

## Method of manufacturing a tetraalkylammonium salt

### Technical Field

The present invention refers to a novel method of manufacturing a specific tetraalkylammonium salt, preferably R-DOTMA (1,2-di-O-octadecenyl-3-trimethylammoniumpropane), in particular the chloride salt, a novel method of manufacturing an intermediate thereof as well as a specific method of freeze-drying.

### Background

R-DOTMA is a lipid used in RNA delivery systems, for example mRNA vaccines. The lipid as such is known for years. However, for pharmaceutical application high standards in view of purity have to be fulfilled. Especially when higher amounts are needed, new method of manufacturing need to be developed, since lab scale methods cannot be transferred to methods suitable to provide amounts necessary for commercial applications.

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Furthermore, known manufacturing methods have several disadvantages like elevated reaction temperatures or the use of excessive monomers, e.g. as disclosed in US 6649780 B1. Further disadvantages are processes which lead to too many or too difficult to separate by-products, making the process unsuitable to be used for the production of high purity pharmaceutical grade products, e.g. as described in EP 3 231 790 A1.

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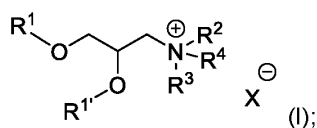
One or more of these objects have been solved by the new method of the present invention, which can be performed at room temperature and requires less equivalent of reagents. The process is thus more sustainable. Moreover, the present inventors have surprisingly found that it can be performed with sustainable solvents and the product can be obtained as a fine powder, when a specific isolation step is additionally applied.

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### Summary of the invention

Therefore, in a first aspect the present invention refers to a method of manufacturing a compound of formula (I)

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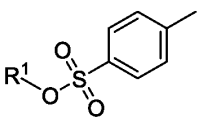
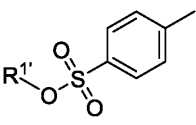
wherein  $R^1$  and  $R^{1'}$  are independently selected from saturated or unsaturated, with up to three  $-C=C-$  bonds,  $C_{6-22}$  hydrocarbon groups;

$R^2$  and  $R^3$  are independently selected from  $-H$  and  $C_{1-6}$  alkyl groups;

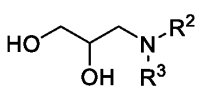
$R^4$  is a  $C_{1-6}$  alkyl group; and

5  $X = F, Cl, \text{ or } Br$ ;

comprising or consisting of the steps:

i) allowing to react  (IIa) and  (IIb);

wherein  $R^1$  and  $R^{1'}$  are the same as defined in formula (I) with

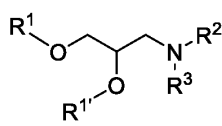
10  (III);

wherein  $R^2$  and  $R^3$  are as defined in formula (I);

in an organic solvent;

in the presence of a potassium base having a  $pK_a$  value in DMSO of 20 to 35 measured by spectrophotometry;

15 ii) thereafter performing an aqueous work-up at a pH value of 1.2 to 2;  
to obtain a compound of formula (IV)

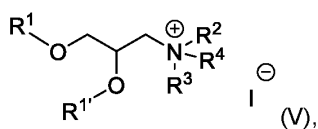
 (IV),

in the organic phase

iii) optionally performing a concentration step;

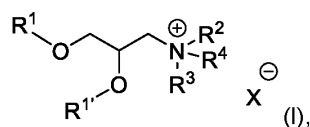
20 iv) optionally performing a chromatography step;

v) allowing to react the compound of formula (IV) or (Iva) obtained after step ii), step iii), or step iv) with  $R^4I$  in order to obtain a compound of formula (V)



wherein  $R^1$  to  $R^4$  and  $R^{1'}$  are defined as in formula (I);

vi) performing ion exchange to obtain a compound of formula (I)



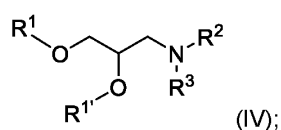
5  $X = F, Cl, \text{ or } Br$

vii) optionally performing chromatography;

viii) optionally performing distillation; and

ix) optionally performing freeze drying.

10 In a second aspect the present invention pertains to a method of manufacturing a compound of formula (IV)



wherein  $R^1$  and  $R^{1'}$  are independently selected from saturated or unsaturated, with up to three  $-C=C-$  bonds,  $C_{6-22}$  hydrocarbon groups;

15  $R^2$  and  $R^3$  are independently selected from  $-H$  and  $C_{1-6}$  alkyl groups;

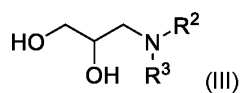
comprising or consisting of the steps:

i) allowing to react

(IIa)

(IIb)

wherein  $R^1$  and  $R^{1'}$  are the same as defined in formula (IV) with

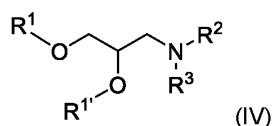


wherein  $R^2$  and  $R^3$  are as defined in formula (IV);

in an organic solvent;

in the presence of a potassium base having a  $pK_a$  value in DMSO of 20 to 35 measured by spectrophotometry;

- 5 ii) thereafter performing an aqueous work-up at an pH value of 1.2 to 2.0;  
to obtain a compound of formula (IV)



in the organic phase;

- iii) optionally performing a concentration step;
- 10 iv) optionally performing a chromatography step.

In a third aspect the present invention refers to a method of freeze drying a lipid comprising or consisting of the steps:

- i) providing the lipid to be freeze-dried in an alcohol solution and
- 15 ii) cooling the solution to a temperature of -25 to 5 °C;
- iii) thereafter stopping the cooling and removing the solvent by vacuum evaporation.

These and other aspects, embodiments, features, and advantages of the invention will become  
apparent to a person skilled in the art through the study of the following detailed description and  
20 claims. Any feature from one aspect of the invention can be used in any other aspect of the invention.  
Furthermore, it will readily be understood that the examples contained herein are intended to  
describe and illustrate the invention but not to limit the invention and that, in particular, the invention  
is not limited to these examples.

## 25 Detailed description of the invention

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

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

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

Numerical ranges that are indicated in the format "from x to y" also include the stated values. If several preferred numerical ranges are indicated in this format, it is self-evident that all ranges that result from the combination of the various endpoints are also included.

