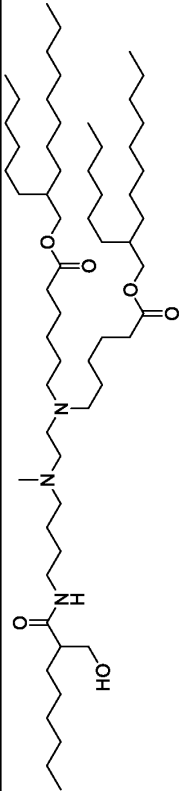
Structure	
I-16	
I-17	
I-18	
I-19	

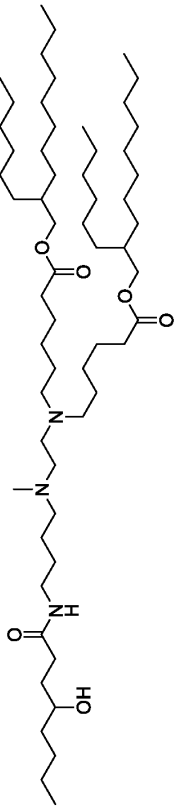
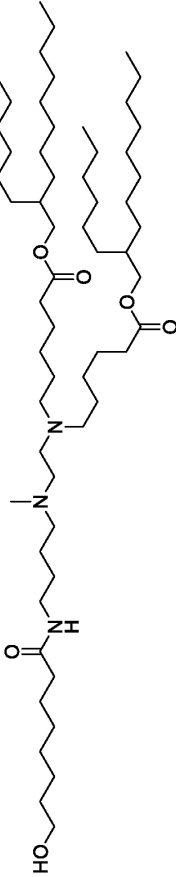
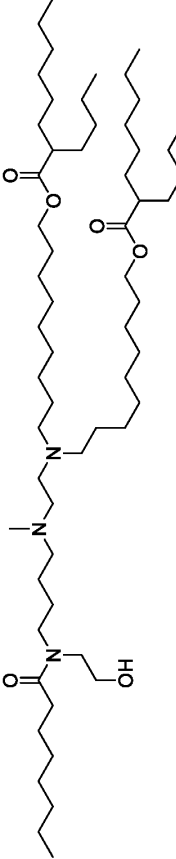
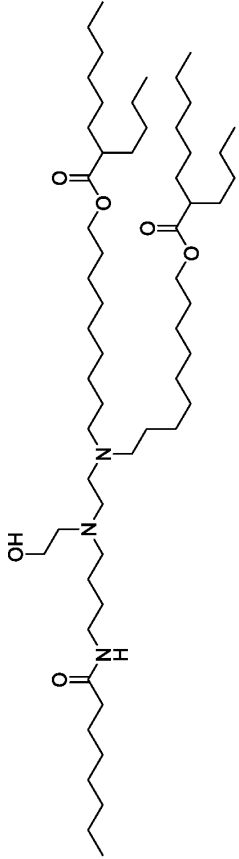
Structure	
I-20	
I-21	
I-22	
I-23	

Structure	
I-24	
I-25	
I-26	
I-27	

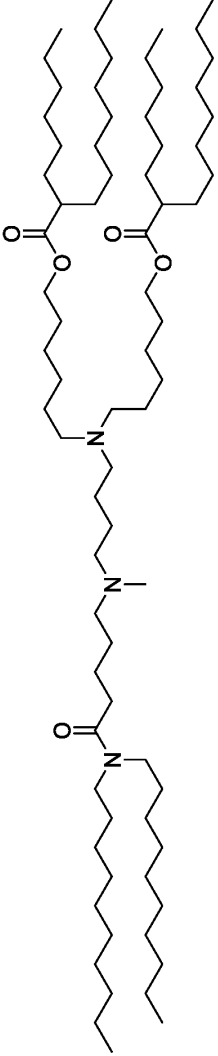
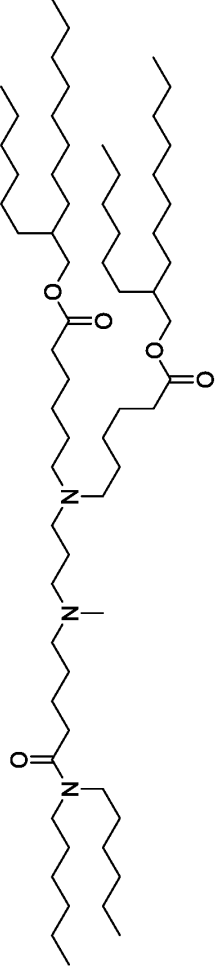
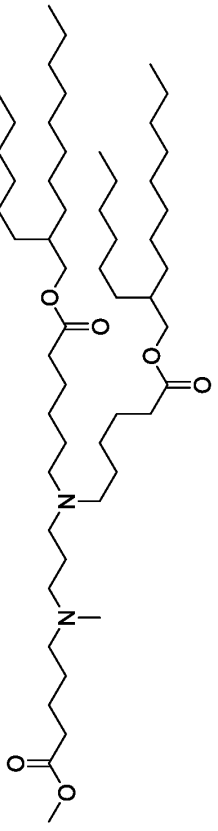
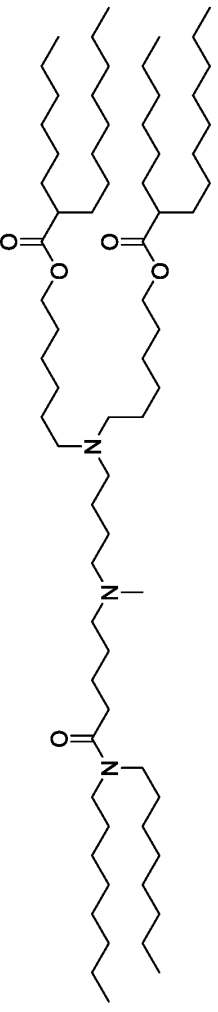
Structure	
I-28	
I-29	
I-30	
I-31	

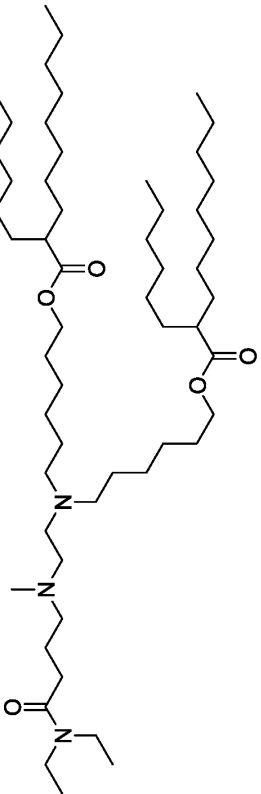
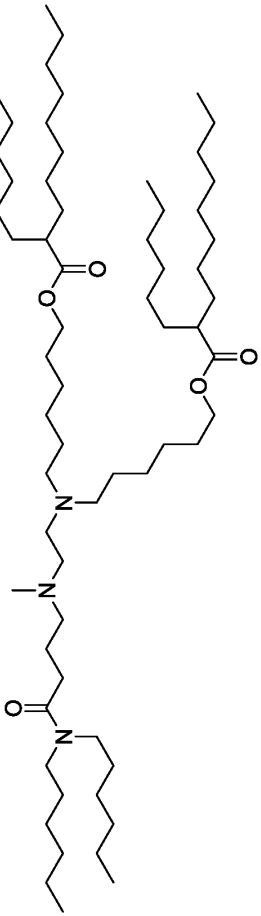
No.	Structure
I-32	
I-33	
I-34	
I-35	

No.	Structure
I-36	
I-37	
I-38	
I-39	

Structure	
I-40	
I-41	
I-42	
I-43	

No.	Structure
I-44	
I-45	
I-46	
I-47	

Structure	
I-48	
I-49	
I-50	
I-51	

No.	Structure
I-52	
I-53	

It is understood that any embodiment of the compounds of Structure (I), as set forth above, and any specific substituent and/or variable in the compound Structure (I), as set forth above, may be independently combined with other embodiments and/or substituents and/or variables of compounds of Structure (I) to form embodiments of the disclosure not specifically set forth above. In addition, in the event that a list of substituents and/or variables is listed for any particular R group, L group, or variables n1-n2 in a particular embodiment and/or claim, it is understood that each individual substituent and/or variable may be deleted from the particular embodiment and/or claim and that the remaining list of substituents and/or variables will be considered to be within the scope of embodiments of the disclosure.

It is understood that in the present description, combinations of substituents and/or variables of the depicted formulae are permissible only if such contributions result in stable compounds.

For the purposes of administration, the compounds of the present disclosure (typically in the form of lipid nanoparticles in combination with a therapeutic agent) may be administered as a raw chemical or may be formulated as pharmaceutical compositions. Pharmaceutical compositions of the present disclosure comprise a compound of Structure (I) and one or more pharmaceutically acceptable carrier, diluent, or excipient. The compound of Structure (I) is present in the composition in an amount which is effective to form a lipid nanoparticle and deliver the therapeutic agent, *e.g.*, for treating a particular disease or condition of interest.

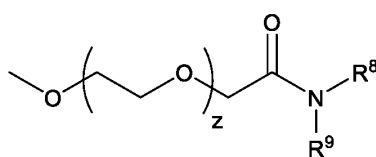
Appropriate concentrations and dosages can be readily determined by one skilled in the art.

An embodiment provides a composition comprising a compound of Structure (I) and a therapeutic agent. In some embodiments, the composition further comprises one or more excipient selected from neutral lipids, steroids, and polymer conjugated lipids.

In some embodiments, the therapeutic agent comprises a nucleic acid. In certain embodiments, the nucleic acid is selected from antisense and messenger RNA.

In certain embodiments, the composition comprises one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE, and SM. In some embodiments, the neutral lipid is DSPC. In some embodiments, the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1. In certain embodiments, the steroid is cholesterol. In some embodiments, the molar ratio of the compound to cholesterol ranges from about 2:1 to 1:1. In certain embodiments, the polymer conjugated lipid is a pegylated lipid. In various embodiments, the polymer conjugated lipid is a pegylated lipid. For example, some embodiments include a pegylated diacylglycerol (PEG-DAG) such as

1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanolamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl-1-O-(ω -methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypropylcarbamate such as ω -methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoxyl)propyl)carbamate or 2,3-di(tetradecanoxyl)propyl-N-(ω -methoxy(polyethoxy)ethyl)carbamate. In some embodiments, the molar ratio of the compound to the pegylated lipid ranges from about 100:1 to about 10:1 or from about 100:1 to about 25:1. In some embodiments, the pegylated lipid is PEG-DMG. In certain embodiments, the pegylated lipid has the following Structure (II):



(II)

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R⁸ and R⁹ are each independently an unbranched or branched, alkyl, alkenyl, or alkynyl containing from 10 to 30 carbon atoms, wherein the alkyl, alkenyl, or alkynyl is optionally interrupted by one or more ester bonds; and
z has a mean value ranging from 30 to 60.

In some embodiments, R⁸ and R⁹ are each independently unbranched alkyl chains containing from 12 to 16 carbon atoms. In some embodiments, the average z is about 45 (*e.g.*, 43, 44, 45, 46, or 47). In some embodiments, the average z is about 43-47. In some embodiments, the average z is about 40-50.

Synthesis of pegylated lipids can be found in US Patent No. 9,738,593, the disclosure of which is hereby incorporated by reference.

Administration of the compositions of the disclosure can be carried out via any of the accepted modes of administration of agents for serving similar utilities. The pharmaceutical compositions of the disclosure may be formulated into preparations in solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suspensions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Typical routes of administering such pharmaceutical compositions include, without limitation, oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intradermal, intrasternal injection or infusion techniques. Pharmaceutical compositions of the

disclosure are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of the disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington: The Science and Practice of Pharmacy*, 20th Edition (Philadelphia College of Pharmacy and Science, 2000). The composition to be administered will, in any event, contain a therapeutically effective amount of a compound of the disclosure, or a pharmaceutically acceptable salt thereof, for treatment of a disease or condition of interest in accordance with the teachings of this disclosure.

A pharmaceutical composition of the disclosure may be in the form of a solid or liquid. In one aspect, the carrier(s) are particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) may be liquid, with the compositions being, for example, an oral syrup, injectable liquid, or an aerosol, which is useful in, for example, inhalatory administration.

When intended for oral administration, the pharmaceutical composition is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the pharmaceutical composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer, or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, gum tragacanth or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent.

When the pharmaceutical composition is in the form of a capsule, for example, a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or oil.

The pharmaceutical composition may be in the form of a liquid, for example, an elixir, syrup, solution, emulsion, or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred composition contain, in addition to the present compounds, one or more of a sweetening agent,

preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer, and isotonic agent may be included.

The liquid pharmaceutical compositions of the disclosure, whether they be solutions,
5 suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such
10 as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose; agents to act as cryoprotectants such as sucrose or trehalose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable
15 pharmaceutical composition is preferably sterile.

A liquid pharmaceutical composition of the disclosure intended for either parenteral or oral administration should contain an amount of a compound of the disclosure such that a suitable dosage will be obtained.

The pharmaceutical composition of the disclosure may be intended for topical
20 administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a
25 transdermal patch or iontophoresis device.

The pharmaceutical composition of the disclosure may be intended for rectal administration, in the form, for example, of a suppository, which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter, and
30 polyethylene glycol.

The pharmaceutical composition of the disclosure may include various materials, which modify the physical form of a solid or liquid dosage unit. For example, the composition may include materials that form a coating shell around the active ingredients. The materials that form

the coating shell are typically inert, and may be selected from, for example, sugar, shellac, and other enteric coating agents. Alternatively, the active ingredients may be encased in a gelatin capsule.

The pharmaceutical composition of the disclosure in solid or liquid form may include an agent that binds to the compound of the disclosure and thereby assists in the delivery of the compound. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, or a protein.

The pharmaceutical composition of the disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of compounds of the disclosure may be delivered in single phase, bi-phasic, or tri-phasic systems to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, sub-containers, and the like, which together may form a kit. One skilled in the art, without undue experimentation may determine preferred aerosols.

The pharmaceutical compositions of the disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining the lipid nanoparticles of the disclosure with sterile, distilled water or other carrier so as to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of the disclosure to facilitate dissolution or homogeneous suspension of the compound in the aqueous delivery system.

The compositions of the disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific therapeutic agent employed; the metabolic stability and length of action of the therapeutic agent; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy.

Compositions of the disclosure may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation of a composition of the disclosure and one or more additional active agents, as well as administration of the composition of the disclosure and each active agent in its own separate pharmaceutical dosage formulation. For

example, a composition of the disclosure and the other active agent can be administered to the patient together in a single oral dosage composition such as a tablet or capsule, or each agent administered in separate oral dosage formulations. Where separate dosage formulations are used, the compounds of the disclosure and one or more additional active agents can be administered at
5 essentially the same time, *i.e.*, concurrently, or at separately staggered times, *i.e.*, sequentially; combination therapy is understood to include all these regimens.

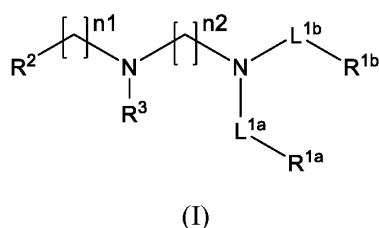
Preparation methods for the above compounds and compositions are described herein below and/or known in the art.

It will be appreciated by those skilled in the art that in the process described herein the
10 functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include *t*-butoxycarbonyl,
15 benzyloxycarbonyl, and the like. Suitable protecting groups for mercapto include -C(O)-R" (where R" is alkyl, aryl or arylalkyl), *p*-methoxybenzyl, trityl and the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and
20 P.G.M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

It will also be appreciated by those skilled in the art, although such protected derivatives of compounds of this disclosure may not possess pharmacological activity as such, they may be
25 administered to a mammal and thereafter metabolized in the body to form compounds of the disclosure which are pharmacologically active. Such derivatives may therefore be described as "prodrugs". All prodrugs of compounds of this disclosure are included within the scope of the disclosure.

Furthermore, all compounds of the disclosure which exist in free base or acid form can be
30 converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to one skilled in the art. Salts of the compounds of the disclosure can be converted to their free base or acid form by standard techniques.

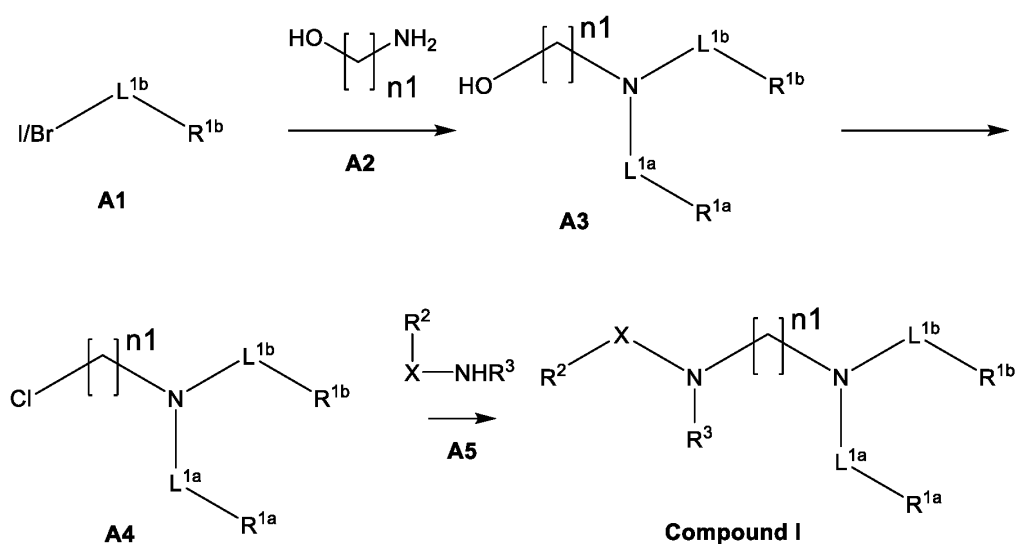
The following Reaction Scheme illustrates methods to make compounds of this disclosure, *i.e.*, compounds of Structure (I):



- 5 wherein R^{1a} , R^{1b} , R^2 , R^3 , L^{1a} , L^{1b} , $n1$, and $n2$ are as defined herein. It is understood that one skilled in the art may be able to make these compounds by similar methods or by combining other methods known to one skilled in the art. It is also understood that one skilled in the art would be able to make, in a similar manner as described below, other compounds of Structure (I) not specifically illustrated below by using the appropriate starting components and modifying the
- 10 parameters of the synthesis as needed. In general, starting components may be obtained from sources such as Sigma Aldrich, Lancaster Synthesis, Inc., Maybridge, Matrix Scientific, TCI, and Fluorochem USA, etc. or synthesized according to sources known to those skilled in the art (see, *e.g.*, Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, 5th edition (Wiley, December 2000)) or prepared as described in this disclosure.

15

GENERAL REACTION SCHEME 1



Embodiments of the compounds of Structure (I) can be prepared according to General Reaction Scheme 1 ("Method A"). R^{1a} , R^{1b} , R^2 , R^3 , L^{1a} , L^{1b} , and X in General reaction Scheme 1 are as defined herein.

Referring to General Reaction Scheme 1, compound starting materials A1 can be purchased from commercial sources or prepared according to methods familiar to one of ordinary skill in the art. Reaction of A1 under appropriate base conditions (*e.g.*, potassium carbonate and cesium carbonate) with compound A2 was heated at 70 °C to afford the compound A3. A3 is then treated with a dropwise addition of thionyl chloride in an ice bath to convert the hydroxyl group into a chlorine compound A4, which is then reacted with amine A5 using appropriate conditions (*e.g.*, heat) to yield a compound of structure (I) as shown.

It should be noted that various alternative strategies for preparation of compounds of structure (I) are available to those of ordinary skill in the art. For example, other compounds of structure (I) can be prepared according to analogous methods using the appropriate starting material and as shown in the Examples that follow. The use of protecting groups as needed and other modification to the above General Reaction Scheme will be readily apparent to one of ordinary skill in the art. The following examples are provided for purpose of illustration and not limitation.

15

EXAMPLE 1

LUCIFERASE MRNA IN VIVO EVALUATION USING LIPID NANOPARTICLE COMPOSITIONS

A lipid of structure (I), DSPC, cholesterol and PEG-lipid are solubilized in ethanol at a molar ratio of 50:10:38.5:1.5 or 47.5:10:40.7:1.8. Lipid nanoparticles (LNP) are prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 40:1. Briefly, the mRNA is diluted to 0.2 mg/mL in 10 to 50 mM citrate buffer, pH 4 or 10 to 25 mM acetate buffer, pH 4. Syringe pumps are used to mix the ethanolic lipid solution with the mRNA aqueous solution at a ratio of about 1:5 to 1:3 (vol/vol) with total flow rates above 15 mL/min. The ethanol is then removed, and the external buffer replaced with PBS by dialysis. Finally, the lipid nanoparticles are filtered through a 0.2 µm pore sterile filter.

Studies are performed in 6–8-week-old female C57BL/6 mice (Charles River) or 8–10-week-old CD-1 (Harlan) mice (Charles River) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC). Varying doses of mRNA-lipid nanoparticle are systemically administered by tail vein injection and animals euthanized at a specific time point (*e.g.*, 4 hours) post-administration. Liver and spleen are collected in pre-weighed tubes, weights determined, immediately snap frozen in liquid nitrogen, and stored at -80 °C until processing for analysis.

