

Cyclohexane lipidoids for nucleic acid transfection and use thereofField of Art

The invention relates to novel ionizable lipidoids and to the use of these compounds for the transfection and administration of nucleotides and nucleic acids and their synthetic analogues into cells and tissues.

Background Art

The development of nucleic acid (NA)-based therapies has experienced an unprecedented renaissance in recent years. Due to the high efficacy and at the same time the low risk of adverse side effects compared to previously tested therapeutic deoxyribonucleic acids (DNA), ribonucleic acid (RNA) therapies are now gaining ground. Several such drugs have already reached clinical use, for example patisiran for hereditary transthyretin amyloidosis, eteplirsen for certain types of Duchenne muscular dystrophy, or nusinersen for the treatment of spinal muscular atrophy. All of these diseases are life-threatening and there is no alternative treatment for them. Potential drugs targeting ribonucleic acid (RNA) or its use can be divided into three categories based on whether they target NA or proteins or encode proteins. The first category includes single-stranded antisense oligonucleotides of 13-25 nucleotides (nt) that block the translation of messenger RNA (mRNA) or RNA splicing (nusinersen, eteplirsen); and small interfering RNAs (siRNA, 21-23nt) that degrade mRNA (patisiran). Therapeutic RNA molecules that target proteins use a type of molecule known as an RNA aptamer. It is designed to modulate the function of a particular protein. An example of such a drug is pegaptanib, used to treat neovascular age-related macular degeneration, which was the first drug approved of its kind in 2004. Therapies utilising mRNA are mainly used for the preparation of so-called personalized vaccines against cancer or vaccines against infectious diseases (*e.g.*, Zika virus). In viral diseases, candidates for a prophylactic vaccine based on mRNA against rabies and pandemic influenza have been shown to induce safe antibody production in healthy volunteers. Protein-replacing mRNA therapies are also in the preclinical stages of development, for example, for the treatment of hemophilia.

Molecular technologies enabling direct genome editing, especially those based on the CRISPR-Cas9 system, are now booming. CRISPR technology is a tool that allows to change DNA sequences and modify gene function. Potential applications include the repair of genetic defects, the treatment and prevention of the spread of diseases or the improvement of agricultural crops. The CRISPR-Cas9 system has been tested in a number of preclinical and clinical studies, including HIV treatment, treatment of hematological malignancies, and genetic disorders, including sickle cell disease and β -thalassemia. RNA editing is then enabled by the ADAR system (adenosine deaminase acting on RNA), which so far seems to be safer from a clinical point of view. Molecular technologies for direct genome editing can be delivered to the site of action in the form of mRNA that encodes the appropriate enzyme responsible for editing.

A key factor enabling the safe use of all these technologies (or others, based on NA) is their safe and efficient transport to the site of action. The critical step is the penetration of negatively charged NAs through the phospholipid membrane of the cell; the process of deliberately introducing NAs into eukaryotic cells is

called transfection. In recent decades, there has been an intensive development of carriers (so-called vectors) to efficiently transport NAs across the cell membrane while protecting the NAs from degradation *in vivo* (Stewart, M. P.: *Chem. Rev.* **2018**, *118*, 7409–7531).

Both viral and non-viral (physical, chemical) vectors are used for NA transfection. Although approximately 70 % of clinical trials in the field of gene therapy have so far been performed using viral vectors, this approach carries numerous risks (carcinogenicity, induction of an immune response, tissue nonspecificity, limited NA incorporation capacity, and manufacturing complexity). Physical methods (*e.g.*, electroporation) are difficult to use systemically in human medicine.

In contrast, synthetic chemical vectors usually have lower immunogenicity, are able to transport larger amounts of genetic material, and because they are composed of well-defined molecules, their structure can be adjusted as needed to increase their efficiency and suppress toxicity. Cationic polymers or cationic lipids are used as chemical vectors, which form a complex with negatively charged NAs. This complex can penetrate the cell membrane and at the same time protects NAs from degradation in the extracellular environment.

From a structural point of view, so-called lipid nanoparticles (LNPs) are currently the most promising and clinically advanced form of these complexes. In them, cationic lipids are usually formulated with a PEGylated lipid that prevents aggregation, affects particle size and transfection efficiency, with a helper lipid and cholesterol, which are necessary for stable NA encapsulation, as shown, for example, in an siRNA transfection system (Kulkarni, J.: *Nanoscale*, **2019**, *11*, 21733–21739). LNPs can accommodate NA molecules ranging in size from a few nucleotide units to millions.

Synthetic cationic lipids and lipidoids (synthetic molecules similar to lipids that differ in a larger number of hydrophobic chains) are formed by a cationic and a hydrophobic domain. To date, a large number of these substances have been developed with high structural variability in both domains, both by targeted design and by testing combinatorially generated libraries.

Lipids and lipidoids such as D-Lin-MC3-DMA, C12-200, cKK-E12, SA2-SC8 and others have been specially developed for siRNA transfection (Dong, Y.: *Adv. Drug Deliv. Rev.* **2019**, *144*, 133–147). A formulation containing D-Lin-MC3-DMA was recently (August 2018) introduced into clinical practice under the name Onpattro (formerly Patisiran), making it the first approved siRNA drug in history (Zhang, X.: *J. Clin. Pharmacol.* **2020**, *60* (1), 37-49). However, formulations developed for siRNA may not be effective for mRNA, and therefore, targeted optimization is necessary (Cullis, P.: *Mol. Ther.* **2017**, *25* (7), 1467–1475).

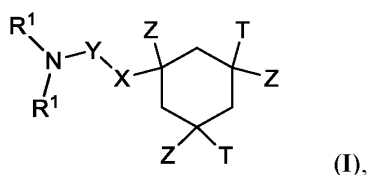
Ionizable lipids and lipidoids such as D-Lin-MC3-DMA, C12-200, cKK-E12, and TT3 are used to transfect mRNA (Zhong, Z.: *Nano Today* **2018**, *23*, 16–39; Kowalski, P.: *Mol. Ther.* **2019**, *27* (4), 1-19; Li, B.: *Nano Lett.* **2015**, *15*, 8099–8107). Ionizable lipids are also used for DNA transfection. Again, it should be emphasized that transfection systems optimized for small-molecule (siRNA) transfection are not always suitable for DNA transfection, and even formulations developed for mRNA may not be effective for DNA (Buck, J.: *ACS Nano* **2019**, *13*, 3754–3782).

Because, despite their enormous therapeutic potential, very few synthetic vectors based on ionizable lipids have been brought to the stage of clinical use so far, it is necessary to develop new systems with higher efficiency and also with very low *in vivo* toxicity.

Disclosure of the Invention

The present invention provides a solution to the problem of the efficiency of transfection and targeted delivery of nucleotides and nucleic acids and their synthetic analogs using ionizable (cationic) lipids and the problem of the toxicity of these lipids to the target organism or cell. We have surprisingly found that, if cyclohexane is used as the central core of the ionizable lipidoid, the transfection efficiency of such lipidoids is significantly increased in comparison to previously known solutions. At the same time, these cyclohexane-containing lipidoids exhibit extremely low cytotoxicity at relevant doses. Specific properties of the cyclohexane core, which is used as the central structural motif of the new ionizable lipidoids, are steric complexity in its vicinity and a wide range of substitutions in comparison to the ionizable lipids and lipidoids known so far.

The object of the present invention are ionizable lipidoids of general formula I



wherein

X is selected from a group comprising -C(=O)NH-, -C(=O)O-, -C(=S)O-, -C(=O)S-, -C(=S)S-, -C(=O)NHNH-, -CH₂-, -O-, -OC(=O)-, -S-, -SC(=O)-, -NH-, -NHNH-, -NHC(=O)-, -NHNHC(=O)-, -C≡C-, -CH=CH-, a five-membered heterocycle containing at least 2 nitrogen atoms, -CH₂C(=O)NH-, -CH₂C(=O)O-, -CH₂C(=S)O-, -CH₂C(=S)S-, -CH₂C(=O)NHNH-, -N=CH-, -CH=N-, -NH-N=CH-, and -CH=N-NH-;

Y is selected from a group comprising alkylene C₂-C₁₀ chains, wherein in the said alkylene chain, one or more -CH₂- groups may optionally be replaced with one or more O and/or S atoms; preferably, Y is selected from a group comprising alkylene C₂-C₁₀ chains, in particular alkylene C₃-C₆ chains, preferably propylene (-(CH₂)₃-), pentylene (-(CH₂)₅-), hexylene (-(CH₂)₆-);

R¹ are the same or different from each other, each R¹ being independently selected from the group comprising alkyl C₁-C₄₆, alkenyl C₂-C₄₆, alkynyl C₂-C₄₆, wherein said alkyl, alkenyl or alkynyl may be linear or branched, and wherein in the said alkyl, alkenyl or alkynyl, one or more -CH₂- groups may optionally be replaced with one or more groups selected from -CH(OH)-, -OC(=O)-, -C(=O)O-, -S-S-, -C(=O)NH-, -NHC(=O)-, -O-, and -S-;

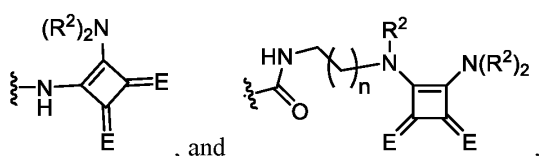
wherein if said alkyl, alkenyl or alkynyl is branched, then one or more >CH- groups may optionally be replaced with >C(OH)-;

and wherein if R¹ is alkyl C₁-C₄, then one hydrogen from the terminal -CH₃ group may be substituted with Z;

with the proviso that at least one R¹ contains at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms; more preferably, at least two R¹ substituents contain at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms;

Z are the same or different from each other, each Z being independently selected from the group

comprising hydrogen, -OH, -CH₃, -CH₂OH, -NH₂, -C(=O)NH₂, -CONH(CH₂)₂OH, -CON[(CH₂)₂OH]₂, -CONHCH(CH₂OH)₂, -CONHCH₂CH(-OH)CH₂OH, -CONH(CH₂)₂C(=O)NH₂, -CON[CH₂C(=O)NH₂]₂, -CONHCH[C(=O)NH₂]₂, -CONH(CH₂)₂NHC(=O)NH₂, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-COO⁻, -COO(CH₂)₂-O-P(=O)(O⁻)-O(CH₂)₂-N⁺(CH₃)₃, -N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -N⁺(CH₃)₂-(CH₂)₂-COO⁻,



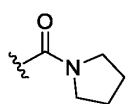
wherein

R² is independently selected from hydrogen and -CH₃;

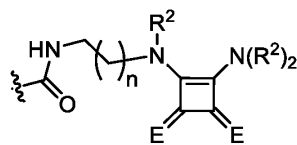
E is independently selected from O and S atoms;

n is an integer within the range of from 1 to 5;

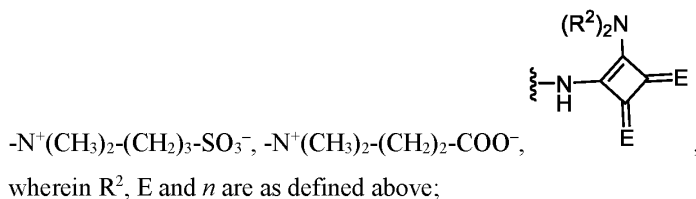
and T are the same or different from each other, each T being independently selected from the group comprising -X-Y-N(R¹)₂, -C(=O)O(C₁-C₃ alkyl), -C(=O)OCH₂CH₂OH, -C(=O)NH₂,



-C(=O)OH, -CONH(CH₂)₂OH, -CON[(CH₂)₂OH]₂, -CONHCH(CH₂OH)₂, -CONHCH₂CH(-OH)CH₂OH, -CONH(CH₂)₂C(=O)NH₂, -CON[CH₂C(=O)NH₂]₂, -CONHCH[C(=O)NH₂]₂, -CONH(CH₂)₂NHC(=O)NH₂, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-COO⁻,



-COO(CH₂)₂-O-P(=O)(O⁻)-O(CH₂)₂-N⁺(CH₃)₃, -OH, -O(C₁-C₃ alkyl), -NH₂, -NHC(=O)CH₃, -NHS(=O)₂CH₃, -NHC(=O)N(CH₃)₂, -NHC(=O)NH(CH₃), -NHC(=S)N(CH₃)₂, -NHC(=S)NH(CH₃), -NHC(=N-CN)NH₂, -NHC(=N-CN)NH(CH₃), -NHC(=N-CN)N(CH₃)₂, -NHC[=N-S(=O)₂NH₂]NH₂,



and/or if Z is -OH or -CH₂OH, and T is -C(=O)OH, then Z together with T and three carbon atoms between them may form a cyclic lactone containing 4 to 5 carbon atoms. The invention also encompasses the pharmaceutically acceptable salts, addition salts and solvates of the above defined lipidoids.

The term “alkyl” means a saturated hydrocarbon chain, which may be straight, branched or cyclic or cycle-containing, and which is derived from an alkane by removal of one hydrogen atom.

The term “alkenyl” means a hydrocarbon chain containing at least one double bond between carbon atoms, and which is derived from an alkene by removal of one hydrogen atom. The hydrocarbon chain may be straight, branched or cyclic or cycle-containing.

The term “alkynyl” means a hydrocarbon chain containing at least one triple bond between carbon atoms, and optionally also one or more double bonds between carbon atoms, and which is derived from an alkyne by removal of one hydrogen atom. The hydrocarbon chain may be straight, branched or cyclic or cycle-containing.

The term “alkylene” means a bivalent saturated hydrocarbon chain, which may be linear, branched, cyclic or cycle-containing, preferably it is linear. The chain has two valencies, i.e. it is derived from an alkane by removal of two hydrogen atoms from different carbon atoms, and it binds as a linker or bridge via two single bonds.

The terms “branched alkyl”, “branched alkenyl”, “branched alkynyl” mean an alkyl, alkenyl or alkynyl comprising from one to five branches (hydrocarbon chains) attached to the main hydrocarbon chain.

When the molecule of general formula **I** has a positive charge, the compound includes a counterion, which may be a pharmaceutically acceptable anion of an organic or inorganic acid, to form a pharmaceutically acceptable salt. Such an anion may be selected, for example, from the group comprising acetate, aspartate, benzenesulphonate, benzoate, besylate, bicarbonate, bitartrate, bromide, camsylate, carbonate, chloride, citrate, decanoate, edetate, esylate, fumarate, gluceptate, gluconate, glutamate, glycolate, hexanoate, iodide, lactate, malate, maleate, mandelate, mesylate, methanesulphate, napsylate, nitrate, octanoate, oleate, palmoate, pantothenate, phosphate, polygalacturonate, propionate, salicylate, stearate, succinate, sulphate, tartrate, and tosylate.

When the compound of formula **I** contains chiral centers, then the formula **I** includes pure enantiomers as well as mixtures of enantiomers, including the racemate.

Formula I includes compounds of formula I in free form, as well as in the form of salts, addition salts (with acids or bases) and/or solvates, including hydrates or alcohol solvates.

In one embodiment, X is selected from a group consisting of -C(=O)NH-, -C(=O)O-, -C(=O)NHNH-, -OC(=O)-, -O-, -NHC(=O)-, -NHNHC(=O)-, and a five-membered heterocycle containing at least 2 nitrogen atoms. Preferably X is -C(=O)NH- or -NHC(=O)-.

The R¹ chains may be the same within the whole molecule or different from each other or R¹ chains are the same for one nitrogen atom but not within the whole molecule. Preferably R¹ chains are the same within the whole molecule, or all nitrogen atoms are substituted identically (either with two identical R¹ or with two different R¹), for synthetic simplicity. At least one R¹ substituent is fatty chain of at least 8 carbon atoms, and R¹ are selected from the group comprising alkyl C₁-C₄₆, alkenyl C₂-C₄₆, alkynyl C₂-C₄₆, wherein said alkyl, alkenyl or alkynyl may be linear or branched, and wherein in the said alkyl, alkenyl or alkynyl, one or more -CH₂- groups may optionally be replaced with one or more groups selected from -CH(OH)-, -OC(=O)-, -C(=O)O-, -S-S-, -C(=O)NH-, -NHC(=O)-, -O-, and -S-;

wherein if said alkyl, alkenyl or alkynyl is branched, then one or more >CH- groups may optionally be replaced with >C(OH)-;

wherein if R¹ is alkyl C₁-C₄, then one hydrogen from the terminal -CH₃ group may be substituted with Z.

In one embodiment, R¹ are independently selected from the group comprising alkyl C₁-C₄₆, and alkenyl C₂-C₄₆, wherein in the said alkyl or alkenyl, one or more -CH₂- groups may optionally be replaced with one or more groups selected from -CH(OH)-, -OC(=O)-, -C(=O)O-.

Preferably, R¹ are independently selected from the group comprising linear or branched alkyl C₈-C₂₀, and linear or branched alkenyl C₈-C₂₀, wherein in the said alkyl or alkenyl, one or more -CH₂- groups may optionally be replaced with one or more groups selected from -OC(=O)-, -C(=O)O-. More preferably, R¹ is selected from the group comprising linear or branched alkyl C₁₀-C₁₅, which may optionally be replaced with one or more groups selected from -OC(=O)-, -C(=O)O-; and linear or branched alkenyl C₁₂-C₁₈, which may optionally be replaced with one or more groups selected from -OC(=O)-, -C(=O)O-.

Preferably, alkenyl chain contains from one to five carbon-carbon double bonds.

In one embodiment, all R¹ in the molecule are the same.

In one embodiment, each nitrogen atom in the molecule, bearing R¹ groups, is substituted identically by two identical R¹ (but R¹ bound to different nitrogen atoms within the same lipidoid structure may not be identical).

In one embodiment, two different R¹ are bound to one nitrogen atom.

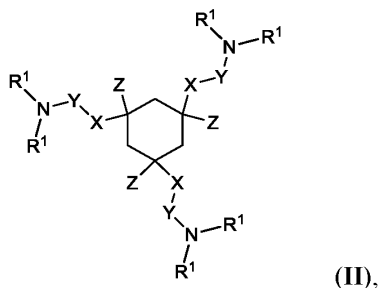
At least one R¹ in the lipidoid structure must contain at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms in order to enable for the lipid-like properties of the

molecule. Preferably, two R^1 in the lipidoid structure contain at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms.

In one embodiment, one substituent T is $-X-Y-N(R^1)_2$.

In another embodiment, both substituents T are $-X-Y-N(R^1)_2$.

In the latter case, the resulting molecule of the lipidoid has a general formula II



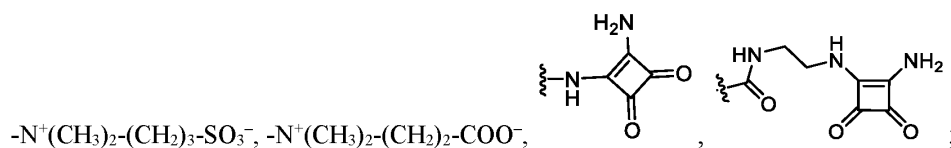
wherein X, Y, Z and R^1 are as defined above.

The structure of the general formula II preferably has the following substituents:

X is selected from a group comprising $-C(=O)NH-$, $-C(=O)O-$, $-C(=S)O-$, $-C(=O)S-$, $-C(=S)S-$, $-C(=O)NHNH-$, $-CH_2-$, $-O-$, $-OC(=O)-$, $-S-$, $-SC(=O)-$, $-NH-$, $-NHNH-$, $-NHC(=O)-$, $-NHNHC(=O)-$, $-C\equiv C-$, $-CH=CH-$, a five-membered heterocycle containing at least 2 nitrogen atoms, $-CH_2C(=O)NH-$, $-CH_2C(=O)O-$, $-CH_2C(=S)O-$, $-CH_2C(=S)S-$, $-CH_2C(=O)NHNH-$, $-N=CH-$, $-CH=N-$;

Y is an alkylene chain C_2-C_{10} , wherein one or more $-CH_2-$ groups may optionally be replaced with one or more O and/or S atoms;

Z are the same or different from each other, each Z being selected from the group consisting of hydrogen atom, $-OH$, $-CH_3$, $-CH_2OH$, $-NH_2$, $-C(=O)NH_2$, $-CONH(CH_2)_2OH$, $-CON[(CH_2)_2OH]_2$, $-CONHCH(CH_2OH)_2$, $-CONHCH_2CH(OH)CH_2OH$, $-CONH(CH_2)_2C(=O)NH_2$, $-CON[CH_2C(=O)NH_2]_2$, $-CONHCH[C(=O)NH_2]_2$, $-CONH(CH_2)_2NHC(=O)NH_2$,



and R^1 are the same or different from each other, each R^1 being selected from the group consisting of alkyl C_8-C_{20} , alkenyl C_8-C_{20} , alkynyl C_8-C_{20} , wherein in the said alkyl, alkenyl or alkynyl, one or more $-CH_2-$ groups may optionally be replaced with one or more groups selected from $-CH(OH)-$, $-OC(=O)-$, $-C(=O)O-$, $-S-S-$, $-C(=O)NH-$, $-NHC(=O)-$, $-O-$, $-S-$.