10 "One or more", as used herein, relates to at least one and comprises 1, 2, 3, 4, 5, 6, 7, 8, 9 or more of the referenced species. Similarly, "at least one" means one or more, i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9 or more. "At least one", as used herein in relation to any component, refers to the number of chemically different molecules, i.e. to the number of different types of the referenced species, but not to the total number of molecules. For example, "at least one therapeutic agent" means that at least one type of  
15 molecule falling within the definition for a therapeutic agent is used but that also two or more different types of therapeutic agents falling within this definition can be present, but does not mean that only one or more molecules of one type of therapeutic agents are present.

"Essentially free of" according to the present invention with regard to compounds means that the compound can only be present in an amount, which does not influence the characteristics of the composition, in particular the respective compound is present in less than 3 wt.-%, preferably 1 wt.-%, more preferably 0.01 wt.-%, based on the total weight of the composition or is not present at all.  
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The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include  
25 fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

A "cationic lipid" refers to a lipid capable of being positively charged. Exemplary cationic lipids include one or more amine group(s) which bear the positive charge. Preferred cationic lipids are ionizable such that they can exist in a positively charged or neutral form depending on pH. The ionization of  
30 the cationic lipid affects the surface charge of the lipid nanoparticle under different pH conditions.

The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH value.

Embodiments disclosed herein are also meant to encompass the in vivo metabolic products of the compounds according to the present invention. Such products may result from, for example, the

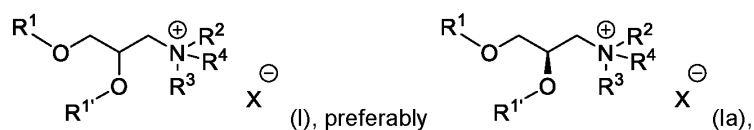
oxidation, reduction, hydrolysis, amidation, esterification, and the like of the administered compound, primarily due to enzymatic processes. Accordingly, embodiments of the disclosure include compounds produced by a process comprising administering a compound of this disclosure to a mammal for a period of time sufficient to yield a metabolic product thereof.

- 5 The compounds of the present invention including their pharmaceutically acceptable salts may contain one or more stereocenters and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)- or, as (D)- or (L)- for amino acids. The present invention is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (-), (R)- and (S)-, or (D)- and (L)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using
- 10 conventional techniques, for example, chromatography and fractional crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high pressure liquid chromatography (HPLC). When the compounds
- 15 described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers. Likewise, all tautomeric forms are also intended to be included.

- A "stereoisomer" refers to a compound made up of the same atoms bonded by the same bonds but having different three-dimensional structures, which are not interchangeable. The present invention
- 20 contemplates various stereoisomers and mixtures thereof and includes "enantiomers", which refers to two stereoisomers whose molecules are non-superimposable mirror images of one another.

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

- 25 In particular, the present invention refers to a method of manufacturing a compound of formula (I)



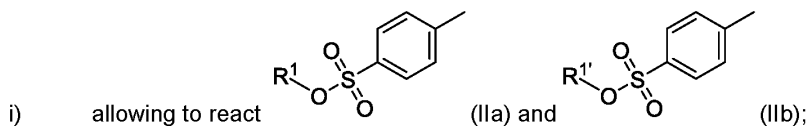
wherein  $R^1$  and  $R^{1'}$  are independently selected from saturated or unsaturated, with up to three  $-C=C-$  bonds,  $C_{6-22}$  hydrocarbon groups;

$R^2$  and  $R^3$  are independently selected from  $-H$  and  $C_{1-6}$  alkyl groups;

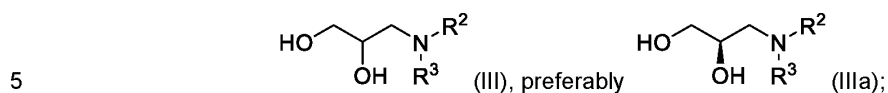
- 30  $R^4$  is a  $C_{1-6}$  alkyl group; and

$X = F, Cl, \text{ or } Br$ ;

comprising or consisting of the steps:



wherein R<sup>1</sup> and R<sup>1'</sup> are the same as defined in formula (I) with



wherein R<sup>2</sup> and R<sup>3</sup> are as defined in formula (I);

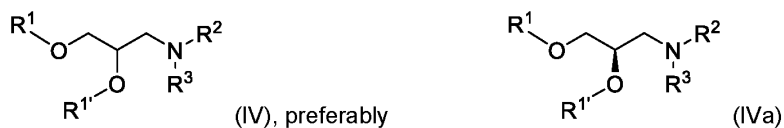
in an organic solvent, preferably in tetrahydrofuran or a tetrahydrofuran-based solvent, more preferably methyl tetrahydrofuran, most preferably 2-methyl tetrahydrofuran;

10 in the presence of a potassium base having a pK<sub>a</sub> value in DMSO (dimethyl sulfoxide) of 20 to 35 measured by spectrophotometry, preferably potassium tert-butoxide (KOtBu), potassium bis(trimethylsilyl)amide (KHMDs), KNH<sub>2</sub>, KOH, and KH, more preferably KHMDs;

preferably at a temperature of 20 to 30 °C, more preferably 25 to 28 °C

15 ii) thereafter performing an aqueous work-up at a pH value of 1.2 to 2, preferably 1.3 to 1.8, more preferably 1.6;

to obtain a compound of formula (IV)



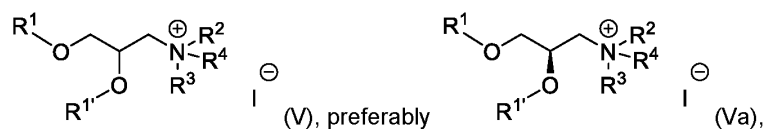
in the organic phase

20 iii) optionally performing a concentration step;

iv) optionally performing a chromatography step, preferably in a solvent mixture of cyclohexane and ethyl acetate;