For liver, approximately 50 mg is dissected for analyses in a 2 mL FastPrep tubes (MP Biomedicals, Solon OH). ¼" ceramic sphere (MP Biomedicals) is added to each tube and 500 µL of Glo Lysis Buffer – GLB (Promega, Madison WI) equilibrated to room temperature is added to liver tissue. Liver tissues are homogenized with the FastPrep24 instrument (MP Biomedicals) at 2×6.0 m/s for 15 seconds. Homogenate is incubated at room temperature for 5 minutes prior to a 1:4 dilution in GLB and assessed using SteadyGlo Luciferase assay system (Promega). Specifically, 50 µL of diluted tissue homogenate is reacted with 50 µL of SteadyGlo substrate, shaken for 10 seconds followed by 5-minute incubation and then quantitated using a CentroXS³ LB 960 luminometer (Berthold Technologies, Germany). The amount of protein assayed is determined by using the BCA protein assay kit (Pierce, Rockford, IL). Relative luminescence units (RLU) are then normalized to total µg protein assayed. To convert RLU to ng luciferase a standard curve is generated with QuantiLum Recombinant Luciferase (Promega).

The FLuc mRNA (L-6107) from Trilink Biotechnologies will express a luciferase protein, originally isolated from the firefly, *photinus pyralis*. FLuc is commonly used in mammalian cell culture to measure both gene expression and cell viability. It emits bioluminescence in the presence of the substrate, luciferin. This capped and polyadenylated mRNA is fully substituted with 5-methylcytidine and pseudouridine.

EXAMPLE 2

IMMUNOGLOBULIN G (IGG) mRNA IN VIVO EVALUATION USING

LIPID NANOPARTICLE COMPOSITIONS

A lipid of structure (I), DSPC, cholesterol and PEG-lipid are solubilized in ethanol at a molar ratio of 50:10:38.5:1.5 or 47.5:10:40.7:1.8. Lipid nanoparticles (LNP) are prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 40:1. Briefly, the mRNA is diluted to 0.2 mg/mL in 10 to 50 mM citrate buffer, pH 4 or 10 to 25 mM acetate buffer, pH 4. Syringe pumps are used to mix the ethanolic lipid solution with the mRNA aqueous solution at a ratio of about 1:5 to 1:3 (vol/vol) with total flow rates above 15 mL/min. The ethanol is then removed, and the external buffer replaced with PBS by dialysis. Finally, the lipid nanoparticles are filtered through a 0.2 µm pore sterile filter.

Studies are performed in 6–8-week-old CD-1/ICR mice (Envigo) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC). Varying doses of mRNA-lipid nanoparticle are systemically administered by tail vein injection and animals euthanized at a specific time point (e.g., 24 hours) post-

administration. The whole blood is collected, and the serum subsequently separated by centrifuging the tubes of the whole blood at 2000 x g for 10 minutes at 4 °C and stored at -80 °C until use for analysis.

For immunoglobulin G (IgG) ELISA (Life Diagnostics Human IgG ELISA kit), the
5 serum samples are diluted at 100 to 15000 folds with 1 × diluent solution. 100 µL of diluted serum is dispensed into anti-human IgG coated 96-well plate in duplicate alongside human IgG standards and incubated in a plate shaker at 150 rpm at 25 °C for 45 minutes. The wells are washed 5 times with 1x wash solution using a plate washer (400 µL/well). 100 µL of HRP conjugate is added into each well and incubated in a plate shaker at the same condition above.
10 The wells are washed 5 times again with 1 × wash solution using a plate washer (400 µL/well). 100 µL of TMB reagent is added into each well and incubated in a plate shaker at the same condition above. The reaction is stopped by adding 100 µL of Stop solution to each well. The absorbance is read at 450 nm (A450) with a microplate reader. The amount of human IgG in mouse serum is determined by plotting A450 values for the assay standard against human IgG
15 concentration.

EXAMPLE 3

DETERMINATION OF pK_a OF FORMULATED LIPIDS

As described elsewhere, the pK_a of formulated lipids is correlated with the effectiveness of LNPs for delivery of nucleic acids (*see* Jayaraman et al, *Angewandte Chemie, International*
20 *Edition* (2012), 51(34), 8529-8533; Semple et al, *Nature Biotechnology* 28, 172–176 (2010)). In some embodiments, the preferred range of pK_a is ~5 to ~7. The pK_a of each lipid is determined in lipid nanoparticles using an assay based on fluorescence of 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS). Lipid nanoparticles comprising compound of structure (I)/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 or 47.5:10:40.7:1.8 mol%) in PBS at a
25 concentration of 0.4 mM total lipid are prepared using the in-line process as described in Example 1. TNS is prepared as a 100 µM stock solution in distilled water. Vesicles are diluted to 24 µM lipid in 2 mL of buffered solutions containing 10 mM HEPES, 10 mM MES, 10 mM ammonium acetate, and 130 mM NaCl, where the pH ranged from 2.5 to 11. An aliquot of the TNS solution is added to give a final concentration of 1 µM and following vortex mixing
30 fluorescence intensity is measured at room temperature in a SLM Aminco Series 2 Luminescence Spectrophotometer using excitation and emission wavelengths of 321 nm and 445

nm. A sigmoidal best fit analysis is applied to the fluorescence data and the pK_a is measured as the pH giving rise to half-maximal fluorescence intensity.

EXAMPLE 4

DETERMINATION OF EFFICACY OF LIPID NANOPARTICLE FORMULATIONS

CONTAINING VARIOUS CATIONIC LIPIDS USING AN IN VIVO LUCIFERASE/IGG MRNA EXPRESSION RODENT MODEL

Representative compounds of the disclosure shown in Table 2 were formulated using the following molar ratio: 50% cationic lipid / 10% distearoylphosphatidylcholine (DSPC) / 38.5% Cholesterol / 1.5% PEG lipid 2-[2-(ω -methoxy(polyethyleneglycol₂₀₀₀)ethoxy]-*N,N*-ditetradecylacetamide) or 47.5% cationic lipid / 10% DSPC / 40.7% Cholesterol / 1.8% PEG lipid. Activity was determined by measuring luciferase expression in the liver 4 hours following administration via tail vein injection as described in Example 1 or by measuring the amount of human IgG in mouse serum as described in Example 2. The activity was compared at a dose of 1.0 or 0.5 or 0.3 mg mRNA/kg and expressed as ng luciferase/g liver measured 4 hours after administration, as described in Example 1 or as μ g IgG/mL serum measured 24 hours after administration, as described in Example 2. Compound numbers in Table 2 refer to the compound numbers of Table 1.

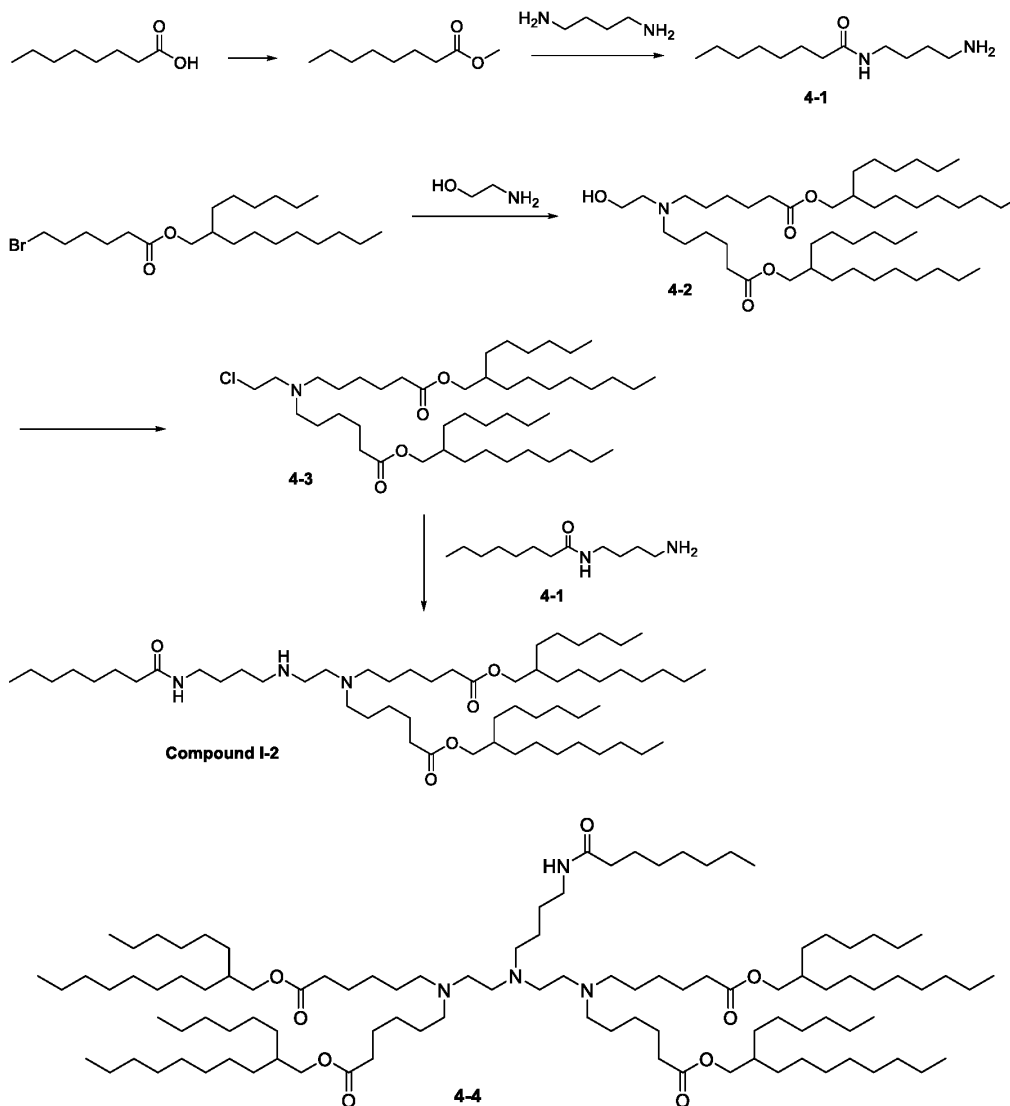
Table 2. Novel Cationic Lipids and Associated Activity

Cmp. No.	pKa	Liver Luc @ 0.3 mg/kg (ng/g)	Liver Luc @ 1.0 mg/kg (ng /g)	Serum IgG @ 0.3 mg/kg (μ g/mL)	Serum IgG @ 1.0 mg/kg (μ g/mL)
I-1	6.64	5497 $\pm$ 1457	13160 $\pm$ 5805	-	-
I-3	6.70	31 $\pm$ 6* Determined at 0.5 mg/kg	-	-	-
I-4	6.70	7747 $\pm$ 1565	24964 $\pm$ 8175	-	-
I-5	6.68	6538 $\pm$ 11243	43609 $\pm$ 9207	-	-
I-6	6.56	12241 $\pm$ 3108	67044 $\pm$ 44033	-	-

Cmp. No.	pKa	Liver Luc @ 0.3 mg/kg (ng/g)	Liver Luc @ 1.0 mg/kg (ng /g)	Serum IgG @ 0.3 mg/kg (µg/mL)	Serum IgG @ 1.0 mg/kg (µg/mL)
I-7	6.57	10812 ± 2335	44836 ± 12026	-	-
I-8	6.13	16110 ± 2654	56927 ± 16125	-	-
I-10	6.33	9794 ± 1750	43271 ± 18103	-	-
I-11	6.27	16601 ± 5871	66075 ± 18868	-	-
I-47	6.45	-	-	15.3 ± 3.7	61.8 ± 11.5
I-48	6.37	-	-	4.4 ± 1.1	21.8 ± 5.0
I-49	6.34	-	-	42.4 ± 11.7	120.6 ± 31.7
I-50	6.46	-	-	15.4 ± 2.6	91.5 ± 19.7
I-51	6.40	-	-	31.9 ± 6.5	131.5 ± 18.1
I-52	6.71	-	-	6.4 ± 2.6	35.4 ± 11.1

EXAMPLE 5

SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-((4-OCTANAMIDOBUTYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-2)



Synthesis of Compound 4-1

To an ice-salt-cooled solution of octanoic acid (50 mmol, 7.21 g, 7.92 mL) in 200 mL of methanol is added slowly (ca 15 min) acetyl chloride (10 mL) under Ar. Stirred for 20 min before removing the cooling bath. The solution is allowed to stand overnight at room temperature (20 h). Methanol was removed under reduced pressure. To the residue was added saturated sodium bicarbonate (100 mL) and hexanes (200 mL). The hexane extract was washed with brine (70 mL), dried over sodium sulfate, filtered, and concentrated to give methyl octanoate as a colorless oil. The methyl octanoate was dried on high vacuum line (oil pump) overnight (6.439 g, 40.7 mmol, 93%) and used next without further purification.

To a solution of 1,4- butanediamine (5 eq. 100 mmol, 8.8 g) in 50 ml of methanol at reflux was slowly (15 min) added methyl octanoate (20 mmol, 3.16 g) in methanol (20 mL). Reflux was continued for 48 h. The solvent was then evaporated under reduced pressure and the residue was taken up in a mixture of water (70 mL) and ethyl acetate (100 mL). A small amount of citric acid was added. The two phases were separated and the aqueous was extracted with ethyl acetate twice. The combined ethyl acetate extracts were washed with water and brine, dried over sodium sulfate and filtered. Concentration gave a mixture of oil and white solid. The residue was dissolved in DCM and MeOH (19:1). The solution was passed through a pad of silica gel (1.5 cm height x 6.5 cm width) and washed with a mixture of MeOH (5%) and DCM until TLC showed that all di-acylated product came out. Then the pad was washed with a mixture of CHCl₃/EtOH/water/NH₃ (30/25/3/2, 225 mL) to elute the desired product **4-1**. Product containing fractions were combined and concentrated to dryness (white solid, 2.52 g, 11.8 mmol, 59%).

Synthesis of Compound 4-2

To a solution of 2-aminoethanol (333 mg, 5.46 mmol) in 35ml of anhydrous THF, 2-hexyldecyl 6-bromohexanoate (1.9 eq, 4.37 g, 10.4 mmol), potassium carbonate (1.9 eq, 1.44 g, 10.4 mmol, MW138.21), cesium carbonate (0.3 eq, 534 mg, 1.64 mmol, MW325.82) and sodium iodide (30 mg) were added and the mixture was heated at 70 C for 6 days in a sealed pressure flask. The solvent was evaporated under reduced pressure and the residue was taken up in a mixture of hexanes and ethyl acetate (ca 6%) and washed with water and brine. The organic layer was separated and dried over anhydrous sodium sulphate. The dried extract was filtered through a pad of silica gel under reduced pressure. The pad then was washed with Hexane - EtOAc-Et₃N (95:5:0 to 80:20:1). The fractions containing the desired product were combined and concentrated to dryness. This gave a colorless oil (1.766 g, 2.39 mmol, 46%).

Synthesis of Compound 4-3

To an ice-cooled solution of **4-2** (2.16 g, 2.93 mmol) of in 8 mL of CHCl₃, was added thionyl chloride (3 eq, 8.79 mmol, 1.05 g) in 35 mL of chloroform dropwise (ca 1-2 min), under an argon atmosphere. After the completion of addition, the ice bath was removed, and the reaction mixture was stirred for 16 h at room temperature (20 C). Concentration of the mixture gave a thick and dark oil. The residue was dissolved in a mixture of chloroform and Et₃N (0.6

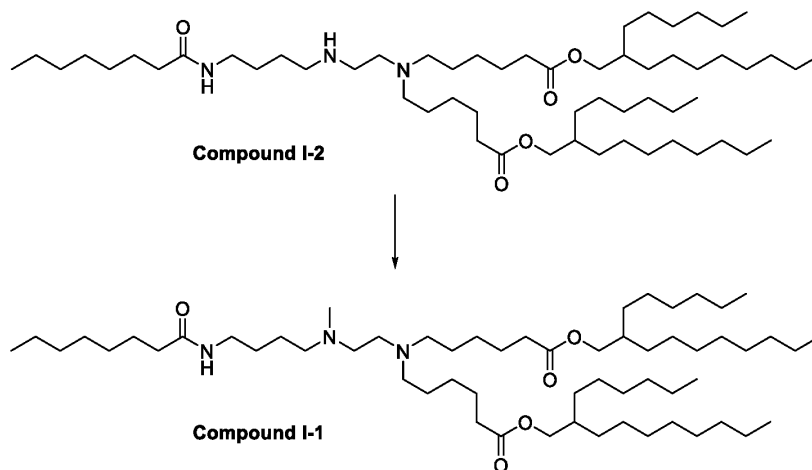
mL) and was purified by flash column chromatography on silica gel (0 to 1% MeOH in chloroform). This gave the desired product as a brown oil (1.786 g, 2.36 mmol, 80%).

Synthesis of Compound I-2

A solution of **4-3** (1 eq, 522 mg, 0.67 mmol), **4-1** (3 eq, 2.07 mmol, 444 mg) and *N,N*-diisopropylethylamine (3 equiv. 2.07 mmol, 267 mg, 0.36 mL; MW129.25, d 0.742) in acetonitrile (15 mL) were sealed and heated at 65 C overnight. The reaction mixture was cooled and concentrated. The residue was taken in a mixture of ethyl acetate and hexane (80:20) was filtered through a short silica gel column (2.5 cm h x 3 cm w). Then the column was eluted with the same solvent mixture. The fractions containing a less polar product were combined and concentrated (433 mg, slightly yellow oil, dialkylated product **4-4**). Then the column was eluted with a mixture of DCM-MeOH-NH₃ (95:5:0.5). All fractions containing the desired product were combined and concentrated; the residue (oil/solid) was taken up in a mixture of hexane and ethyl acetate (ca 98:2) and filtered. The filtrate was concentrated and gave a viscous brown oil (compound **I-2**). The yellow oil (444 mg) was further purified by flash dry column chromatography on silica gel (MeOH in chloroform, 1 to 5%). The fractions containing the desired product were combined and concentrated (288 mg, 0.23 mmol, yellow oil). ¹H NMR (400 MHz, CDCl₃) δ: 6.24-6.13 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.32-3.19 (m, 2H), 2.64-2.59 (m, 4H), 2.54-2.50 (m, 2H), 2.44-2.34 (m, 4H), 2.30 (t, 7.5 Hz, 4H), 2.18-2.09 (m, 2H), 1.67-1.50 (m, 13H), 1.47-1.38 (m, 4H), 1.38-1.08 (m, 60H), 0.90-0.86 (m, 15H). ESI-MS: MW for C₅₈H₁₁₅N₃O₅ [M+H]⁺ Calc. 934.9; Found 935.1.

EXAMPLE 6

SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-(METHYL(4-OCTANAMIDOBUTYL)AMINO)ETHYL)AZANEDIYL)DIHEXOANOATE (COMPOUND I-1)

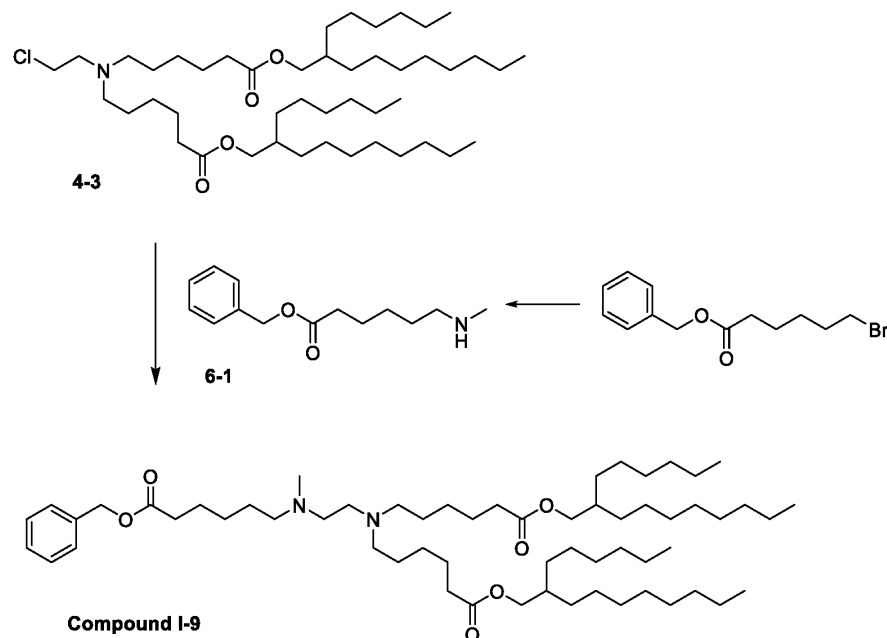


5 Synthesis of Compound I-1

To a solution of compound **I-2** (223 mg, 0.23 mmol) in THF (5 mL) was added formaldehyde solution (ca 6 mmol, 500 mg of 37 wt. % solution in water) at RT. The resulting mixture was stirred for 30 min before introducing sodium triacetoxyborohydride (5 eq., 1.2 mmol, 243 mg). After being stirred overnight, the mixture was taken up in a mixture of hexane and ethyl acetate (100 mL) and washed with saturated sodium bicarbonate solution. After dried over sodium sulfate, the solution was filtered through a short column of silica gel (230-400 mesh grade silica gel, 2.5 cm h x 3 cm w) under reduced pressure. Then the column was eluted with a mixture of hexane, ethyl acetate and Et₃N (80:20:1, 200 mL). Then the column was eluted with a mixture of DCM and MeOH (97:3, 100 mL). All fractions were combined and concentrated (220 mg, yellow oil). The crude product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform, 0 to 5%). This gave the desired product as a colorless oil (165 mg, 016 mmol, 42%). ¹H NMR (400 MHz, CDCl₃) δ: 6.18-6.10 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.29-3.18 (m, 2H), 2.55-2.50 (m, 2H), 2.44-2.38 (m, 6H), 2.37-2.33 (m, 2H), 2.30 (t, 7.5 Hz, 4H), 2.21 (s, 3H), 2.16-2.10 (m, 2H), 1.67-1.58 (m, 8H), 1.55-1.48 (m, 4H), 1.48-1.39 (m, 4H), 1.34-1.08 (m, 60H), 0.90-0.85 (m, 15H). ESI-MS: MW for C₅₉H₁₁₇N₃O₅ [M+H]⁺ Calc. 948.9; Found 949.1.