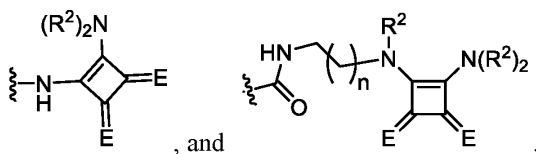
The linker X is formed by a reaction of attachment of the amine moieties of the molecule to the central cyclohexane core. These may therefore be different linkers, depending on the reaction chosen, *e.g.* the formation of ester, amide and their analogues, via click reaction (*e.g.* azido-alkyne cycloaddition), etc. X is thus selected from the group comprising -C(=O)NH-, -C(=O)O-, -C(=S)O-, -C(=O)S-, -C(=S)S-, -C(=O)NHNH-, -CH₂-, -O-, -OC(=O)-, -S-, -SC(=O)-, -NH-, -NHNH-, -NHC(=O)-, -NHNHC(=O)-, -C≡C-, -CH=CH-, a five-membered heterocycle containing at least 2 nitrogen atoms, -CH₂C(=O)NH-, -CH₂C(=O)O-, -CH₂C(=S)O-, -CH₂C(=S)S-, -CH₂C(=O)NHNH-, -N=CH-, -CH=N-, -NH=N=CH-, and -CH=N-NH-. Preferably, X is selected from -C(=O)NH-, -NHC(=O)-, a five-membered heterocycle containing at least 2 nitrogen atoms, -OC(=O)-, and -C(=O)O-.

The linker Y is an alkylene chain providing at least a minimum distance of the amine from the linker X and the cyclohexane core. Y is a C₂-C₁₀ alkylene chain, preferably a C₂-C₈ alkylene chain, wherein one or more -CH₂- groups may optionally be replaced with one or more O and/or S atoms.

The substituent Z may further modify the properties of the compound of formula **I** and **II**.

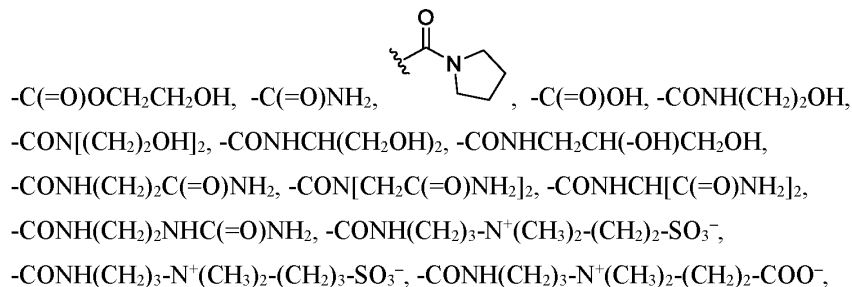
In one embodiment, Z is selected from the group consisting of

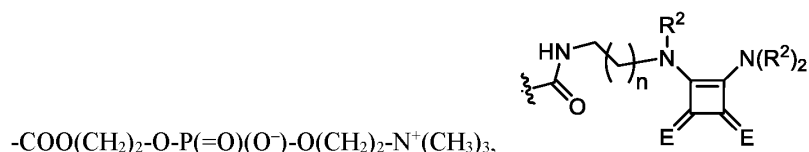
hydrogen, -OH, -CH₃, -CH₂OH, -NH₂, -C(=O)NH₂, -CONH(CH₂)₂OH, -CON[(CH₂)₂OH]₂, -CONHCH(CH₂OH)₂, -CONHCH₂CH(OH)CH₂OH, -CONH(CH₂)₂C(=O)NH₂, -CON[CH₂C(=O)NH₂]₂, -CONHCH[C(=O)NH₂]₂, -CONH(CH₂)₂NHC(=O)NH₂, -N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -N⁺(CH₃)₂-(CH₂)₂-COO⁻,



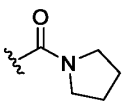
wherein R², E and n are as defined above. Preferably Z is H, -CH₃, -CH₂OH, or if Z is -OH or -CH₂OH, and T is -C(=O)OH, then Z together with T and three carbon atoms between them may form a cyclic lactone containing 4 to 5 carbon atoms.

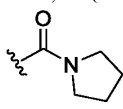
The substituent T may further modify the properties of the compound of formula **I** and **II**. In one embodiment, T is selected from the group consisting of -X-Y-N(R¹)₂, -C(=O)O(C₁-C₃ alkyl),



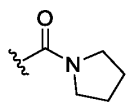


wherein R^2 , E and n are as defined above. More preferably, T is selected from the group consisting of -X-

Y-N(R^1)₂, -C(=O)O(C₁-C₃ alkyl), , -C(=O)OH. Most preferably, T is selected from the group

consisting of -X-Y-N(R^1)₂, -C(=O)OCH₃, , -C(=O)OH.

In one specific embodiment, the lipidoid of formula I has Z selected from the group consisting of hydrogen, -OH, -CH₃, -CH₂OH;

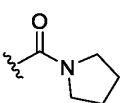
and T selected from the group consisting of -X-Y-N(R^1)₂, -C(=O)O(C₁-C₃ alkyl), , -C(=O)OH;

and/or if Z is -OH or -CH₂OH, and T is -C(=O)OH, then Z together with T and three carbon atoms between them may form a cyclic lactone containing 4 to 5 carbon atoms. Preferably, lipidoid of formula I has the following substituents:

X is -C(=O)NH- or -NHC(=O)-;

Y is selected from -(CH₂)₃-, -(CH₂)₄-, -(CH₂)₅-, and -(CH₂)₆-;

R^1 is selected from the group comprising linear or branched alkyl C₁₀-C₁₅, which may optionally be replaced with one or more groups selected from -OC(=O)-, -C(=O)O-; and linear or branched alkenyl C₁₂-C₁₈, which may optionally be replaced with one or more groups selected from -OC(=O)-, -C(=O)O-;

T is selected from -X-Y-N(R^1)₂, -C(=O)OCH₃, , and -C(=O)OH;

Z is selected from H, -CH₃, and -CH₂OH.

Specifically, the following lipidoids are preferred:

cis,cis-N¹,N³,N⁵-Tris(6-(didodecylamino)hexyl)cyclohexane-1,3,5-tricarboxamide (3);

cis,cis-N¹,N³,N⁵-Tris(6-(di((hexyloxycarbonyl)butyl)amino)hexyl)cyclohexane-1,3,5-tricarboxamide (7);

cis,cis-N¹,N³,N⁵-Tris(6-(didodecylamino)hexyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide (8);

cis,cis-N¹,N³,N⁵-Tris(3-(didodecylamino)propyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide (11);

cis,cis-N¹,N³,N⁵-Tris(6-(di((9Z,12Z)-octadeca-9,12-dien-1-yl)amino)hexyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide (15);

Hexa(octan-2-yl) *cis,cis*-6,6',6'',6''',6''''-((((cyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate (19);

Hexakis(3-methylhexyl) *cis,cis*-7,7',7'',7''',7''''-((((cyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexaheptanoate (23);

Hexakis((E)-3,7-dimethylocta-2,6-dien-1-yl) *cis,cis*-5,5',5'',5''',5''''-((((cyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexapentanoate (27);

Hexakis((Z)-3,7-dimethylocta-2,6-dien-1-yl) *cis,cis*-5,5',5'',5''',5''''-((((cyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexapentanoate (31);

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-(((5-(methoxycarbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate (32);

Tetrakis(3-methylhexyl) *cis,cis*-7,7',7'',7'''-(((5-(methoxycarbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetraheptanoate (33);

cis,cis-3,5-Bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyle)cyclohexane-1-carboxylic acid (35);

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-(((5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate (36);

Hexakis(3-methylhexyl) *cis,cis*-7,7',7'',7''',7''''-((((1,3,5-trimethylcyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexaheptanoate (40);

cis,cis-6,6',6'',6''',6''''-((((1,3,5-Trimethylcyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate (41);

cis,cis-3,5-Bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyle)-1,3,5-trimethylcyclohexane-1-carboxylic acid (42);

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris((benzyloxy)methyl)-5-(bis(6-(octan-2-yloxy)-6-oxohexyl)carbamoyle)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate (45);

cis,cis-1,3,5-Tris((benzyloxy)methyl)-3,5-bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyle)cyclohexane-1-carboxylic acid (46);

Hexa(octan-2-yl) *cis,cis*-6,6',6'',6''',6''''-((((1,3,5-tris(hydroxymethyl)cyclohexane-1,3,5-tricarbonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate (47);

cis,cis-3,5-Bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyle)-1,3,5-tris(hydroxymethyl)cyclohexane-1-carboxylic acid (48);

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris((benzyloxy)methyl)-5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate (49);

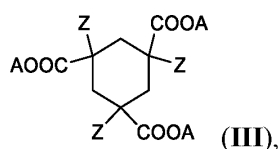
Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris(hydroxymethyl)-5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate (50);

cis,cis-*N*¹,*N*⁷-Bis(6-(didodecylamino)hexyl)-5,7-bis(hydroxymethyl)-4-oxo-3-oxabicyclo[3.3.1]nonane-1,7-dicarboxamide (52);

cis,cis-N,N',N''-(Cyclohexane-1,3,5-triyl)tris(6-(didodecylamino)hexanamide) (56).

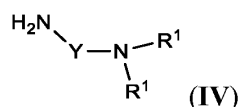
Compounds of formula **I** and **II** are prepared by the corresponding reaction of a cyclohexane precursor substituted with groups Z at positions 1, 3, 5 and precursor groups of linker X at positions 1; 1 and 3; or 1, 3 and 5 with an tertiary amine of general formula X'-Y-N(R¹)₂, wherein X' is a precursor group of the linker X. Preferably, X' is -NH₂ or activated carboxylic group. The tertiary amine of general formula X'-Y-N(R¹)₂ can be prepared by reactions and procedures known to those skilled in the art, and some suitable amines are also commercially available.

In one embodiment, the compounds of formula **I** are preferably prepared by reacting a compound of formula **III**,



wherein A is H, halogen or benzyl,

with diamine of formula **IV**,



optionally in the presence of an alcohol selected from MeOH, BnOH, and optionally in the presence of pyrrolidine, a condensing agent and/or a base, optionally followed or preceded by hydrogenolysis. In formulas **III** and **IV**, R¹, Y and Z are as described above for formula **I**.

In a more specific example, the compounds of formula **II** are preferably prepared by reacting a compound of formula **III**, wherein A is hydrogen, with a diamine of formula **IV** in the presence of a condensing agent and a base, or by reacting a compound of formula **III** wherein A is a halogen, with a diamine of formula **IV** in the presence of a base. In formulas **III** and **IV**, R¹, Y and Z are as described above for formula **II**.

Another object of the present invention is a transfection agent comprising at least one lipidoid of general formula **I** or **II**, and at least one helper lipid. The transfection agent may be prepared in the form of a solution by dissolving and mixing the components, or it can be prepared in the form of particles by means of techniques used in conventional nanoparticle technology, such as microfluidic mixing. The particles are generally understood to mean nanoparticles with dimensions in the range of from 1 to 500 nm. Preferably, the dimensions of the nanoparticles are in the range of from 30 to 250 nm, even more preferably from 40 to 150 nm.

In one embodiment, the transfection agent contains at least one lipidoid of general formula **I** in an amount of 10 to 50 mol. %, and at least one helper lipid in an amount of 50 to 90 mol. %. Preferably, the transfection agent contains at least one lipidoid of general formula **I** in an amount of 15 to 40 mol. %, and at least one helper lipid in an amount of 60 to 85 mol. %. In some preferred embodiments, the transfection agent comprises at least one lipidoid of general formula **I** in an amount of 15 to 40 mol. %, cholesterol in an amount of 30 to 55 mol. %, and at least one other helper lipid in an amount of 20 to 50 mol. %.

In a particularly preferred embodiment, the transfection agent contains at least one lipidoid of general formula **I** in an amount of 15 to 40 mol. %, cholesterol in an amount of 30 to 55 mol. %, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine in an amount of 20 to 45 mol. % and 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethyleneglycol-2000 in an amount of 0.5 to 5 mol. %.

The present invention also relates to a transfection particle comprising at least one lipidoid of formula **I**, at least one nucleic acid and/or a part thereof and/or nucleic acid derivative, and preferably also at least one helper lipid. The transfection particles can be prepared, for example, by mixing a solution of a lipidoid of general formula **I**, optionally containing helper lipids, with a solution of the nucleic acid and/or a part thereof and/or nucleic acid derivative. Mixing can be performed by means of techniques used in conventional nanoparticle technology, for example, by microfluidic mixing.

The weight ratio of the total amount of nucleic acid and/or a part thereof and/or nucleic acid derivative to the total amount of lipidoid of general formula **I** and helper lipids in the transfection particle is preferably in the range of from 1:2 to 1: 500, more preferably from 1:5 to 1:100. Specifically and for illustration: in the particles which are prepared in the examples herein below, this ratio was around 1:9 for mRNA and around 1:68 for siRNA.

Transfection particles are usually nanoparticles, which is generally understood to mean particles with dimensions in the range of from 1 to 500 nm. Typically, the dimensions of the transfection nanoparticles are in the range of from 50 to 250 nm, more preferably from 40 to 150 nm.

The structure of the transfection particles was observed by cryogenic transmission electron microscopy, and the observation showed that the transfection particles were compact layered lipid nanoparticles containing nucleic acid inside.

Helper lipids in transfection reagents and transfection particles are mainly neutral lipids, sterols or lipid conjugates of lipids with hydrophilic polymers.

Neutral lipids have a zero net charge under physiological conditions, and they can exist in an uncharged form or electroneutral zwitterionic form. Neutral lipids can be selected from e.g. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-

glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Cyanine 5).

Sterols may, for example, be selected from cholesterol, β -sitosterol, stigmasterol, campesterol, fucosterol, avenasterol, fecosterol, brassicasterol, ergosterol, and 9,11-dehydroergosterol. Preferably, the sterol is cholesterol.

Lipid conjugates with hydrophilic polymers comprise a lipid portion and a polymer portion such as poly(ethyleneglycol), poly(2-ethyl-2-oxazoline), poly(2-methyl-2-oxazoline), poly(glycerol), poly(sarcosine). Preferably, the polymer portion consists of poly(ethyleneglycol) with a molecular weight which can range from about 500 to about 10,000 Da, more preferably from about 1,000 to about 5,000 Da. Lipid conjugates with hydrophilic polymers may, for example, be selected from 1,2-dimyristoyl-*rac*-glycero-3-methoxy poly(ethyleneglycol)-2000, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethyleneglycol)-2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000, and similar conjugates. A lipid conjugate with hydrophilic polymers may be preferably 1,2-dimyristoyl-*rac*-glycero-3-methoxy poly(ethyleneglycol)-2000.

The nucleic acid or a part thereof contains one or more nucleotides and/or deoxynucleotides. The nucleic acid or a part thereof may be a therapeutic, diagnostic or prophylactic agent, or may provide labeling for the cells or tissues into which they are transfected. The compounds of formula **I** thus have predominantly therapeutic or biotechnological uses.

The term “nucleic acid or a part thereof” is understood to mean one or more types of nucleic acids selected preferably from oligonucleotides (1-100 nucleotides, *e.g.* aptamers), cyclic dinucleotides (*e.g.* 2',3'-cGAMP), antisense oligonucleotides, deoxyribonucleic acid (single-stranded DNA, double-stranded DNA, cDNA, plasmid DNA encoding a gene or genes), ribonucleic acid, typically messenger RNA (mRNA), transfer RNA (tRNA), small interfering RNA (siRNA), double-stranded RNA, micro-RNA (miRNA), piwi-RNA (piRNA), antisense RNA (asRNA), guide RNA (gRNA) for the CRISPR system and their combinations (typically *e.g.* gRNA and mRNA encoding Cas9 nuclease, Cas13a/C2c2 and Cas13b, or analogous nucleases, suitable for use in CRISPR, CRISPRi and other variations and subsequent modification of the host cell or tissue genome or modification of the host cell or tissue transcriptome). Furthermore, all nucleic acids (NA) disclosed herein may be formed or modified with synthetic base analogs, for example, to increase their stability in biological systems. Synthetic NA analogs involve, in particular, the following substitutions: 2'-O-methyl, 2'-O-methoxy-ethyl, 2'-fluoro, a methylene bridge between the 2'-oxygen and the 4'-carbon of the pentose ring (a so-called locked nucleic acid), phosphorylation at the 5' and/or 3' end of the strand, boranophosphonates, or phosphorothioates.

The present invention further includes the use of lipidoids of formula **I** or transfection agents or transfection particles for transfecting cells or tissues with nucleic acid and/or a part thereof and/or nucleic acid derivative *in vitro*. In addition, the invention includes lipidoids of formula **I** or transfection particles for use in transfecting cells or tissues with nucleic acid and/or a part thereof and/or nucleic acid derivative *in vivo* (excluding the transfection of human embryos for industrial or commercial use, and excluding the modification of a human germ line).

Transfection particles containing lipidoids of general formula **I** are useful in a number of biological applications in basic research, especially for the transfection of cell cultures or animals to deliver active nucleic acid and subsequent silencing or activation of a chromosomal gene or genes, genome editing (gene excision, gene insertion or mutation introduction) or transcriptome editing, or enabling the expression of a given protein encoded by the nucleic acid inserted by means of a transfection particle, so-called “*in trans*”. In veterinary and human medicine, transfection particles containing lipidoids of the general formula **I** can preferably be used for therapeutic or prophylactic purposes. Particles containing therapeutic nucleic acid can be administered to an animal or human to silence or activate chromosomal gene(s), to silence or activate immunogens, to inhibit or activate signaling pathways, to edit the genome (gene excision, gene insertion or mutation introduction) or the transcriptome, or enable the expression of protein(s) encoded by the nucleic acid.

The invention also provides lipidoids of general formula **I** or transfection agents or transfection particles for use as medicaments, in particular for gene therapy. In particular, they are suitable for use in the treatment of malignancies and/or genetic disorders.

The lipidoids of general formula (**I**) or the transfection agents or the transfection particles according to the present invention are also suitable for use as a prophylactic vaccines, preferably for the prevention of infectious diseases.

The lipidoids of general formula **I** or transfection particles can be formulated for therapeutic, cosmetic or biotechnological use in the form of preparations with pharmaceutically acceptable excipients. The formulations may be in liquid or solid form. Liquid forms include solutions, suspensions, dispersions, adapted *e.g.* for injection or oral administration, gels, ointments. Solid forms include, for example, capsules, tablets, coated tablets, powders, suppositories, and other forms.

The liquid formulations can be nebulized by inert gases. Nebulized suspensions may be breathed in directly, or from the nebulizing device, or the nebulizing device can be attached to a face mask or breathing machine. The solid forms may be administered using dry-powder inhalers. Suspension or dry powder formulations can be administered orally or nasally from appropriate devices.

For application to the skin or mucous membranes, the preparations can be also prepared in the form of a cream, gel, ointment, paste, balm, liquid and others, and may be applied directly to the site of action.

Pharmaceutically acceptable excipients include solvents, solubility control agents, pH-adjusting agents, carriers, fillers, binders, glidants, disintegrants, preservatives, sorbents, viscosity control agents, agents that affect sensory properties such as taste, odor or the color of the formulation.