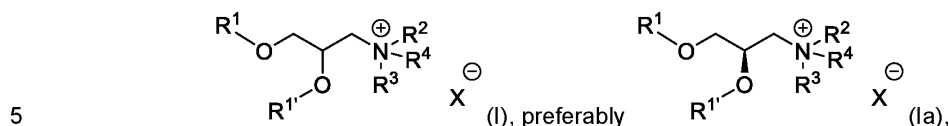
v) allowing to react the compound of formula (IV) or (IVa) obtained after step ii), step iii), or step iv)

with R<sup>4</sup>I in order to obtain a compound of formula (V)



wherein R<sup>1</sup> to R<sup>4</sup> and R<sup>1'</sup> are defined as in formula (I);

vi) performing ion exchange, preferably via ion exchange chromatography, to obtain a compound of formula (I)



X = F, Cl, or Br

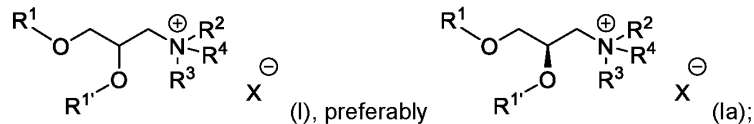
vii) optionally performing chromatography;

viii) optionally performing distillation; and

ix) optionally performing freeze drying.

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In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein R<sup>1</sup> and R<sup>1'</sup> are independently selected from saturated or unsaturated, with up to three -C=C- bonds, C<sub>6-22</sub> hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>12-18</sub> hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>16-18</sub> hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond, C<sub>16-18</sub> hydrocarbon groups or most preferably an unsaturated with one -C=C- bond C<sub>18</sub> hydrocarbon group;

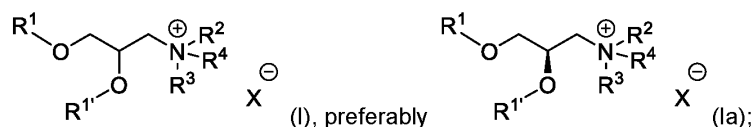
R<sup>2</sup> and R<sup>3</sup> are independently selected from -H and C<sub>1-6</sub> alkyl groups, preferably R<sup>2</sup> and R<sup>3</sup> are the same, more preferably R<sup>2</sup> and R<sup>3</sup> are selected from C<sub>1-3</sub> alkyl groups most preferably R<sup>2</sup> and R<sup>3</sup> are methyl;

R<sup>4</sup> is a C<sub>1-6</sub> alkyl group; preferably R<sup>4</sup> is selected from C<sub>1-3</sub> alkyl groups; more preferably R<sup>4</sup> is methyl; and

X = F, Cl, or Br,

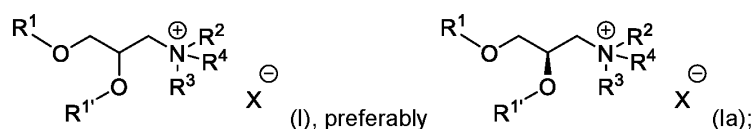
preferably X = F or Cl, more preferably X = Cl.

In one embodiment the method of manufacturing leads to a compound of formula (I)



- wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to three
- 5 -C=C- bonds,  $C_{6-22}$  hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds,  $C_{12-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds,  $C_{16-18}$  hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one -C=C- bond  $C_{18}$  hydrocarbon group;
- 10  $R^2$  and  $R^3$  are independently selected from -H and  $C_{1-6}$  alkyl groups, preferably  $R^2$  and  $R^3$  are the same, more preferably  $R^2$  and  $R^3$  are selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;
- $R^4$  is a  $C_{1-6}$  alkyl group; preferably  $R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and
- 15  $X = F, Cl, \text{ or } Br$ ,
- preferably  $X = F$  or  $Cl$ , more preferably  $X = Cl$ .

In one embodiment the method of manufacturing leads to a compound of formula (I)

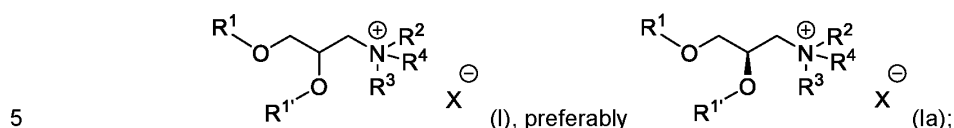


- 20 wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to three -C=C- bonds,  $C_{6-22}$  hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds,  $C_{12-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds,  $C_{16-18}$  hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one -C=C- bond  $C_{18}$  hydrocarbon group;
- 25  $R^2$  and  $R^3$  are the same and selected from -H and  $C_{1-6}$  alkyl groups, more preferably  $R^2$  and  $R^3$  are selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;
- $R^4$  is a  $C_{1-6}$  alkyl group; preferably  $R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

X = F, Cl, or Br,

preferably X = F or Cl, more preferably X = Cl.

In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to three -C=C- bonds,  $C_{12-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds,  $C_{16-18}$  hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one -C=C- bond  $C_{18}$  hydrocarbon group;

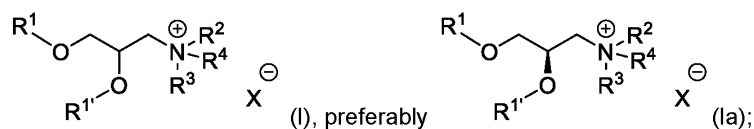
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$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

X = F or Cl, preferably X = Cl.

15 In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to two -C=C- bonds,  $C_{12-18}$  hydrocarbon groups; preferably saturated or unsaturated, with up to two -C=C- bonds,  $C_{16-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with one -C=C- bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one -C=C- bond  $C_{18}$  hydrocarbon group;

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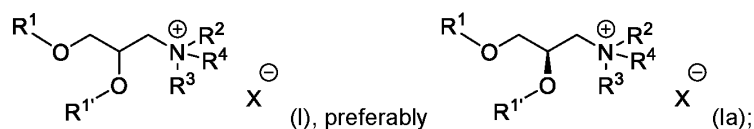
$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

X = F or Cl, preferably X = Cl.

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In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{12-18}$  hydrocarbon groups; preferably saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{16-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

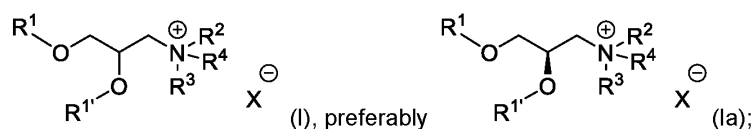
$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

$X = Cl$ .