EXAMPLE 7

**BIS(2-HEXYLDECYL) 6,6'-((2-((6-(BENZYLOXY)-6-
OXOHEXYL)(METHYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-9)**



5 Synthesis of Compound 6-1

A mixture of methylamine (5 eq, 75 mmol, 37.5 mL, 2M solution in THF), benzyl 6-bromohexanoate (4.2 g, 14.7 mmol), NaI (40 mg) and *N,N*-diisopropylethylamine (2.6 mL) was sealed in a pressure flask and stirred at RT overnight. After concentration of the reaction mixture, the residue was purified by flash dry column chromatography on silica gel (DCM/MeOH/Et₃N, 99:1:0 to 80:20:1). This gave the desired product as a slightly yellow oil (1.95 g, 8.3 mmol, 56%).

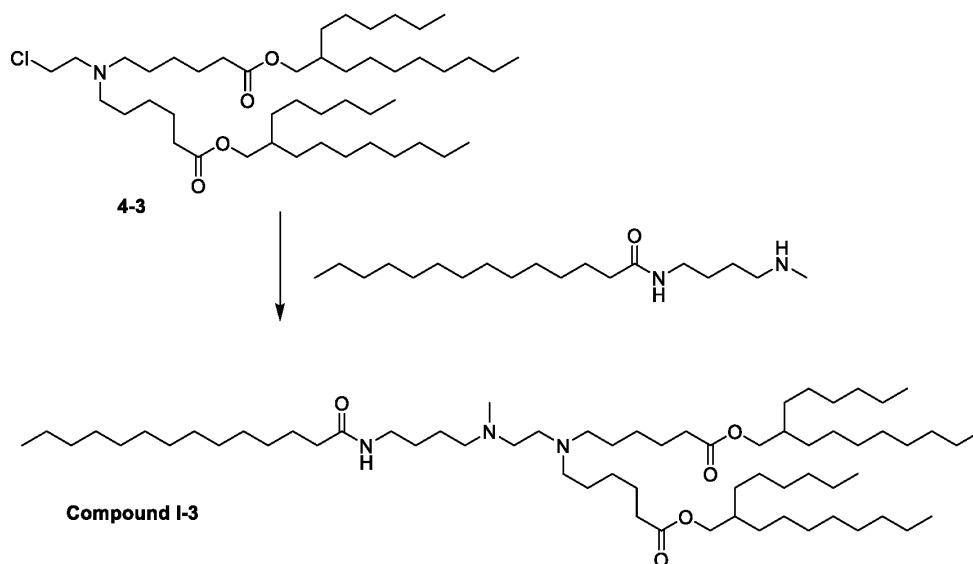
Synthesis of Compound I-9

A mixture of 4-3 (1.141 g, 1.51 mmol), 6-1 (3 eq, 4.53 mmol, 1.066 g), *N,N*-diisopropylethylamine (3 equiv., 4.53 mmol, 584 mg, 0.788 mL), and sodium iodide (60 mg) in acetonitrile (20 mL) was sealed and heated at 76 C (oil bath) for 24 h. Next day the reaction mixture was cooled and concentrated. The crude product was purified by flash dry column chromatography on silica gel (230-400 mesh grade silica gel, hexane/EtOAc/Et₃N, from 90:10:0 to 80:20:1). This gave the desired product as light-yellow oil (1.20 g, 1.25 mmol, 83%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 7.39-7.29 (m, 5H), 5.11 (s, 2H), 3.96 (d, 5.8 Hz, 4H),

2.54-2.49 (m, 2H), 2.43-2.27 (m, 14H), 2.20 (s, 3H), 1.70-1.58 (m, 8H), 1.51-1.38 (m, 6H), 1.36-1.18 (m, 54H), 0.90-0.85 (m, 12H).

EXAMPLE 8

5 SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-(METHYL(4-TETRADECANAMIDOBUTYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-3)

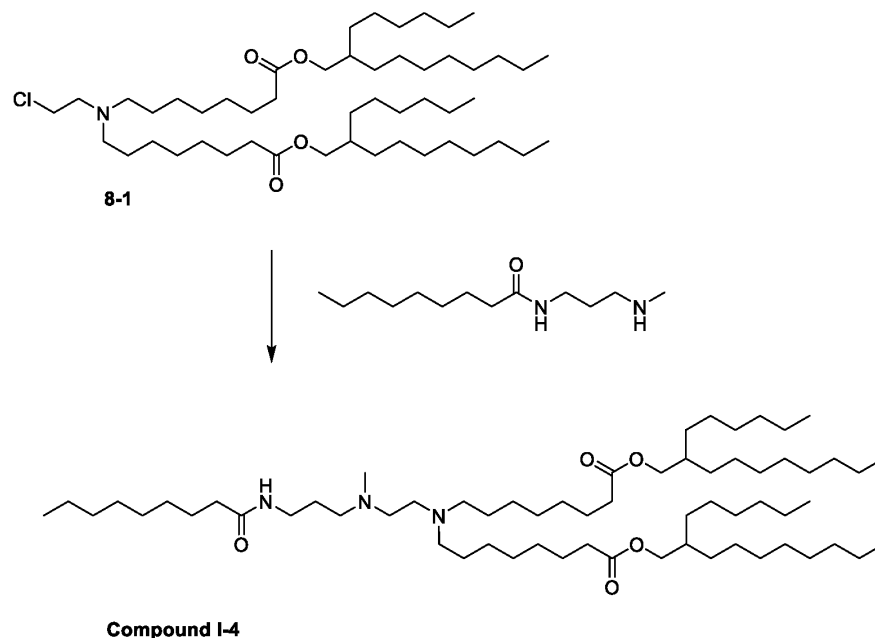


Synthesis of Compound I-3

A mixture of **4-3** (372 mg, 0.49 mmol), N-(4-(methylamino)butyl)tetradecanamide (343 mg, 1.1 mmol), *N,N*-diisopropylethylamine (5 equiv., 2.1 mmol, 271 mg, 0.37 mL), and sodium iodide (10 mg) in acetonitrile (10 mL) was sealed and heated at 76 °C (oil bath) for 24 h. Next day the mixture was cooled and concentrated. The residue was taken up in a mixture of hexane/EtOAc/Et₃N/MeOH (70:30:1:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated. The product (418 mg) was further purified by flash dry column chromatography on silica gel (MeOH in chloroform with a trace of Et₃N, 0 to 5%). This gave the desired product as colorless oil (294 mg, 0.28 mmol, 58%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 6.16-6.08 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.28-3.19 (m, 2H), 2.55-2.50 (m, 2H), 2.44-2.32 (m, 8H), 2.30 (t, 7.5 Hz, 4H), 2.21 (s, 3H), 2.17-2.09 (m, 2H), 1.67-1.58 (m, 8H), 1.55-1.37 (m, 8H), 1.36-1.08 (m, 72H), 0.90-0.86 (m, 15H). ESI-MS: MW for C₆₅H₁₂₉N₃O₅ [M+H]⁺ Calc. 1033.0; Found 1033.2.

EXAMPLE 9

SYNTHESIS OF BIS(2-HEXYLDECYL) 8,8'-((2-(METHYL(3-NONANAMIDOPROPYL)AMINO)ETHYL)AZANEDIYL)DIOCTANOATE (COMPOUND I-4)



5 Synthesis of Compound 8-1

This intermediate was prepared in a similar manner to the preparation of compound **4-3** in Example 5.

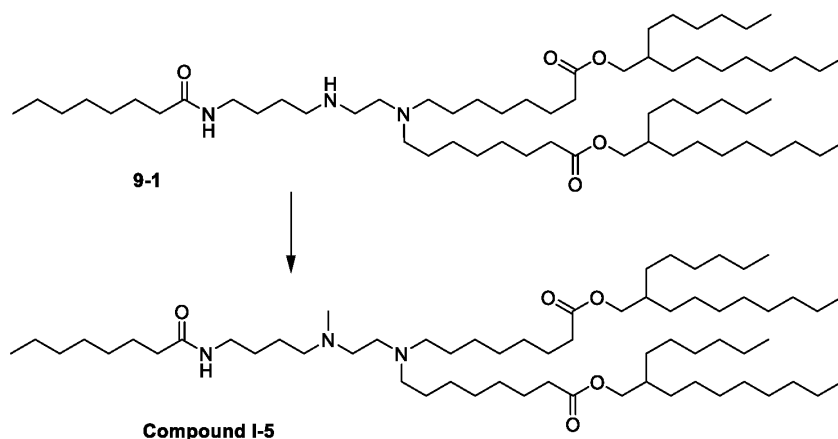
Synthesis of Compound I-4

A mixture of **8-1** (344 mg, 0.42 mmol), *N*-(3-(methylamino)propyl)nonanamide (3 eq.
 10 1.27 mmol, 290 mg), *N,N*-diisopropylethylamine (0.37 mL), and sodium iodide (10 mg) in acetonitrile (10 mL) was sealed and heated at 80 °C for 24 h. Next day the mixture was cooled and concentrated. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (70:30:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined
 15 and concentrated, 332 mg, yellow oil. The product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform with a trace of Et₃N, 0 to 5%). This gave the desired product as slightly yellow oil (96 mg, 0.095 mmol, 23%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 7.11-7.05 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.37-3.30 (m, 2H), 2.55-2.49 (m, 2H), 2.48-2.36 (m, 8H), 2.29 (t, 7.5 Hz, 4H), 2.22 (s, 3H), 2.16-2.09 (m, 2H), 1.67-1.58 (m,

10H), 1.46-1.37 (m, 4H), 1.34-1.08 (m, 70H), 0.90-0.85 (m, 15H). ESI-MS: MW for $C_{63}H_{125}N_3O_5$ $[M+H]^+$ Calc. 1005.0; Found 1005.2.

EXAMPLE 10

SYNTHESIS OF BIS(2-HEXYLDECYL) 8,8'-((2-(METHYL(4-OCTANAMIDOBUTYL)AMINO)ETHYL)AZANEDIYL)DIOCTANOATE (COMPOUND I-5)



Synthesis of Compound 9-1

This intermediate was prepared in a similar manner to the preparation of compound **I-2** in Example 5.

10 Synthesis of Compound I-5

To a solution of **9-1** (122 mg, 0.12 mmol) in THF (6 mL) was added formaldehyde HCHO solution (460 mg of 37 wt. % solution in water) at RT. The resulting mixture was stirred for 30 min before introducing sodium triacetoxyborohydride (5 eq., 0.6 mmol, 122 mg). The resulting mixture was stirred at RT overnight. The mixture was taken in hexane and washed with saturated sodium bicarbonate solution. The organic phase was dried over Na_2SO_4 and concentrated to give yellow oil, 122 mg. The oil was taken up in a mixture of hexane/EtOAc/ Et_3N (70:30:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated, 106 mg, yellow oil. The product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform with a trace of Et_3N , 0 to 5%). This gave the desired product as colorless oil (43 mg). 1H NMR (400 MHz, $CDCl_3$ at 7.26 ppm) δ : 6.21-6.14 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.27-3.20 (m, 2H),

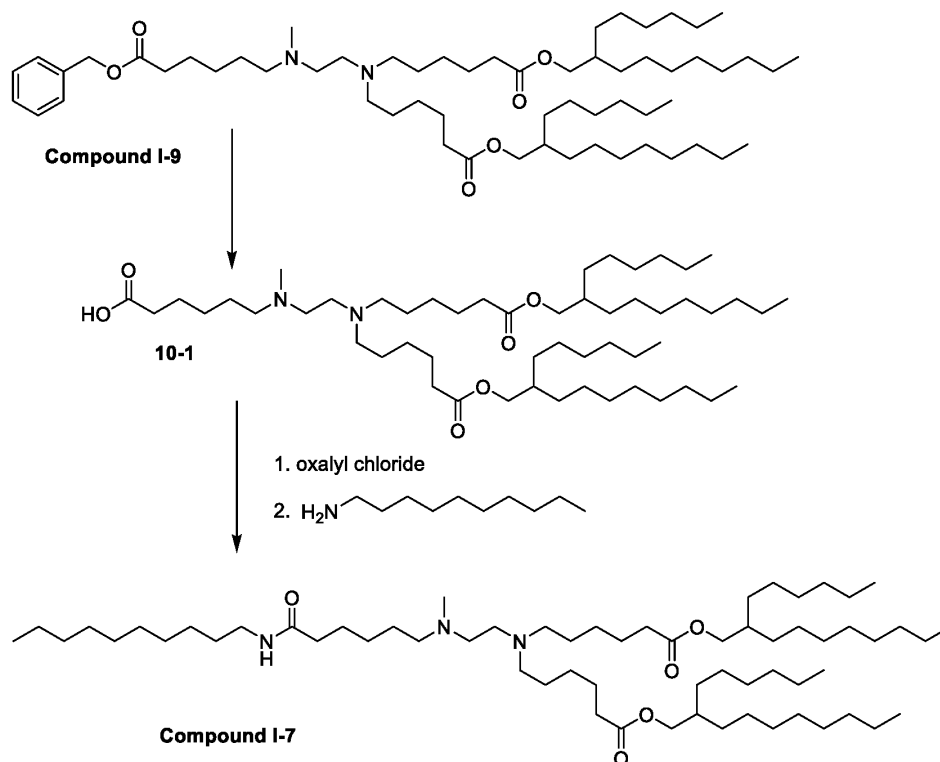
2.55-2.50 (m, 2H), 2.45-2.33 (m, 8H), 2.29 (t, 7.5 Hz, 4H), 2.22 (s, 3H), 2.16-2.10 (m, 2H), 1.67-1.58 (m, 8H), 1.55-1.48 (m, 4H), 1.46-1.37 (m, 4H), 1.35-1.18 (m, 68H), 0.90-0.85 (m, 15H).

ESI-MS: MW for $C_{63}H_{125}N_3O_5$ $[M+H]^+$ Calc. 1005.0; Found 1005.2.

EXAMPLE 11

5

SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-((6-(DECYLAMINO)-6- OXOHEXYL)(METHYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-7)



Synthesis of Compound 10-1

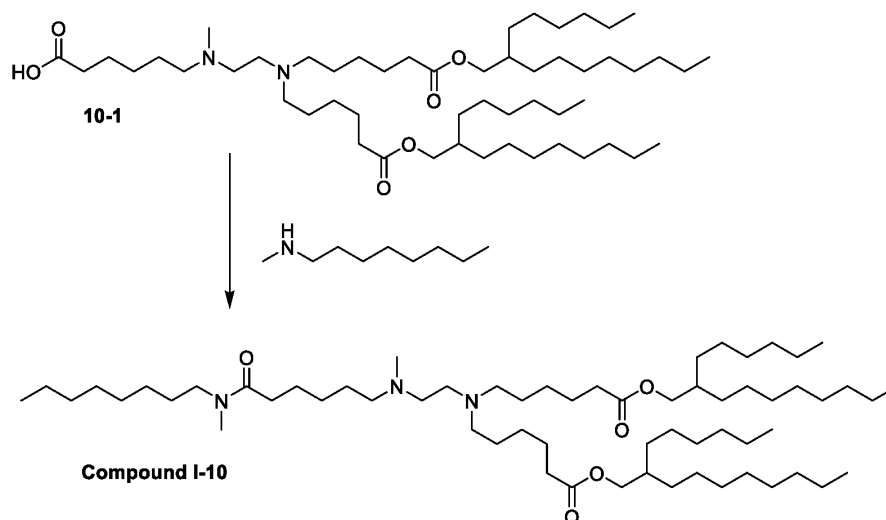
To a solution of compound **I-9** (prepared according to the procedures of Example 10,
10 1.019 g, 1.07 mmol) in EtOH/EtOAc (1:10 mL) was added 10% Pd/C (25 mg) and the mixture stirred under hydrogen. The reaction was monitored by TLC. Another two portions of Pd/C (30 mg each) were added during reaction. After 9 days, TLC showed approximately 90% completion of the reaction. The mixture was filtered through a pad of diatomaceous earth (*e.g.*, Celite®), and the pad was washed with DCM. The filtrate was concentrated to give a yellow oil. The crude
15 product was purified by flash dry column chromatography on silica gel (0 to 10% methanol in DCM). This gave the desired product as yellow oil (751 mg, 0.87 mmol, 81%).

Synthesis of Compound I-7

To a solution of **10-1** (250 mg, 0.29 mmol) in DCM (6 mL) and DMF (about 0.01 mL) was added oxalyl chloride (5 eq, 1.45 mmol, 184 mg, 0.13 mL) at RT. After stirring at RT for 16 h, the mixture was concentrated under reduced pressure. The residual oil was dissolved in 5 mL of DCM and added to a solution of 1-decylamine (1.5 eq, 0.44 mmol, 68 mg) and triethylamine (0.12 mL) in DCM (5 mL). The resulting mixture was stirred at RT for 30 min and then was concentrated. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated, 254 mg, brownish oil. The product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform with a trace of Et₃N, 0 to 5%). This gave the desired product as colorless oil (218 mg, 0.22 mmol, 75%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 5.54-5.46 (m, 1H, NHCO), 3.96 (d, 5.8 Hz, 4H), 3.27-3.19 (m, 2H), 2.55-2.50 (m, 2H), 2.44-2.37 (m, 6H), 2.35-2.27 (m, 6H), 2.21 (s, 3H), 2.19-2.13 (m, 7.6 Hz, 2H), 1.69-1.57 (m, 8H), 1.52-1.39 (m, 8H), 1.34-1.08 (m, 68H), 0.90-0.85 (m, 15H). ESI-MS: MW for C₆₃H₁₂₅N₃O₅ [M+H]⁺ Calc. 1005.0; Found 1005.2.

EXAMPLE 12

**SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-(METHYL(6-(METHYL(OCTYL)AMINO)-6-
OXOHEXYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-10)**



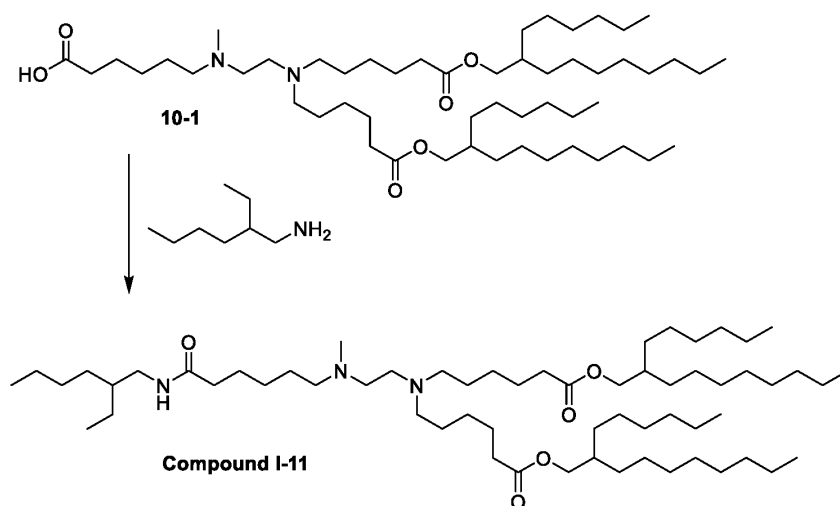
Synthesis of Compound I-10

To a solution of **10-1** (prepared according to the procedures of Example 11, 250 mg, 0.29 mmol) in DCM (6 mL) and DMF (0.01 mL) was added oxalyl chloride (5 eq, 1.45 mmol, 184 mg, 0.13 mL) at RT. After stirring at RT for 16 h, the mixture was concentrated under reduced pressure. The residual oil was dissolved in 5 mL of DCM and added to a solution of N-methyloctylamine (1.5 eq, 0.44 mmol, 63 mg) and triethylamine (0.12 mL) in DCM (5 mL). The resulting mixture was stirred at RT for 30 min and then was concentrated. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) was filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated to give a brownish oil. The product (brownish oil) was further purified by column chromatography on silica gel (0 to 5% MeOH in DCM). This gave the desired product as slightly yellow oil (146 mg, 0.15 mmol, 51%).

¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ : 3.96 (d, 5.8 Hz, 4H), 3.34, 3.32 (2 sets of triplet, ratio=1:1, 7.5 Hz, 2H), 2.95, 2.90 (2 sets of singlet, ratio=1:1, 3H), 2.55-2.50 (m, 2H), 2.43-2.36 (m, 8H), 2.35-2.27 (m, 6H), 2.21 (s, 3H), 1.70-1.54 (m, 8H), 1.54-1.38 (m, 8H), 1.37-1.08 (m, 64H), 0.91-0.85 (m, 15H). ESI-MS: MW for C₆₂H₁₂₃N₃O₅ [M+H]⁺ Calc. 991.0; Found 991.1.