Furthermore, lipidoids of general formula **I** or the transfection agents or transfection particles can preferably be used for the purposes of the cosmetics industry in order to deliver the active substance to the site of action. The transfection particles with the active substance can be prepared in the form of a cream, gel, ointment, paste, balm, liquid or the like, and used as make-up, hair cosmetics or a personal hygiene product.

Brief Description of Drawings

Figure 1. Synthetic scheme of lipidoids **3** and **7**.

Figure 2. Synthetic scheme of lipidoids **8** and **11**.

Figure 3. Synthetic scheme of lipidoid **15**.

Figure 4. Synthetic scheme of lipidoids **19** and **23**.

Figure 5. Synthetic scheme of lipidoids **27** and **31**.

Figure 6. Synthetic scheme of lipidoids **32**, **33**, **35** and **36**.

Figure 7. Synthetic scheme of precursors **39** and **44** and lipidoids **40-42**.

Figure 8. Synthetic scheme of lipidoids **47**, **48**, **50** and **52**.

Figure 9. Synthetic scheme of lipidoid **56**.

Figure 10. Functional delivery of mRNA-LNP (**B40**) in murine liver in comparison to benchmark LNP (**B45**) analyzed by histology.

Figure 11. Functional delivery of mRNA-LNP (**B40**) in murine liver in comparison to benchmark LNP (**B45**) analyzed by PCR amplification of the genomic DNA. Lanes marked from 1 to 4 relate to **B40**. Lanes marked from 5 to 8 relate to **B45**.

Examples

List of abbreviations:

eq.	equivalent
R_f	retention factor
TLC	thin-layer chromatography
RVE	rotary vacuum evaporator
rt	room temperature
v/v	volume/volume
br s	broad signal

s	singlet
d	doublet
t	triplet
m	multiplet
dd	doublet of doublets
<i>J</i>	interaction constant
δ	chemical shift
HRMS	high-resolution mass spectrometry
EI	electron ionization
ESI	electrospray ionization
MALDI	matrix-assisted laser desorption/ionisation
GC-MS	gas chromatography – mass spectrometry
IR	infrared spectroscopy
NMR	nuclear magnetic resonance
CE5	95:5 (v/v) cyclohexane-ethylacetate mixture
CE20	80:20 (v/v) cyclohexane-ethylacetate mixture
CE50	50:50 (v/v) cyclohexane-ethylacetate mixture
D1	75:22:3 (v/v/v) dichloromethane-methanol-25% aqueous NH ₃ mixture
D2	175:22:3 (v/v/v) dichloromethane-methanol-25% aqueous NH ₃ mixture
D3	275:22:3 (v/v/v) dichloromethane-methanol-25% aqueous NH ₃ mixture
D4	375:22:3 (v/v/v) dichloromethane-methanol-25% aqueous NH ₃ mixture
TFA	trifluoroacetic acid
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DCM	dichloromethane
ACN	acetonitrile
DIC	diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
PyBroP	bromotripyrrolidinophosphonium hexafluorophosphate
TCE	1,1,2,2-tetrachloroethane
LNP	lipid nanoparticles
NA	nucleic acid
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
siRNA	small interfering RNA
tRNA	transfer RNA

miRNA	micro RNA
ssDNA/RNA	single-stranded DNA/RNA
dsDNA/RNA	double-stranded DNA/RNA
DMG-PEG ₂₀₀₀	1,2-dimyristoyl- <i>rac</i> -glycero-3-methoxypolyethyleneglycol-2000
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE-Cy5	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine- <i>N</i> -(Cyanine 5)
Lip2000	Lipofectamine [®] 2000 (Invitrogen)
h	hour

Example 1

*N*¹,*N*¹-Didodecylethan-1,6-diamine 2

A 500 ml round-bottom flask equipped with a calcium chloride drying tube and magnetic stirrer was filled with a solution of *N*¹-*tert*-butyloxycarbonyl-1,6-diaminohexane (5.0 g, 23.11 mmol) in DCM (100 ml) and cooled to 0 °C in an ice bath. With intensive stirring, n-dodecylaldehyde (15.38 ml, 69.34 mmol, 3 eq.) was added, followed by sodium triacetoxyborohydride (14.70 g, 69.34 mmol, 3 eq.) in three portions over 10 minutes. The cooling bath was removed, and the reaction mixture was stirred at room temperature for 2 h. The progress of the reaction was monitored by TLC using an 80:20 (v/v) hexane-ethylacetate mobile phase on a TLC plate pre-saturated with ammonia (detection with ninhydrin). After completion of the reaction, aqueous NaOH solution (1 M, 200 ml) was added, the reaction mixture was stirred for 15 min, then poured into a separatory funnel and diluted with water (300 ml). The product was extracted with DCM (300 ml, 2x50 ml), the combined organic phase was washed with brine (50 ml), dried over anhydrous sodium sulphate, filtered through an S2 frit, and the solvents were evaporated in an RVE. The dark oily residue was purified by silica gel column chromatography using a linear gradient of ethyl acetate in hexane (10-30 %). Amine 1 (3.67 g, 28.7 %) was obtained as a yellowish oil.

Trifluoroacetic acid (10 ml) was added to a solution of compound 1 in DCM (10 ml), cooled to 0 °C with stirring in an ice bath, and the reaction mixture was left at 0 °C for 3 h. The solution was then poured into a 1 l separatory flask, diluted with 20% aqueous Na₂CO₃ (300 ml), and the product was extracted with DCM (250 ml, 2 x 50 ml). The combined organic phase was washed with brine (100 ml), dried over anhydrous sodium sulphate, filtered through an S2 frit, and the solvents were evaporated in an RVE. The crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (0-70 %). The diamine 2 (2.17 g, 72.2% yield; *R*_f 0.31 in mobile phase D2 on a TLC plate pre-saturated with ammonia, detection with ninhydrin) was obtained in the form of a yellowish oil.

¹H NMR (600 MHz, CDCl₃): δ = 2.73, 2.65, 2.57, 1.56, 1.52, 1.51, 1.36, 1.31, 1.28, 1.25–1.29, 1.24, 0.87 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 53.51, 53.20, 41.62, 32.40, 31.89, 29.63, 29.61, 29.57, 29.44, 29.32, 27.39, 27.09, 26.53, 25.65, 25.02, 22.66, 14.10 ppm. IR (film): ν_{max}/cm⁻¹ = 3374 w and 3294 w (v

NH₂), 2797 m (v_s N-CH₂), 2956 s (v_{as} CH₃), 2924 vs (v_{as} CH₂), 2853 s (v_s CH₂), 1467 m and 1455 m, sh (β_s CH₂ and δ_{as} CH₃), 1378 w and 1367 w (δ_s CH₃), 721 m (β_{as} CH₂). **HRMS** (ESI): m/z calculated for C₃₀H₆₅N₂ [M + H]⁺ 453.51423; found 453.51340.

***cis,cis*-N¹,N³,N⁵-Tris(6-(didodecylamino)hexyl)cyclohexane-1,3,5-tricarboxamide 3**

DMF (2 μl) and thionylchloride (300 μl) were added to *cis,cis*-1,3,5-cyclohexanetricarboxylic acid (17 mg, 0.079 mmol) and the suspension was stirred for 30 min at 70 °C in a sealed vial, gradually clarifying the reaction mixture to a homogeneous colorless solution. Excess SOCl₂ was blown off with a stream of dry nitrogen at 70 °C, the residue was dried under vacuum (10 min), and cooled to rt. A solution of N¹,N¹-didodecylhexane-1,6-diamine **2** (142 mg, 0.315 mmol, 4 eq.) and DIPEA (137 μl, 0.786 mmol, 10 eq.) in a mixture of DCM (1.5 ml) and DMF (0.5 ml), was added *via* septum using a syringe and the reaction mixture was stirred for 10 min. Subsequently, the reaction mixture was sorbed onto chromatographic silica gel (10 g), and the solvents were removed in an RVE. The residue was purified by silicagel column chromatography (40 g) using a linear gradient of D1 in DCM (0-55%). Lipidoid **3** was obtained (81 mg, 67.7% yield; *R_f* 0.36 in mobile phase D2, detection with ninhydrin) in the form of a yellowish semi-solid. **¹H NMR** (600 MHz, CDCl₃): δ = 7.80, 3.20, 3.02–2.96, 2.60, 2.07, 1.81–1.74, 1.37, 1.325, 1.28–1.23, 0.87 ppm. **¹³C NMR** (150.9 MHz, CDCl₃): δ = 175.90, 52.85, 52.22, 42.83, 39.42, 31.87, 31.66, 29.58, 29.48, 29.43, 29.30, 29.08, 26.84, 26.18, 26.00, 23.50, 23.16, 22.65, 14.09 ppm. **IR** (CCl₄): ν_{max}/cm⁻¹ = 3293 m (v NH), 1640 s (amide I) and 1550 m (amide II), 2964 s, sh (v_{as} CH₃), 2871 m, sh (v_s CH₃), 2927 vs (v_{as} CH₂), 2856 s, sh (v_s CH₂), 1468 m and 1460 m, 1445 w (β_s CH₂ and δ_{as} CH₃), 1378 w (δ_s CH₃), 721 w (β_{as} CH₂ and δ_{as} CH₂); **HRMS** (MALDI): m/z calculated for C₉₉H₁₉₉N₆O₃ [M + H]⁺ 1520.5598; found 1520.5598.

Example 2

N¹,N¹-Di((hexyloxycarbonyl)butyl)hexane-1,6-diamine 6

1-hexanol (2.48 ml, 19.89 mmol, 1.2 eq), DMAP (61 mg, 0.50 mmol, 0.03 eq.) and DIC (3.37 ml, 21.54 mmol, 1.3 eq.) were added to a solution of 5-bromopentanoic acid (3.00 g, 16.57 mmol) in DCM (60 ml) and the reaction mixture was stirred for 1 h at rt. Subsequently, the reaction mixture was sorbed onto chromatographic silica gel (16 g), the solvents were removed in an RVE, and the residue was purified by silicagel column chromatography (80 g) using a linear gradient of ethyl acetate in cyclohexane (0-10 %). Hexyl 5-bromopentanoate **4** (3.938 g, 89.6% yield; *R_f* 0.31 in mobile phase CE5, detection KMnO₄) was obtained as a colourless liquid.

Hexyl 5-bromopentanoate **4** (1.53 g, 5.78 mmol, 2.5 eq.) and anhydrous K₂CO₃ (3.19 g, 23.11 mmol, 10 eq.) were added to a solution of N¹-tert-butyloxycarbonyl-1,6-diaminohexane (0.50 g, 2.31 mmol) in anhydrous ACN (10 ml) and the reaction mixture was stirred vigorously at 35 °C for 2 days. Subsequently, the reaction mixture was sorbed onto chromatographic silicagel (16 g), the solvents were removed in an RVE, and the residue was purified by silicagel column chromatography (40 g) using a linear gradient of ethyl acetate in cyclohexane (0-100 %). Amine **5** was obtained (1.080 g, 79.9% yield; *R_f* 0.31 in mobile

phase CE50 on a TLC plate pre-saturated with ammonia, detection with ninhydrin) in the form of slightly yellowish oil.

Trifluoroacetic acid (4 ml) was added to a solution of compound **5** in DCM (4 ml) cooled to 0 °C with stirring in an ice bath, and the reaction mixture was left at 0 °C for 1 h. The solution was then poured into a 500 ml separatory flask, diluted with 20% aqueous NaHCO₃ (200 ml), and the product was extracted with DCM (150 ml, 2 x 50 ml). The combined organic phase was washed with brine (50 ml), dried over anhydrous sodium sulfate, filtered through an S2 frit, sorbed onto chromatographic silicagel (16 g), the solvents removed in an RVE, and the residue was purified by silicagel column chromatography (40 g) using a linear gradient of D1 in DCM (0-80 %). Diamine **6** was obtained (0.788 g, 86.9% yield; *R_f* 0.14 in mobile phase D2, detection with ninhydrin) as a yellowish oil. ¹H NMR (600 MHz, CDCl₃): δ = 4.05, 3.63, 3.21, 3.15, 2.79, 2.68, 2.60, 2.51, 2.42, 2.32, 1.61, 1.50, 1.36–1.28, 0.88 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 173.62, 64.52, 53.68, 53.29, 41.22, 40.26, 34.03, 31.41, 28.58, 25.75, 25.57, 22.80, 22.51, 13.98 ppm. HRMS (ESI): *m/z* calculated for C₂₈H₅₇O₄N₂ [M + H]⁺ 485.43128; found 485.43052.

cis,cis-N¹,N³,N⁵-Tris(6-(di((hexyloxycarbonyl)butyl)amino)hexyl)cyclohexane-1,3,5-tricarboxamide 7

Lipidoid **7** was prepared from *cis,cis*-1,3,5-cyclohexanetricarboxylic acid (21 mg, 0.097 mmol), *N¹,N¹*-di((hexyloxycarbonyl) butyl)hexane-1,6-diamine **6** (188 mg, 389 μmol, 4 eq.) and DIPEA (169 μl, 0.971 mmol, 10 eq.) according to the procedure described for lipidoid **3** in Example 1. Lipidoid **7** (55 mg, 35%; *R_f* 0.42 in mobile phase D2, detection with ninhydrin) was obtained as a yellowish semi-solid. ¹H NMR (600 MHz, CDCl₃): δ = 6.23, 4.05, 3.205, 2.65, 2.34, 2.30, 2.085, 1.63–1.60, 1.49, 1.33–1.29, 0.88 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 174.35, 173.37, 64.60, 53.35, 52.88, 43.98, 39.12, 33.79, 31.94, 31.40, 29.21, 28.57, 26.62, 26.33, 25.56, 22.58, 22.51, 13.98 ppm. IR (CCl₄): ν_{max}/cm⁻¹ = 1736 vs (ν_s C=O), 1173 m, 1075 w (ν_s C-O), 3293 m (ν NH), 1640 s (amide I) and 1551 m (amide II), 2955 s (ν_{as} CH₃), 2933 vs (ν_{as} CH₂), 2875 m, sh (ν_s CH₃), 2860 m (ν_s CH₂), 1467 m and 1460 m (β_s CH₂ and δ_{as} CH₃), 1379 w (δ_s CH₃), 724 w (β_{as} CH₂ and γ_{as} CH₂). HRMS (MALDI): *m/z* calculated for C₉₃H₁₇₅N₆O₁₅ [M + H]⁺ 1616.3110; found 1616.3095.

Example 3

cis,cis-N¹,N³,N⁵-Tris(6-(didodecylamino)hexyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide 8

Lipidoid **8** was prepared from *cis,cis*-1,3,5-trimethyl-1,3,5-cyclohexanetricarboxylic acid (20 mg, 0.077 mmol), *N¹,N¹*-di(dodecyl)hexane-1,6-diamine **2** (140 mg, 310 μmol, 4 eq.) and DIPEA (135 μl, 0.774 mmol, 10 eq.) according to the procedure described for lipidoid **3** in Example 1. Lipidoid **8** (64 mg, 53 %; *R_f* 0.43 in mobile phase D2, detection with ninhydrin) was obtained in the form of a slightly yellowish solid oil. IR (CCl₄): ν_{max}/cm⁻¹ = 3288 w, br (ν NH), 1651 m (amide I), 1585 w, br, sh and 1559 m, br (amide II), 2956 s (ν_{as} CH₃), 2927 vs (ν_{as} CH₂), 2878 m, sh (ν_s CH₃), 2855 s (ν_s CH₂), 1468 m, and 1459 m, sh, 1448

m, sh (β_s CH₂ and δ_{as} CH₃), 1378 w (δ_s CH₃); 721 w (β_{as} CH₂ and γ_{as} CH₂). **HRMS** (MALDI): m/z calculated for C₁₀₂H₂₀₅N₆O₃ [M + H]⁺ 1562.6068; found 1562.6011.

Example 4

***N*¹,*N*¹-Didodecylpropane-1,3-diamine 10**

Amine **9** was prepared from *N*¹-*tert*-butoxycarbonyl-1,3-diaminopropane (6.0 g, 34.43 mmol), *n*-dodecylaldehyde (22.91 ml, 103.30 mmol, 3 eq.) and sodium triacetoxyborohydride (21.89 g, 103.3 mmol, 3 eq.) according to the procedure described for compound **1** in Example 1. Amine **9** was obtained as a yellowish oil (7.72 g, 43.9 %).

The deprotection of amine **9** was performed according to the procedure described for compound **2** in Example 1; diamine **10** (4.26 g, 68.6 %; *R*_f 0.35 in mobile phase D2 on a TLC plate pre-saturated with ammonia, detection with ninhydrin) was obtained in the form of a yellowish oil. ¹H NMR (600 MHz, CDCl₃): δ = 3.07, 2.70, 2.50, 1.81, 1.46, 1.28, 1.26, 1.25–1.29, 1.24, 0.87 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 53.70, 53.30, 41.18, 31.90, 29.64, 29.62, 29.60, 29.58, 29.48, 29.33, 27.42, 25.71, 23.87, 22.67, 14.10 ppm. **IR** (film): $\nu_{\max}/\text{cm}^{-1}$ = 3361 w and 3274 w (ν NH₂), 2803 m (ν_s N-CH₂), 2954 s (ν_{as} CH₃), 2924 vs (ν_{as} CH₂), 2853 s (ν_s CH₂), 1467 m and 1456 m, sh (β_s CH₂ and δ_{as} CH₃), 1378 w and 1364 w (δ_s CH₃), 720 m (β_{as} CH₂). **HRMS** (ESI): m/z calculated for C₂₇H₅₉N₂ [M + H]⁺ 411.46728; found 411.46652.

***cis,cis-N*¹,*N*³,*N*⁵-Tris(3-(didodecylamino)propyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide 11**

Lipidoid **11** was prepared from *cis,cis*-1,3,5-trimethyl-1,3,5-cyclohexanetricarboxylic acid (20 mg, 0.077 mmol), *N*¹,*N*¹-di(dodecyl)propane-1,3-diamine **10** (127 mg, 310 mmol, 4 eq.) and DIPEA (135 μ l, 0.774 mmol, 10 eq.) according to the procedure described for lipidoid **3** in Example 1. Lipidoid **11** (41 mg, 37% yield; *R*_f 0.36 in mobile phase D2, detection with ninhydrin) was obtained in the form of a slightly yellowish solid oil. **IR** (CCl₄): $\nu_{\max}/\text{cm}^{-1}$ = 3303 w, vbr (ν NH), 1679 s, sh (amide I), 1513 w, br, sh (amide II), 2956 s (ν_{as} CH₃), 2927 vs (ν_{as} CH₂), 2878 m, sh (ν_s CH₃), 2855 s (ν_s CH₂), 1464 m, sh (β_s CH₂ and δ_{as} CH₃), 1380 w (δ_s CH₃); 721 w (β_{as} CH₂ and γ_{as} CH₂). **HRMS** (MALDI): m/z calculated for C₉₃H₁₈₇N₆O₃ [M + H]⁺ 1436.4665; found 1436.4629.

Example 5

Linoleylaldehyde 12

Dess-Martin periodinane (4.45 g, 10.49 mmol, 1.3 eq.) was added to a solution of linoleylalcohol (2.50 ml, 8.07 mmol) in DCM (120 ml) cooled to 0 °C with an ice bath and the mixture was stirred at 0 °C for 4 h. The reaction was then quenched by the addition of sodium thiosulfate solution (20 g Na₂S₂O₃·5H₂O/100 ml H₂O) and saturated aqueous sodium bicarbonate solution (50 ml), and stirred at rt for 1 h until the initially milky solution turned clear. The solution was poured into a 1000 ml separatory flask, diluted with water (150 ml), and the product was extracted with DCM (150 ml, 2x50 ml). The combined organic phase was

washed with brine (150 ml), dried over anhydrous sodium sulfate, filtered through an S2 frit, and the solvents were evaporated on a RVE. The crude product was purified by silicagel column chromatography (isocratic conditions, 5 % ethyl acetate in cyclohexane). Aldehyde **12** (1.271 g, 59.6% yield; R_f 0.36 in mobile phase CE5, detection with KMnO_4) was obtained as a colorless oil.