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In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{16-18}$  hydrocarbon groups; preferably saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

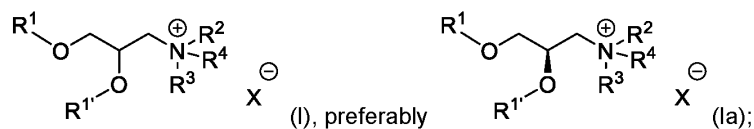
$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

$X = Cl$ .

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In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

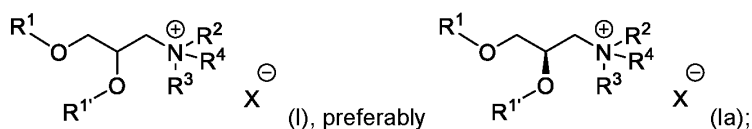
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$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; more preferably  $R^4$  is methyl; and

$X = Cl$ .

- 5 In one embodiment the method of manufacturing leads to a compound of formula (I)



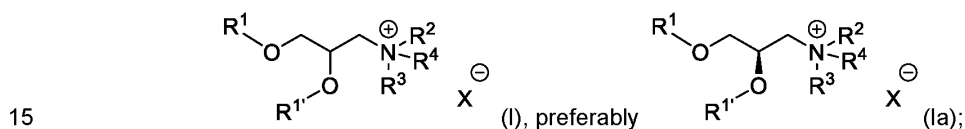
wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

- 10  $R^2$  and  $R^3$  are methyl;

$R^4$  is methyl; and

$X = Cl$ .

In one embodiment the method of manufacturing leads to a compound of formula (I)



wherein  $R^1$  and  $R^{1'}$  are the same and are an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

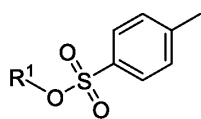
$R^2$  and  $R^3$  are methyl;

$R^4$  is methyl; and

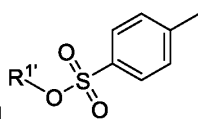
- 20  $X = Cl$ .

In one embodiment the compound of formula (I) is 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt).

- 25 In preferred embodiments  $R^1$  and  $R^{1'}$  in formulae



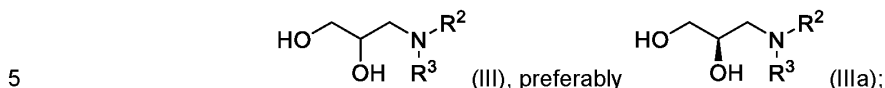
(IIa) and



(IIb) are the same as defined in any of the above-

mentioned embodiments for formula (I). In preferred embodiments (IIa) and (IIb) are the same.

In preferred embodiments  $R^2$  and  $R^3$  in formulae (III) and (IIIa)



are the same as defined in any of the above-mentioned embodiments for formula (I).

In one embodiment the molar ratio of compounds (IIa) and (IIb) to compound (III) is 10 to 1, preferably 2.5 to 1, more preferably 2 to 1.

10

In step (i) of the method of manufacturing a compound of formula (I) an organic solvent is used.

In one embodiment the organic solvent is tetrahydrofuran or a tetrahydrofuran based solvent.

15 Tetrahydrofuran-based solvents are stable under (strong) basic conditions. Due to the polarity of tetrahydrofuran-based solvents, polar as well as non-polar compounds can be dissolved. 2-Methyl tetrahydrofuran is considered as a green solvent, because it can be produced from renewable resources.

In one embodiment the organic solvent is methyl tetrahydrofuran.

In one embodiment the organic solvent is 2-methyl tetrahydrofuran;

20 Step (i) of the method of manufacturing a compound of formula (I) is performed in the presence of a potassium base having a  $pK_a$  value in DMSO of 20 to 35 measured by spectrophotometry.

Potassium bases show the best conversion towards the desired product compared to lithium and sodium bases. A clear tendency is observed – the harder the Lewis acid the lower the conversion. Obviously, lithium binds stronger to the oxygen than the potassium ion. This in turn results in an unreactive species and a lower conversion. This effect was not expected, since lithium and sodium

25 bases are well known for this kind of deprotonation reactions.

In one embodiment the potassium base is selected from potassium tert-butoxide (KOtBu), potassium bis(trimethylsilyl)amide (KHMDs),  $KNH_2$ , KOH, and KH.

In one embodiment the potassium base is KHMDs. Surprisingly, KHMDs performed best in the method of the present invention.

In one embodiment step (i) of the method of manufacturing a compound of formula (I) is performed at a temperature of 20 to 30°C.

- 5 In one embodiment step (i) of the method of manufacturing a compound of formula (I) is performed at a temperature of 25 to 28°C.

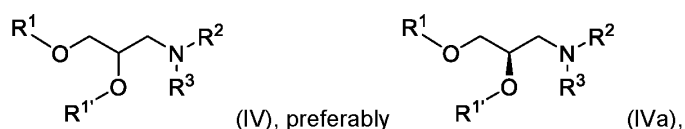
In one embodiment step (i) of the method of manufacturing a compound of formula (I) is performed under inert conditions.

- 10 In one embodiment step (i) of the method of manufacturing a compound of formula (I) is performed under nitrogen.

In one embodiment step (i) of the method of manufacturing a compound of formula (I) is performed under stirring.

- In one embodiment in step (i) of the method of manufacturing a compound of formula (I) the potassium base and the compound of formula (III) are added, preferably from 1 second to 10 minutes, more preferably 5 seconds to 5 minutes, to the reaction vessel and then stirred, preferably for 1 minute to 5 hours, more preferably 5 minutes to 1 hour, and thereafter the compounds of formulae (IIa) and (IIb) are added to the reaction vessel, preferably the mixture thereafter is stirred for 5 minutes to 24 hours, more preferably 2 hours to 18 hours.
- 15

- 20 Thereafter, in the method of manufacturing a compound of formula (I) in step (ii) an aqueous work-up at a pH value of 1.2 to 2.0 is performed to obtain a compound of formula (IV)



in the organic phase.