EXAMPLE 13

**SYNTHESIS OF BIS(2-HEXYLDECYL) 6,6'-((2-((6-((2-ETHYLHEXYL)AMINO)-6-
OXOHEXYL)(METHYL)AMINO)ETHYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-11)**

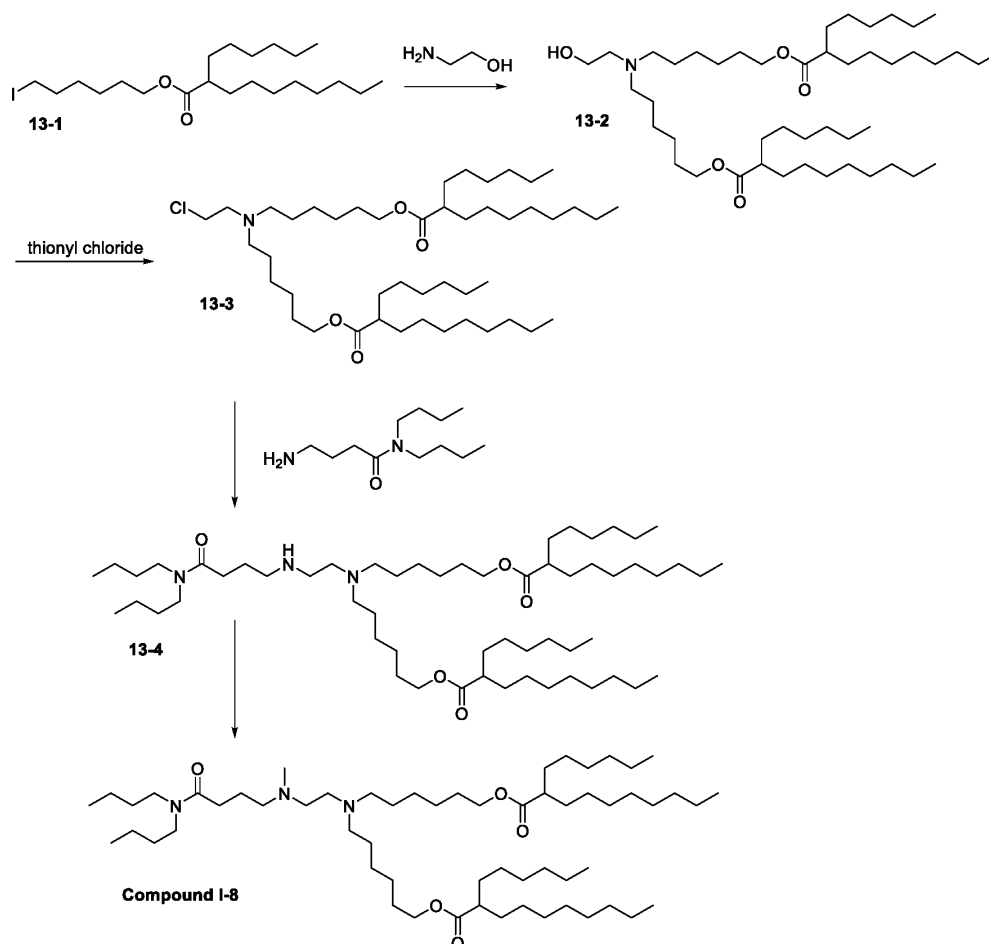


Synthesis of Compound I-11

To a solution of **10-1** (prepared according to the procedures of Example 11, 250 mg, 0.29 mmol) in DCM (6 mL) and DMF (about 0.01 mL) was added oxalyl chloride (5 eq, 1.45 mmol, 184 mg, 0.13 mL) at RT. After stirring at RT for 16 h, the mixture was concentrated under reduced pressure. The residual oil was dissolved in 5 mL of DCM and added to a solution of 2-ethyl-1-hexylamine (1.5 eq, 0.44 mmol, 57 mg) and triethylamine (0.12 mL) in DCM (5 mL). The resulting mixture was stirred at RT for 2 hours and then was concentrated. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated, 258 mg, brownish oil. The product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform with a trace of Et₃N, 0 to 5%). This gave the desired product as slightly yellow oil (184 mg, 0.19 mmol, 65%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 5.43 (s, 1H), 3.96 (d, 5.8 Hz, 4H), 3.25-3.13 (m, 2H), 2.55-2.49 (m, 2H), 2.43-2.37 (m, 6H), 2.35-2.27 (m, 6H), 2.21 (s, 3H), 2.17 (t, 7.6 Hz, 2H), 1.69-1.58 (m, 8H), 1.52-1.38 (m, 7H), 1.37-1.18 (m, 62H), 0.91-0.85 (m, 18H). ESI-MS: MW for C₆₁H₁₂₁N₃O₅ [M+H]⁺ Calc. 976.9; Found 977.1.

EXAMPLE 14

**SYNTHESIS OF ((2-((4-(DIBUTYLAMINO)-4-
OXOBUTYL)(METHYL)AMINO)ETHYL)AZANEDIYL)BIS(HEXANE-6,1-DIYL) BIS(2-
HEXYLDECANOATE) (COMPOUND I-8)**



5

Synthesis of Compound 13-2

A mixture of **13-1** (620 mg, 1.33 mmol), 2-aminoethanol (0.68 mmol, 42 mg), *N,N*-diisopropylethylamine (0.18 mL) and anhydrous acetonitrile (10 mL) was heated for 1 day in a sealed pressure flask (oil bath 80 °C). The reaction was cooled and concentrated under reduced pressure. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) and filtered through a short column of silica gel (230–400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated to give a colorless oil (0.395 g), which was used for the next step without further purification.

Synthesis of Compound 13-3

To an ice-cooled solution of **13-2** (462 mg, 0.62 mmol) in 2 mL of CHCl_3 , was added thionyl chloride (3 eq, 1.88 mmol, 223 mg, 0.137 mL) in 10 mL of chloroform dropwise over a couple of minutes. After the completion of the addition the ice bath was removed, and the reaction mixture was stirred for 16 h at room temperature (20 °C). The reaction was concentrated under reduced pressure. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) and filtered through a pad of silica gel. Then the column was washed with the same solvent mixture. The filtrate was concentrated to give a yellow oil (445 mg), which was used for the next step without further purification.

10 Synthesis of Compound 13-4

A mixture of **13-3** (222 mg, 0.29 mmol), 4-amino-*N,N*-dibutylbutanamide (3 eq. 0.88 mmol, 188 mg), *N,N*-diisopropylethylamine (0.25 mL), and sodium iodide (10 mg) in acetonitrile (10 mL) was sealed and heated at 76 °C for 24 h. The resulting mixture was concentrated. The residue was taken up in a mixture of hexane and ethyl acetate (85:15) was filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with a mixture of hexane/EtOAc/Et₃N (85:15:0 to 70:30:1). All fractions containing the desired product were combined and concentrated to give a yellow oil (109 mg, 0.12 mmol, 40%).

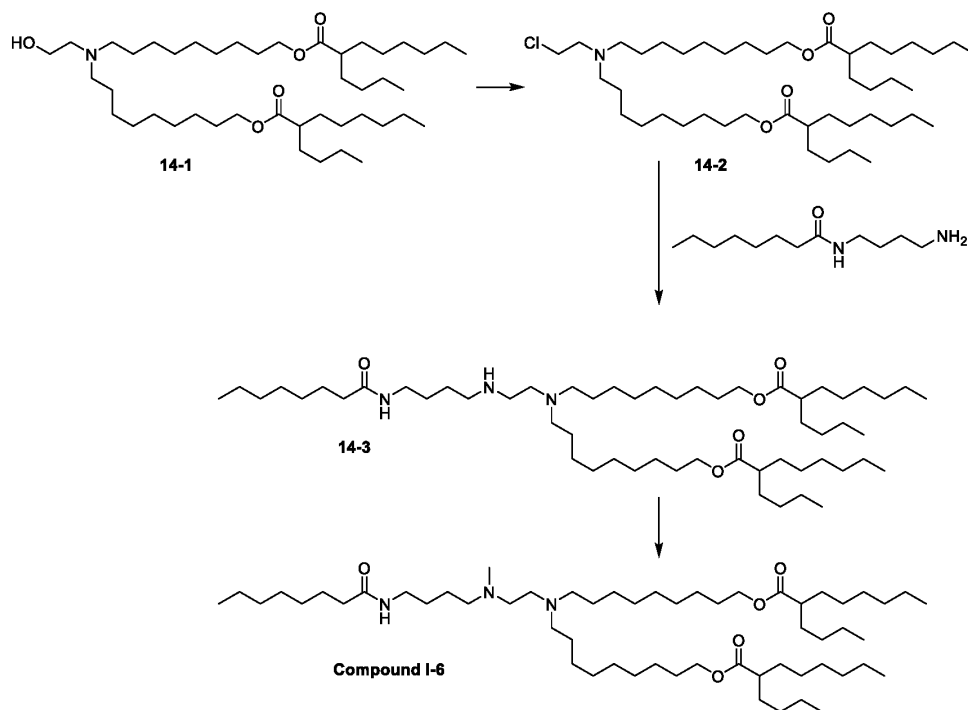
Synthesis of Compound I-8

To a solution of **13-4** (109 mg, 0.12 mmol) in THF (5 mL) was added formaldehyde HCHO solution (250 mg of 37 wt. % solution in water) at RT. The resulting mixture was stirred for 30 min before introducing sodium triacetoxyborohydride (5 eq., 0.6 mmol, 122 mg). After stirring overnight, the crude mixture was taken in a mixture of hexane and ethyl acetate (ca 90:10, 100 mL) and washed with saturated sodium bicarbonate solution. The extract was concentrated to give a yellow oil/solid. The residue was taken up in a mixture of hexane/EtOAc/Et₃N (80:20:1) and filtered through a short column of silica gel (230-400 mesh grade silica gel). Then the column was eluted with the same solvent mixture. All fractions containing the product were combined and concentrated to give a brownish oil (100 mg). The product was further purified by column chromatography on silica gel (0 to 6% MeOH in DCM; 0 to 5% methanol in chloroform). This gave the desired product as slightly yellow oil (30 mg, 0.03 mmol, 26%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ : 4.06 (t, 6.7 Hz, 4H), 3.33-3.26 (m,

2H), 3.25-3.18 (m, 2H), 2.55-2.50 (m, 2H), 2.47-2.35 (m, 8H), 2.35-2.25 (m, 4H), 2.23 (s, 3H), 1.81 (quintet, 7.4 Hz, 2H), 1.67-1.37 (m, 20H), 1.37-1.08 (m, 52H), 0.97-0.85 (m, 18H). ESI-MS: MW for $C_{59}H_{117}N_3O_5$ $[M+H]^+$ Calc. 948.9; Found 949.1.

EXAMPLE 15

5 SYNTHESIS OF ((2-(METHYL(4-OCTANAMIDOBUTYL)AMINO)ETHYL)AZANEDIYL)BIS(NONANE-9,1-DIYL) BIS(2-BUTYLOCTANOATE) (COMPOUND I-6)



Synthesis of Compounds 14-1, 14-2 and 14-3

These intermediates were prepared in a similar manner to the preparation of compounds
10 **13-2**, **13-3** and **13-4** in Example 14.

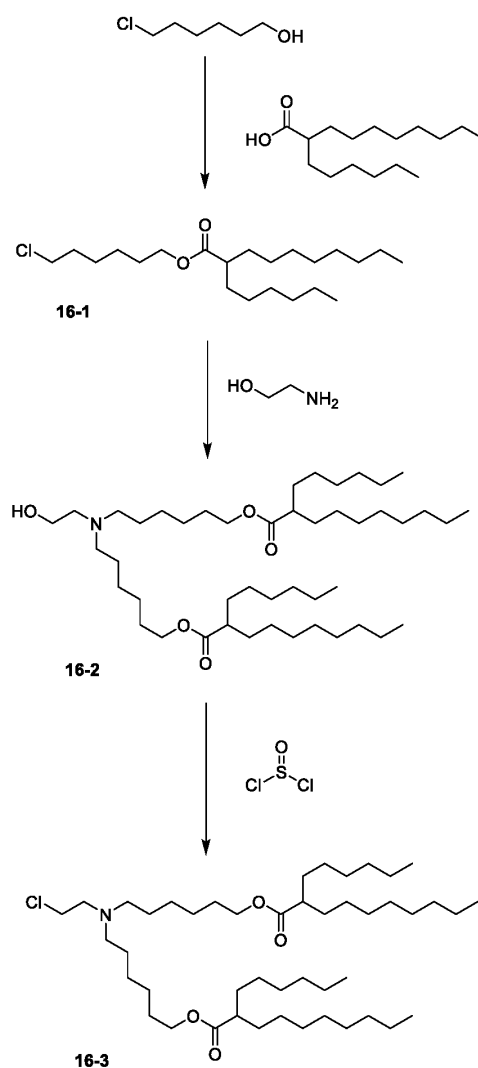
Synthesis of Compound I-6

To a solution of **14-3** (152 mg, 0.17 mmol) in THF (6 mL) was added formaldehyde HCHO solution (420 mg of 37 wt. % solution in water) at RT. The resulting mixture was stirred for 30 min before introducing sodium triacetoxyborohydride (5 eq., 0.6 mmol, 122 mg,
15 MW211.94). After stirring overnight, the crude mixture was taken in a mixture of hexane (60 mL) and washed with saturated sodium bicarbonate solution. The aqueous phase was extracted with hexane. The combined extracts were filtered through a short column of silica gel (230-400

mesh grade silica gel). Then the column was eluted with a mixture of hexane/EtOAc/Et₃N (80:20:1). All fractions containing the product were combined and concentrated to give a brownish oil (129 mg). The product was further purified by column chromatography on silica gel (0 to 5% methanol in chloroform). This gave the desired product as slightly yellow oil (76 mg, 0.08 mmol, 49%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 6.20 (br. 1H, NHCO), 4.06 (t, 6.7 Hz, 4H), 3.28-3.20 (m, 2H), 2.56-2.51 (m, 2H), 2.48-2.26 (m, 10H), 2.22 (s, 3H), 2.16-2.10 (t, 2H), 1.67-1.55 (m, 10H), 1.55-1.50 (m, 4H), 1.48-1.37 (m, 8H), 1.37-1.12 (m, 52H), 0.90-0.85 (m, 15H). ESI-MS: MW for C₅₇H₁₁₃N₃O₅ [M+H]⁺ Calc. 920.9; Found 921.1.

EXAMPLE 16

10 **6-((2-((3-(*N,N*-DIHEXYLCARBAMOYL)PROPYL)-*N*-METHYLAMINO)ETHYL)(6-(1-HEXYLNONYLCARBONYLOXY)HEXYL)AMINO)HEXYL 2-HEXYLDECANOATE (COMPOUND I-53)**



Synthesis of Compound 16-1

6-Chloro-1-hexanol (5.0 g, 4.87 mL, 36.6 mmol, 1 eq), 2-hexyldecanoic acid (14.1 g, 16.1 mL, 54.9 mmol, 1.5 eq) and *N,N*-dimethyl-4-pyridylamine (DMAP; 2.2 g, 18.3 mmol, 0.5 eq) were dissolved in DCM (40 mL). 1-((cyclohexylimino)methyleneamino)cyclohexane (DCC; 5 8.3 g, 40.3 mmol, 1.1 eq) was added in one portion and the reaction mixture was stirred at room temperature overnight. After that, the reaction mixture was diluted with hexanes (200 mL), solids were removed by passing the mixture through a small pad of diatomaceous earth (*e.g.*, Celite®). After removing solvent under vacuum, residue was purified via automated flash chromatography (220 g SiO₂ column; 0-15% EtOAc in hexanes, target elutes with 4-6% EtOAc in hexanes) to 10 give 6-chlorohexyl 2-hexyldecanoate (compound **16-1**; 10.0 g, 73%). ESI-MS: MW for C₂₂H₄₃ClO₂ [M+H]⁺ Calc. 375.33; Found 375.43.

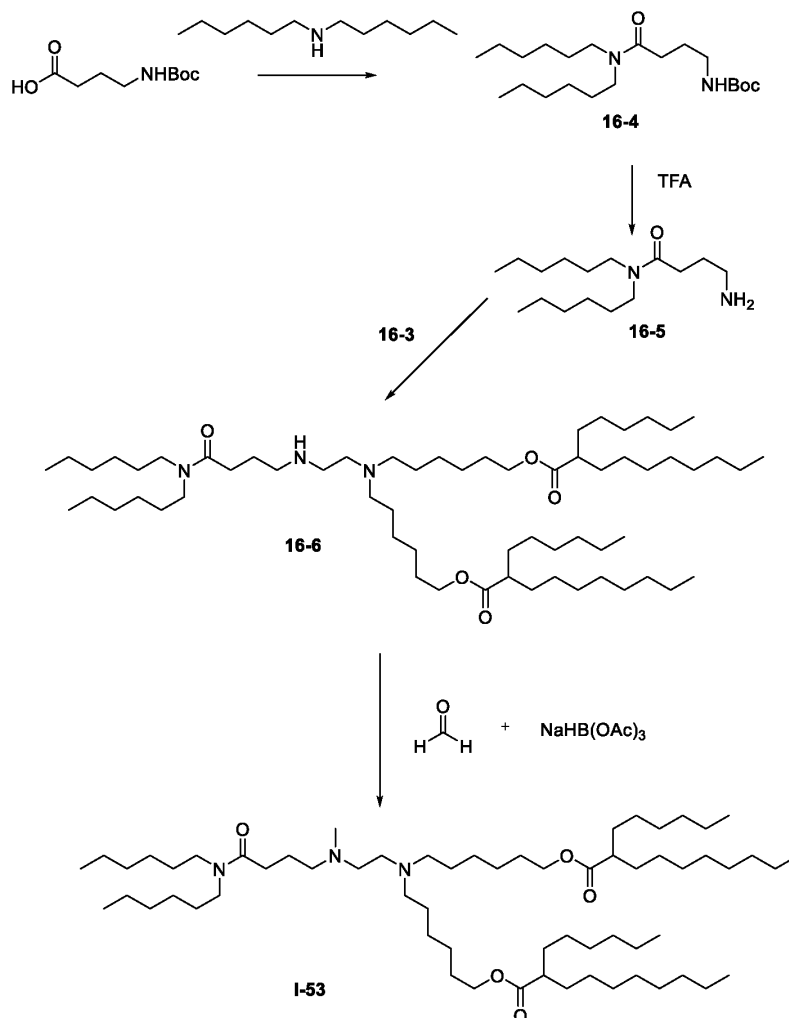
Synthesis of Compound 16-2

6-chlorohexyl 2-hexyldecanoate (compound **16-1**; 2.0 g, 5.33 mmol, 2 eq), 2-aminoethanol (195 mg, 3.2 mmol, 1.2 eq), *N*-ethylbis(isopropyl)amine (DIPEA; 1.4 g, 1.91 mL, 15 10.9 mmol, 4.1 eq) and potassium iodide (930 mg, 5.6 mmol, 2.1 eq) in acetonitrile (6.4 mL) were put in a microwave reactor and heated to 140 °C for 30 minutes. The reaction was done in triplicate (3 × 2.0 g of 6-chlorohexyl 2-hexyldecanoate). After the reaction mixtures were cooled, they were combined, and acetonitrile was removed under vacuum. The residue was partitioned between EtOAc and water, the organic layer was dried over anhydrous Na₂SO₄, and the solvent 20 was removed under vacuum. The residue was purified via automated flash chromatography (80 g SiO₂ column; 0-30% EtOAc in hexanes with 1% Et₃N) to give 6-((6-(1-hexylnonylcarbonyloxy)hexyl)(2-hydroxyethyl)amino)hexyl 2-hexyldecanoate (compound **16-2**; 2.5g, 42%). ESI-MS: MW for C₄₆H₉₁NO₅ [M+H]⁺ Calc. 738.70; Found 738.83.

Synthesis of Compound 16-3

25 To an ice-cooled solution of **16-2** (3.08 g, 4.17 mmol, 1 eq) in DCM (50 mL), SOCl₂ (1.49 g, 908 μL, 12.5 mmol, 3 eq) was added dropwise. After this, the ice bath was removed, and the reaction mixture was stirred overnight at room temperature. DCM and SOCl₂ were removed under vacuum, and the crude dark red oil was purified via automated flash chromatography (80 g SiO₂ column; 0-30% EtOAc in hexanes with 1% Et₃N; target eluted with 8% to 12% EtOAc) to 30 give 6-((2-chloroethyl)(6-(1-hexylnonylcarbonyloxy)hexyl)amino)hexyl 2-hexyldecanoate

(compound **16-3**; 2.8 g, 89%). ESI-MS: MW for $C_{46}H_{90}NClO_4$ $[M+H]^+$ Calc. 756.66; Found 756.80.



Synthesis of Compound 16-4

- 5 4-(tert-Butoxycarbonylamino)butyric acid (1.0 g, 4.92 mmol, 1 eq), dihexylamine (1.19g, 1.49 mL, 6.40 mmol, 1.3 eq), and DIPEA (1.27 g, 1.71 mL, 9.84 mmol, 2 eq) were combined in DCM (15 mL) before adding HATU (2.25 g, 5.90 mmol, 1.2 eq). The resulting mixture was stirred for 1.5 hours at room temperature, then diluted with EtOAc (15 mL) and washed with water (10 mL). The aqueous layer was washed with 2 × 15 mL of EtOAc. The combined organic
- 10 phases were dried over anhydrous Na₂SO₄, and the solvent was removed. The resulting pale yellow oil was purified via automated flash chromatography (80 g SiO₂ column; 0-100% EtOAc in hexanes, target elutes with 29% to 39% EtOAc) to give 3-(*N,N*-

dihexylcarbamoyl)propylamino-tert-butylformylate (compound **16-4**, 1.49 g, 82%) as a clear, pale yellow oil. ESI-MS: MW for $C_{21}H_{42}N_2O_3$ $[M+H]^+$ Calc. 371.32; Found 371.53.