N*¹,*N*¹-Di((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl)hexane-1,6-diamine **14*

Amine **13** was prepared from *N*¹-*tert*-butyloxycarbonyl-1,6-diaminohexane (0.345 g, 1.59 mmol), aldehyde **12** (1.27 g, 4.78 mmol, 3 eq.) and sodium triacetoxyborohydride (1.01 g, 4.78 mmol, 3 eq.) according to the procedure described for compound **1** in Example 1. Amine **13** was obtained as a yellowish oil (1.08 g, 94.9% yield; R_f 0.18 in CE20 mobile phase, detection with ninhydrin).

Deprotection of amine **13** was performed in a mixture of TFA (4 ml) and DCM (5 ml) according to the procedure described for compound **2** in Example 1; diamine **14** (0.594 g, 64.0%; R_f 0.13 in mobile phase D2, detection with ninhydrin) was obtained as a yellowish oil. ¹H NMR (600 MHz, CDCl_3): δ = 5.30–5.40, 2.765, 2.73, 2.67, 2.59, 2.04, 1.51, 1.385, 1.37, 1.34, 1.295, 1.29, 1.28–1.34, 1.28, 0.88 ppm. ¹³C NMR (150.9 MHz, CDCl_3): δ = 130.19, 130.06, 127.99, 127.89, 53.49, 53.45, 41.67, 32.52, 31.50, 29.62, 29.46, 29.20–29.48, 27.37, 27.20, 27.18, 26.52, 25.61, 22.56, 14.06 ppm. IR (CCl_4): $\nu_{\text{max}}/\text{cm}^{-1}$ = 3011 s ($\nu_{\text{as}}=\text{CH}$); 1646–1673 m ($\nu \text{ C}=\text{C}$); 3455 w ($\nu_{\text{as}} \text{ NH}_2$); 3394 ($\nu_{\text{s}} \text{ NH}_2$); 1620 w ($\beta_{\text{s}} \text{ NH}_2$); 1087 m ($\nu \text{ C-NH}_2$); 2957 s, sh ($\nu_{\text{as}} \text{ CH}_3$); 2928 vs ($\nu_{\text{as}} \text{ CH}_2$); 2873 s, sh ($\nu_{\text{s}} \text{ CH}_3$); 2856 vs ($\nu_{\text{s}} \text{ CH}_2$); 2801 m ($\nu_{\text{s}} \text{ N-CH}_2$); 1467 m and 1457 m, sh ($\beta_{\text{s}} \text{ CH}_2$ and $\delta_{\text{as}} \text{ CH}_3$); 1378 m ($\delta_{\text{s}} \text{ CH}_3$); 721 m (β_{as} and $\gamma_{\text{as}} \text{ CH}_2$). HRMS: m/z calculated for $\text{C}_{42}\text{H}_{81}\text{N}_2$ [$\text{M} + \text{H}$]⁺ 613.63943; found 613.63899.

cis,cis-N*¹,*N*³,*N*⁵-Tris(6-(di((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl)amino)hexyl)-1,3,5-trimethylcyclohexane-1,3,5-tricarboxamide **15*

Lipidoid **15** was prepared from *cis,cis*-1,3,5-trimethyl-1,3,5-cyclohexanetricarboxylic acid (17 mg, 0.066 mmol), *N*¹,*N*¹-di((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl)hexane-1,6-diamine **14** (161 mg, 273 mmol, 4 eq.) and DIPEA (115 μl , 0.658 mmol, 10 eq.) according to the procedure described for lipidoid **3** in Example 1. Lipidoid **15** (86 mg, 64% yield; R_f 0.36 in mobile phase D2, detection with ninhydrin) was obtained as a pale yellow solid. IR (CCl_4): $\nu_{\text{max}}/\text{cm}^{-1}$ = 3348 w, br, sh and 3302 w, br ($\nu \text{ NH}$), 1656 m and 1635 m, sh (amide I + $\nu \text{ C}=\text{C}$), 1565 w, sh and 1557 w, br (amide II), 3011 m ($\nu_{\text{as}}=\text{C-H}$), 2956 s ($\nu_{\text{as}} \text{ CH}_3$), 2929 vs ($\nu_{\text{as}} \text{ CH}_2$), 2875 m, sh ($\nu_{\text{s}} \text{ CH}_3$), 2856 s ($\nu_{\text{s}} \text{ CH}_2$), 1467 m and 1450 m, sh, ($\beta_{\text{s}} \text{ CH}_2$ and $\delta_{\text{as}} \text{ CH}_3$), 1379 w ($\delta_{\text{s}} \text{ CH}_3$); 720 w ($\beta_{\text{as}} \text{ CH}_2 + \gamma_{\text{as}} \text{ CH}_2 + \gamma =\text{C-H}$). HRMS (MALDI): m/z calculated for $\text{C}_{138}\text{H}_{253}\text{N}_6\text{O}_3$ [$\text{M} + \text{H}$]⁺ 2042.9829; found 2042.9861.

Example 6

Compound 18

DIC (4.41 mL, 28.7 mmol, 1.6 eq.) and DMAP (88 mg, 0.72 mmol, 0.04 eq.) were added to a solution of 6-bromohexanoic acid (3.50 g, 17.9 mmol) and octan-2-ol (3.51 g, 26.9 mmol, 1.5 eq.) in DCM (30 mL),

and the mixture was stirred overnight at rt. The reaction mixture was then adsorbed onto silica (16 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-10 %) to yield the compound **16** (4.02 g, 77 %; R_f 0.41 in CE5, visualization by KMnO_4) as a colorless oil.

Bromoester **16** (4.00 g, 13.1 mmol, 2.6 eq.) and potassium carbonate (7.23 g, 52.3 mmol, 10 eq.) were added to a solution of *N*-Boc-1,6-hexanediamine (1.13 g, 5.23 mmol, 1 eq.) in anhydrous ACN (10 mL), and the mixture was stirred at 40 °C for 3 days. The reaction mixture was then adsorbed onto silica (16 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-100 %) to yield the compound **17** (2.80 g, 80 %; R_f 0.58 in CE50 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a pale-yellow oil.

Hydrochloric acid in dioxane (4 mL, 4 M) was added to a solution of compound **17** (2.75 g, 4.11 mmol) in anhydrous DCM (4 mL), and the reaction mixture was stirred at rt for 1 h. The solution was then poured into a 500 mL separatory funnel, diluted with saturated aqueous NaHCO_3 (100 mL), and the product was extracted with diethyl ether (100 mL, 2 x 50 mL). The combined organic phases were washed with brine (75 mL), dried over anhydrous sodium sulfate, filtered through an S2 sintered glass, and the solvents were evaporated in an RVE. The crude product was purified by column chromatography on silica using a linear gradient of D1 in DCM (0-70 %). The amine **18** (1.74 g, 74 %; R_f 0.25 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) was obtained as a pale-yellow oil. $^1\text{H NMR}$ (600 MHz, CDCl_3): δ = 4.90-4.77 (m), 2.72-2.62 (m), 2.39-2.29 (m), 2.21 (t, J = 7.5 Hz), 1.64-1.44 (m), 1.47-1.32 (m), 1.30-1.17 (m), 1.15-1.08 (m), 0.86-0.78 (m) ppm. $^{13}\text{C NMR}$ (150.9 MHz, CDCl_3): δ = 173.42, 70.83, 53.89, 41.87, 35.98, 31.77, 29.13, 27.41, 27.17, 26.84, 26.69, 25.40, 25.09, 22.60, 20.04, 14.09 ppm. HRMS (ESI): m/z calculated for $\text{C}_{34}\text{H}_{68}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$ 569.5252; found 569.5247.

Hexa(octan-2-yl) *cis,cis*-6,6',6'',6''',6''''-((((cyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate **19**

Thionylchloride (700 μL) and DMF (6 μL) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (80 mg, 370 μmol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess SOCl_2 was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (2.5 mL) and DIPEA (645 μL , 3.70 mmol). Then, a solution of amine **18** (1.05 g, 1.85 mmol, 5 eq.) in anhydrous TCE (2.5 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-35 %) to yield the target compound **19** (489 mg, 71 %) as a yellow waxy semi-solid. $^1\text{H NMR}$ (600 MHz, CDCl_3): δ = 5.75, 4.88, 3.21, 2.42, 2.27, 2.22, 2.11, 1.62, 1.58, 1.56, 1.47, 1.46, 1.30, 1.28, 1.27, 1.26, 1.19, 0.87 ppm. $^{13}\text{C NMR}$ (150.9 MHz, CDCl_3): 173.88, 173.39, 70.82, 53.87, 53.77,

44.10, 39.44, 35.93, 34.67, 31.86, 31.73, 29.51, 29.09, 27.08, 26.74, 25.36, 25.01, 22.56, 20.01, 14.06 ppm.

HRMS (MALDI): m/z calcd. for $C_{111}H_{211}N_6O_{15}$ $[M + H]^+$ 1868.5927; found 1868.5906.

Example 7

Compound 22

DIC (3.60 mL, 23.0 mmol, 1.6 eq.) and DMAP (70 mg, 0.58 mmol, 0.04 eq.) were added to a solution of 7-bromoheptanoic acid (3.00 g, 14.4 mmol) and 3-methylhexan-1-ol (2.50 g, 21.5 mmol, 1.5 eq.) in DCM (30 mL), and the mixture was stirred overnight at rt. The reaction mixture was then adsorbed onto silica (16 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-10 %) to yield the compound **20** (3.64 g, 83 %; R_f 0.27 in CE5, visualization by $KMnO_4$) as a colorless oil.

Bromoester **20** (3.63 g, 11.8 mmol, 2.6 eq.) and potassium carbonate (6.29 g, 45.5 mmol, 10 eq.) were added to a solution of *N*-Boc-hexane-1,6-diamine (0.98 g, 4.55 mmol, 1 eq.) in anhydrous ACN (10 mL), and the mixture was stirred at 40 °C for 3 days. The reaction mixture was then adsorbed onto silica (16 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-100 %) to yield the compound **21** (2.90 g, 95 %; R_f 0.54 in CE50 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a pale-yellow oil.

Hydrochloric acid in dioxane (4 mL, 4 M) was added to a solution of compound **21** (2.85 g, 4.26 mmol) in anhydrous DCM (4 mL), and the reaction mixture was stirred at rt for 1 h. The solution was then poured into a 500 mL separatory funnel, diluted with saturated aqueous $NaHCO_3$ (100 mL), and the product was extracted with diethyl ether (100 mL, 2 x 50 mL). The combined organic phases were washed with brine (75 mL), dried over anhydrous sodium sulfate, filtered through an S2 sintered glass, and the solvents were evaporated in an RVE. The crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (0-70 %). The amine **22** (2.38 g, 96%; R_f 0.48 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) was obtained as a pale-yellow oil.

1H NMR (400 MHz, $CDCl_3$): δ = 4.08-3.96 (m), 2.63-2.58 (m), 2.33-2.26 (m), 2.21 (t, J = 7.5 Hz), 1.61-1.50 (m), 1.49-1.40 (m), 1.40-1.30 (m), 1.30-1.14 (m), 1.14-1.00 (m), 0.85-0.76 (m) ppm. **^{13}C NMR** (150.9 MHz, $CDCl_3$): 173.85, 62.77, 54.13, 42.19, 38.98, 35.55, 34.35, 33.79, 29.56, 29.15, 27.49, 27.29, 26.88, 25.01, 19.95, 19.48, 14.26 ppm. **HRMS (ESI):** m/z calculated for $C_{34}H_{68}N_2O_4$ $[M + H]^+$ 569.5252; found 569.5250.

Hexakis(3-methylhexyl) *cis,cis*-7,7',7'',7''',7''''',7'''''-((((cyclohexane-1,3,5-tricarboxyl) tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexaheptanoate **23**

Thionylchloride (500 μ L) and DMF (4 μ L) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (60 mg, 278 μ mol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess $SOCl_2$ was removed at 70 °C with a stream of dry nitrogen,

the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (2 mL) and DIPEA (483 μ L, 2.78 mmol, 10 eq.). Then, a solution of amine **22** (632 mg, 1.11 mmol, 4 eq.) in anhydrous TCE (2 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 0–46 %) to yield the target compound **23** (346 mg, 67 %; R_f 0.66 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a yellow waxy semi-solid. ^1H NMR (600 MHz, CDCl_3): δ = 5.75, 4.09, 3.21, 2.41, 2.28, 2.22, 2.11, 1.64, 1.62, 1.61, 1.58, 1.54, 1.48, 1.44, 1.42, 1.33, 1.32, 1.29, 1.28, 1.13, 0.89, 0.88 ppm. ^{13}C NMR (150.9 MHz, CDCl_3): δ = 173.92, 173.87, 62.64, 53.90, 44.10, 39.45, 39.14, 35.51, 34.23, 31.86, 29.54, 29.52, 29.09, 27.22, 27.13, 26.77, 24.97, 19.94, 19.47, 14.26 ppm. HRMS (MALDI): m/z calcd. for $\text{C}_{111}\text{H}_{211}\text{N}_6\text{O}_{15}$ $[\text{M} + \text{H}]^+$ 1868.5927; found 1868.5905.

Example 8

Compound 24

N,N'-Diisopropylcarbodiimide (4.07 mL, 24.9 mmol, 1.6 eq.) was added to a solution containing 6-bromopentanoic acid (3.00 g, 16.6 mmol), DMAP (81 mg, 0.66 mmol, 0.04 eq.), and geraniol (4.36 mL, 24.9 mmol, 1.5 eq.) in anhydrous DCM (150 mL). The mixture was stirred overnight at rt. The reaction mixture was then adsorbed onto silica (20 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (120 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0–10 %) to yield the target compound **24** (4.80 g, 91 %; R_f 0.39 in CE5, visualization by KMnO_4) as a pale-yellow oil. ^1H NMR (400 MHz, CDCl_3) δ 5.37 – 5.30 (m, 1H), 5.11 – 5.05 (m, 1H), 4.60 (d, J = 7.1 Hz, 2H), 3.41 (t, J = 6.6 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 2.15 – 2.01 (m, 4H), 1.95 – 1.86 (m, 2H), 1.83 – 1.74 (m, 2H), 1.70 (s, 3H), 1.68 (s, 3H), 1.60 (s, 3H).

Compound 26

Bromoester **24** (4.80 g, 15.1 mmol, 2.3 eq.) and potassium carbonate (7.27 g, 52.3 mmol, 8 eq.) were added to a solution of *N*-Boc-1,6-hexanediamine (1.42 g, 6.58 mmol, 1 eq.) in anhydrous ACN (30 mL), and the mixture was stirred at 45 °C for 24 h. The reaction mixture was then adsorbed onto silica (20 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (120 g, column pretreated with gaseous NH_3 , elution with a linear gradient of ethyl acetate in cyclohexane, 0–100 %) to yield the compound **25** (2.80 g, 54 %; R_f 0.58 in CE50 on an NH_3 -pretreated TLC plate, visualization by KMnO_4) as a pale-yellow oil.

A flask equipped with a magnetic stir bar was loaded with compound **25** (2.80 g, 4.06 mmol), closed with a rubber septum and flushed with argon. ACN (10 mL) and DCM (20 mL) were added through the septum using a needle and syringe, the stirring was turned on and a solution of 4-toluenesulfonic acid monohydrate (2.32 g, 12.2 mmol, 3 eq.) in ACN (20 mL) was added at rt through the septum over the course of 5 min using a needle and syringe. The reaction mixture was stirred at rt for 6 h and was subsequently neutralized

using a stream of gaseous ammonia. The reaction mixture was then adsorbed onto silica (20 g) and the crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (10-43 %). The amine **26** (810 mg, 34 %; R_f 0.45 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) was obtained as a yellow oil. **HRMS** (ESI): m/z calcd. for $\text{C}_{36}\text{H}_{64}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$ 589.4939; found 589.4936.

Hexakis((E)-3,7-dimethylocta-2,6-dien-1-yl) *cis,cis*-5,5',5'',5''',5''',5''''-((((cyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexapentanoate 27

Thionylchloride (700 μL) and DMF (6 μL) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (45 mg, 208 μmol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess SOCl_2 was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (1.5 mL) and DIPEA (363 μL , 2.08 mmol, 10 eq.). Then, a solution of amine **26** (490 mg, 833 μmol , 4 eq.) in anhydrous TCE (1.0 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 0-31 %) to yield the target compound **27** (262 mg, 65 %; R_f 0.59 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a yellow waxy semi-solid. **^1H NMR** (600 MHz, CDCl_3): δ = 5.72, 5.32, 5.08, 4.58, 3.21, 2.44, 2.41, 2.32, 2.22, 2.11, 2.10, 2.09, 2.03, 1.69, 1.68, 1.61, 1.60, 1.59, 1.58, 1.47, 1.45, 1.29 ppm. **^{13}C NMR** (150.9 MHz, CDCl_3): δ = 173.85, 173.60, 142.16, 131.80, 123.72, 118.32, 62.25, 53.79, 53.45, 44.12, 39.52, 39.42, 34.14, 31.85, 29.48, 27.03, 26.72, 26.29, 25.67, 22.90, 17.68, 16.46 ppm. **HRMS** (MALDI): m/z calcd. For $\text{C}_{117}\text{H}_{199}\text{N}_6\text{O}_{15}$ $[\text{M} + \text{H}]^+$ 1928.4988; found 1928.4967.

Example 9

Compound 28

N,N'-Diisopropylcarbodiimide (4.07 mL, 24.9 mmol, 1.6 eq.) was added to a solution containing 6-bromopentanoic acid (3.00 g, 16.6 mmol), DMAP (81 mg, 0.66 mmol, 0.04 eq.), and nerol (4.38 mL, 24.9 mmol, 1.5 eq.) in anhydrous DCM (60 mL). The mixture was stirred overnight at rt. The reaction mixture was then adsorbed onto silica (20 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (120 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-10 %) to yield the target compound **28** (4.75 g, 90 %; R_f 0.39 in CE5, visualization by KMnO_4) as a pale-yellow oil. **^1H NMR** (401 MHz, CDCl_3) δ 5.35 (t, J = 6.5 Hz, 1H), 5.12 – 5.06 (m, 1H), 4.57 (dd, J = 7.2, 1.1 Hz, 2H), 3.40 (t, J = 6.6 Hz, 2H), 2.34 (t, J = 7.3 Hz, 2H), 2.15 – 2.03 (m, 4H), 1.94 – 1.86 (m, 2H), 1.82 – 1.74 (m, 5H), 1.68 (d, J = 1.4 Hz, 3H), 1.60 (s, 3H).

Compound 30

Bromoester **28** (4.75 g, 15.0 mmol, 2.3 eq.) and potassium carbonate (7.20 g, 52.1 mmol, 8 eq.) were added to a solution of *N*-Boc-1,6-hexanediamine (1.41 g, 6.51 mmol, 1 eq.) in anhydrous ACN (45 mL), and the mixture was stirred at 48 °C for 24 h. The reaction mixture was then adsorbed onto silica (20 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (120 g, column pretreated with gaseous NH₃, elution with a linear gradient of ethyl acetate in cyclohexane, 0-100 %) to yield the compound **29** (3.62 g, 81 %; *R*_f 0.54 in CE50 on an NH₃-pretreated TLC plate, visualization by ninhydrin) as a pale-yellow oil.

A flask equipped with a magnetic stir bar was loaded with compound **29** (3.62 g, 5.25 mmol), closed with a rubber septum and flushed with argon. DCM (10 mL) was added through the septum using a needle and syringe, the stirring was turned on and a solution of 4-toluenesulfonic acid monohydrate (2.50 g, 13.1 mmol, 2.5 eq.) in ACN (25 mL) was added at rt through the septum over the course of 5 min using a needle and syringe. The reaction mixture was stirred at rt for 5 h and was subsequently neutralized using a stream of gaseous ammonia. The reaction mixture was then adsorbed onto silica (20 g) and the crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (10-43 %). The amine **30** (1.36 g, 44 %; *R*_f 0.46 in D2 on an NH₃-pretreated TLC plate, visualization by KMnO₄) was obtained as a yellow oil. **HRMS** (ESI): *m/z* calcd. for C₃₆H₆₄N₂O₄ [M + H]⁺ 589.4939; found 589.4937.