- 25 In one embodiment in the method of manufacturing a compound of formula (I) in step (ii) an aqueous work-up at a pH value of 1.6 is performed.

- A skilled person in the field of organic synthesis is well able to perform an aqueous work-up and knows how to determine and adjust the pH-value. For example, suitable compounds to adjust the pH-value are HCl solution, preferably a 25% aqueous HCl solution and NaOH solution, preferably an 5N aqueous NaOH solution. The aqueous work-up can be completely or partly performed under stirring. Stirring is preferably continuously performed until phase separation is desired. The organic phase is retained and one or more additional work-up steps with an aqueous phase, preferably an
- 30

aqueous NaOH solution, more preferably having a pH-value of about 8.0 to 8.6 can be performed. After the final work-up step the organic phase is retained.

After step (ii) of the method of manufacturing a compound of formula (I) a concentration step (iii) can be optionally performed. A skilled person in the field of organic synthesis is well able to perform a concentration step of an organic phase.

In one embodiment the organic phase is concentrated via evaporation, preferably by a rotary evaporator, at a 55 to 65 °C, preferably 60 °C water bath temperature and a pressure of 200 to 300 mbar, preferably 250 mbar, with a target end pressure of about 20 mbar.

10

After step (ii) or step (iii) in the method of manufacturing a compound of formula (I) a chromatography step can be performed. A skilled person in the field of organic synthesis is well able to perform a chromatography.

In one embodiment the used solvents for the chromatography in step (iii) are cyclohexane and ethyl acetate.

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In one embodiment a linear gradient of cyclohexane to ethyl acetate is used in step (iii).

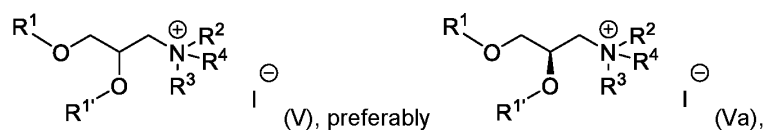
In one embodiment a step gradient of cyclohexane to ethyl acetate is used in step (iii).

The obtained fractions, preferably meeting predefined criteria can be concentrated in a further concentration steps, as described for step (iii) above.

20

In step v) of the method of manufacturing a compound of formula (I) the compound of formula (IV) or (IVa) obtained after step (ii), step (iii), or step (iv) is allowed to react

with R<sup>4</sup>I in order to obtain a compound of formula (V)



25 wherein R<sup>1</sup> to R<sup>4</sup> and R<sup>1'</sup> are defined as in formula (I).

In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed in an organic solvent. In one embodiment the organic solvent is methanol.

In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed under stirring.

In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed in inert atmosphere.

In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed under nitrogen.

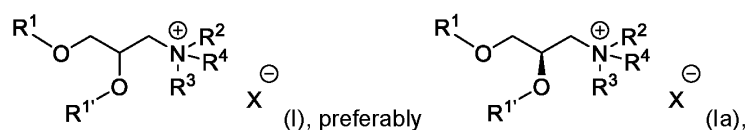
- 5 In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed at 20 to 30 °C.

In one embodiment step (v) of the method of manufacturing a compound of formula (I) is performed at 23 to 28 °C.

- 10 In one embodiment step (v) of the method of manufacturing a compound of formula (I) takes place for 5 min to 24 hours.

In one embodiment step (v) of the method of manufacturing a compound of formula (I) takes place for 1 hour to 10 hours.

- 15 Thereafter, in the method of manufacturing a compound of formula (I) an anion exchange step (vi) is performed to obtain a compound of formula (I)



wherein X = F, Cl, or Br, preferably X = F or Cl, more preferably X = Cl.

- 20 A skilled person in the field of organic synthesis is well able to perform ion exchange of organic compounds.

In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) the ion exchange is performed via ion exchange chromatography.

In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) a concentration step, preferably via rotary evaporation is performed before the ion exchange step.

- 25 In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) the anion exchange resin is a strong base anion resin.

In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) the anion exchange resin is a polystyrene based strong base anion resin.

- 30 In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) the anion exchange resin is a macroporous polystyrene crosslinked with divinylbenzene. A suitable anion

exchange resin is for example commercially available under the tradename Purofine®PFA500Plus from Purolite®.

5 In one embodiment in step (vi) of the method of manufacturing a compound of formula (I) the anion exchanged resin is washed with NaOH, preferably aqueous NaOH 5N, then water, then NaCl, preferably aqueous 10% NaCl, water, and then MeOH, thereafter the compound of formula (V) or (Va) is applied in MeOH and rinsed through the anion exchange resin with MeOH. The obtained solution can be concentrated by rotary evaporation, preferably meeting predefined criteria.

10 In the method of manufacturing a compound of formula (I) a chromatography step (vii) can be performed after the ion exchange step (vi).

A skilled person in the field of organic synthesis is well able to perform a chromatography.

In one embodiment in step (vii) the solvent used for the chromatography is MeOH. The obtained fractions, preferably meeting predefined criteria can be concentrated in a further concentration step, as described for step (iii) above.

15

In the method of manufacturing a compound of formula (I) distillation (viii) can be performed after the chromatography step (vii).

A skilled person in the field of organic synthesis is well able to perform a distillation.

20 In one embodiment the distillation (viii) is performed on a solution containing the compound (I), preferably compound (Ia), dissolved in an alcohol, preferably MeOH.

In one embodiment the distillation (viii) is performed on a solution containing the compound (I), preferably compound (Ia), dissolved in tert-BuOH.

25 In one embodiment the distillation (viii) is performed on a solution containing the compound (I), preferably compound (Ia), dissolved in an alcohol, preferably MeOH, thereafter a further distillation step is performed on a solution containing the compound (I), preferably compound (Ia), dissolved in tert-BuOH.

In one embodiment the distillation (viii) is repeated several times, preferably twice.

30 If the product is applied to freeze-drying right after this MeOH distillation the final product can include residual amounts of MeOH, which can make it sticky and difficult to handle – depending on the amount of residual MeOH. By applying additional tert-BuOH distillations, residual MeOH is removed and the final material is isolated as a fine free-flowing solid.

In the method of manufacturing a compound of formula (I) freeze drying (ix) can be performed.