Synthesis of Compound 16-5

Compound **16-4** (ca. 1.49 g, 4.02 mmol, 1 eq), was dissolved in DCM (35 mL), then
5 TFA (12.1 g, 8.16 mL, 107 mmol, 27 eq) was added. The resulting mixture was stirred overnight at room temperature, then 10 mL of water was added, followed by solid $NaHCO_3$ until evolution of gas was no longer observable. Finally, pH was adjusted to 11 by adding solid K_2CO_3 . The organic and aqueous layers were separated, and the aqueous layer was washed with 3×15 mL of EtOAc. The combined organic phases were dried over anhydrous sodium sulphate, and the
10 solvent was removed to give *N,N*-dihexyl-4-aminobutyramide (compound **16-5**; 987 mg, 91%) as a clear yellow oil. ESI-MS: MW for $C_{16}H_{34}N_2O$ $[M+H]^+$ Calc. 271.27; Found 271.37.

Synthesis of Compound 16-6

A mixture of compound **16-3** (400 mg, 529 μ mol, 1 eq), compound **16-5** (357 mg, 1.32 mmol, 2.5 eq), potassium iodide (87.8 mg, 529 μ mol, 1 eq), and DIPEA (137 mg, 185 μ L, 1.06
15 mmol, 2 eq) in acetonitrile (5 mL) was heated to 140 °C in a microwave reactor for 30 minutes. The resulting mixture was concentrated and partitioned between EtOAc and water, and the aqueous layer was washed with 3×15 mL of EtOAc. The combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed. The resulting yellow oil was purified via automated flash chromatography (40 g SiO_2 column; 0-100% EtOAc in hexanes with 1% Et_3N ,
20 target eluted with 56% to 86% EtOAc with 1% Et_3N) to give 6-((2-(3-(*N,N*-dihexylcarbamoyl)propylamino)ethyl)(6-(1-hexylnonylcarbonyloxy)hexyl)amino)hexyl 2-hexyldecanoate (compound **16-6**; 141 mg, 27%) as a yellow oil. ESI-MS: MW for $C_{62}H_{123}N_3O_5$ $[M+H]^+$ Calc. 990.95; Found 991.29.

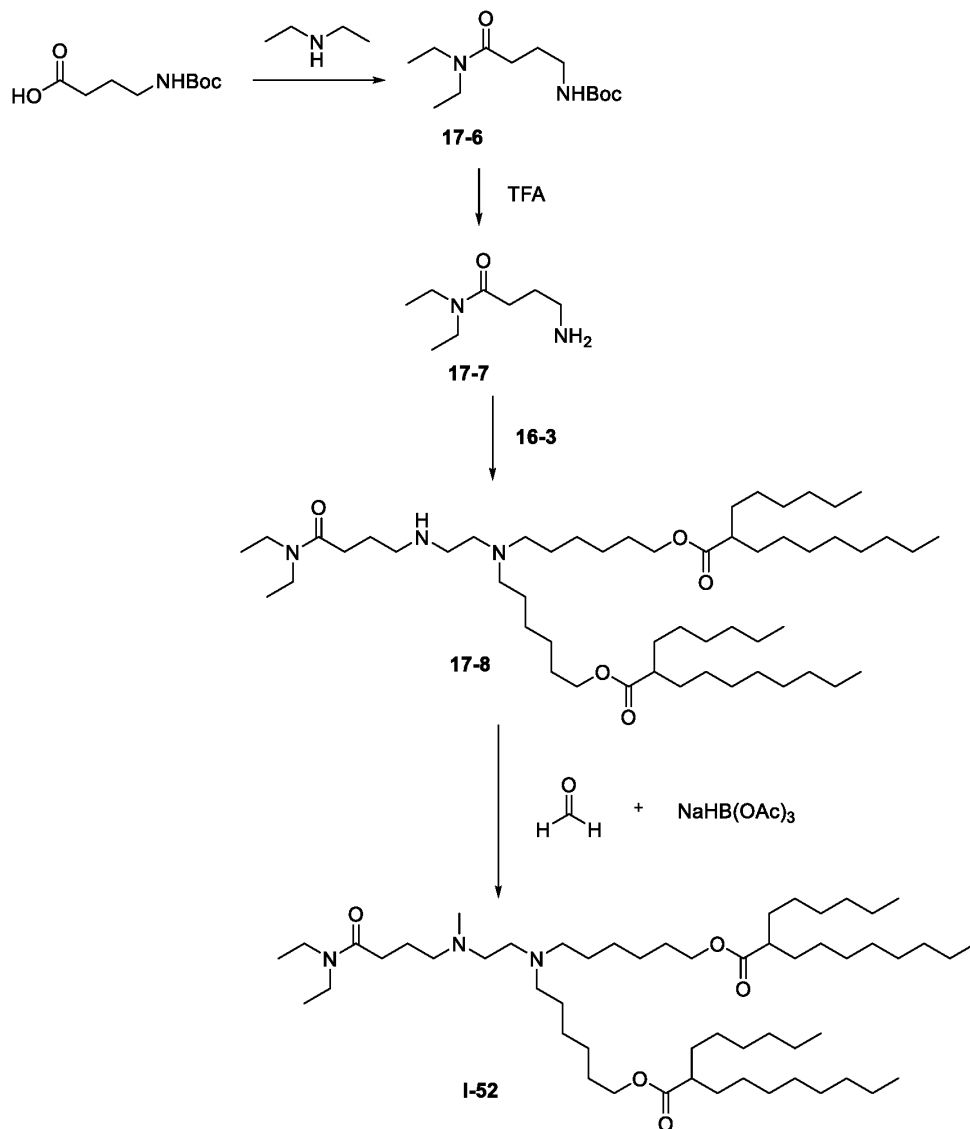
Synthesis of Compound I-53

25 To a solution of compound **16-6** (141 mg, 142 μ mol, 1 eq) in MeOH (1.0 mL) was added a formaldehyde solution in water (13.3 mol/L, 390 μ L, 37 eq). This mixture was stirred at room temperature for 35 minutes before introducing sodium triacetoxyborohydride (127 mg, 598 μ mol, 4.2 eq), then stirred at room temperature for a further 4 hours. The resulting mixture was diluted with EtOAc and washed with a saturated $NaHCO_3$ solution in water (10 mL), and the
30 aqueous layer was washed with 3×15 mL of EtOAc. The combined organic phases were dried

over anhydrous Na₂SO₄, and the solvent was removed. The resulting oil was purified via automated flash chromatography (25 g SiO₂ column; 0-100% EtOAc with 1% Et₃N in hexanes with 1% Et₃N, target eluted with 23% to 44% EtOAc with 1% Et₃N) to give 6-((2-((3-(*N,N*-dihexylcarbamoyl)propyl)-*N*-methyamino)ethyl)(6-(1-hexylnonylcarbonyloxy)hexylamino)-
5 hexyl 2-hexyldecanoate (compound **I-53**; 93.2 mg, 65 %) as a clear, colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 4.09 (t, *J* = 6.7 Hz, 4H), 3.36 – 3.28 (m, 2H), 3.28 – 3.19 (m, 2H), 2.63 – 2.29 (m, 13H), 2.26 (s, 3H), 1.85 (q, *J* = 7.3 Hz, 2H), 1.73 – 1.19 (m, 87H), 0.92 (dt, *J* = 10.2, 6.7 Hz, 18H). ESI-MS: MW for C₆₃H₁₂₅N₃O₅ [M+H]⁺ Calc. 1004.97; Found 1005.25.

EXAMPLE 17

6-((2-((3-(*N,N*-diethylcarbamoyl)propyl)-*N*-methylamino)ethyl)(6-(1-hexylnonylcarbonyloxy)hexyl)amino)hexyl 2-hexyldecanoate (Compound I-52)



5 Synthesis of Compound 17-6

4-(tert-Butoxycarbonylamino)butyric acid (1.02 g, 5.02 mmol, 1eq), diethylamine (477 mg, 675 μL , 6.52 mmol, 1.3 eq), and DIPEA (1.3 g, 1.75 mL, 10.0 mmol, 2 eq) were combined in DCM (15.3 mL) before adding HATU (2.29 g, 6.02 mmol, 1.02 eq). The resulting mixture was stirred for 1.5 hours at room temperature, then diluted with EtOAc (15 mL) and washed with water (10 mL). The aqueous layer was washed with 3×15 mL of EtOAc. The combined organic phases were dried over anhydrous Na_2SO_4 , and the solvent was removed. The resulting pale

yellow oil was purified via automated flash chromatography (80 g SiO₂ column; 0-100% EtOAc in hexanes, target eluted with 78% to 84% EtOAc) to give 3-(*N,N*-diethylcarbamoyl)propylamino-*tert*-butylformylate (compound **17-6**; 1.11 g, 85%) as a pale yellow oil. ESI-MS: MW for C₁₃H₂₆N₂O₃ [M+H]⁺ Calc. 259.20; Found 259.31.

5 Synthesis of Compound 17-7

Compound **17-6** (1.11 g, 4.3 mmol, 1 eq), was dissolved in DCM (25.0 mL), then TFA (9.8 g, 6.58 mL, 85.9 mmol, 20 eq) was added. The resulting mixture was stirred overnight at room temperature, then 10 mL of water was added, followed by solid NaHCO₃ until evolution of gas was no longer observable. Finally, pH was adjusted to 11 by adding solid K₂CO₃. The organic and aqueous layers were separated, and the aqueous layer was washed 3 × 15 mL of EtOAc. The combined organic phases were dried over anhydrous Na₂SO₄, and the solvent was removed to give *N,N*-diethyl-4-aminobutyramide (compound **17-7**; 370 mg, 54%) as a clear, pale yellow oil. ESI-MS: MW for C₈H₁₈N₂O [M+H]⁺ Calc. 159.15; Found 159.14.

Synthesis of Compound 17-8

A mixture of compound **16-3** (413 mg, 546 μmol, 1 eq), compound **17-7** (345 mg, 2.18 mmol, 4 eq), potassium iodide (94.5 mg, 569 μmol, 1.04 eq), and DIPEA (139 mg, 188 μL, 1.08 mmol, 1.97 eq) in acetonitrile (3.2 mL) was heated to 140 °C in a microwave reactor for 30 minutes. The resulting mixture was concentrated and partitioned between EtOAc and water, and the aqueous layer was washed with 3 × 15 mL of EtOAc. The combined organic phases were dried over anhydrous Na₂SO₄, and the solvent was removed. The resulting yellow oil was purified via automated flash chromatography (40 g SiO₂ column; 0-100% EtOAc with 1% Et₃N in hexanes with 1% Et₃N; target eluted with 100% EtOAc with 1% Et₃N) to give 6-((2-(3-(*N,N*-diethylcarbamoyl)propylamino)ethyl)(6-(1-hexylnonylcarbonyloxy)hexyl)amino)hexyl 2-hexyldecanoate (compound **17-8**; 241 mg, 50%) as a brown-orange oil. ESI-MS: MW for C₅₄H₁₀₇N₃O₅ [M+H]⁺ Calc. 878.82; Found 879.11.

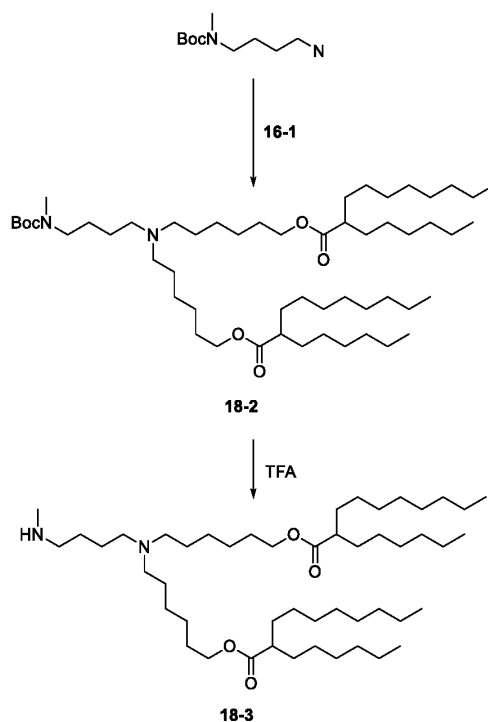
Synthesis of Compound I-52

To a solution of compound **17-8** (240 mg, 273 μmol, 1 eq) in MeOH (4.5 mL) was added a formaldehyde solution in water (13.3 mol/L, 614 μL, 30 eq). This mixture was stirred at room temperature for 30 minutes before introducing sodium triacetoxyborohydride (251 mg, 1.18 mmol, 4.3 eq), then stirred at room temperature overnight. The resulting mixture was

concentrated, diluted with EtOAc, and washed with a saturated NaHCO₃ solution in water (10 mL), and the aqueous layer was washed with 4 × 15 mL of EtOAc. The combined organic phases were dried over anhydrous Na₂SO₄, and the solvent was removed. The resulting oil was purified via automated flash chromatography (40 g column; 0-100% EtOAc with 1% Et₃N in hexanes with 1% Et₃N, target eluted with 40% to 44% EtOAc with 1% Et₃N) to give 6-((2-((3-(*N,N*-diethylcarbamoyl)propyl)-*N*-methylamino)ethyl)(6-(1-hexylnonylcarbonyloxy)hexyl)amino)-hexyl 2-hexyldecanoate (compound **I-52**; 182 mg, 75%) as a clear, slightly yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 4.05 (s, 4H), 3.48 – 3.20 (m, 4H), 2.58 – 2.27 (m, 18H), 2.23 (s, 3H), 1.87 – 1.21 (m, 70H), 1.16 (t, *J* = 7.1 Hz, 3H), 1.10 (t, *J* = 7.1 Hz, 3H), 0.92 – 0.82 (m, 12H). ESI-MS: MW for C₅₅H₁₀₉N₃O₅ [M+H]⁺ Calc. 892.84; Found 893.10.

EXAMPLE 18

((4-((5-(DIHEXYLAMINO)-5-OXOPENTYL)(METHYL)AMINO)BUTYL)AZANEDIYL)BIS(HEXANE-6,1-DIYL) BIS(2-HEXYLDECAOATE) (COMPOUND I-47)



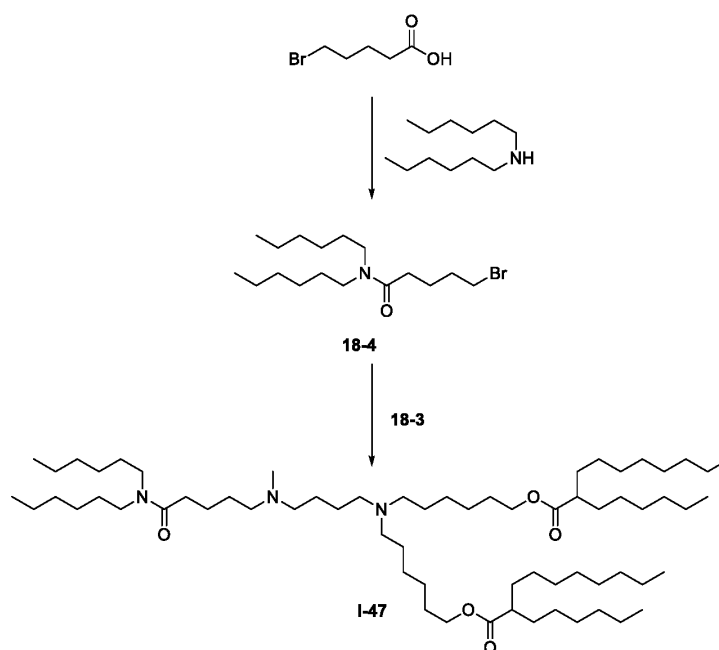
15 Synthesis of Compound 18-2

6-chlorohexyl 2-hexyldecanoate (compound **16-1**; 1.2 g, 3.25 mmol, 2 eq), *tert*-butyl (4-aminobutyl)(methyl)carbamateamine (395 mg, 1.995 mmol, 1.2 eq), *N*-ethylbis(isopropyl)amine (DIPEA; 862 mg, 1.16 mL, 6.67 mmol, 4.1 eq) and KI (567 mg, 3.42 mmol, 2.1 eq) were mixed

in acetonitrile (3.9 mL) and reacted in a microwave reactor (140 °C, 30 min). After cooling down, solvent was removed under vacuum, residue was partitioned between EtOAc and water. The organic layer was dried over anhydrous Na₂SO₄. After removing solvent under vacuum, residue was purified via automated flash chromatography (40 g SiO₂ column; 0-50% EtOAc in
 5 hexanes with 1% Et₃N, target elutes with 15-20% EtOAc) to give ((4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (compound **18-2**; 460 mg, 32%). ESI-MS: MW for C₅₄H₁₀₆N₂O₆ [M+H]⁺ Calc. 879.81; Found 880.08. TLC (MeOH : DCM = 1: 9) R_f = 0.5.

Synthesis of Compound 18-3

10 ((4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (compound **18-2**; 460 mg, 523 μmol, 1 eq) was dissolved in DCM (7.3 mL); TFA (895 mg, 0.6 mL, 7.85 mmol, 15 eq) was added and mixture stirred at room temperature overnight. After that, 10 mL of water was added, followed by solid NaHCO₃. When no bubbles were observed upon addition of fresh amount of NaHCO₃, pH was adjusted to 11 by addition of
 15 1M NaOH. Layers were separated; DCM layer was dried over anhydrous Na₂SO₄. Removal of solvent under vacuum yielded crude 6-((6-(1-hexylnonylcarbonyloxy)hexyl)(4-(methylamino)butyl)amino)hexyl 2-hexyldecanoate (compound **18-3**; 0.4 g, 98%) that was used further as is. ESI-MS: MW for C₄₉H₉₈N₂O₄ [M+H]⁺ Calc. 779.76; Found 779.98.



Synthesis of Compound 18-4

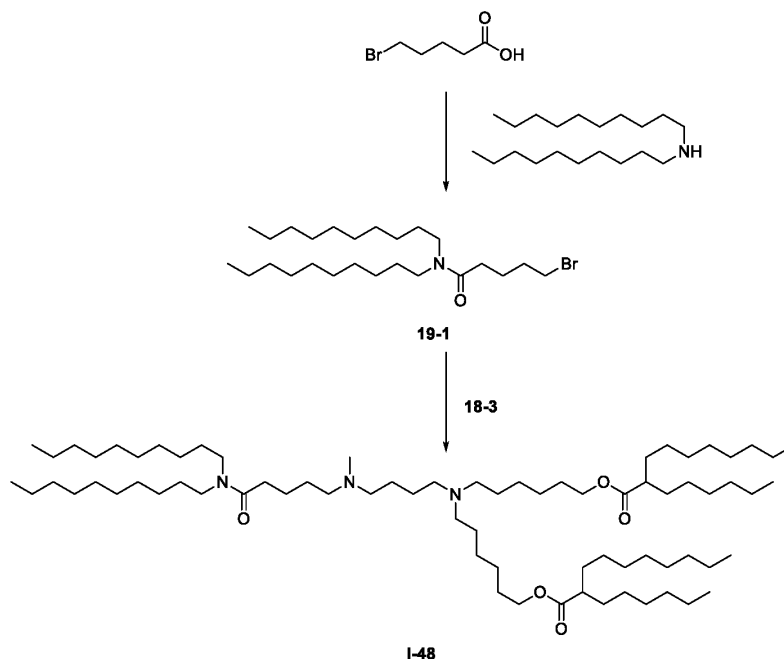
5-Bromovaleric acid (219 mg, 1.21 mmol, 1 eq), dihexylamine (269 mg, 338 μ L, 1.45 mmol, 1.2 eq) and DIPEA (313 mg, 423 μ L, 2.42 mmol, 2 eq) were dissolved in DCM (11 mL). To this solution, 1,1,3,3-tetramethyl-2-(3*H*-1,2,3,4-tetraazainden-3-yl)-3-isoureaium hexafluorido-phosphate(1-) (HATU; 598 mg, 1.57 mmol, 1.3 eq) was added in one portion. The reaction mixture was stirred for 15 minutes at room temperature. After that, the reaction mixture was washed with saturated aqueous NaHCO₃ solution (5 mL). The water layer was extracted with DCM (2 $\times$ 5 mL). The combined organic layer was dried with anhydrous Na₂SO₄ and concentrated under vacuum. The residue was purified via automated flash chromatography (12 g SiO₂ column; 0-50% EtOAc in hexanes, target elutes with 25-30% EtOAc) to give 5-bromo-*N,N*-dihexylpentanamide (compound **18-4**; 290 mg, 68%). ESI-MS: MW for C₁₇H₃₄BrNO [M+H]⁺ Calc. 348.19; Found 348.30.

Synthesis of Compound I-47

Compound **18-3** (290 mg, 373 μ mol, 1.3 eq), *N,N*-dihexyl-5-bromovaleramide (compound **18-4**; 99.8 mg, 287 μ mol, 1 eq) and DIPEA (74.1 mg, 0.1 mL, 576 μ mol, 2 eq) were mixed in acetonitrile (745 μ L) and reacted in a microwave reactor (140 °C, 30 min). After cooling down, solvent was removed under vacuum, residue was partitioned between EtOAc and water. The organic layer was dried over anhydrous Na₂SO₄. After removing solvent under vacuum, residue was purified via automated flash chromatography (12 g SiO₂ column; 0-50% EtOAc in Hex with 1% Et₃N, target elutes with 18-31% EtOAc) to give ((4-((5-(dihexylamino)-5-oxopentyl)(methyl)amino)butyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (compound **I-47**; 99 mg, 33%). ¹H NMR (400 MHz, CDCl₃) δ 4.06 (t, *J* = 6.7 Hz, 4H), 3.33 – 3.24 (m, 2H), 3.24 – 3.14 (m, 2H), 2.45 – 2.26 (m, 14H), 2.19 (s, 3H), 1.72 – 1.47 (m, 19H), 1.47 – 1.16 (m, 73H), 0.93 – 0.82 (m, 18H). ESI-MS: MW for C₆₆H₁₃₁N₃O₅ [M+H]⁺ Calc. 1047.01; Found 1047.27.