Hexakis((Z)-3,7-dimethylocta-2,6-dien-1-yl) *cis,cis*-5,5',5'',5''',5''''',5'''''-((((cyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexapentanoate 31

Thionylchloride (700 μL) and DMF (6 μL) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (65 mg, 301 μmol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess SOCl₂ was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (2 mL) and DIPEA (524 μL, 3.01 mmol, 10 eq.). Then, a solution of amine **30** (796 mg, 1.35 mmol, 4.5 eq.) in anhydrous TCE (2.0 mL) was added and the reaction mixture was stirred for 30 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-29 %) to yield the target compound **31** (351 mg, 51 %) as a yellow waxy semi-solid. **¹H NMR** (600 MHz, CDCl₃): δ = 5.69, 5.34, 5.08, 4.55, 3.21, 2.41, 2.38, 2.30, 2.22, 2.10, 2.06, 1.75, 1.67, 1.60, 1.59, 1.58, 1.47, 1.28 ppm. **¹³C NMR** (150.9 MHz, CDCl₃): δ = 173.81, 173.58, 142.44, 132.12, 123.56, 119.22, 60.96, 53.82, 53.48, 44.12, 39.44, 34.17, 32.15, 31.85, 29.50, 27.07, 26.75, 26.63, 25.68, 23.50, 22.91, 17.65 ppm. **HRMS** (MALDI): *m/z* calcd. for C₁₁₇H₁₉₉N₆O₁₅ [M + H]⁺ 1928.4988; found 1928.4963.

Example 10**Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((5-(methoxycarbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate 32**

Thionylchloride (700 μ L) and DMF (6 μ L) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (60 mg, 278 μ mol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess SOCl₂ was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (2 mL) and DIPEA (483 μ L, 2.78 mmol, 10 eq.). Then, a solution of amine **18** (632 mg, 1.11 mmol, 4 eq.) in anhydrous TCE (1.0 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-29 %) to yield compound **32** (242 mg, 65 %; *R*_f 0.68 in D2 on an NH₃-pretreated TLC plate, visualization by ninhydrin) as a yellow oil. The product **32** is a result of imperfect MeOH removal from amine **18**. ¹H NMR (600 MHz, CDCl₃): δ = 5.68, 5.29, 4.88, 3.67, 3.22, 2.43, 2.40, 2.27, 2.20, 2.19, 2.18, 2.09, 1.62, 1.59, 1.57, 1.56, 1.48, 1.47, 1.46, 1.30, 1.28, 1.27, 1.19, 0.87 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 174.64, 173.75, 173.38, 70.83, 53.82, 53.76, 51.84, 43.99, 42.09, 39.43, 35.93, 34.66, 31.81, 31.73, 31.20, 29.50, 29.09, 27.06, 26.72, 25.36, 24.99, 22.56, 20.00, 14.05 ppm. HRMS (MALDI): *m/z* calcd. for C₇₈H₁₄₆N₄O₁₂ [M + H]⁺ 1332.1010; found 1332.0987.

Example 11**Tetrakis(3-methylhexyl) *cis,cis*-7,7',7'',7'''-((((5-(methoxycarbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetraheptanoate 33**

Thionylchloride (700 μ L) and DMF (6 μ L) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (55 mg, 254 μ mol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess SOCl₂ was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (2 mL) and DIPEA (443 μ L, 2.54 mmol, 10 eq.). Then, a solution of MeOH (10.3 μ L, 254 μ mol, 1.0 eq.) in TCE (0.5 mL) was added at 0 °C. The reaction mixture was allowed to warm to room temperature and after stirring for 1 h, amine **22** (376 mg, 661 μ mol, 2.6 eq.) in anhydrous TCE (2.0 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-29 %) to yield the target compound **33** (87 mg, 26 %; *R*_f 0.75 in D2 on an NH₃-pretreated TLC plate, visualization by ninhydrin) as a pale-yellow oil. ¹H NMR (600 MHz, CDCl₃): δ = 5.68, 5.29, 4.09, 3.67, 3.22, 2.43, 2.40, 2.28, 2.20, 2.18, 2.09, 1.64, 1.61, 1.57, 1.53, 1.48, 1.45, 1.42, 1.34, 1.31, 1.28, 1.13, 0.89, 0.88 ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 174.65, 173.91, 173.76, 62.85, 53.85, 51.85, 43.98, 42.09, 39.42, 39.15,

35.52, 34.22, 31.89, 31.19, 29.55, 29.51, 29.06, 27.20, 27.10, 26.72, 24.95, 19.94, 19.47, 14.26 ppm.

HRMS (MALDI): m/z calcd. for $C_{78}H_{147}N_4O_{12}$ $[M + H]^+$ 1332.1010; found 1332.0984.

Example 12

Compound 34

Thionylchloride (1.2 mL) and DMF (10 μ L) were added to *cis,cis*-cyclohexane-1,3,5-tricarboxylic acid (160 mg, 740 μ mol), and the suspension was stirred for 3 h at 70 °C in a closed vial; during this time the suspension turned into a clear solution. Excess $SOCl_2$ was removed at 70 °C with a stream of dry nitrogen, the residue was dried at rt *in vacuo* (20 min), and the obtained solid was dissolved under argon in anhydrous TCE (3 mL) and DIPEA (1.29 mL, 7.40 mmol, 10 eq.). Then, a solution of BnOH (76 μ L, 740 μ mol, 1.0 eq.) in TCE (0.5 mL) was added at 0 °C. The reaction mixture was allowed to warm to room temperature and after stirring for 2 h, amine **18** (1.09 g, 1.92 mmol, 2.6 eq.) in anhydrous TCE (3.0 mL) was added and the reaction mixture was stirred for 40 min at rt. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-29 %) to yield the compound **34** (150 mg, 14 %; R_f 0.67 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a pale-yellow oil. **HRMS (MALDI):** m/z calcd. for $C_{84}H_{151}N_4O_{12}$ $[M + H]^+$ 1408.1323; found 1408.1283.

cis,cis-3,5-Bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyl)cyclohexane-1-carboxylic acid **35**

A pear-shaped flask was equipped with a magnetic stir bar and loaded with palladium on carbon (10% Pd/C, 25 mg). The flask was closed with a rubber septum, connected to a Schlenk line via a needle, and the atmosphere in the flask was exchanged for argon via 3 cycles of vac-Ar. Subsequently, a solution of compound **34** (150 mg, 107 μ mol) in MeOH (3 mL) was added through the septum using a needle and syringe and the stirring was turned on. The flask was connected to a continuous stream of hydrogen using a second needle and the main argon inlet into the Schlenk line was closed (excess hydrogen continuously released from the flask through the Schlenk line bubbler). The reaction mixture was vigorously stirred for 4 h at rt. Then the flask was thoroughly flushed with argon and the palladium on carbon was removed by filtering through a PTFE syringe filter. The volatiles were then removed *in vacuo* yielding the product **35** (110 mg, 79 %, R_f 0.27 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as a colorless oil. **1H NMR** (600 MHz, $CDCl_3$): δ = 6.59, 4.88, 3.20, 3.15, 2.71, 2.67, 2.27, 2.24, 2.20, 2.13, 2.02, 1.64, 1.63, 1.60, 1.58, 1.57, 1.56, 1.54, 1.47, 1.45, 1.32, 1.28, 1.27, 1.26, 1.18, 0.87 ppm. **^{13}C NMR** (150.9 MHz, $CDCl_3$): δ = 180.09, 175.19, 173.10, 70.94, 53.01, 52.06, 44.36, 44.32, 39.22, 35.91, 34.43, 32.48, 32.16, 31.72, 29.15, 29.08, 26.72, 26.62, 26.30, 25.36, 24.69, 24.11, 22.55, 19.99, 14.06 ppm. **HRMS (MALDI):** m/z calcd. for $C_{77}H_{145}N_4O_{12}$ $[M + H]^+$ 1318.0854; found 1318.0842.

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate 36

A vial equipped with a magnetic stir bar was loaded with compound **35** (60 mg, 45.5 μ mol), NH_4Cl (12.2 mg, 228 μ mol, 5 eq.), and flushed with argon via piercing the septum with a needle. Subsequently, anhydrous DMF (1.5 mL) and DIPEA (48 μ L, 273 μ mol, 6 eq.) were added using a needle and syringe. The resulting suspension was stirred for 10 min at rt, then a solution of PyBroP (53 mg, 114 μ mol, 2.5 eq.) in anhydrous DMF (0.5 mL) was added and the reaction mixture was stirred at rt for 90 min. The reaction mixture was then evaporated *in vacuo*, redissolved in DCM, adsorbed onto silica (10 g), and the DCM was removed *in vacuo*. The crude product was purified by flash chromatography on silica (elution with a linear gradient of D1 in DCM, 5-29 %) to yield the compound **36** (18 mg, 29 %; R_f 0.42 in D2 on an NH_3 -pretreated TLC plate, visualization by ninhydrin) as the main product in the form of a pale-yellow oil. The compound **36** is a product of pyrrolidine impurity in PyBroP resulting from PyBroP hydrolysis. ^1H NMR (600 MHz, CDCl_3): δ = 6.08, 4.88, 3.48, 3.43, 3.35, 3.25, 3.26, 3.20, 3.16, 2.72, 2.50, 2.32, 2.28, 2.15, 1.98, 1.95, 1.85, 1.69, 1.68, 1.66, 1.65, 1.60, 1.57, 1.50, 1.46, 1.34, 1.28, 1.26, 1.19, 0.88 ppm. ^{13}C NMR (150.9 MHz, CDCl_3): δ = 174.39, 173.14, 172.79, 70.97, 53.37, 47.44, 47.40, 47.22, 47.19, 46.43, 46.30, 46.27, 45.90, 44.03, 41.39, 39.09, 35.92, 34.44, 32.08, 31.73, 31.20, 29.10, 26.70, 26.14, 25.37, 24.69, 24.24, 22.57, 20.00, 14.06. HRMS (MALDI): m/z calcd. for $\text{C}_{81}\text{H}_{152}\text{N}_5\text{O}_{11}$ $[\text{M} + \text{H}]^+$ 1371.1483; found 1371.1467.

Example 13

Trimethyl *cis,cis*-1,3,5-cyclohexanetricarboxylate 37

To a solution of *cis,cis*-1,3,5-cyclohexanetricarboxylic acid (9.90 g, 45.8 mmol) in anhydrous methanol (150 mL) placed in a round-bottom flask equipped with a calcium chloride drying tube and magnetic stirrer was slowly added thionyl chloride (15.29 mL, 210.8 mmol, 4.6 eq.) through a rubber septum via syringe. After the violent exothermic reaction ceased, the solution was stirred at room temperature for 12 h. The solvent was then removed *in vacuo*, the oily residue was poured into a 500 mL separatory flask, diluted with saturated aqueous NaHCO_3 (250 mL), and the product was extracted with diethyl ether (2x100 mL). The combined organic phase was washed with brine (100 mL), dried over anhydrous sodium sulfate, filtered through an S2 sintered glass, and the solvents were evaporated in an RVE to give **37** as a pale-yellow oil. ^1H NMR (400 MHz, CDCl_3): δ = 3.68 (s, 9H), 2.44 – 2.32 (m, 3H), 2.31 – 2.21 (m, 3H), 1.59 – 1.45 (m, 3H) ppm. HRMS (ESI): m/z calculated for $\text{C}_{12}\text{H}_{19}\text{O}_6$ $[\text{M} + \text{H}]^+$ 259.11761; found 259.11766.

Trimethyl *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylate 38

Diisopropylamine was distilled from sodium hydroxide before use. A solution of diisopropylamine (2.19 mL, 15.5 mmol, 4 eq.) in anhydrous diethyl ether (10 mL) was added dropwise to the solution of 2.0 M *n*-butyllithium (7.74 mL, 15.5 mmol, 4 eq.) at 0 °C to generate lithium diisopropylamide *in situ*. A solution of **37** (1.00 g, 3.87 mmol; 1 eq.) in anhydrous diethyl ether (10 mL) was added dropwise to the reaction solution at 0 °C, and the mixture was stirred for 2 h at 0 °C. Dimethyl sulfate (2.20 mL, 23.2 mmol,

6 eq.) was added, and the stirring continued overnight at rt. The product was washed with water, 1 M HCl and brine, dried over sodium sulfate, filtered through an S2 sintered glass, and the solvents were evaporated in an RVE. GC-MS analysis exhibited that ratio of two isomers *cis,cis/cis,trans* = 7:1 in the crude product. The fractional crystallization in the mixture of diethyl ether and pentane afforded *cis,cis* isomer **38** (225 mg, 19 %) as white crystals. ¹H NMR (400 MHz, CDCl₃): δ = 3.65 (s, 9H), 2.74 (d, *J* = 13.4 Hz, 3H), 1.21 (s, 9H), 0.97 (d, *J* = 14.8 Hz, 3H) ppm. HRMS (EI): *m/z* calcd. for C₁₅H₂₄O₆ [M]⁺ 300.1567; found 300.1565.

***cis,cis*-1,3,5-Trimethylcyclohexane-1,3,5-tricarboxylic acid 39**

Trimethyl *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylate **38** (220 mg, 0.73 mmol, 1 eq.) was dissolved in methanol (3.0 mL). A solution of lithium hydroxide monohydrate (369 mg, 6.59 mmol, 9 eq.) in water (2.0 mL) was added, and the reaction mixture was stirred at rt overnight. Methanol was removed by evaporation in an RVE. The solution was cooled in an ice bath, and the pH was adjusted to 1 with concentrated HCl. A white precipitate formed immediately and was collected by suction filtration to yield **39** (60 mg, 32 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 11.96 (br s, 3H), 2.52 (d, 3H, partial overlap with solvent signal), 1.20 (d, *J* = 14.7 Hz, 3H), 1.19 (s, 9H) ppm.

Hexakis(3-methylhexyl) *cis,cis*-7,7',7'',7''',7''',7''''-((((1,3,5-trimethylcyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexaheptanoate 40

Thionylchloride (0.50 mL) and DMF (2 μL) was added to *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid **39** (34 mg, 0.13 mmol), and the suspension was stirred overnight at 70 °C in a closed vial. Excessive SOCl₂ was blown out with a stream of dry nitrogen, the residue was dried *in vacuo* (10 min), and after cooling down to rt the residue was dissolved in anhydrous TCE (0.5 mL). Then, a solution of amine **22** (337 mg, 0.59 mmol, 4.5 eq.) and DIPEA (229 μL, 1.32 mmol, 10 eq.) in anhydrous TCE (1.00 mL) was added, and the reaction mixture was stirred for 15 min at rt. The reaction mixture was then adsorbed onto silica (4 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (40 g, elution with a linear gradient of D1 in DCM, 0-30 %) to yield the target compound **40** (6 mg, 5 %; *R*_f 0.26 in D4, visualization by ninhydrin) as a viscous pale-yellow oil. HRMS (MALDI): *m/z* calcd. for C₁₁₄H₂₁₆N₆O₁₅ [M + H]⁺ 1910.6396; found 1910.6368.

Example 14

Hexa(octan-2-yl) *cis,cis*-6,6',6'',6''',6''',6''''-((((1,3,5-trimethylcyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate 41 and *cis,cis*-3,5-Bis((6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyl)-1,3,5-trimethylcyclohexane-1-carboxylic acid 42

Thionylchloride (0.50 mL) and DMF (2 μL) was added to **39** (28 mg, 0.11 mmol), and the suspension was stirred for 2 h at 70 °C in a closed vial. Excessive SOCl₂ was blown out with a stream of dry nitrogen, the residue was dried *in vacuo* (10 min), and after cooling down to rt the residue was dissolved in anhydrous

TCE (0.25 mL). Then, a solution of amine **18** (278 mg, 0.49 mmol, 4.5 eq.) and DIPEA (180 μ L, 1.08 mmol, 10 eq.) in anhydrous TCE (0.75 mL) was added, and the reaction mixture was stirred for 15 min at rt. The reaction mixture was then adsorbed onto silica (4 g), and the solvents were evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (40 g, elution with a linear gradient of D1 in DCM, 0-30 %) to yield the target compound **41** (49 mg, 24 %; R_f 0.30 in D4, visualization by ninhydrin) as a viscous pale-yellow oil and compound **42** (52 mg, 45 %; R_f 0.28 in D4, visualization by ninhydrin) as a pale-yellow oil.

41 ^1H NMR (600 MHz, CDCl_3): δ = 7.98, 4.88, 3.12, 2.88, 2.75, 2.68, 2.27, 1.64, 1.56, 1.46, 1.45, 1.28, 1.27, 1.26, 1.23, 1.19, 1.12, 0.87 ppm. ^{13}C NMR (150.9 MHz, CDCl_3): δ = 184.26, 177.45, 173.06, 70.96, 52.45, 51.68, 43.03, 43.00, 42.38, 38.83, 35.92, 34.43, 34.17, 33.21, 31.72, 29.08, 28.95, 22.56, 19.99, 14.05 ppm. HRMS (MALDI): m/z calcd. for $\text{C}_{114}\text{H}_{216}\text{N}_6\text{O}_{15}$ $[\text{M} + \text{H}]^+$ 1910.6396; found 1910.6364.

42 HRMS (MALDI): m/z calcd. for $\text{C}_{80}\text{H}_{150}\text{N}_4\text{O}_{12}$ $[\text{M} + \text{H}]^+$ 1360.1323; found 1360.1875.

Example 15

Trimethyl *cis,cis*-1,3,5-tris((benzyloxy)methyl)cyclohexane-1,3,5-tricarboxylate **43**

Diisopropylamine was distilled from sodium hydroxide before use. A solution of diisopropylamine (6.56 mL, 46.5 mmol, 4 eq.) in anhydrous diethyl ether (30 mL) was added dropwise to the solution of 2.0 M *n*-butyllithium (23.2 mL, 46.5 mmol, 4 eq.) at 0 °C to generate lithium diisopropylamide *in situ*. A solution of trimethyl cyclohexane-1,3,5-tricarboxylate **37** (3.00 g, 11.6 mmol; 1 equiv.) in anhydrous diethyl ether (30 mL) was added dropwise to the reaction solution at 0 °C, and the mixture was stirred for 2 h at 0 °C. Benzyl chloromethyl ether (9.69 mL, 69.7 mmol, 6 eq.) was added, and the stirring continued overnight at rt. The product was washed with water, 1 M HCl and brine, dried over sodium sulphate, filtered through an S2 sintered glass, and the solvent was evaporated in an RVE. The crude product was purified by flash chromatography on silica (200 g, elution with a linear gradient of ethyl acetate in cyclohexane, 0-20 %). The fractional crystallization in the mixture of diethyl ether (3.0 mL) and pentane (18.0 mL) afforded *cis,cis* isomer **43** (1.52 mg, 22 %) as white crystals. ^1H NMR (400 MHz, CDCl_3): δ = 7.36–7.22 (m, 15H, partial overlap with solvent signal), 4.46 (s, 6H), 3.69 (s, 9H), 3.39 (s, 6H), 2.68 (d, J = 14.2 Hz, 3H), 1.18 (d, J = 14.9 Hz, 3H) ppm. HRMS (ESI): m/z calcd. for $\text{C}_{36}\text{H}_{42}\text{O}_9$ $[\text{M} + \text{Na}]^+$ 641.2721; found 641.2719.

cis,cis-1,3,5-Tris((benzyloxy)methyl)cyclohexane-1,3,5-tricarboxylic acid **44**

Compound **43** (250 mg, 0.40 mmol, 1 eq.) was dissolved in methanol (4.0 mL). A solution of lithium hydroxide monohydrate (152 mg, 3.64 mmol, 9 eq.) in water (2.0 mL) was added, and the reaction mixture was stirred at rt overnight. Methanol was removed by evaporation in an RVE. The solution was cooled in an ice bath, and the pH was adjusted to 1 with concentrated HCl. A white precipitate formed immediately and was collected by suction filtration to yield **44** (200 mg, 86 %). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 12.12 (br s, 3H), 7.37–7.18 (m, 15H), 4.39 (s, 6H), 3.39 (s, 6H), 2.36 (d, J = 14.9 Hz, 3H), ppm.