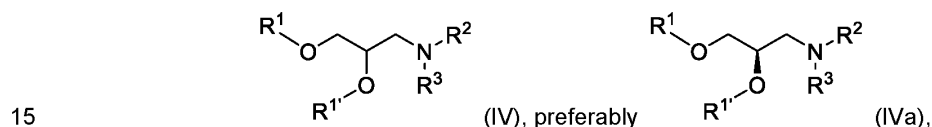
In one embodiment freeze drying (ix) is performed on a solution containing the compound (I), preferably compound (Ia), dissolved in alcohol, preferably tert-BuOH.

In one embodiment freeze drying (ix) is performed by cooling the solution to a temperature from -25 to 5 °C.

In one embodiment freeze drying (ix) is performed in two steps, in the first step the solution is cooled to 0 to 10 °C, preferably 2 to 8 °C, thereafter, in the second step the solution is cooled to -15 to -25 °C, preferably -18 to -20 °C.

In one embodiment after the cooling the solvent, preferably the alcohol, more preferably tert-BuOH, is removed by vacuum evaporation. In one embodiment the pressure is reduced to 1 mbar. In one embodiment thereafter the vessel is flushed with inert gas, preferably nitrogen or argon, more preferably argon.

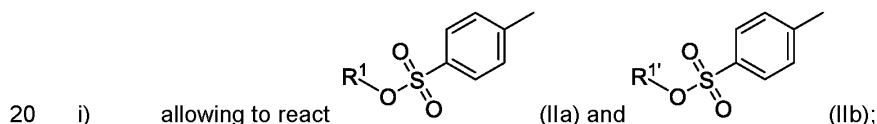
Furthermore, the present invention refers to a method of manufacturing a compound of formula (IV)



wherein  $R^1$  and  $R^{1'}$  are independently selected from saturated or unsaturated, with up to three  $-C=C-$  bonds,  $C_{6-22}$  hydrocarbon groups;

$R^2$  and  $R^3$  are independently selected from  $-H$  and  $C_{1-6}$  alkyl groups;

comprising or consisting of the steps:



wherein  $R^1$  and  $R^{1'}$  are the same as defined in formula (IV) with



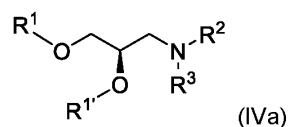
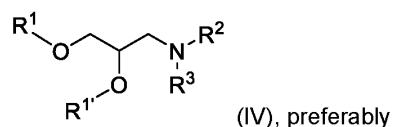
wherein  $R^2$  and  $R^3$  are as defined in formula (IV);

in an organic solvent;

25 in the presence of a potassium base having a  $pK_a$  value in DMSO of 20 to 35 measured by spectrophotometry;

ii) thereafter performing an aqueous work-up at an pH value of 1.2 to 2;

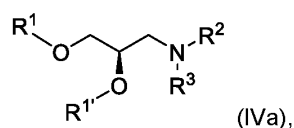
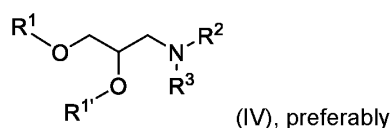
to obtain a compound of formula (IV)



in the organic phase;

- 5 iii) optionally performing a concentration step;
- iv) optionally performing a chromatography step.

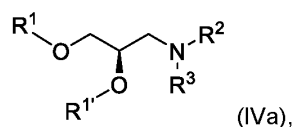
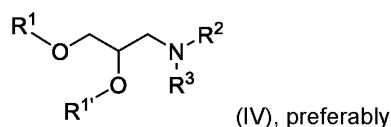
In one embodiment the method of manufacturing leads to a compound of formula (IV)



- 10 wherein R<sup>1</sup> and R<sup>1'</sup> are independently selected from saturated or unsaturated, with up to three -C=C- bonds, C<sub>6-22</sub> hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>12-18</sub> hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>16-18</sub> hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond, C<sub>16-18</sub> hydrocarbon groups or most preferably an unsaturated with one -C=C- bond C<sub>18</sub> hydrocarbon group;
- 15

R<sup>2</sup> and R<sup>3</sup> are independently selected from -H and C<sub>1-6</sub> alkyl groups, preferably R<sup>2</sup> and R<sup>3</sup> are the same, more preferably R<sup>2</sup> and R<sup>3</sup> are selected from C<sub>1-3</sub> alkyl groups most preferably R<sup>2</sup> and R<sup>3</sup> are methyl.

- 20 In one embodiment the method of manufacturing leads to a compound of formula (IV)

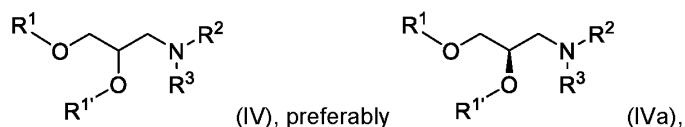


- wherein R<sup>1</sup> and R<sup>1'</sup> are the same and selected from saturated or unsaturated, with up to three -C=C- bonds, C<sub>6-22</sub> hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>12-18</sub> hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>16-18</sub> hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C-
- 25

bond, C<sub>16-18</sub> hydrocarbon groups or most preferably an unsaturated with one -C=C- bond C<sub>18</sub> hydrocarbon group;

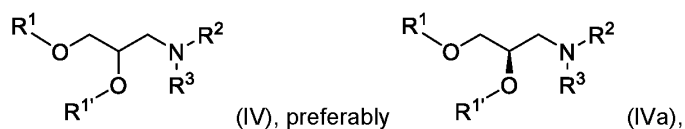
- R<sup>2</sup> and R<sup>3</sup> are independently selected from -H and C<sub>1-6</sub> alkyl groups, preferably R<sup>2</sup> and R<sup>3</sup> are the same, more preferably R<sup>2</sup> and R<sup>3</sup> are selected from C<sub>1-3</sub> alkyl groups most preferably R<sup>2</sup> and R<sup>3</sup> are methyl.