EXAMPLE 19

((4-((5-(DIDECYLAMINO)-5-OXOPENTYL)(METHYL)AMINO)BUTYL)AZANEDIYL)BIS(HEXANE-6,1-DIYL) BIS(2-HEXYLDECANOATE) (COMPOUND I-48)



5 Synthesis of Compound 19-1

5-Bromovaleric acid (200 mg, 1.10 mmol, 1 eq), dodecylamine (395 mg, 1.33 mmol, 1.2 eq) and DIPEA (286 mg, 386 μ L, 2.21 mmol, 2 eq) were dissolved in DCM (10 mL). To this solution, HATU (546 mg, 1.44 mmol, 1.3 eq) was added in one portion. The reaction mixture was stirred for 15 minutes at room temperature. After that, the reaction mixture was washed with saturated aqueous NaHCO₃ solution (5 mL). The water layer was extracted with DCM (2 $\times$ 5 mL). The combined organic layer was dried with anhydrous Na₂SO₄ and concentrated under vacuum. The residue was purified via automated flash chromatography (12 g SiO₂ column; 0-30% EtOAc in hexanes, target elutes with 11% EtOAc) to give 5-bromo-N,N-didecylpentanamide (compound **19-1**; 380 mg, 75%). ESI-MS: MW for C₂₅H₅₀BrNO [M+H]⁺ Calc. 460.31; Found 460.42.

Synthesis of Compound I-48

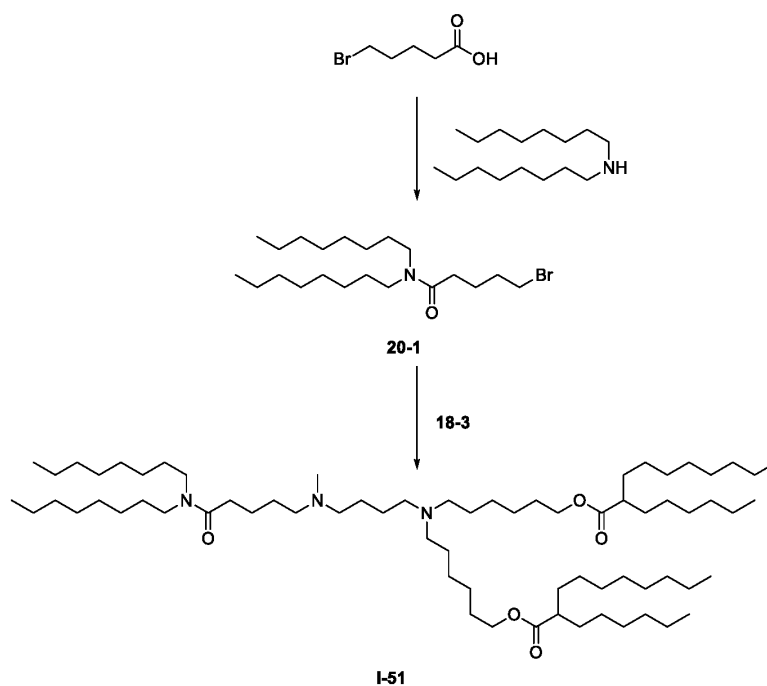
Compound **18-3** (290 mg, 373 μ mol, 1.3 eq), 5-bromo-N,N-didecylpentanamide (compound **19-1**; 132 mg, 287 μ mol, 1 eq) and DIPEA (74.1 mg, 0.1 mL, 576 μ mol, 2 eq) were mixed in acetonitrile (745 μ L) and reacted in a microwave reactor (140 $^{\circ}$ C, 30 min). After

cooling down, solvent was removed under vacuum, residue was partitioned between EtOAc and water. The organic layer was dried over anhydrous Na_2SO_4 . After removing solvent under vacuum, residue was purified via automated flash chromatography (12 g SiO_2 column; 0-50% EtOAc in hexanes with 1% Et_3N , target elutes with 25-31% EtOAc) to give ((4-((5-
 5 (didecylamino)-5-oxopentyl)(methyl)amino)butyl)-azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (compound **I-48**; 125 mg, 38%). ^1H NMR (400 MHz, CDCl_3) δ 4.06 (t, $J = 6.7$ Hz, 4H), 3.34 – 3.24 (m, 2H), 3.24 – 3.15 (m, 2H), 2.41 – 2.25 (m, 14H), 2.20 (s, 3H), 1.77 – 1.19 (m, 105H), 0.88 (tt, $J = 7.1, 2.3$ Hz, 18H). ESI-MS: MW for $\text{C}_{74}\text{H}_{147}\text{N}_3\text{O}_5$ $[\text{M}+\text{H}]^+$ Calc. 1159.14; Found 1159.29.

10

EXAMPLE 20

((4-((5-(DIOCTYLAMINO)-5-OXOPENTYL)(METHYL)AMINO)BUTYL)AZANEDIYL)BIS(HEXANE-6,1-DIYL) BIS(2-HEXYLDECANOATE) (COMPOUND I-51)

**Synthesis of Compound 20-1**

15 5-Bromovaleric acid (200 mg, 1.10 mmol, 1 eq), dihexylamine (293 mg, 367 μL , 1.22 mmol, 1.1 eq) and DIPEA (286 mg, 386 μL , 2.21 mmol, 2 eq) were dissolved in DCM (10 mL). To this solution, HATU (546 mg, 1.44 mmol, 1.3 eq) was added in one portion. The reaction mixture was stirred for 15 minutes at room temperature. After that, the reaction mixture was

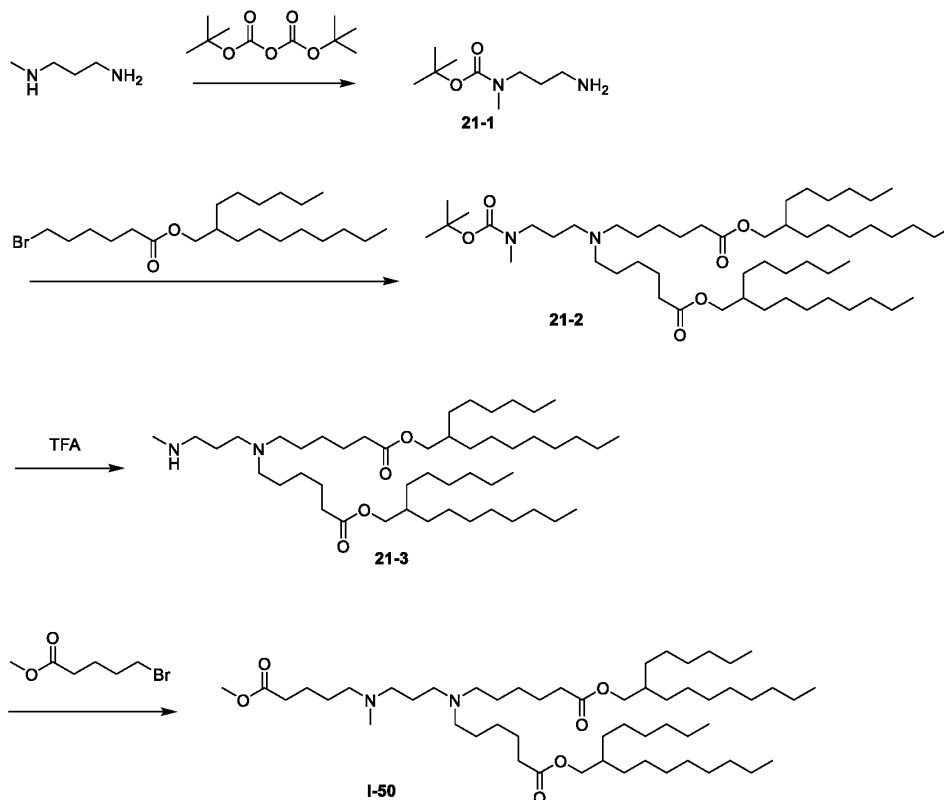
washed with saturated aqueous NaHCO₃ solution (5 mL). The water layer was extracted with DCM (2 × 5 mL). The combined organic layer was dried with anhydrous Na₂SO₄ and concentrated under vacuum. The residue was purified via automated flash chromatography (12 g SiO₂ column; 0-50% EtOAc in hexanes, target elutes with 25-32% EtOAc) to give 5-bromo-*N,N*-dioctylpentanamide (compound **20-1**; 325 mg, 72 %). ESI-MS: MW for C₂₁H₄₂BrNO [M+H]⁺ Calc. 404.25; Found 404.43.

Synthesis of Compound I-51

Compound **18-3** (217 mg, 278 μmol, 1.5 eq), *N,N*-dioctyl-5-bromovaleramide (compound **20-1**; 75.0 mg, 185 μmol, 1 eq) and DIPEA (47.9 mg, 64.8 μL, 371 μmol, 2 eq) were mixed in acetonitrile (556 μL) and reacted in a microwave reactor (140 °C, 30 min). After cooling down, solvent was removed under vacuum, residue was partitioned between EtOAc and water. The organic layer was dried over anhydrous Na₂SO₄. After removing solvent under vacuum, residue was purified via automated flash chromatography (12 g SiO₂ column; 0-50% EtOAc in hexanes with 1% Et₃N, target elutes with 34-38% EtOAc) to give ((4-((5-(dioctylamino)-5-oxopentyl)(methylamino)-butyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (compound **I-51**; 110 mg, 54%). ¹H NMR (400 MHz, CDCl₃) δ 4.06 (t, *J* = 6.7 Hz, 4H), 3.40 – 3.24 (m, 2H), 3.19 (dd, *J* = 9.2, 6.4 Hz, 2H), 2.55 – 2.25 (m, 14H), 2.19 (s, 3H), 1.77 – 1.11 (m, 98H), 0.95 – 0.77 (m, 18H). ESI-MS: MW for C₇₀H₁₃₉N₃O₅ [M+H]⁺ Calc. 1103.08; Found 1103.32.

EXAMPLE 21

BIS(2-HEXYLDECYL) 6,6'-((3-((5-METHOXY-5-OXOPENTYL)(METHYL)AMINO)PROPYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-50)



5 Synthesis of Compound 21-1

To a solution of *N*-methyl-1,3-diaminopropane (15 mmol, 1.32 g, 1.57 mL) in dichloromethane (25 mL) was added a solution of di-*tert*-butyl dicarbonate (15 mmol, 3.27 g) in dichloromethane (25 mL) dropwise at 0-5 °C. After addition, the temperature was allowed to rise to room temperature. Gradually, white precipitation appeared. Stirring was continued at room temperature for 16h. The white solid was filtered off and washed with DCM. Concentration of the filtrate gave the desired product as a colorless oil (2.31 g, 82%) and was used for the next step without further purification.

Synthesis of Compound 21-2

A mixture of 2-hexyldecyl 6-bromohexanoate (2 eq, 3.56 g, 8.5 mmol), anhydrous acetonitrile (35 mL), *N,N*-diisopropylethylamine (1.85 mL) and compound **21-1** (800 mg, 4.25 mmol) and sodium iodide (0.1 eq, 64 mg). The mixture was heated at 75 °C for 18h in a sealed

pressure flask. TLC ($\text{CHCl}_3/\text{MeOH} = 19:1$; $\text{CHCl}_3/\text{EtOH}/\text{H}_2\text{O}/\text{NH}_4\text{OH} = 30:25:3:2$; Hexane/Ethyl acetate = 9:1) showed there were still lots of the starting bromide and no starting amine. Another 430 mg of compound **21-1** was added and heating was continued for another day. The reaction mixture was concentrated. The residue was taken up in hexane (100 mL) and
5 filtered through a pad of silica gel. The pad was then eluted with a gradient mixture of hexane, EtOAc and Et_3N (95:5:0 to 80:20:1). The desired product was obtained as brownish oil (2.381 g, 65%) and used for the next step without further purification.

Synthesis of Compound 21-3

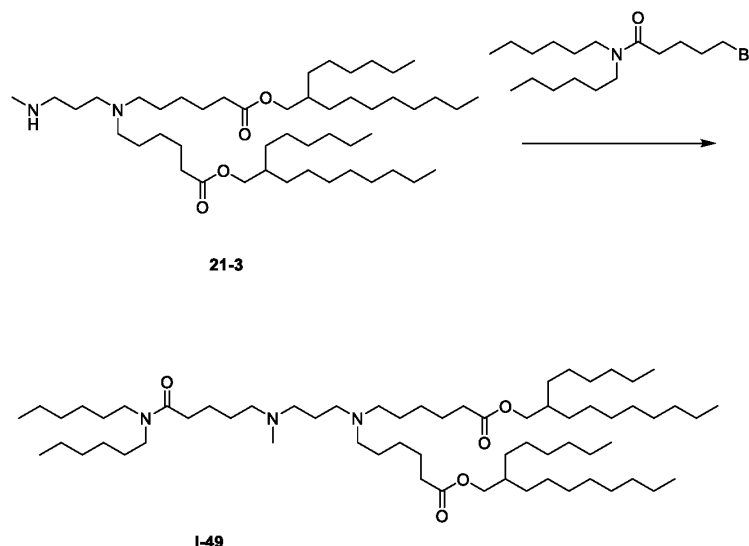
To a solution of compound **21-2** (2.381 g, ca 2.75 mmol) in CH_2Cl_2 (15 mL) was added
10 TFA (55 mmol, 4.2 mL). After stirring at room temperature for 16 h, the reaction mixture was diluted with DCM and washed with saturated bicarbonate solution. The aqueous phase was extracted with DCM. The combined organic phase was dried over sodium sulfate and concentrated. The residue was taken up in hexane (100 mL) and filtered through a pad of silica gel. The pad was then eluted with a gradient mixture of hexane and EtOAc (95:5:0 to 80:20) and
15 then with a gradient mixture of DCM, MeOH, and Et_3N (85:15:1). The desired product was obtained as brownish oil (1.14 g, 60%). MS (ESI): m/z 765.93 (M+1).

Synthesis of Compound I-50

A mixture of methyl 5-bromovalerate (1 eq, 102 mg, 0.52 mmol), compound **21-3** (1 eq., 400 mg, 0.52 mmol), *N,N*-diisopropylethylamine (1.5 equiv. 0.14 mL), and sodium iodide (0.5
20 eq, 39 mg) and anhydrous acetonitrile (10 mL). The mixture was heated at 80 °C for 18h in a sealed pressure flask and then was concentrated. The residue was taken up in a mixture of hexane, EtOAc and Et_3N (80:20:1) and filtered through a pad of silica gel. The pad was then washed with the same solvent mixture. The filtrate was concentrated to give the crude product as brown oil (200 mg). The product was further purified by column chromatography on silica gel (0
25 to 5% methanol in chloroform). This gave the desired product as colorless oil (113 mg, 0.13 mmol, 25%). ^1H NMR (400 MHz, CDCl_3 at 7.26 ppm) δ : 3.96 (d, 5.8 Hz, 4H), 3.66 (s, 3H), 2.42-2.27 (m, 16H), 2.18 (s, 3H), 1.68-1.57 (m, 10H), 1.53-1.38 (m, 6H), 1.36-1.18 (m, 52H), 0.92-0.85 (m, 12H). MS (ESI): m/z (M+1), 880.10

EXAMPLE 22

**BIS(2-HEXYLDECYL) 6,6'-((3-((5-(DIHEXYLAMINO)-5-
OXOPENTYL)(METHYL)AMINO)PROPYL)AZANEDIYL)DIHEXANOATE (COMPOUND I-49)**



5 Synthesis of Compound I-49

A mixture of 5-bromo-*N,N*-diheptylpentanamide (1 eq, 182 mg, 0.52 mmol), compound **21-3** (prepared according to the procedures of Example 21, 1 eq., 400 mg, 0.52 mmol) *N,N*-diisopropylethylamine (1.5 equiv. 0.14 mL) and sodium iodide (0.5 eq, 39 mg) and anhydrous acetonitrile (10 mL). The mixture was heated at 80°C for 18h in a sealed pressure flask (oil bath 80 °C) and then was concentrated. The residue was taken up in a mixture of hexane, EtOAc and Et₃N (80:20:1) and filtered through a pad of silica gel. The pad was then washed with the same solvent mixture. The filtrate was concentrated to give the crude product as brown oil (320 mg). The product was further purified by column chromatography on silica gel (0 to 5% methanol in chloroform). This gave the desired product as colorless oil (167.6 mg, 0.16 mmol, 27%). ¹H NMR (400 MHz, CDCl₃ at 7.26 ppm) δ: 3.96 (d, 5.8 Hz, 4H), 3.31-3.25 (m, 2H), 3.22-3.17 (m, 2H), 2.42-2.27 (m, 16H), 2.19 (s, 3H), 1.68-1.57 (m, 10H), 1.55-1.38 (m, 10H), 1.36-1.20 (m, 64H), 0.92-0.85 (m, 18H). MS (ESI): *m/z* 1033.3 (M+1).

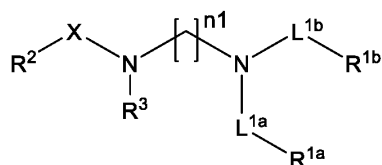
The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety.

Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications, and publications to provide yet further embodiments. These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the
5 specific embodiments disclosed in the specification and the claims but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

U.S. Provisional Patent Application No. 63/404,463, filed September 7, 2022, to which the present application claims priority, is hereby incorporated herein by reference in its entirety.

CLAIMS

1. A compound having the following Structure (I):



(I)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein:

L^{1a} and L^{1b} are each independently optionally substituted C_3 - C_{12} alkyl;

R^{1a} is $-C(=O)OR^{4a}$ or $-O(C=O)R^{4a}$;

R^{1b} is $-C(=O)OR^{4b}$ or $-O(C=O)R^{4b}$;

R^2 is $-NR^6(C=O)R^5$, $-(C=O)N(R^6)R^5$ or $-(C=O)OR^7$;

R^3 and R^6 are each independently hydrogen or optionally substituted C_1 - C_{12} alkyl;

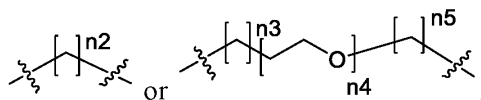
R^{4a} , R^{4b} , and R^5 are each independently optionally substituted alkyl;

R^7 is optionally substituted C_1 - C_6 alkyl or optionally substituted arylalkyl;

n_1 is 2, 3, 4, 5 or 6; and

X is C_2 - C_6 alkylene or C_4 - C_{20} alkyleneoxide.

2. The compound of claim 1, wherein X is:



wherein:

n_2 is 2, 3, 4, 5, or 6;

n_3 is 0, 1, 2, 3, or 4;

n_4 is 2, 3, or 4; and

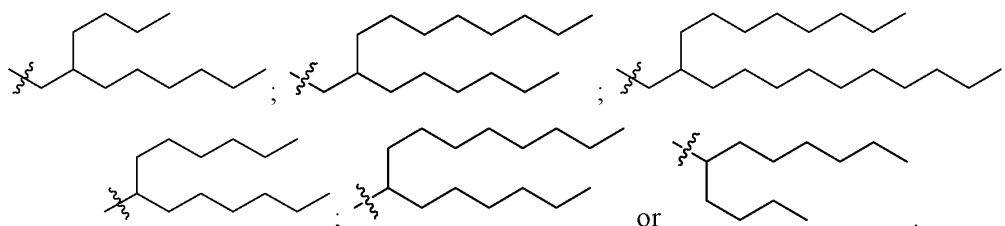
n_5 is 2, 3, 4, or 5.

3. The compound of claim 1 or 2, wherein L^{1a} is C_5 - C_9 alkyl.
4. The compound of any one of claims 1-3, wherein L^{1b} is C_5 - C_9 alkyl.
5. The compound of any one of claims 1-4, wherein L^{1a} is C_5 -, C_6 -, C_7 -, or C_9 -alkyl.

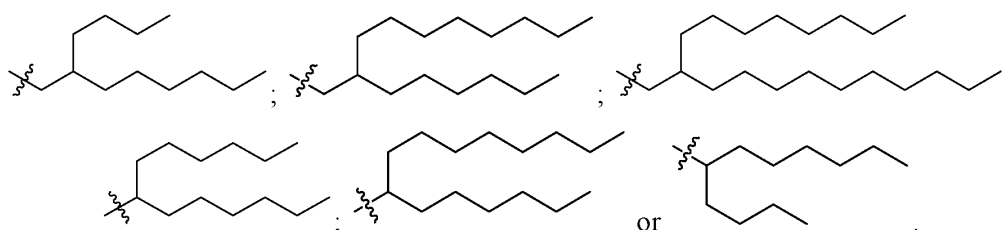
6. The compound of any one of claims 1-5, wherein L^{1b} is C₅-, C₆-, C₇-, or C₉-alkyl.
7. The compound of any one of claims 1-6, wherein L^{1a} is unsubstituted.
8. The compound of any one of claims 1-7, wherein L^{1b} is unsubstituted.
9. The compound of any one of claims 1-8, wherein L^{1a} is unbranched.
10. The compound of any one of claims 1-9, wherein L^{1b} is unbranched.
11. The compound of any one of claims 1-10, wherein R^{1a} is -O(C=O)R^{4a}.
12. The compound of any one of claims 1-10, wherein R^{1a} is -(C=O)OR^{4a}.
13. The compound of any one of claims 1-12, wherein R^{1b} is -O(C=O)R^{4b}.
14. The compound of any one of claims 1-12, wherein one of R^{1b} is -(C=O)OR^{4b}.
15. The compound of any one of claims 1-14, wherein R^{4a} is C₈-C₂₄-alkyl.
16. The compound of any one of claims 1-15, wherein R^{4a} is C₁₁-C₁₆-alkyl.
17. The compound of any one of claims 1-16, wherein R^{4a} is C₁₁-alkyl.
18. The compound of any one of claims 1-16, wherein R^{4a} is C₁₅-alkyl.
19. The compound of any one of claims 1-16, wherein R^{4a} is C₁₆-alkyl.
20. The compound of any one of claims 1-19, wherein R^{4b} is C₈-C₂₄-alkyl.
21. The compound of any one of claims 1-20, wherein R^{4b} is C₁₀-C₁₈-alkyl.
22. The compound of any one of claims 1-21, wherein R^{4b} is C₁₁-C₁₆-alkyl.