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris((benzyloxy)methyl)-5-(bis(6-(octan-2-yloxy)-6-oxohexyl)carbamoyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate 45 and *cis,cis*-1,3,5-Tris((benzyloxy)methyl)-3,5-bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyl)cyclohexane-1-carboxylic acid 46

Thionylchloride (0.50 mL) and DMF (2 μ L) was added to **44** (100 mg, 0.17 mmol, 1 eq.), and the suspension was stirred overnight at 70 °C in a closed vial. Excessive SOCl₂ was blown out with a stream of dry nitrogen, the residue was dried *in vacuo* (10 min), and after cooling down to rt the residue was dissolved in anhydrous TCE (0.5 mL). Then, a solution of amine **18** (444 mg, 0.78 mmol, 4.5 eq.) and DIPEA (302 μ L, 1.73 mmol, 10 eq.) in anhydrous TCE (1.50 mL) was added, and the reaction mixture was stirred for 15 min at rt. The reaction mixture was then adsorbed onto silica (4 g), and the solvent was evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of D1 in DCM, 0-20 %) to yield the target compound **45** (46 mg, 12 %; *R_f* 0.46 in D4, visualization by ninhydrin) as a viscous pale-yellow oil and compound **46** (37 mg, 13 %; *R_f* 0.39 in D4, visualization by ninhydrin) as a pale-yellow oil.

45 HRMS (MALDI): m/z calcd. for C₁₃₅H₂₃₄N₆O₁₈ [M + H]⁺ 2228.7652; found 2228.7702.

46 HRMS (MALDI): m/z calcd. for C₁₀₁H₁₆₈N₄O₁₅ [M]⁺ 1677.2506; found 1677.2479.

Hexa(octan-2-yl) *cis,cis*-6,6',6'',6''',6''''',6'''''-((((1,3,5-tris(hydroxymethyl)cyclohexane-1,3,5-tricarboxonyl)tris(azanediyl))tris(hexane-6,1-diyl))tris(azanetriyl))hexahexanoate 47

Following the procedure outlined for **35**, the target compound **47** was prepared from lipidoid **45** (20 mg, 0.008 mmol, 1 eq.), palladium on carbon (10% Pd/C, 19 mg, 0.018 mmol, 2 eq.) and acetic acid (80 μ L, 1.73 mmol) to yield lipidoid **47** (5 mg, 30 %) as a thick pale-yellow oil. **HRMS (MALDI):** m/z calcd. for C₁₁₄H₂₁₆N₆O₁₈ [M + H]⁺ 1958.6244; found 1958.6314.

***cis,cis*-3,5-Bis((6-(bis(6-(octan-2-yloxy)-6-oxohexyl)amino)hexyl)carbamoyl)-1,3,5-tris(hydroxymethyl)cyclohexane-1-carboxylic acid 48**

Following the procedure outlined for **35**, the target compound **48** was prepared from lipidoid **46** (15 mg, 0.009 mmol, 1 eq.), palladium on carbon (19 mg, 0.018 mmol, 2 eq.) and acetic acid (80 μ L, 1.73 mmol) to yield lipidoid **48** (7 mg, 59 %) as a thick pale-yellow oil. **HRMS (MALDI):** m/z calcd. for C₈₀H₁₅₀N₄O₁₅ [M]⁺ 1407.1098; found 1407.1042.

Example 16

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris((benzyloxy)methyl)-5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate 49

Compound **44** (100 mg, 0.17 mmol, 1 eq.) was dissolved in anhydrous DMF (1.0 mL) and DMAP (3 mg, 0.02 mmol, 0.15 eq.) and DIPEA (450 μ L, 2.60 mmol, 10 eq.) were added. A solution of PyBroP (363 mg, 0.78 mmol, 4.5 eq.) in anhydrous DMF (1.0 mL) was added dropwise, and the reaction mixture was stirred

for 1.5 h at rt under argon atmosphere. Then, a solution of amine **18** (444 mg, 0.78 mmol, 4.5 eq.) in anhydrous DMF (1.0 mL) was added, and the reaction mixture was stirred for 1 h at rt under argon atmosphere. The reaction mixture was then adsorbed onto silica (4 g), and the solvent was evaporated *in vacuo*. The crude product was purified by flash chromatography on silica (80 g, elution with a linear gradient of D1 in DCM, 0-30 %) to yield the target compound **49** (146 mg, 51 %; R_f 0.58 in D3, visualization by ninhydrin) as a pale-yellow oil. **HRMS** (MALDI): m/z calcd. for $C_{105}H_{175}N_5O_{14}$ $[M + Na]^+$ 1753.3028; found 1753.3044.

Tetra(octan-2-yl) *cis,cis*-6,6',6'',6'''-((((1,3,5-tris(hydroxymethyl)-5-(pyrrolidine-1-carbonyl)cyclohexane-1,3-dicarbonyl)bis(azanediyl))bis(hexane-6,1-diyl))bis(azanetriyl))tetrahexanoate **50**

Following the procedure outlined for **35**, the target compound **50** was prepared from lipidoid **49** (70 mg, 0.04 mmol, 1 eq.), palladium on carbon (86 mg, 0.40 mmol, 2 eq.) and acetic acid (80 μ L, 1.73 mmol) to yield lipidoid **50** (57 mg, 96 %) as a thick pale-yellow oil. **HRMS** (MALDI): m/z calcd. for $C_{84}H_{157}N_5O_{14}$ $[M + Na]^+$ 1483.1619; found 1483.1656.

Example 17

cis,cis*- N^1,N^7 -Bis(6-(didodecylamino)hexyl)-5,7-bis(hydroxymethyl)-4-oxo-3-oxabicyclo[3.3.1]nonane-1,7-dicarboxamide **52*

PyBroP (0.323 g, 0.694 mmol, 5 eq.), *N,N*-diisopropylethylamine (DIPEA, 0.483 mL, 2.77 mmol, 20 eq.) and amine **10** (0.314 g, 0.694 mmol, 5 eq.) were added to a solution of triacid **44** (80 mg, 0.139 mmol) in anhydrous TCE (4 mL), and the reaction mixture was stirred at rt for 12 h. The reaction mixture was then adsorbed onto silica (10 g), and the solvents were evaporated *in vacuo*. The crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (20-80%). Diamide **51** (16 mg, 8.0 %; R_f 0.56 in mobile phase D3, detection with ninhydrin) was obtained in the form of a viscous yellowish oil.

Palladium on charcoal (10 %) was added to a solution of diamide **51** in a methanol-ethyl acetate mixture (10+10 mL) under argon atmosphere. The reaction mixture was then stirred under hydrogen atmosphere at rt for 12 h. The suspension was filtered through a pad of celite and evaporated to yield lipidoid **52** as a colorless oil (5.5 mg, 42.9 %, R_f 0.61 in mobile phase D3, detection with ninhydrin) **HRMS** (MALDI): m/z calcd. for $C_{72}H_{141}N_4O_6$ $[M + H]^+$ 1158.0846; found 1158.0822.

Example 18

6-(Didodecylamino)hexanoic acid **53**

A 500 mL round-bottom flask equipped with a calcium chloride drying tube and magnetic stirrer was filled with a solution of 6-aminohexanoic acid (2.00 g, 15.2 mmol) in ACN (150 mL), and sodium triacetoxymethylborohydride (12.93 g, 61.0 mmol, 4 eq.) was added. With intensive stirring, *n*-dodecylaldehyde (10.16 mL, 45.7 mmol, 3 eq.) was slowly added through a rubber septum via syringe, and the resulting

white suspension was stirred at room temperature for 5 d. The reaction mixture was then adsorbed onto silica (25 g), and the solvents were evaporated *in vacuo*. The crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (0-100%). Amino acid **53** (3.203 g, 44.9 %) was obtained as a colourless oil. ¹H NMR (400 MHz, CDCl₃): δ = 2.88-2.84 (m, 6H), 2.24 (t, 2H), 1.62-1.72 (m, 2H), 1.42-1.26 (m, 44H) 0.89 (t, 6H) ppm. ¹³C NMR (150.9 MHz, CDCl₃): δ = 178.65, 51.37, 36.22, 31.90, 29.61-29.20, 27.07, 26.74, 25.45, 23.78, 23.31, 22.67, 14.10 ppm. HRMS (ESI): m/z calculated for C₃₀H₆₀O₂N [M - H]⁻ 466.46295; found 466.46286.

cis,cis-1,3,5-Triaminocyclohexane trihydrobromide **55**

The synthesis was carried out according to Bowen, T. et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 6, No. 7, 1996, 807-810 and slightly modified. *cis,cis*-1,3,5-Cyclohexanetricarboxylic acid (2.0 g, 9.25 mmol) was suspended in toluene (75 mL), and DIPEA (4.83 mL, 27.75 mmol, 3 eq.) was added followed by diphenyl phosphoryl azide (5.97 mL, 27.75 mmol, 3 eq.). The mixture was stirred for 0.5 h at rt and then heated to 90 °C for 0.5 h. Benzyl alcohol (3.20 mL, 30.81 mmol, 3.33 eq.) was added and the solution was heated to 90 °C for 18 h. After cooling to rt, the product was collected by vacuum filtration, washed with minimal cold toluene, and dried under vacuum to yield 0.386 g (7.85 %) of *cis,cis*-1,3,5-tri(*N*-benzyloxycarbonyl)cyclohexane **54**.

Compound **54** (0.386 g, 0.726 mmol) was suspended in a 33% solution of HBr in acetic acid (5 mL), and stirred for 12 h at rt. The yellow suspension was then diluted with diethyl ether (20 mL), and filtered through an S2 sintered glass to give trihydrobromide **55** as a yellow solid (0.248 g, 91.8 %).

cis,cis-*N,N,N'*-(Cyclohexane-1,3,5-triyl)tris(6-(didodecylamino)hexanamide) **56**

PyBroP (0.752 g, 1.61 mmol, 6 eq.), DIPEA (0.937 mL, 5.38 mmol, 20 eq.) and acid **53** (0.755 g, 1.61 mmol, 6 eq.) were added to a solution of triamine salt **55** (0.100 g, 0.269 mmol) in a mixture of anhydrous TCE (3 mL) and anhydrous DMF (3 mL), and the reaction mixture was stirred at rt for 12 h. The reaction mixture was then adsorbed onto silica (10 g), and the solvents were evaporated *in vacuo*. The crude product was purified by silica gel column chromatography using a linear gradient of D1 in DCM (20-30%). Lipidoid **56** (113 mg, 28.4 %; *R_f* 0.65 in mobile phase D2, detection with ninhydrin) was obtained in the form of a viscous yellowish oil. HRMS (ESI): m/z calculated for C₉₆H₁₉₅O₆N₃ [M + H]⁺ 1478.5134; found 1478.5162.

Example 19

Preparation of transfection reagents

Reagents were generated by mixing the individual components listed in Table 1 up to Table 4. All tables contain the final molar concentrations in the transfection reagent. Stock 5 mM solutions of the individual components in 99.7% ethanol (v/v) were used for the preparation of **A01** to **A16**, **A21**, **A22**, **A24**, **A28**, and

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A29. Stock 5 mM solutions of lipidoids, DOPE and DMG-PEG2000, and stock 10 mM solution of cholesterol, all in 99.7% ethanol (v/v) were used for the preparation of **A17** to **A20**, **A23**, and **A25** to **A27**. The DOPE-Cy5 stock solution had a concentration of 0.79 mM and was prepared in chloroform. **TT3** is a lipid according to WO 2016/187531, used herein for comparison. Benchmark **D-Lin-MC3-DMA** lipid (MedChemExpress Europe) was also used for comparison.

Table 1. Composition of transfection reagents A01-A06.

Compound	Concentration of individual components in transfection reagents (mM)					
	A01	A02	A03	A04	A05	A06
3	1.1	—	—	—	—	—
7	—	1.1	—	—	—	—
8	—	—	1.1	—	—	—
11	—	—	—	1.1	—	—
15	—	—	—	—	1.1	—
TT3	—	—	—	—	—	1.1
cholesterol	2.18	2.18	2.18	2.18	2.18	2.18
DOPE	1.64	1.64	1.64	1.64	1.64	1.64
DMG-PEG ₂₀₀₀	0.075	0.075	0.075	0.075	0.075	0.075

Table 2. Composition of transfection reagents A07-A12.

[illegible]

Table 3. Composition of transfection reagents A13-A22.

[illegible]

41	—	—	—	—	—	—	—	—	—	1.1
cholesterol	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18
DOPE	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64
DMG-PEG ₂₀₀₀	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075

Table 4. Composition of transfection reagents **A23-A29**.

Compound	Concentration of individual components in transfection reagents (mM)						
	A23	A24	A25	A26	A27	A28	A29
42	1.64	—	—	—	—	—	—
47	—	1.1	—	—	—	—	—
48	—	—	1.64	—	—	—	—
50	—	—	—	1.64	—	—	—
52	—	—	—	—	1.64	—	—
56	—	—	—	—	—	1.1	—
D-Lin-MC3-DMA	—	—	—	—	—	—	2.5
cholesterol	2.18	2.18	2.18	2.18	2.18	2.18	1.93
DOPE	1.64	1.64	1.64	1.64	1.64	1.64	—
DSPC	—	—	—	—	—	—	0.5
DMG-PEG ₂₀₀₀	0.075	0.075	0.075	0.075	0.075	0.075	0.075

Example 20**Preparation of lipid nanoparticles (LNP) containing nucleic acids**

The siRNA-containing LNPs (siRNA-LNPs) were prepared as follows: 300 μ l of a solution of each of the **A01-A06** transfection reagents prepared in Example 19 was mixed with a solution of 1.2 nmol siRNA (catalog number 4392420, Ambion) in 300 μ l 10 mM citrate buffer (pH 3.0) using a “Y” microfluidic device with two inputs and one output for sampling. The lipid mixture and the siRNA solution were injected separately into each inlet by a linear pump at a constant flow rate of 300 μ l/min. The resulting 600 μ l nanoparticle solution was collected and immediately diluted by the addition of 600 μ l PBS; the corresponding nanoparticle samples designated **B01-B06** were thus formed from the transfection reagents **A01-A06**.

DNA encoding the fluorescent protein mKate2 was amplified from the plasmid pmKate2-C (Evrogen) using the primers (5'-ATCAACATATGGTGAGCGAGCTG-3' (SEQ ID NO. 1); 5'-AAGAATTCCTATCATCTGTGCCCCAG-3' (SEQ ID NO. 2)) and cloned into the pET24a vector (Invitrogen) under the T7 promoter. Messenger RNA (mRNA) encoding mKate2 was transcribed *in vitro* using the Ampliscribe T7-Flash transcription kit (Lucigen) according to the manufacturer's protocol. The RNA cap analog ARCA (Jena Bioscience) was added to the *in vitro* transcription reaction, and the poly(A) terminus was synthesized using poly(A) polymerase (New England Biolabs) according to the standard protocol.

The mRNA-containing LNPs (mRNA-LNPs) were prepared as follows: 300 μ l of a solution of each of the **A07-A29** transfection reagents prepared in Example 19 were mixed with a solution of 120 μ g of mRNA in 300 μ l of 10 mM citrate buffer (pH 3.0), the preparation was then analogous to siRNA-LNP. Thus, the corresponding nanoparticle samples designated **B07-B29** were generated from the transfection reagents

A07-A29. Nanoparticle samples designated **B08a** and **B11a** were generated from the transfection reagents **A02** and **A05**, respectively.

Each of the LNP samples (**B01-B29**) was prepared in triplicate. The hydrodynamic diameter of freshly formed LNPs was measured using dynamic light scattering (NanoZS Zetasizer, Malvern, Worcestershire, UK) at a scattering angle of 173° at 25 °C. The hydrodynamic diameter of siRNA-LNPs ranged from 67 to 110 nm, and the hydrodynamic diameter of mRNA-LNPs ranged from 82 to 288 nm (**Tab. 5**). In this form, the particles were used for subsequent biological tests.

Table 5. Hydrodynamic diameter of LNPs including standard deviation measured by dynamic light scattering.

LNP	Diameter (nm)	LNP	Diameter (nm)
B01	83.3 ± 4.0	B16	143.2 ± 6.2
B02	109.9 ± 12.7	B17	190.8 ± 2.5
B03	100.1 ± 6.3	B18	190.4 ± 2.1
B04	81.7 ± 8.8	B19	194.8 ± 5.2
B05	72.3 ± 13.2	B20	194.4 ± 6.2
B06	67.1 ± 3.5	B21	179.4 ± 10.5
B07	90.4 ± 1.3	B22	261.0 ± 11.4
B08	153.5 ± 11.9	B23	221.0 ± 12.7
B09	117.0 ± 7.6	B24	217.1 ± 13.8
B10	90.5 ± 3.9	B25	287.8 ± 10.5
B11	157.7 ± 3.5	B26	165.1 ± 11.9
B12	81.5 ± 5.4	B27	242.1 ± 3.9
B13	174.3 ± 7.8	B28	227.9 ± 10.5
B14	144.6 ± 17.8	B29	119.1 ± 4.5
B15	124.5 ± 14.3		

Example 21

Efficiency of siRNA and mRNA incorporation into lipid nanoparticles

The efficiency of packaging of the siRNA prepared in Example 20 (which caused the degradation of mRNA encoding tyrosyl-DNA phosphodiesterase (*TDP2*)) into siRNA-LNP **B01-B06** was determined using a Qubit microRNA Assay Kit (Life Technologies) according to the manufacturer's protocol. The efficiency of the packaging of the mRNA (encoding the fluorescence protein mKate2) into mRNA-LNP **B07-B29** prepared in Example 20 was determined using a Qubit RNA HS Assay Kit (Life Technologies) according to the manufacturer's protocol. The efficiency of incorporation was determined by comparing the concentration of siRNA and mRNA freely available in the nanoparticle solution and the concentration of siRNA and mRNA released from the nanoparticles after their decomposition. LNPs were decomposed with buffer containing Triton X-100 (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, 2% Triton X-100). High

packaging efficiency of siRNA was demonstrated, ranging from 76 to 91%. The packaging efficiency of mRNA ranged from 60 to 96% (**Tab. 6**).

Table 6. Efficiency of packaging siRNA against tyrosyl-DNA phosphodiesterase 2 (*TDP2*) and efficiency of packaging mRNA encoding fluorescent protein mKate2 into LNPs, including standard deviations from triplicates.

LNP	siRNA packaging (%)		
B01	76.1	±	4.9
B02	91.1	±	1.3
B03	77.4	±	4.6
B04	85.0	±	6.9
B05	75.6	±	2.21
B06	89.9	±	0.1

LNP	mRNA packaging (%)			LNP	mRNA packaging (%)		
B07	88.3	±	1.6	B19	67.0	±	2.8
B08	86.2	±	0.9	B20	64.2	±	0.2
B09	70.4	±	6.8	B21	65.0	±	0.1
B10	86.9	±	0.6	B22	60.8	±	3.2
B11	64.3	±	3.4	B23	63.7	±	0.4
B12	83.2	±	3.2	B24	68.7	±	3.3
B13	85.5	±	0.5	B25	78.8	±	1.3
B14	94.0	±	0.1	B26	61.1	±	1.1
B15	96.0	±	1.0	B27	75.0	±	0.1
B16	86.5	±	6.5	B28	70.0	±	0.1
B17	75.0	±	11.0	B29	77.0	±	2.0
B18	65.1	±	0.1				

Example 22

Cellular toxicity of LNPs

A human cell line derived from embryonic kidney cells expressing SV40 large T antigen (HEK293T), and human hepatocyte carcinoma cell line (HepG2) were cultured in 96-well plates (5×10^4 cells in 100 μ l of culture medium per well) in Dulbecco's modified medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were transfected with 2 μ l of LNPs **B07-B12** generated in triplicates in Example 20 (the final total concentration of all lipid components in the well was 20 μ M) or 10 μ l of LNPs (the final total concentration of all lipid components in the well was 100 μ M) and subsequently incubated for 24 hours. The cytotoxicity of LNPs was analyzed in a CellTiterGlo 2.0 cell viability assay (Promega, USA). Cell viability was normalized to non-transfected cells (control). The results are summarized in **Tables 7 and 8**.

For both cell lines used, none of the new LNPs exhibited significant toxicity compared to the 100 μ M mixture with TT3 lipid, with which the viability of HEK293T and HepG2 cells is reduced to only 27 and 15% (Tab. 8).

Table 7. Cytotoxicity of LNPs expressed as cell viability (%) after the addition of 20 μ M transfection mixture **B07-B12** for individual cell line types.