In one embodiment the method of manufacturing leads to a compound of formula (IV)



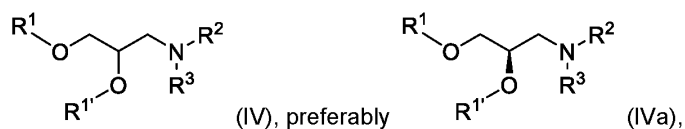
- wherein R<sup>1</sup> and R<sup>1'</sup> are the same and selected from saturated or unsaturated, with up to three -C=C- bonds, C<sub>6-22</sub> hydrocarbon groups; preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>12-18</sub> hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>16-18</sub> hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond, C<sub>16-18</sub> hydrocarbon groups or most preferably an unsaturated with one -C=C- bond C<sub>18</sub> hydrocarbon group;
- R<sup>2</sup> and R<sup>3</sup> are the same and selected from -H and C<sub>1-6</sub> alkyl groups, more preferably R<sup>2</sup> and R<sup>3</sup> are selected from C<sub>1-3</sub> alkyl groups most preferably R<sup>2</sup> and R<sup>3</sup> are methyl.

In one embodiment the method of manufacturing leads to a compound of formula (I)



- wherein R<sup>1</sup> and R<sup>1'</sup> are the same and selected from saturated or unsaturated, with up to three -C=C- bonds, C<sub>12-18</sub> hydrocarbon groups; more preferably saturated or unsaturated, with up to three -C=C- bonds, C<sub>16-18</sub> hydrocarbon groups; most preferably saturated or unsaturated, with one -C=C- bond, C<sub>16-18</sub> hydrocarbon groups or most preferably an unsaturated with one -C=C- bond C<sub>18</sub> hydrocarbon group;
- R<sup>2</sup> and R<sup>3</sup> are the same and selected from C<sub>1-3</sub> alkyl groups most preferably R<sup>2</sup> and R<sup>3</sup> are methyl.

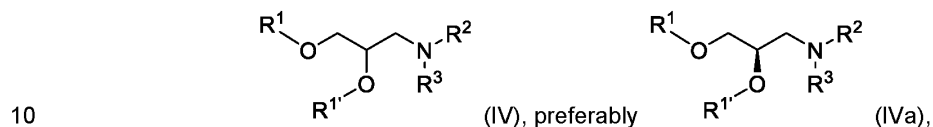
In one embodiment the method of manufacturing leads to a compound of formula (IV)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{12-18}$  hydrocarbon groups; preferably saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{16-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl.

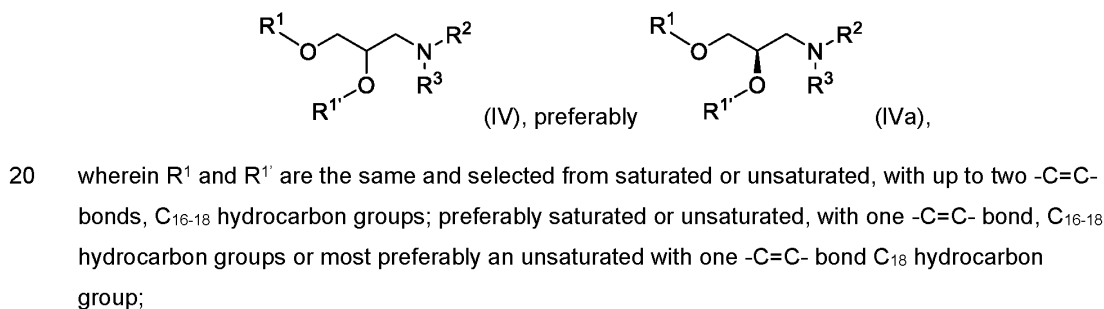
In one embodiment the method of manufacturing leads to a compound of formula (IV)



wherein  $R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{12-18}$  hydrocarbon groups; preferably saturated or unsaturated, with up to two  $-C=C-$  bonds,  $C_{16-18}$  hydrocarbon groups; more preferably saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups or most preferably an unsaturated with one  $-C=C-$  bond  $C_{18}$  hydrocarbon group;

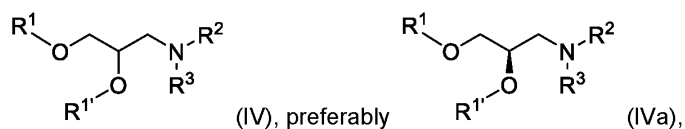
$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl.

In one embodiment the method of manufacturing leads to a compound of formula (IV)



$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups most preferably  $R^2$  and  $R^3$  are methyl.

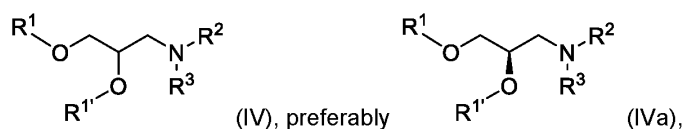
In one embodiment the method of manufacturing leads to a compound of formula (IV)



wherein  $\text{R}^1$  and  $\text{R}^{1'}$  are the same and selected from saturated or unsaturated, with one  $\text{-C=C-}$  bond,  $\text{C}_{16-18}$  hydrocarbon groups or preferably an unsaturated with one  $\text{-C=C-}$  bond  $\text{C}_{18}$  hydrocarbon group;

- 5  $\text{R}^2$  and  $\text{R}^3$  are the same and selected from  $\text{C}_{1-3}$  alkyl groups most preferably  $\text{R}^2$  and  $\text{R}^3$  are methyl.

In one embodiment the method of manufacturing leads to a compound of formula (IV)

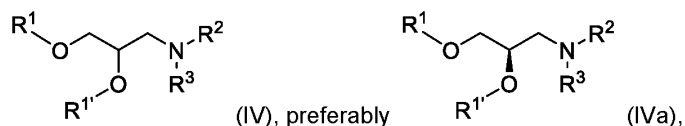


wherein  $\text{R}^1$  and  $\text{R}^{1'}$  are the same and selected from saturated or unsaturated, with one  $\text{-C=C-}$  bond,  $\text{C}_{16-18}$  hydrocarbon groups or preferably an unsaturated with one  $\text{-C=C-}$  bond  $\text{C}_{18}$  hydrocarbon group;

- 10  $\text{R}^2$  and  $\text{R}^3$  are methyl.

$\text{R}^2$  and  $\text{R}^3$  are methyl.