23. The compound of any one of claims 1-22, wherein R^{4b} is C_{11} -alkyl.
24. The compound of any one of claims 1-22, wherein R^{4b} is C_{15} -alkyl.
25. The compound of any one of claims 1-22, wherein R^{4b} is C_{16} -alkyl.
26. The compound of any one of claims 1-25, wherein R^{4a} is branched.
27. The compound of any one of claims 1-26, wherein R^{4b} is branched.
28. The compound of any one of claims 1-27, wherein R^{4a} is unsubstituted.
29. The compound of any one of claims 1-28, wherein R^{4b} is unsubstituted.

30. The compound of any one of claims 1-29, wherein R^{4a} has one of the following structures:

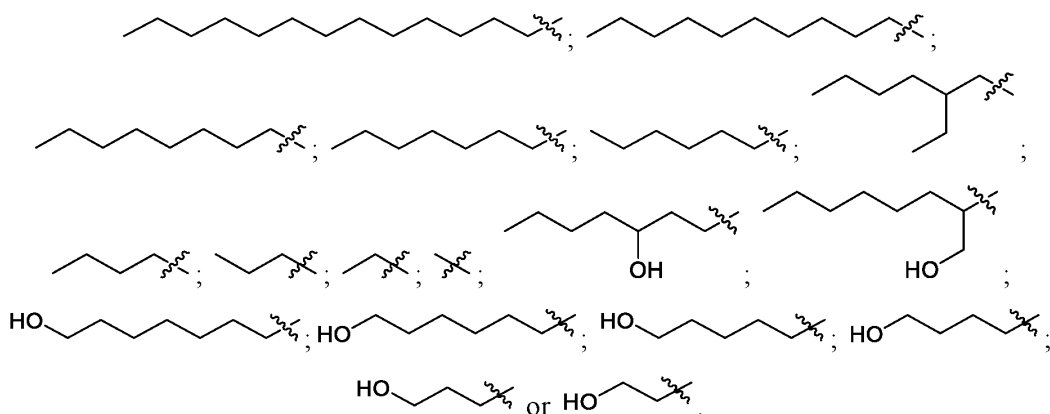


31. The compound of any one of claims 1-30, wherein R^{4b} has one of the following structures:



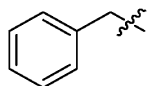
32. The compound of any one of claims 1-31, wherein R^2 is $-NR^6(C=O)R^5$.
33. The compound of any one of claims 1-31, wherein R^2 is $-(C=O)N(R^6)R^5$.

34. The compound of any one of claims 1-33, wherein R^5 is C_2 - C_{16} -alkyl.
35. The compound of any one of claims 1-34, wherein R^5 is C_4 - C_{13} -alkyl.
36. The compound of any one of claims 1-35, wherein R^5 is C_4 -, C_7 -, C_8 -, C_{10} -, or C_{13} -alkyl.
37. The compound of any one of claims 1-36, wherein R^5 is unsubstituted.
38. The compound of any one of claims 1-36, wherein R^5 is substituted.
39. The compound of claim 38, wherein R^5 is substituted with hydroxyl.
40. The compound of any one of claims 1-39, wherein R^5 is branched.
41. The compound of any one of claims 1-39, wherein R^5 is unbranched.
42. The compound of any one of claims 1-41 wherein R^5 has one of the following structures:



43. The compound of any one of claims 1-42, wherein R^6 is C_1 - C_{10} alkyl.
44. The compound of any one of claims 1-43, wherein R^6 is C_1 -, C_2 -, C_3 -, C_6 -, C_8 -, or C_{10} -alkyl.

45. The compound of any one of claims 1-44, wherein R^6 is unbranched.
46. The compound of any one of claims 1-45, wherein R^6 is methyl, ethyl, n-butyl, n-hexyl, n-octyl, or n-decyl.
47. The compound of any one of claims 1-46, wherein R^6 is unsubstituted.
48. The compound of any one of claims 1-46, wherein R^6 is substituted.
49. The compound of any one of claims 1-42, wherein R^6 is C_1 - C_6 alkyl substituted with one or more hydroxyl.
50. The compound of any one of claims 1-42, wherein R^6 is C_2 -, C_3 -, C_4 -, or C_6 - alkyl substituted with one or more hydroxyl.
51. The compound of any one of claims 1-42, wherein R^6 is hydrogen.
52. The compound of any one of claims 1-51, wherein R^2 is $-(C=O)OR^7$.
53. The compound of claim 52, wherein R^7 is C_1 - C_3 alkyl or C_7 - C_{16} arylalkyl.
54. The compound of any one of claims 52 or 53, wherein R^7 is unsubstituted.
55. The compound of any one of claims 52-54, wherein R^7 is $-CH_3$ or has the following structure:



56. The compound of any one of claims 1-55, wherein R^3 is optionally substituted C_1 - C_6 alkyl.
57. The compound of any one of claims 1-56, wherein R^3 is optionally substituted methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl.

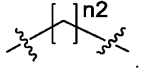
58. The compound of any one of claims 1-57, wherein R^3 is optionally substituted methyl.

59. The compound of any one of claims 1-56, wherein R^3 is C_1 - C_6 alkyl substituted with one or more hydroxyl.

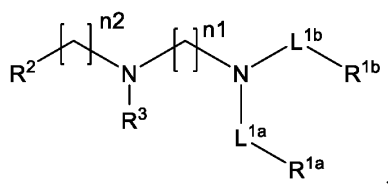
60. The compound of any one of claims 1-56, wherein R^3 is C_2 - or C_4 - alkyl substituted with one or more hydroxyl.

61. The compound of any one of claims 1-56, wherein R^3 is unsubstituted.

62. The compound of any one of claims 1-55, wherein R^3 is hydrogen.

63. The compound of any one of claims 2-62, wherein X is .

64. The compound of any one of claims 2-63, wherein the compound has the following structure (II):

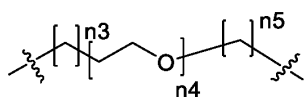


(II)

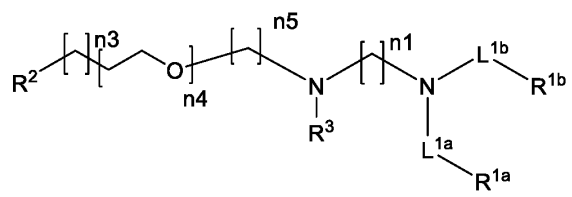
or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof.

65. The compound of any one of claims 2-64, wherein n_2 is 3, 4, or 5.

66. The compound of any one of claims 2-62, wherein X is



67. The compound of claim 66, wherein the compound has the following structure (III):



(III)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof.

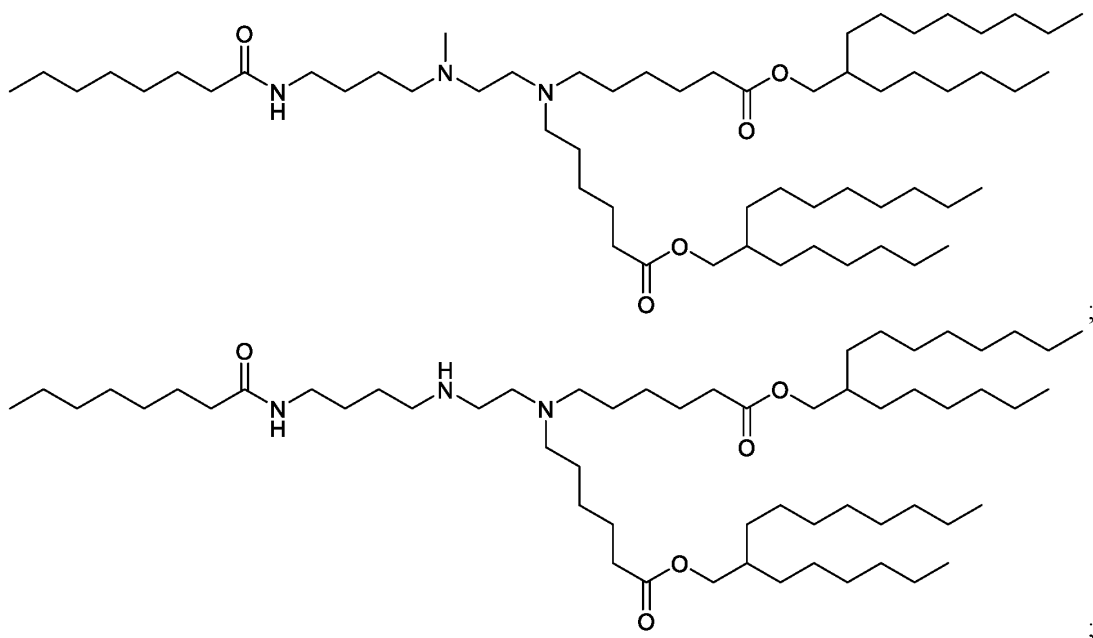
68. The compound of any one of claims 66 or 67 wherein n_3 is 0 or 1.

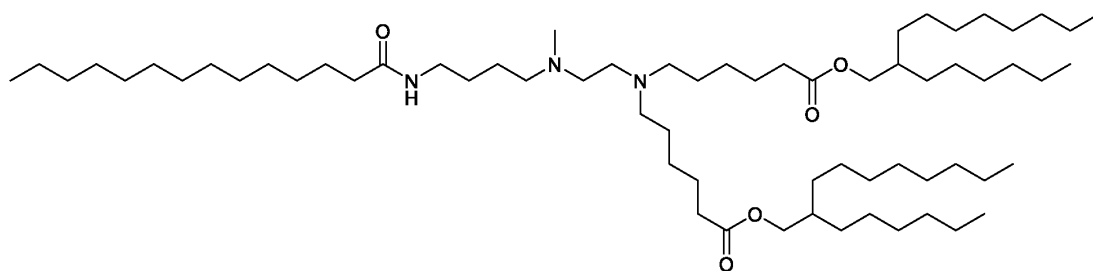
69. The compound of any one of claims 66-68, wherein n_4 is 2 or 3.

70. The compound of any one of claims 66-69, wherein n_5 is 3.

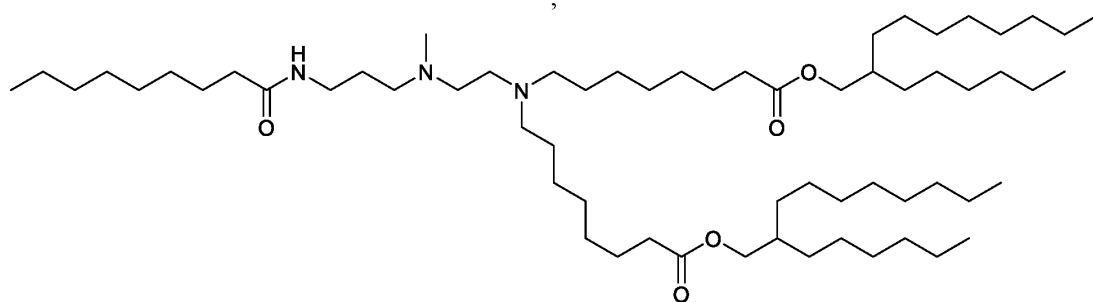
71. The compound of any one of claims 1-70, wherein n_1 is 2.

72. The compound of claim 1, wherein the compound has one of the following structures:

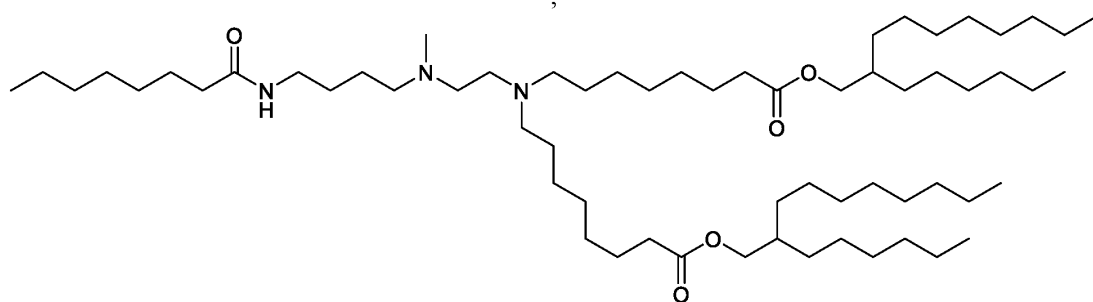




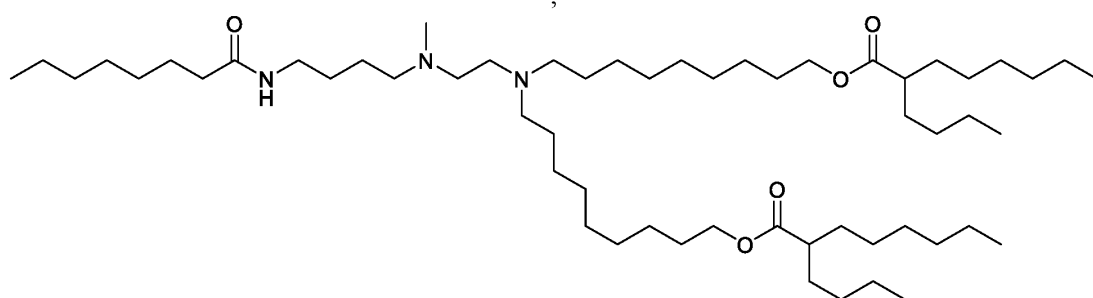
;



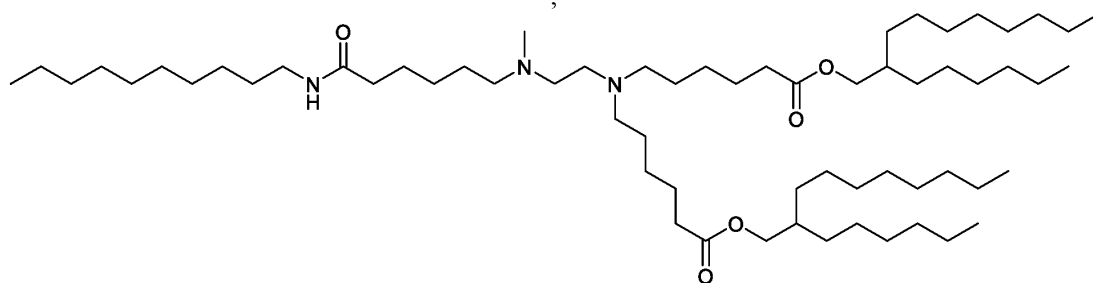
;

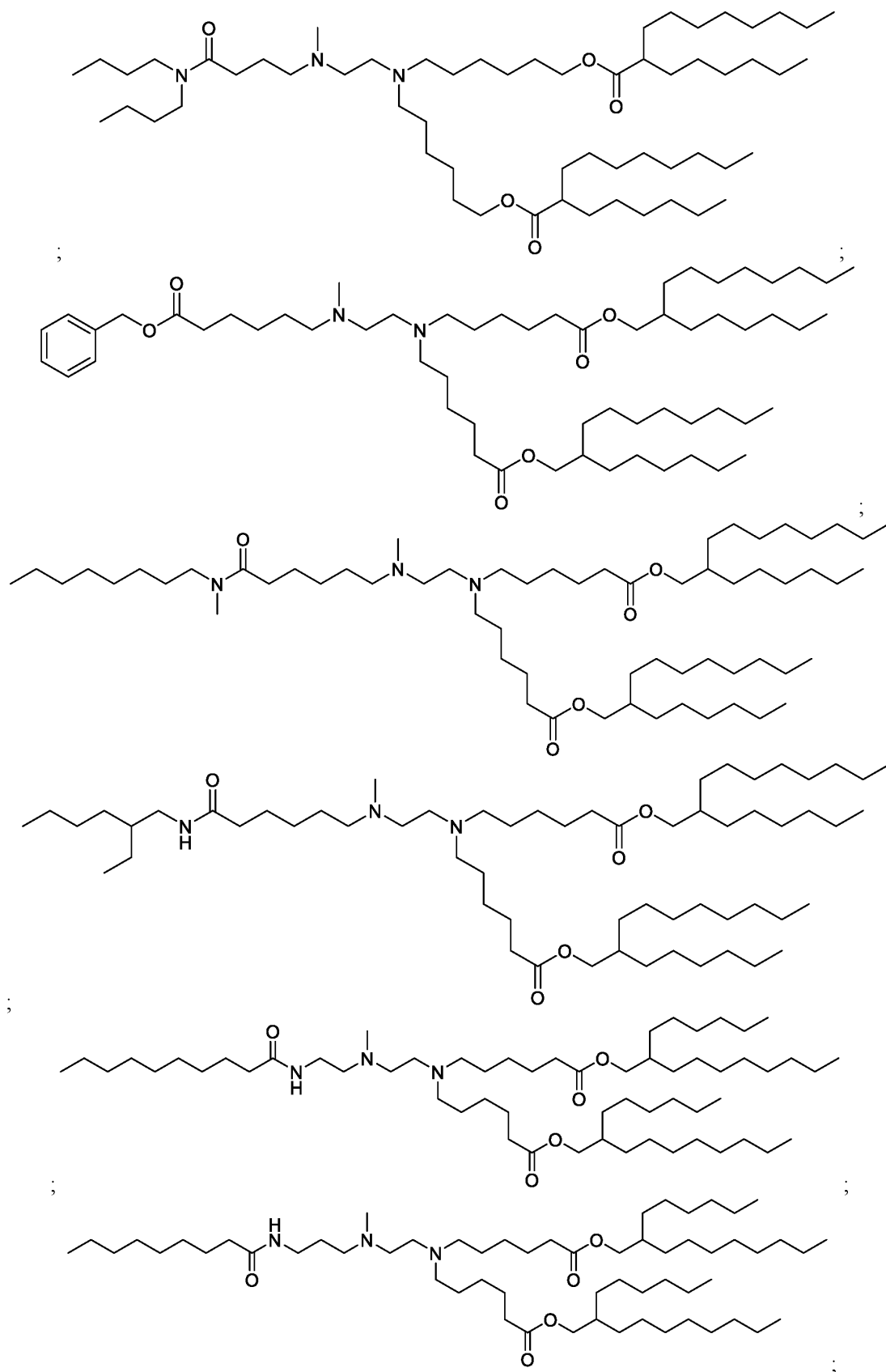


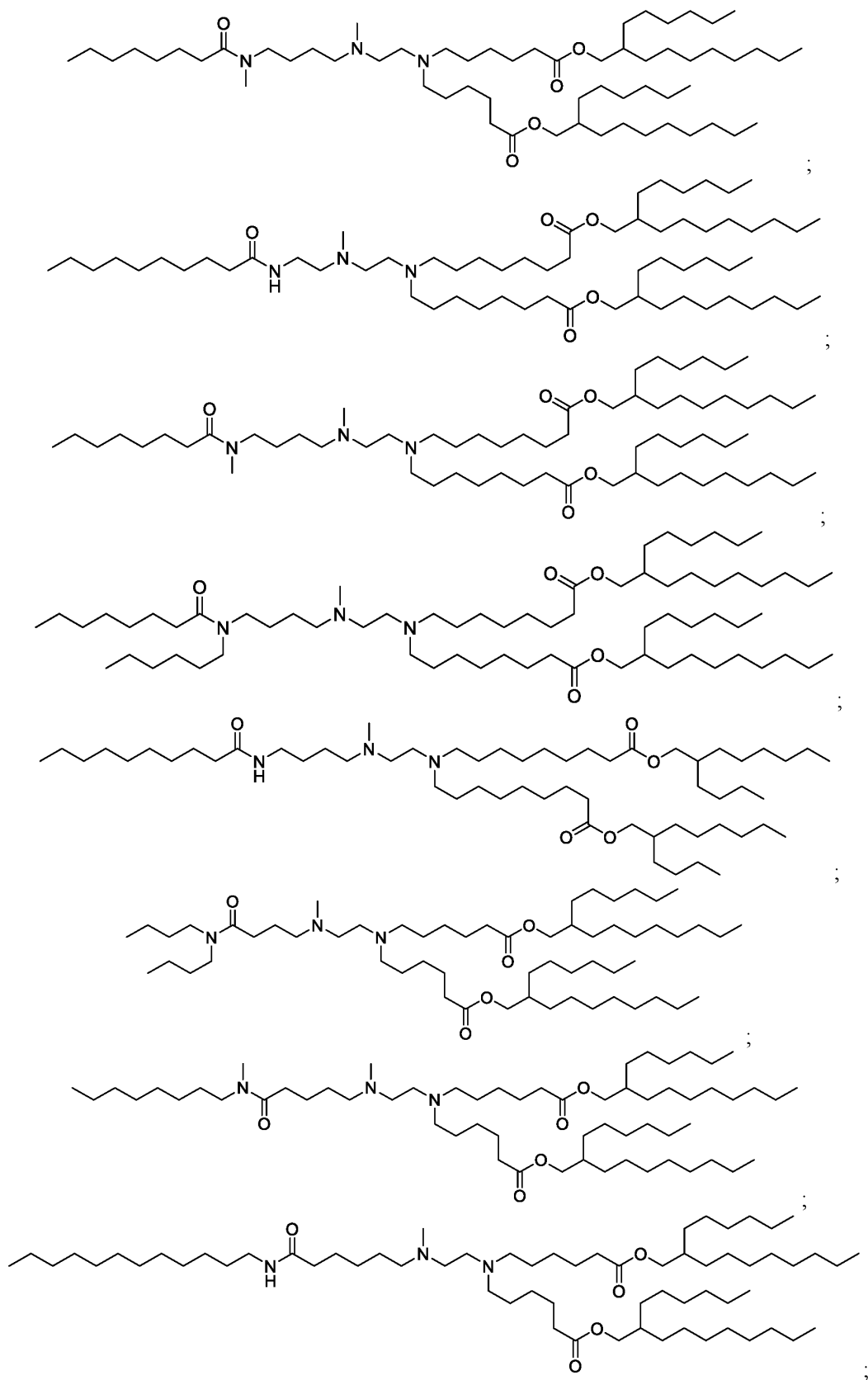
;