LNP	HEK293T			HepG2		
Control	100.00	$\pm$	4.29	100.00	$\pm$	3.44
B07	91.85	$\pm$	4.34	93.49	$\pm$	4.25
B08	95.90	$\pm$	6.14	100.85	$\pm$	6.35
B09	102.19	$\pm$	5.75	103.10	$\pm$	7.13
B10	100.49	$\pm$	3.12	100.93	$\pm$	5.87
B11	103.51	$\pm$	5.79	101.74	$\pm$	6.09
B12	102.55	$\pm$	5.67	104.55	$\pm$	4.98

Table 8. Cytotoxicity of LNPs expressed as cell viability (%) after the addition of 100 μ M transfection mixture **B07-B12** for individual cell line types.

LNP	HEK293T			HepG2		
Control	100.00	$\pm$	4.29	100.00	$\pm$	3.44
B07	99.86	$\pm$	3.95	107.75	$\pm$	7.27
B08	103.07	$\pm$	2.05	117.75	$\pm$	3.71
B09	106.79	$\pm$	2.32	113.40	$\pm$	5.38
B10	70.88	$\pm$	3.77	95.77	$\pm$	3.37
B11	108.84	$\pm$	3.31	110.43	$\pm$	1.19
B12	27.29	$\pm$	2.38	14.92	$\pm$	4.19

Example 23

Transfection of siRNA using new LNPs *in vitro*

The siRNA-LNP particles containing small interfering RNA (siRNA, catalog number 4392420, Ambion) that causes degradation of mRNA encoding tyrosyl-DNA phosphodiesterase 2 (*TDP2*) were prepared according to Example 20. Lipofectamine RNAiMax (Invitrogen) was used as a control transfection reagent specifically for siRNA transfection. The human cell line HEK293T and the cell line derived from human multiple myeloma, which is very difficult to transfect with available transfection reagents, were used for siRNA-LNP knock-down (Brito J.L.R., Brown N., Morgan G.J. (2010) The transfection of siRNAs in Multiple Myeloma Cell Lines. In: Min WP., Ichim T. (eds) RNA Interference. Methods in Molecular Biology (Methods and Protocols), vol 623. Humana Press). Cells were cultured in 96-well plates (5×10^4 cells in 100 μ l culture medium per well) in DMEM medium supplemented with 10% FBS at 37 °C in 5% CO₂. Cells were transfected with 2 μ l siRNA-LNP (with a final total concentration of all lipid components of 20 μ M and a final siRNA concentration of 16 nM) and subsequently incubated for 24 hours. Transfections were performed in biological triplicates. RNA was isolated using an RNAeasy Plus Micro

Kit (Qiagen). cDNA was prepared using TATAA GrandScript cDNA Supermix (TATAAbiocenter) according to the manufacturer's recommendations. Quantitative RT-PCR was performed using a LightCycler 480 (Roche Life Science). The primers for amplifying mRNA encoding TDP2 were as follows: 5'-CGAGAGGAGGGTCTCAAAGAG-3' (SEQ ID NO. 3) and 5'-ATTTCGGGAAGGCTGCTGTC-3' (SEQ ID NO. 4). mRNA encoding GAPDH was used to normalize the data (Primers: 5'-AATCCCATCACCATCTTCCA-3' (SEQ ID NO. 5) and 5'-TGGACTCCACGACGTACTCA-3' (SEQ ID NO. 6)).

In all these cases, the new siRNA-LNPs significantly reduced the level of *TDP2* mRNA in the cells compared to the commercial transfection reagent RNAiMax. In the HEK293T cell line, the new LNPs **B02-B05** have almost 3-fold higher efficiency than RNAiMax. In the OPM-2 myeloma line, **B03** reduces mRNA expression 13-fold better than a commercial siRNA transfection reagent (Tab. 9).

Table 9. Reduction of endogenously expressed *TDP2* mRNA in cells by new siRNA-LNPs (**B01-B06**) compared to commercial transfection reagent RNAiMax in the HEK293T and OPM-2 cell lines. Statistics were evaluated by Student's unpaired t-test. P values are based on the Lipofectamine RNAiMax control transfection mixture; p values <0.001 are marked with the letter "a", p values <0.05 are marked with the letter "c".

LNP	HEK293T	p	OPM-2	p
Control	1.00 ± 0.10		1.00 ± 0.10	
B01	0.24 ± 0.17		0.52 ± 0.05	
B02	0.04 ± 0.02	c	0.11 ± 0.01	a
B03	0.05 ± 0.03	c	0.04 ± 0.01	a
B04	0.06 ± 0.02	c	0.08 ± 0.02	a
B05	0.05 ± 0.01	c	0.05 ± 0.01	a
B06	0.05 ± 0.01	c	0.07 ± 0.02	a
RNAiMax	0.15 ± 0.06	—	0.52 ± 0.02	—

Example 24

Transfection of mRNA using new LNPs *in vitro*

A human cell line derived from embryonic kidney cells expressing the large SV40 T antigen (HEK293T), liver carcinoma cells (HepG2) and a human osteosarcoma-derived cell line (U2OS) were cultured in 96-well plates (5×10^4 cells in 100 μ l culture medium per well) in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were transfected with 2 μ l of the mRNA-LNP **B07** to **B12**, prepared in Example 20 and subsequently incubated for 24 hours. The final total concentration of the lipid components in the well was 20 μ M and the amount of mRNA that encodes the fluorescent protein mKate2 was 100 ng. Lipofectamine® 2000 was used as a control transfection reagent.

Transfections were performed in three biological replicates, with each biological replicate having three technical replicates. The Cy5 and mKate2 fluorescence was detected using the BD LSR Fortessa cytometer, and the percentage of cells positive for Cy5 fluorescence, indicating LNP entry into cells (**Tab. 10**), percentage of cells expressing the fluorescent protein mKate2 and the fluorescence intensity of the mKate2 (**Tab. 11**) were calculated. For fluorescence intensity, the data were normalized to the commercial transfection reagent Lipofectamine® 2000. According to Cy5 fluorescence, it is evident that all LNPs enter cells with an efficiency exceeding 90 % (**Tab. 10**). Furthermore, the new LNPs **B11** produce 2.13-fold more fluorescent mKate2 protein from transfected mRNA than cells transfected with Lipofectamine® 2000 transfection control (**Tab. 11**).

Table 10. Transfection efficiency of new mRNA-LNPs (**B07-B12**) expressed as a percentage of cells positive for Cy5 for cell lines HEK293T and HepG2. The transfection mixtures contain a lipid labeled with the fluorescent dye Cy5, which makes it possible to observe the entry of LNPs into the cells.

LNP	HEK293T	HepG2
B07	99.28 ± 0.10	98.59 ± 0.15
B08	99.19 ± 0.09	94.73 ± 0.88
B09	97.83 ± 0.63	90.52 ± 2.39
B10	97.79 ± 0.36	92.93 ± 0.77
B11	98.59 ± 0.47	96.49 ± 1.70
B12	97.94 ± 0.94	95.19 ± 0.98

Table 11. Transfection efficiency of new mRNA-LNPs expressed as a percentage of cells expressing fluorescent mKate2 protein and fluorescence intensity of mKate2 from mRNA transfected with particles for U2OS cell line. Statistics were evaluated by Student's unpaired t-test. The p values are related to the control transfection mixture Lipofectamine2000; p values <0.001 are indicated with the letter “a”.

LNP	% of cells expressing mKate2		p	Fluorescence of mKate2		p
B07	28.76	± 1.51		0.10	± 0.00	
B08	88.62	± 0.60	a	1.08	± 0.07	
B09	88.38	± 1.00	a	1.79	± 0.23	a
B10	84.46	± 1.06	a	0.69	± 0.08	
B11	92.32	± 2.18	a	2.13	± 0.19	a
B12	90.63	± 0.92	a	1.86	± 0.31	a
Lip2000	47.67	± 1.25	—	1.00	± 0.05	—

Example 25

Transfection of mRNA using new LNPs *in vitro*

A human cell line derived from embryonic kidney cells expressing the large SV40T antigen (HEK293T) was cultured in 96-well plates (2.5×10^4 cells in 100 µl culture medium per well) in Dulbecco's modified

medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were transfected with mRNA-LNPs **B08a**, **B11a**, **B13** to **B18**, **B20** to **B25** and **B29** prepared in Example 20 and then incubated for 24 hours. The final total concentration of all lipid components in the well was 20 µM and the amount of mRNA encoding the fluorescent protein mKate2 was 100 ng. **B29** LNPs containing benchmark D-Lin-MC3-DMA lipid were used as a control. Transfections were performed in triplicate. The percentage of cells expressing the fluorescent protein mKate2 and the fluorescence intensity of the mKate2 (**Tab. 12**) were analyzed in a BD LSR Fortessa cytometer at a wavelength of 561-610/20 nm. For fluorescence intensity, data were normalized to the benchmark D-Lin-MC3-DMA containing LNPs (**B29**). It is evident that all new LNPs transfected the HEK293T cells more efficiently, as documented by significantly higher % of transfected cells as well as by significantly increased fluorescence intensity compared to the benchmark D-Lin-MC3-DMA LNPs. In particular, the new LNPs **B22** produce 13.66-fold more fluorescent mKate2 protein from transfected mRNA than cells transfected with **B29** LNPs containing the benchmark D-Lin-MC3-DMA lipid (**Tab. 12**).

Table 12. Transfection efficiency of new mRNA-LNPs for HEK293T cell line shown as a percentage of cells expressing fluorescent mKate2 protein from mRNA delivered by LNPs. Statistics were evaluated by Student's unpaired t-test. The p values are related to the control D-Lin-MC3-DMA (**B29**) LNPs; p values <0.0001 are indicated with the letter “a”, p <0.001 as “b”, p <0.01 as “c”, respectively.

LNP	% of cells transfected	p (D-Lin-MC3-DMA)	Normalized fluorescence of mKate2	p (D-Lin-MC3-DMA)
B08a	86.2 ± 2.1	a	3.72 ± 0.11	a
B11a	76.0 ± 3.8	b	3.10 ± 0.16	b
B13	94.0 ± 0.7	a	5.27 ± 0.25	a
B14	77.1 ± 5.3	b	1.70 ± 0.12	b
B15	83.5 ± 3.8	b	1.98 ± 0.31	c
B16	69.5 ± 1.3	b	1.37 ± 0.05	b
B17	85.4 ± 3.2	b	3.79 ± 0.67	c
B18	82.8 ± 1.5	a	3.99 ± 0.39	b
B20	86.6 ± 9.1	c	7.78 ± 1.63	c
B21	56.5 ± 2.3	c	2.41 ± 0.21	b
B22	93.3 ± 1.5	a	13.66 ± 1.46	a
B23	89.7 ± 5.7	b	7.89 ± 1.35	b
B24	72.4 ± 1.3	b	1.88 ± 0.09	a
B25	62.2 ± 4.2	c	1.58 ± 0.13	c
B29	29.9 ± 4.2	—	1.00 ± 0.02	—

Example 26

Transfection efficacy of new mRNA-LNPs monitored by Cre-based recombination

Mouse embryonic fibroblast cells (MEFs), derived from the mouse model with a global dual Cre reporter (Mazumdar, M.D.: *Genesis* **2007**, 45:593-605), were seeded (2.5 × 10⁴ cells in 100 µl culture medium per well) on a 96-well plate and cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO₂ for 24

hours. Messenger RNA (mRNA) encoding Cre recombinase was prepared *in vitro* analogously to Example 20 from a linearized plasmid and packaged into LNP as follows: A 300 μ l sample of transfection reagents **A13** to **A25**, **A02** and **A27** and the control transfection reagent **A29** including the benchmark D-Lin-MC3-DMA lipid, and 120 μ g of mRNA in 300 μ l of 10 mM citrate buffer (pH 3.0) were assembled into LNP using a microfluidic device analogously to Example 20. The resulting mRNA-LNPs were immediately diluted in 600 μ l PBS; the corresponding nanoparticles labeled **B31** to **B43** (formed from transfection reagent **A13** to **A25**), nanoparticles **B30** (formed from transfection reagent **A02**), nanoparticles **B44** (formed from transfection reagent **A27**) and analogously, the benchmark nanoparticles labeled **B45** were thus formed from transfection reagent **A29**.

Cells were transfected with the respective mRNA-LNPs (the final total concentration of all lipid components in the well was 20 μ M) carrying 100 ng of mRNA encoding the Cre recombinase and subsequently incubated for 48 hours. **B45** LNPs containing benchmark D-Lin-MC3-DMA lipid were used as a control. Transfections were performed in quadruplicates. The percentage of cells expressing GFP was analyzed using a BD LSR Fortessa cytometer (at a wavelength of 488 – 530/30 nm). It is again evident that all new LNPs transfected the mT/mG cells more efficiently, as documented by a significantly higher percentage of cells expressing GFP compared to cells transfected with the benchmark D-Lin-MC3-DMA LNPs (**B45**). Interestingly, the percentage of cells expressing GFP was always higher than 95% for all new LNPs tested (except **B44**), whereas LNPs containing the benchmark D-Lin-MC3-DMA lipid showed only 21% of recombined cells (**Tab. 13**).

Table 13. Comparison of the efficacy of new Cre mRNA-LNPs to convert td Tomato into GFP in MEFs derived from the mT/mG reporter mouse embryos. Statistics were evaluated using Student's unpaired t-test. The p values are related to the control D-Lin-MC3-DMA (**B45**) LNPs; p values <0.0001 are indicated with the letter “a”.

LNP	% cells expressing GFP	p (D-Lin-MC3-DMA)
B30	99.05 $\pm$ 0.10	a
B31	99.83 $\pm$ 0.07	a
B32	99.60 $\pm$ 0.01	a
B33	95.50 $\pm$ 0.04	a
B34	99.75 $\pm$ 0.13	a
B35	99.13 $\pm$ 0.32	a
B36	99.00 $\pm$ 0.17	a
B37	98.85 $\pm$ 0.45	a
B38	99.48 $\pm$ 0.10	a
B39	95.50 $\pm$ 1.25	a
B40	99.13 $\pm$ 0.22	a
B41	98.00 $\pm$ 0.43	a
B42	99.80 $\pm$ 0.06	a
B43	99.73 $\pm$ 0.04	a
B44	69.05 $\pm$ 2.00	a
B45	21.25 $\pm$ 2.35	—
untreated	0.325 $\pm$ 0.248	a

Example 27

Biodistribution of new mRNA-LNPs *in vivo*

The Messenger RNA (mRNA) encoding Cre recombinase was prepared *in vitro* analogously to Example 20 from a linearized plasmid and packaged into LNP as follows: A 300 µl sample of transfection reagent **A22** and the control transfection reagent **A29** that includes the benchmark lipid D-Lin-MC3-DMA and 120 µg of mRNA in 300 µl of 10 mM citrate buffer (pH 3.0) were assembled into LNP using a microfluidic device analogously to Example 20. The resulting mRNA-LNPs were immediately diluted in 600 µl PBS; the corresponding **B40** labeled nanoparticles were formed from the transfection reagent **A22**. Analogously, the benchmark nanoparticles labeled **B45** were thus formed from the transfection reagent **A29**. The mRNA-LNPs were dialyzed in PBS and filtered. Endotoxin levels were < 2 EU/ml. The mRNA-LNPs (**B40** and **B45**) were administered intravenously at a concentration of 2.0 mg mRNA/kg in each case to 4 mice with a global dual Cre reporter (Mazumdar, M.D.: *Genesis* **2007**, 45:593-605) (breeding BIOCEV, Vestec) enabling the analysis of successful recombination. In cells to which mRNA-LNPs carrying Cre recombinase mRNA were successfully delivered, chromosomal recombination and subsequent excision of the membrane red protein gene (the so-called red tomato) and “turning on” the transcription of membrane green protein (GFP) gene occurred. Mice, including non-particulate control mice, were sacrificed 5 days after particulate application, and specific organs (liver, heart, kidney, lungs, and spleen) were subjected to histological analysis according to a standardized protocol. Analysis of histological images revealed a complete distribution of the **B40** nanoparticles in the liver, leading to a 50-80% conversion of cells expressing the red membrane protein to cells expressing the green membrane protein 5 days after application. In the case of the D-Lin-MC3-DMA lipid containing LNPs **B45**, the conversion was about 20% (**Fig. 10**).

Histological analysis was further complemented by the evaluation of tdTomato/GFP conversion in the liver of the mT/mG mice by genomic DNA (gDNA) PCR analysis. Briefly, 50 ng of the gDNA template isolated from the respective liver samples was used in the PCR reaction with subsequent primers: (5'-AACGTGCTGGTTATTGTGCTG-3' (SEQ ID NO. 7); 5'-AAGTCGTGCTGCTTCATGTG-3' (SEQ ID NO. 8)). In **Fig. 11**, electrophoretic analysis of the resulting gDNA PCR amplification of the liver samples shows the PCR product of 530 bp in the case of the active Cre recombination at the tdTomato/tGFP locus, while the PCR product of 2943 bp (upper band) in the case no Cre recombination occurred. The designated **B40** mRNA-LNPs showed complete Cre recombination in all 4 mice tested, while in the case of the benchmark LNPs (**B45**) the recombination was complete only in 50% of the tested mice.

Example 28

Efficacy of new mRNA-LNPs *in vivo*

Messenger RNA (mRNA) encoding hEPO (human erythropoietin) was prepared *in vitro* analogously to Example 20 from a linearized plasmid and packaged in LNP as follows: A 300 µl sample of transfection reagent **A23** or the control transfection reagent **A29** that includes the benchmark lipid D-Lin-MC3-DMA and 120 µg of mRNA in 300 µl of 10 mM citrate buffer (pH 3.0) were assembled into LNP using a

microfluidic device analogously to Example 20. The resulting mRNA-LNPs were immediately diluted in 600 μ l PBS; the corresponding **B46** labeled nanoparticles were thus formed from the transfection reagent **A23**. Analogously, the benchmark nanoparticles labeled **B47** were thus formed from the transfection reagent **A29**. The mRNA-LNPs were dialyzed in PBS and filtered. Endotoxin levels were < 0.3 EU/ml. The mRNA-LNPs (**B46** and **B47**) were administered intravenously at a concentration of 0.5 mg mRNA/kg in each case to 3 C57B1/6 mice (BIOCEV, Vestec), wherein three control C57B1/6 mice were administered with PBS. Six hours after administration, blood was collected from mice via the tail vein and allowed to clot at room temperature in serum separator tubes. The tubes were then centrifuged at 7000 rpm for 7 min and the sera samples were aliquoted and stored at -80°C until analysis. hEPO concentrations were determined using an hEPO ELISA assay (Catalog #DEP00; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Hematology profiles were determined using a standardized protocol. Nanoparticles designated as **B46** showed higher, yet statistically not significant, production of the human EPO protein 6 hours after administration compared to control **B47** LNPs. Importantly, LNPs containing new lipidoid (**B46**) did not alter hematology profile as opposed to control **B47** LNPs. The blood test after the new LNP administration remained comparable to PBS control (**Tab. 14**).

Table 14. Comparison of the *in vivo* efficacy of new human EPO mRNA-LNPs in protein production six hours after administration.

LNP	hEPO [mIU/ml]	WBS# [$\times 10^9/\text{L}$]	Neu# [$\times 10^9/\text{L}$]	Lym# [$\times 10^9/\text{L}$]
B46	750 $\pm$ 514	4.91 $\pm$ 0.57	0.90 $\pm$ 0.19	3.58 $\pm$ 0.73
B47	465 $\pm$ 154	10.06 $\pm$ 2.59	4.89 $\pm$ 2.66	4.23 $\pm$ 0.43
PBS		4.40 $\pm$ 1.12	1.02 $\pm$ 0.84	3.09 $\pm$ 0.33

Example 29

Efficacy of new siRNA-LNPs *in vivo*

Transfection reagents **A02**, **A14**, **A16**, **A17**, **A22**, **A23** and **A29** from Example 19 was used to form siRNA-LNPs designated as **B48** to **B54**, respectively, with siRNA targeting mouse apolipoprotein B (*ApoB*) gene, a hepatocyte-expressed gene involved in cholesterol transport (catalogue number 238055 Apob mouse siPOOL-40 kit, siTOOLS Biotech GmbH) and alternatively siRNA-LNPs (**B55** to **B60**) with control non-targeted siRNA-LNPs (enclosed in 238055 Apob mouse siPOOL-40 kit, siTOOLS Biotech GmbH), assembled as described in Example 20. The hydrodynamic diameter measured by dynamic light scattering ranged from 103 nm to 154 nm and the efficiency of packaging of the siRNA pool ranged from 59% to 99%. The siRNA-LNPs were dialyzed to PBS. Endotoxin levels were < 2 EU/ml. Mice were fasted for 4 hours before plasma collection by retroorbital bleed. The siRNA-LNPs targeting *ApoB* were administered intravenously to 3 C57B1/6 mice (BIOCEV, Czech Center of Phenogenomics, Vestec) at a concentration of 16 μ g of

siRNA, wherein the control 3 mice were administered with 16 µg of non-targeting siRNA-LNPs and another 3 mice were administered PBS control. All mice were sacrificed 2 days after LNP application. Plasma levels of cholesterol, triglycerides and LDL-C were measured by using automated systems at the Czech Center of Phenogenomics according to standardized protocol.