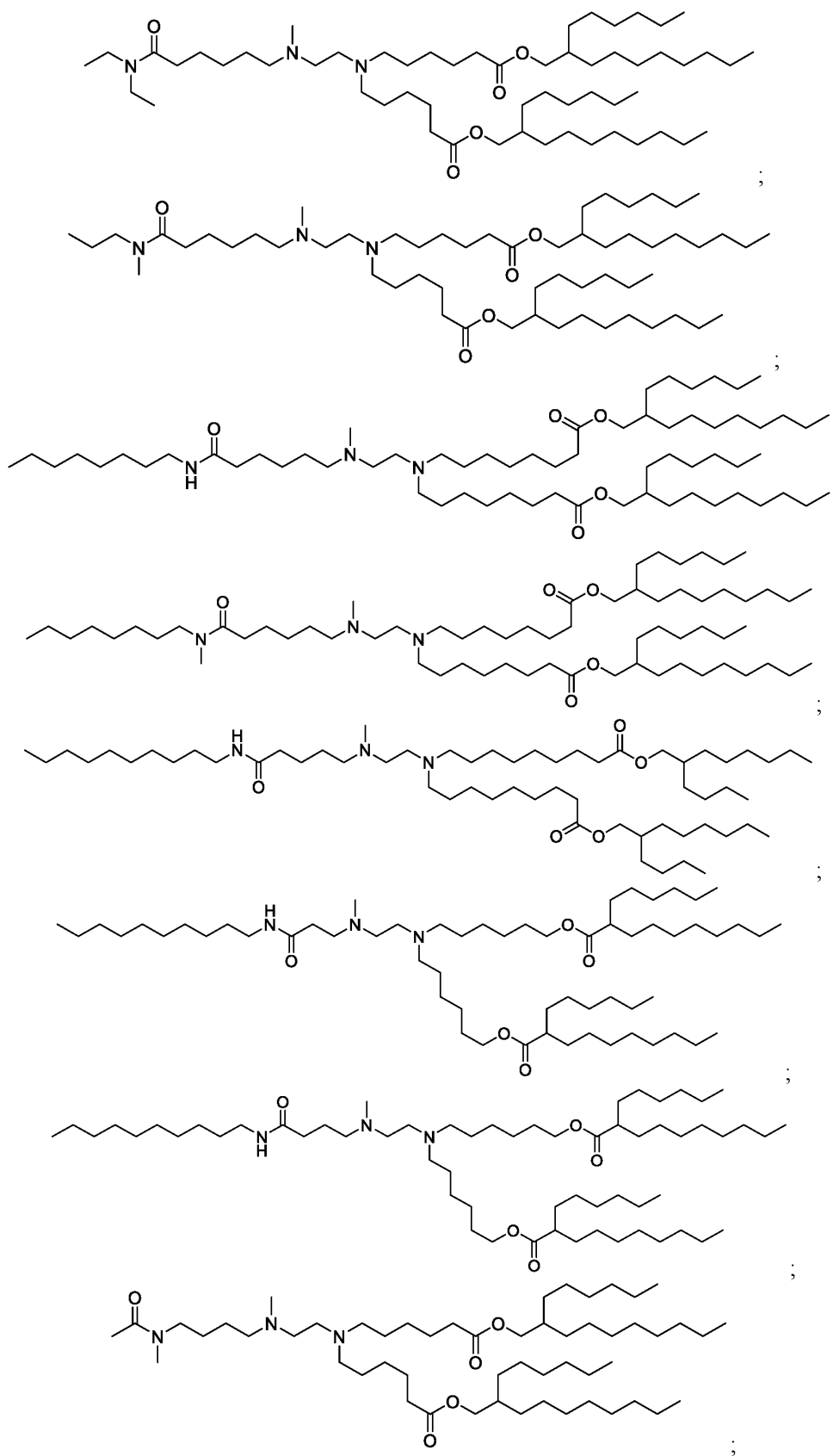


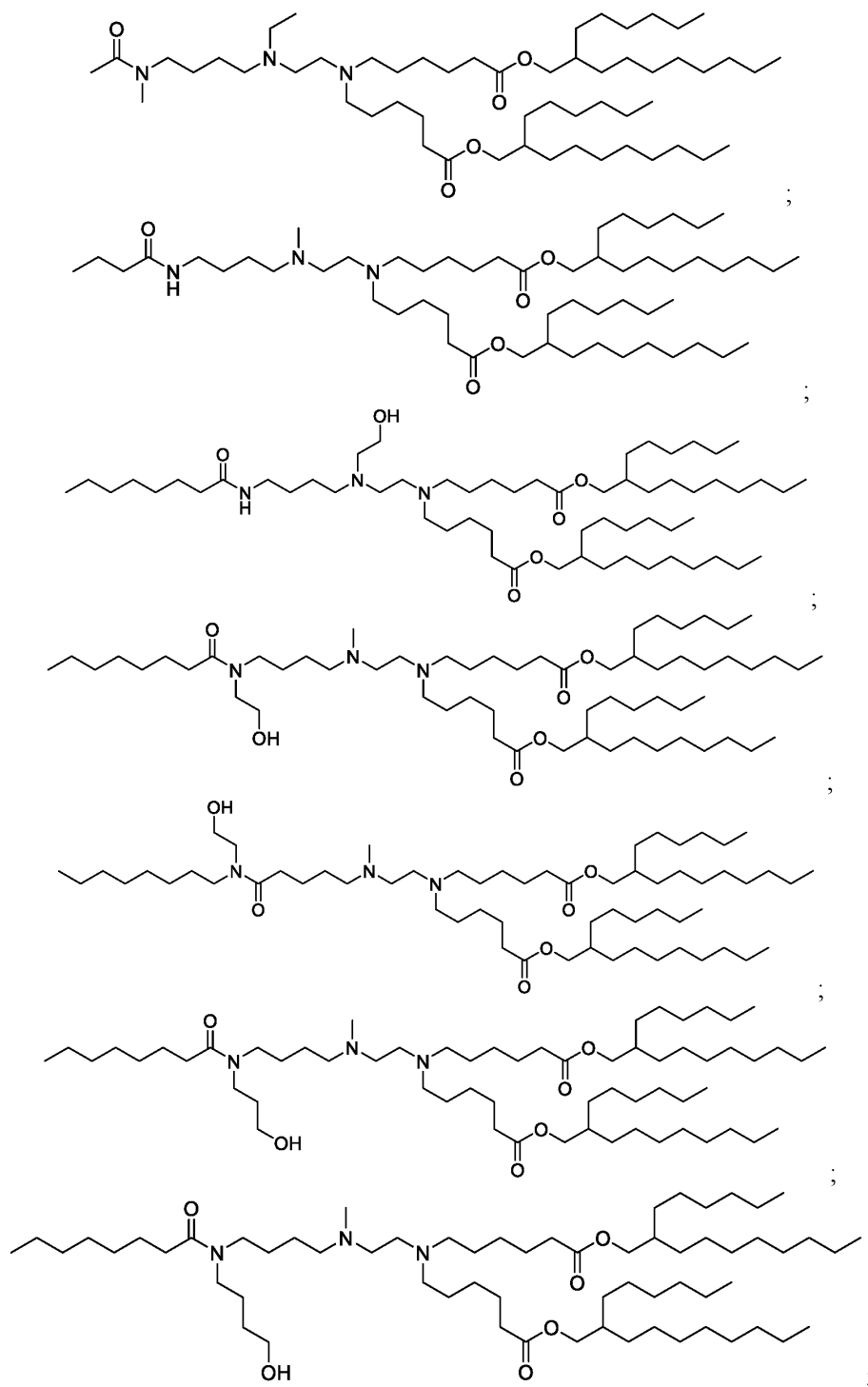
;

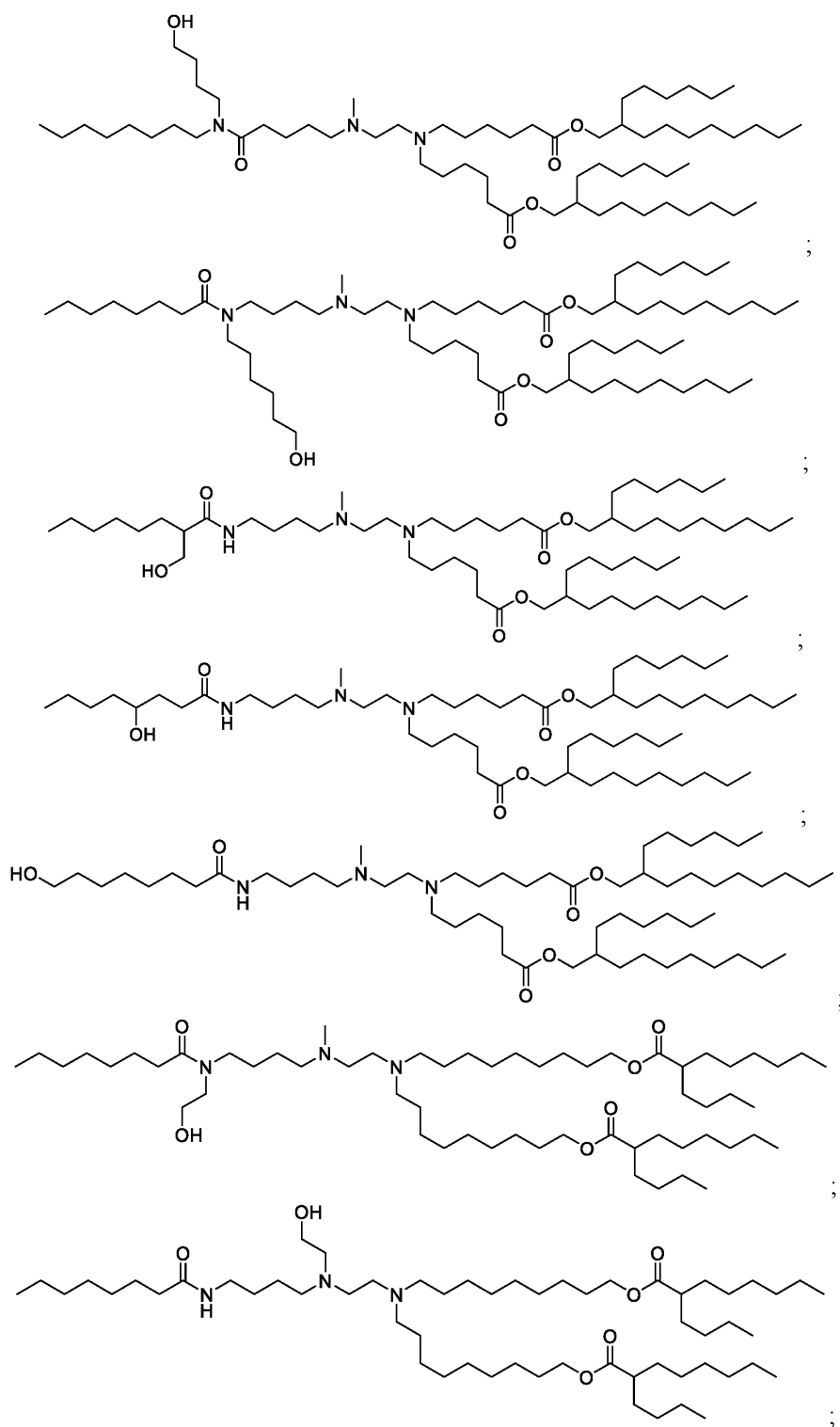


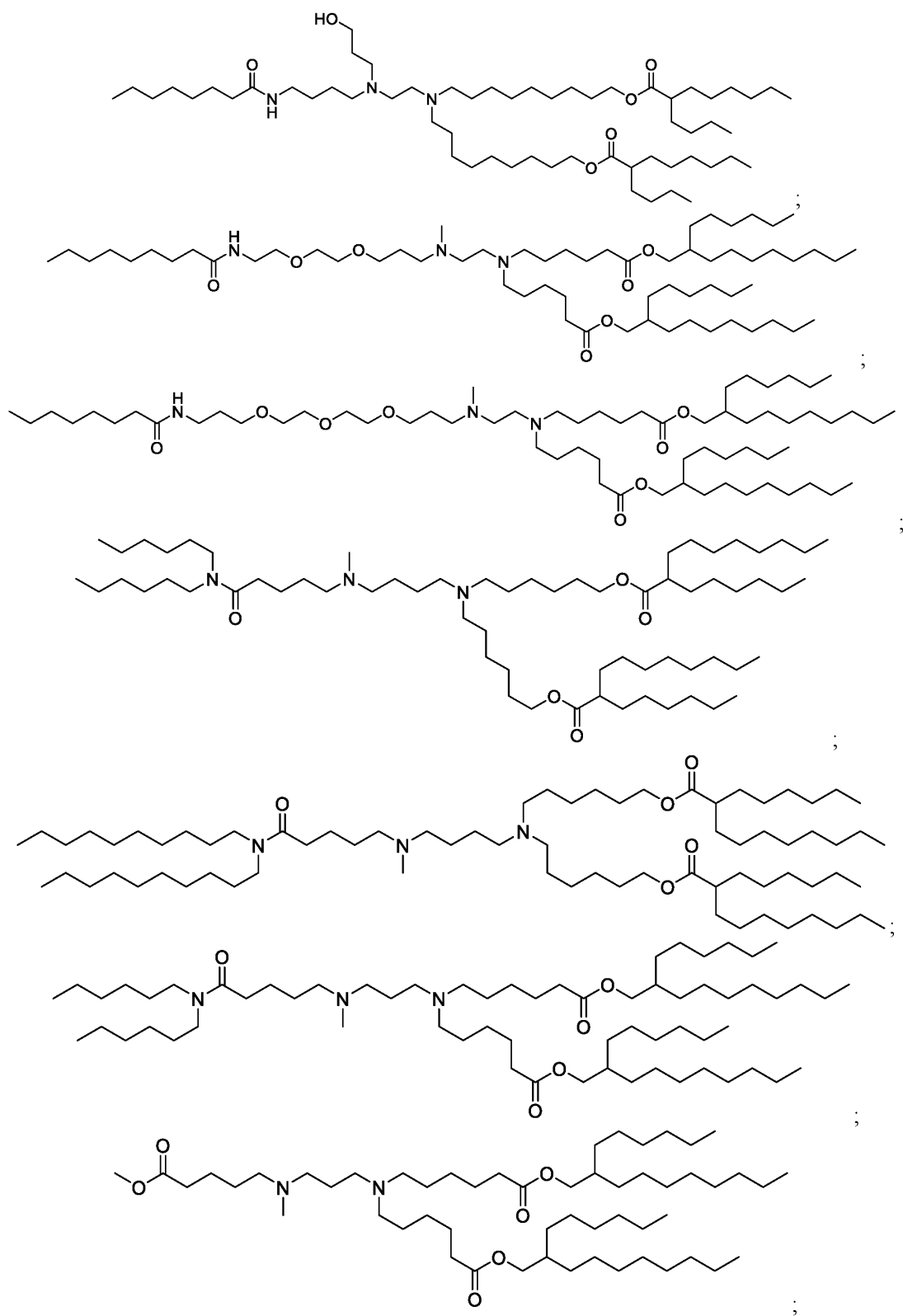


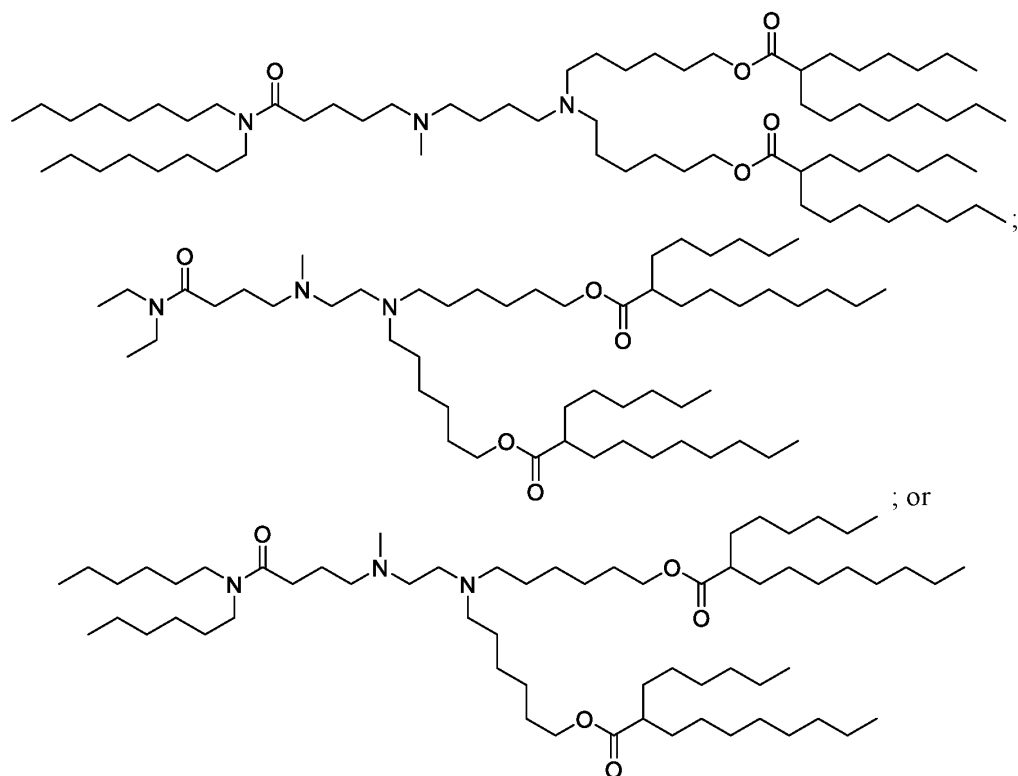












or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof.

73. A composition comprising the compound of any one of claims 1-72 and a therapeutic agent.

74. A lipid nanoparticle comprising the compound of any one of claims 1-72 and a therapeutic agent.

75. The composition or lipid nanoparticle of claim 73 or 74, further comprising one or more excipient selected from neutral lipids, steroids, and polymer conjugated lipids.

76. The composition or lipid nanoparticle of claim 73 or 74, wherein the composition comprises one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE, and SM.

77. The composition or lipid nanoparticle of claim 76, wherein the neutral lipid is DSPC.

78. The composition or lipid nanoparticle of any one of claims 75-77, wherein the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1.

79. The composition or lipid nanoparticle of any one of claims 75-78, wherein the steroid is cholesterol.

80. The composition or lipid nanoparticle of claim 79, wherein the molar ratio of the compound to cholesterol ranges from about 2:1 to 1:1.

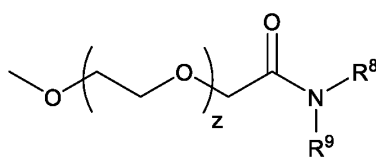
81. The composition or lipid nanoparticle of any one of claims 75-80, wherein the polymer conjugated lipid is a pegylated lipid.

82. The composition or lipid nanoparticle of claim 81, wherein the molar ratio of the compound to the pegylated lipid ranges from about 100:1 to about 10:1 or from about 100:1 to about 25:1.

83. The composition or lipid nanoparticle of any one of claims 75-82, wherein the pegylated lipid is PEG-DMG, PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer or a PEG dialkyoxypropylcarbamate.

84. The lipid nanoparticle of claim 83, wherein the pegylated lipid is PEG-DMG.

85. The composition or lipid nanoparticle of any one of claims 75-82, wherein the pegylated lipid has the following Structure (II):



(II)

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R⁸ and R⁹ are each independently an unbranched or branched, alkyl, alkenyl, or alkynyl containing from 10 to 30 carbon atoms, wherein the alkyl, alkenyl, or alkynyl is optionally interrupted by one or more ester bonds; and

z has a mean value ranging from 30 to 60.

86. The composition or lipid nanoparticle of claim 85, wherein R⁸ and R⁹ are each independently unbranched alkyl chains containing from 12 to 16 carbon atoms.

87. The composition or lipid nanoparticle of any one of claims 85 or 86, wherein the average z is about 45.

88. The composition or lipid nanoparticle of any one of claims 73-87, wherein the therapeutic agent comprises a nucleic acid.

89. The composition or lipid nanoparticle of claim 88, wherein the nucleic acid is selected from antisense and messenger RNA.

90. A pharmaceutical composition comprising the lipid nanoparticle of any one of claims 74-89, and a pharmaceutically acceptable excipient.

91. A method for administering a therapeutic agent to a patient in need thereof, the method comprising preparing or providing the pharmaceutical composition of claim 90 and administering the composition to the patient.

92. A method for inducing expression of a protein in a patient in need thereof, comprising administering the pharmaceutical composition of claim 90 to the patient, wherein the lipid nanoparticle comprises an mRNA encoding the protein.

93. The method of claim 92, wherein the protein is an antigen and the method is for inducing an immune response in the patient.

94. The method of claim 92, wherein the protein is an antigen and the method is for vaccinating the patient against a pathogen.

95. The method of claim 92, wherein the protein is for gene editing.