Clinical biochemistry of plasma markers such as total cholesterol, triglycerides and LDL-C, affected by *ApoB* knock down, were significantly decreased compared to control animals, demonstrating thus the efficient delivery of *ApoB* siRNA by novel LNPs into the liver (Tab. 15).

Table 15. Clinical biochemistry of plasma markers indicating efficient *ApoB* knockdown in the liver. Statistics were evaluated by Student's unpaired t-test. The p1 values are always relative to the control mice injected with PBS; the p2 values are always relative to the mice injected with respective (B55 to B60) LNPs with control non-targeted siRNA; values of $p < 0.0001$ are marked as “a”, $p < 0.001$ are marked as “b”, $p < 0.01$ are marked as “c”, and $p < 0.05$ are marked as “d”. Those cases that were not evaluated are marked with “n.e.”.

LNP	Total cholesterol, mmol/L	p1	p2	Triglycerides, mmol/L	p1	p2	LDL-C, mmol/L	p1	p2
PBS control	2.27 ± 0.20			0.60 ± 0.12			0.45 ± 0.05		
B48	0.82 ± 0.02	a	a	0.71 ± 0.07			0.21 ± 0.02	b	
B49	0.52 ± 0.06	a	a	0.19 ± 0.01	b	b	0.15 ± 0.03	b	d
B50	0.85 ± 0.45	c	d	0.42 ± 0.13		c	0.33 ± 0.20		d
B51	0.51 ± 0.10	a	b	0.25 ± 0.01	c	a	0.22 ± 0.05	c	d
B52	0.50 ± 0.21	a	b	0.27 ± 0.01	c	a	0.19 ± 0.08	c	d
B53	0.62 ± 0.15	a	b	0.3 ± 0.01	c	a	0.29 ± 0.02	c	
B55	2.36 ± 0.61		n.e.	0.52 ± 0.01		n.e.	0.64 ± 0.14		n.e.
B56	2.47 ± 0.20		n.e.	0.65 ± 0.10		n.e.	0.63 ± 0.10	d	n.e.
B57	2.41 ± 0.50		n.e.	0.80 ± 0.20		n.e.	0.8 ± 0.22	d	n.e.
B58	2.46 ± 0.76		n.e.	0.92 ± 0.14	d	n.e.	0.78 ± 0.24	d	n.e.
B59	2.50 ± 0.77		n.e.	0.79 ± 0.08		n.e.	0.77 ± 0.32		n.e.
B60	2.56 ± 0.76		n.e.	1.00 ± 0.07	c	n.e.	0.73 ± 0.19	d	n.e.

The toxicity of new siRNA-LNPs was further evaluated by analyzing the clinical biochemistry of plasma markers that indicate organ failure and the hematology profile compared to saline-injected controls (PBS) and control LNPs (B54) containing the lipid D-Lin-MC3-DMA. In particular, new siRNA-LNPs designated as B49 showed a better toxicology profile when compared to the control B54 LNPs (Tab. 16).

Table 16. Clinical biochemistry of plasma markers and hematology profile of new siRNA-LNPs designated as **B49** compared to control siRNA-LNPs containing the benchmark lipid D-Lin-MC3-DMA (**B54**). Statistics were evaluated by Student's unpaired t-test. The p1 values are always relative to the control mice injected with PBS; the p2 values are always relative to the mice injected with control **B54** LNPs; values of $p < 0.01$ are marked as “c” and $p < 0.05$ are marked as “d”.

LNP		B49	p1	p2	B54	p1	PBS
Total bilirubin, $\mu\text{mol/L}$	2.8 $\pm$	1.06			2.5 $\pm$ 0.34		2.14 $\pm$ 0.43
AST, U/L	66 $\pm$	12.06			150 $\pm$ 76.14		74.6 $\pm$ 7.78
ALT, U/L	25 $\pm$	3.79			75 $\pm$ 47.52		23.8 $\pm$ 1.6
HBDH, U/L	81.03 $\pm$	18.8		d	148.7 $\pm$ 15.2	c	63.2 $\pm$ 8.24
LDH, U/L	310.1 $\pm$	73.82			567 $\pm$ 61.8	c	231.2 $\pm$ 30.45
WBC, $\times 10^9/\text{L}$	3.17 $\pm$	0.91			6.58 $\pm$ 1.43		4.35 $\pm$ 0.44
Neutrophils, %	34 $\pm$	7	d		51 $\pm$ 11	c	18 $\pm$ 2
Neutrophils, $\times 10^9/\text{L}$	1.18 $\pm$	0.55			3.65 $\pm$ 1.37	d	0.79 $\pm$ 0.09
Lymphocytes, %	64 $\pm$	6	d		45 $\pm$ 1	c	77 $\pm$ 2
Lymphocytes, $\times 10^9/\text{L}$	1.93 $\pm$	0.41			2.69 $\pm$ 0.36		3.35 $\pm$ 0.38

Industrial applicability

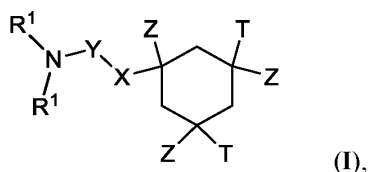
The transfection particles containing lipidoids of the present invention are useful for a variety of biological applications in basic research, particularly cell culture or animal transfections to deliver active NA and subsequently silence or activate the chromosomal gene or genes, edit the genome or transcriptome, or allow the expression of protein encoded in the NA inserted using a transfection particle.

In veterinary and human medicine, the transfection particles containing lipidoids of general formula **I** can preferably be used for therapeutic or prophylactic purposes. Therapeutic NA-containing particles can be administered to an animal or human to silence or activate chromosomal gene(s), silence or activate immunogens, inhibit or activate signaling pathways, edit the genome or transcriptome, or allow expression of the NA-encoded protein(s).

The lipidoids of the formula **I** or transfection agents or transfection particles can also be used as medicaments, in particular for gene therapy, and are suitable for use in the treatment of malignancies and/or genetic disorders. They can also be formulated for cosmetic or biotechnological use.

C L A I M S

1. Lipidoids of general formula (I)



wherein

X is selected from a group comprising -C(=O)NH-, -C(=O)O-, -C(=S)O-, -C(=O)S-, -C(=S)S-, -C(=O)NHNH-, -CH₂-, -O-, -OC(=O)-, -S-, -SC(=O)-, -NH-, -NHNH-, -NHC(=O)-, -NHNHC(=O)-, -C≡C-, -CH=CH-, a five-membered heterocycle containing at least 2 nitrogen atoms, -CH₂C(=O)NH-, -CH₂C(=O)O-, -CH₂C(=S)O-, -CH₂C(=S)S-, -CH₂C(=O)NHNH-, -N=CH-, -CH=N-, -NH-N=CH-, and -CH=N-NH-;

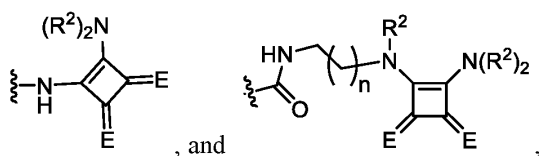
Y is selected from a group comprising alkylene C₂-C₁₀ chains, wherein in the said alkylene chain, one or more -CH₂- groups may optionally be replaced with one or more O and/or S atoms;

R¹ are the same or different from each other, each R¹ being independently selected from the group comprising alkyl C₁-C₄₆, alkenyl C₂-C₄₆, alkynyl C₂-C₄₆, wherein said alkyl, alkenyl or alkynyl may be linear or branched, and wherein in the said alkyl, alkenyl or alkynyl, one or more -CH₂- groups may optionally be replaced with one or more groups selected from -CH(OH)-, -OC(=O)-, -C(=O)O-, -S-S-, -C(=O)NH-, -NHC(=O)-, -O-, and -S-; wherein if said alkyl, alkenyl or alkynyl is branched, then one or more >CH- groups may optionally be replaced with >C(OH)-;

wherein if R¹ is alkyl C₁-C₄, then one hydrogen from the terminal -CH₃ group may be substituted with Z;

with the proviso that at least one R¹ contains at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms;

Z are the same or different from each other, each Z being independently selected from the group comprising hydrogen, -OH, -CH₃, -CH₂OH, -NH₂, -C(=O)NH₂, -CONH(CH₂)₂OH, -CON[(CH₂)₂OH]₂, -CONHCH(CH₂OH)₂, -CONHCH₂CH(-OH)CH₂OH, -CONH(CH₂)₂C(=O)NH₂, -CON[CH₂C(=O)NH₂]₂, -CONHCH[C(=O)NH₂]₂, -CONH(CH₂)₂NHC(=O)NH₂, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -CONH(CH₂)₃-N⁺(CH₃)₂-(CH₂)₂-COO⁻, -COO(CH₂)₂-O-P(=O)(O⁻)-O(CH₂)₂-N⁺(CH₃)₃, -N⁺(CH₃)₂-(CH₂)₃-SO₃⁻, -N⁺(CH₃)₂-(CH₂)₂-COO⁻,



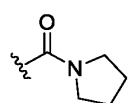
wherein

R^2 is independently selected from hydrogen and $-CH_3$;

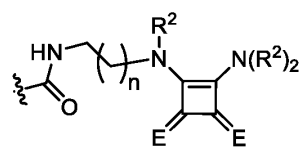
E is independently selected from O and S atoms;

n is an integer within the range of from 1 to 5;

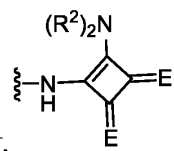
and T are the same or different from each other, each T being independently selected from the group comprising $-X-Y-N(R^1)_2$, $-C(=O)O(C_1-C_3 \text{ alkyl})$, $-C(=O)OCH_2CH_2OH$, $-C(=O)NH_2$,



, $-C(=O)OH$, $-CONH(CH_2)_2OH$, $-CON[(CH_2)_2OH]_2$, $-CONHCH(CH_2OH)_2$,
 $-CONHCH_2CH(OH)CH_2OH$, $-CONH(CH_2)_2C(=O)NH_2$, $-CON[CH_2C(=O)NH_2]_2$,
 $-CONHCH[C(=O)NH_2]_2$, $-CONH(CH_2)_2NHC(=O)NH_2$, $-CONH(CH_2)_3-N^+(CH_3)_2-(CH_2)_2-SO_3^-$,
 $-CONH(CH_2)_3-N^+(CH_3)_2-(CH_2)_3-SO_3^-$, $-CONH(CH_2)_3-N^+(CH_3)_2-(CH_2)_2-COO^-$,



, $-COO(CH_2)_2-O-P(=O)(O^-)-O(CH_2)_2-N^+(CH_3)_3$,
 $-OH$, $-O(C_1-C_3 \text{ alkyl})$, $-NH_2$, $-NHC(=O)CH_3$, $-NHS(=O)_2CH_3$, $-NHC(=O)N(CH_3)_2$,
 $-NHC(=O)NH(CH_3)$, $-NHC(=S)N(CH_3)_2$, $-NHC(=S)NH(CH_3)$, $-NHC(=N-CN)NH_2$,
 $-NHC(=N-CN)NH(CH_3)$, $-NHC(=N-CN)N(CH_3)_2$, $-NHC[=N-S(=O)_2NH_2]NH_2$,



, $-N^+(CH_3)_2-(CH_2)_3-SO_3^-$, $-N^+(CH_3)_2-(CH_2)_2-COO^-$,

wherein R^2 , E and n are as defined above;

and/or if Z is $-OH$ or $-CH_2OH$, and T is $-C(=O)OH$, then Z together with T and three carbon atoms between them may form a cyclic lactone containing 4 to 5 carbon atoms;

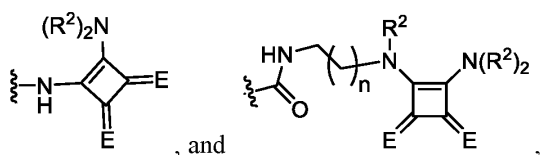
and pharmaceutically acceptable salts, addition salts and solvates thereof.

2. The lipidoid according to claim 1, wherein X is selected from a group consisting of $-C(=O)NH-$, $-C(=O)O-$, $-C(=O)NHNH-$, $-OC(=O)-$, $-O-$, $-NHC(=O)-$, $-NHNHC(=O)-$, a five-membered heterocycle containing at least 2 nitrogen atoms.

3. The lipidoid according to claim 1 or 2, wherein R^1 are independently selected from the group consisting of alkyl C_1 - C_{46} , and alkenyl C_2 - C_{46} , wherein in the said alkyl or alkenyl, one or more $-CH_2-$ groups may optionally be replaced with one or more groups selected from $-CH(OH)-$, $-OC(=O)-$, $-C(=O)O-$.

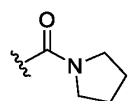
4. The lipidoid according to claim 1, 2 or 3, wherein all R^1 in the molecule are the same or all nitrogen atoms in the molecule, bearing R^1 groups, are substituted identically either by two identical R^1 or by two different R^1 .

5. The lipidoid according to any one of the preceding claims, wherein Z is selected from the group consisting of hydrogen, $-OH$, $-CH_3$, $-CH_2OH$, $-NH_2$, $-C(=O)NH_2$, $-CONH(CH_2)_2OH$, $-CON[(CH_2)_2OH]_2$, $-CONHCH(CH_2OH)_2$, $-CONHCH_2CH(OH)CH_2OH$, $-CONH(CH_2)_2C(=O)NH_2$, $-CON[CH_2C(=O)NH_2]_2$, $-CONHCH[C(=O)NH_2]_2$, $-CONH(CH_2)_2NHC(=O)NH_2$, $-N^+(CH_3)_2-(CH_2)_3-SO_3^-$, $-N^+(CH_3)_2-(CH_2)_2-COO^-$,

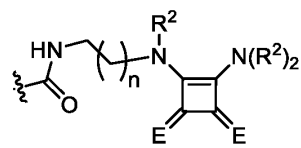


wherein R^2 , E and n are as defined in claim 1.

6. The lipidoid according to any one of the preceding claims, wherein T is selected from the group consisting of $-X-Y-N(R^1)_2$, $-C(=O)O(C_1-C_3 \text{ alkyl})$, $-C(=O)OCH_2CH_2OH$, $-C(=O)NH_2$,



$-C(=O)OH$, $-CONH(CH_2)_2OH$, $-CON[(CH_2)_2OH]_2$, $-CONHCH(CH_2OH)_2$, $-CONHCH_2CH(OH)CH_2OH$, $-CONH(CH_2)_2C(=O)NH_2$, $-CON[CH_2C(=O)NH_2]_2$, $-CONHCH[C(=O)NH_2]_2$, $-CONH(CH_2)_2NHC(=O)NH_2$, $-CONH(CH_2)_3-N^+(CH_3)_2-(CH_2)_2-SO_3^-$, $-CONH(CH_2)_3-N^+(CH_3)_2-(CH_2)_2-COO^-$,

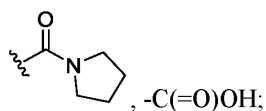


$-COO(CH_2)_2-O-P(=O)(O^-)-O(CH_2)_2-N^+(CH_3)_3$,

wherein R^2 , E and n are as defined in claim 1.

7. The lipidoid according to any one of the preceding claims, wherein at least one substituent T is $-X-Y-N(R^1)_2$; preferably both substituents T are $-X-Y-N(R^1)_2$.

8. The lipidoid according to any one of the preceding claims, wherein Z is selected from the group consisting of hydrogen, $-OH$, $-CH_3$, $-CH_2OH$;
and T is selected from the group consisting of $-X-Y-N(R^1)_2$, $-C(=O)O(C_1-C_3 \text{ alkyl})$,



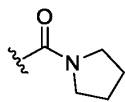
and/or if Z is $-OH$ or $-CH_2OH$, and T is $-C(=O)OH$, then Z together with T and three carbon atoms between them may form a cyclic lactone containing 4 to 5 carbon atoms.

9. The lipidoid according to any one of the preceding claims, wherein:

X is $-C(=O)NH-$ or $-NHC(=O)-$;

Y is selected from $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, and $-(CH_2)_6-$;

R^1 is selected from the group comprising linear or branched alkyl $C_{10}-C_{15}$, which may optionally be replaced with one or more groups selected from $-OC(=O)-$, $-C(=O)O-$; and linear or branched alkenyl $C_{12}-C_{18}$, which may optionally be replaced with one or more groups selected from $-OC(=O)-$, $-C(=O)O-$;



T is selected from $-X-Y-N(R^1)_2$, $-C(=O)OCH_3$, and $-C(=O)OH$;

Z is selected from H, $-CH_3$, and $-CH_2OH$.

10. The lipidoid according to any one of the preceding claims, wherein the lipidoid of general formula (I) contains at least two R^1 substituents, each of them containing at least 8 carbon atoms, preferably at least 10 carbon atoms, more preferably at least 12 carbon atoms.

11. A transfection agent comprising at least one lipidoid of general formula (I) according to any one of claims 1 to 10, and at least one helper lipid; preferably the helper lipid is selected from the group comprising cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*rac*-glycero-3-methoxy poly(ethyleneglycol)-2000, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethyleneglycol)-2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000.

12. The transfection agent according to claim 11, comprising at least one lipidoid of general formula (I) according to any one of claims 1 to 10 in an amount of from 10 to 50 mol. %, and at least one helper lipid in an amount of from 50 to 90 mol. %.
13. The transfection agent according to claim 11 or 12, comprising at least one lipidoid of general formula (I) according to any one of claims 1 to 10 in an amount of from 15 to 40 mol. %, cholesterol in an amount of from 30 to 55 mol. %, and at least one helper lipid in an amount of from 20 to 50 mol. %; preferably the helper lipid is selected from the group comprising 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*rac*-glycero-3-methoxy poly(ethyleneglycol)-2000, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethyleneglycol)-2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethyleneglycol)-2000.
14. A transfection particle comprising at least one lipidoid of general formula (I) according to any one of claims 1 to 10, at least one nucleic acid and/or a part thereof and/or nucleic acid derivative; and preferably also at least one helper lipid.
15. Use of the lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 for *in vitro* transfection of cells or tissues with nucleic acid and/or a part thereof and/or nucleic acid derivative.
16. Use according to claim 15 for silencing or activating chromosomal gene(s), silencing or activating immunogenes, inhibiting or activating signaling pathways, editing genome or transcriptome, or enabling the expression of the protein(s) encoded by the nucleic acid.
17. The lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 for use in transfection of cells or tissues with nucleic acid and/or a part thereof and/or nucleic acid derivative *in vivo*, excluding the transfection of human embryos for use in industrial or commercial purposes and excluding the modification of a human germ line.

18. The lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 for use according to claim 16 for silencing or activating chromosomal gene(s), silencing or activating immunogenes, inhibiting or activating signaling pathways, editing genome or transcriptome, or enabling the expression of the protein(s) encoded by the nucleic acid.
19. The lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 for use as a medicament, in particular for gene therapy, preferably for the treatment of malignancies and/or genetic disorders.
20. The lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 for use as a prophylactic vaccine, preferably for the prevention of infectious diseases.
21. Use of the lipidoid of general formula (I) according to any one of claims 1 to 10, or the transfection agent according to claims 11, 12 or 13, or the transfection particle according to claim 14 in cosmetic preparations for delivering the active ingredient to the site of action.