In one embodiment the method of manufacturing leads to a compound of formula (IV)



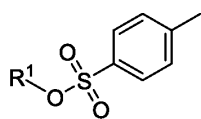
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wherein  $\text{R}^1$  and  $\text{R}^{1'}$  are the same and are an unsaturated with one  $\text{-C=C-}$  bond  $\text{C}_{18}$  hydrocarbon group;

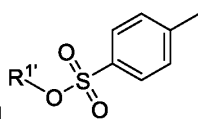
$\text{R}^2$  and  $\text{R}^3$  are methyl.

- 20 In one embodiment the compound of formula (IV) is 1,2-dioleyloxy-3-dimethylaminopropane.

In preferred embodiments  $\text{R}^1$  and  $\text{R}^{1'}$  in formulae



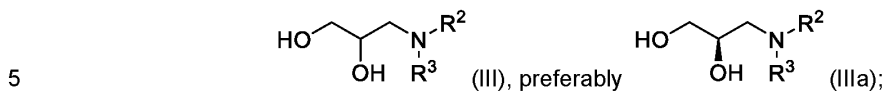
(IIa) and



(IIb) are the same as defined in any of the above-

mentioned embodiments for formula (IV). In preferred embodiments (IIa) and (IIb) are the same.

In preferred embodiments  $R^2$  and  $R^3$  in formulae (III) and (IIIa)



are the same as defined in any of the above-mentioned embodiments for formula (IV).

In one embodiment the molar ratio of compounds (IIa) and (IIb) to compound (III) is 10 to 1, preferably 2.5 to 1, more preferably 2 to 1.

10

In step (i) of the method of manufacturing a compound of formula (IV) an organic solvent is used.

In one embodiment the organic solvent is tetrahydrofuran or a tetrahydrofuran-based solvent.

In one embodiment the organic solvent is methyl tetrahydrofuran.

In one embodiment the organic solvent is 2-methyl tetrahydrofuran;

15

Step (i) of the method of manufacturing a compound of formula (IV) is performed in the presence of a potassium base having a  $pK_a$  value in DMSO of 20 to 35 measured by spectrophotometry.

In one embodiment the potassium base is selected from potassium tert-butoxide (KOt-Bu), potassium bis(trimethylsilyl)amide (KHMDs),  $KNH_2$ , KOH, and KH.

20 In one embodiment the potassium base is KHMDs.

In one embodiment step (i) of the method of manufacturing a compound of formula (IV) is performed at a temperature of 20 to 30°C.

In one embodiment step (i) of the method of manufacturing a compound of formula (IV) is performed at a temperature of 25 to 28°C.

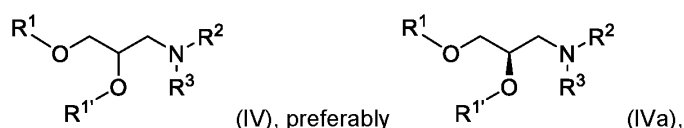
25 In one embodiment step (i) of the method of manufacturing a compound of formula (IV) is performed under inert conditions.

In one embodiment step (i) of the method of manufacturing a compound of formula (IV) is performed under nitrogen.

In one embodiment step (i) of the method of manufacturing a compound of formula (IV) is performed under stirring.

- 5 In one embodiment in step (i) of the method of manufacturing a compound of formula (IV) the potassium base and the compound of formula (III) are added, preferably from 1 second to 10 minutes, more preferably 1 second to 5 minutes, to the reaction vessel and then stirred, preferably for 1 minute to 5 hours, more preferably 5 minutes to 1 hour, and thereafter the compounds of formulae (IIa) and (IIb) are added to the reaction vessel, preferably the mixture thereafter is stirred for 5 minutes to 24  
10 hours, more preferably 2 hours to 18 hours.

Thereafter, in the method of manufacturing a compound of formula (I) in step (ii) an aqueous work-up at a pH value of 1.2 to 2.0 is performed to obtain a compound of formula (IV)



- 15 in the organic phase.

In one embodiment in the method of manufacturing a compound of formula (IV) in step (ii) an aqueous work-up at a pH value of 1.6 is performed.

- A skilled person in the field of organic synthesis is well able to perform an aqueous work-up and knows how to determine and adjust the pH-value. For example suitable compounds to adjust the pH-value are HCl solution, preferably a 25% aqueous HCl solution and NaOH solution, preferably an 5N  
20 aqueous NaOH solution. The aqueous work-up can be completely or partly performed under stirring. Stirring is preferably continuously performed until phase separation is desired. The organic phase is retained and one or more additional work-up steps with an aqueous phase, preferably an aqueous NaOH solution, more preferably having an pH-value of about 8.0 to 8.6 can be performed. After the  
25 final work-up step the organic phase is retained.

After step (ii) of the method of manufacturing a compound of formula (IV) a concentration step (iii) can be optionally performed. A skilled person in the field of organic synthesis is well able to perform a concentration step of an organic phase.

- 30 In one embodiment the organic phase is concentrated via evaporation, preferably by a rotary evaporator, at 55 to 65 °C, preferably 60 °C water bath temperature and a pressure of 200 to 300, preferably 250 mbar, with a target end pressure of about 20 mbar.

After step (ii) or step (iii) in the method of manufacturing a compound of formula (IV) a chromatography step can be performed. A skilled person in the field of organic synthesis is well able to perform a chromatography.

- 5 In one embodiment the used solvents for the chromatography in step (iii) are cyclohexane and ethyl acetate.

In one embodiment a linear gradient of cyclohexane to ethyl acetate is used in step (iii).

In one embodiment a step gradient of cyclohexane to ethyl acetate is used in step (iii).

- 10 The obtained fractions, preferably meeting predefined criteria can be concentrated in a further concentration step, as described for step (iii) above.

Moreover, the present invention refers to a method of freeze drying a lipid, preferably a cationic or cationizable lipid, comprising or consisting of the steps:

- 15 providing the lipid to be freeze-dried in an alcohol solution, wherein the lipid is preferably a cationic lipid, more preferably a compound according to formula (I), most preferably a compound according to formula (Ia), and

cooling the solution to a temperature of -25 to 5 °C;

thereafter stopping the cooling and removing the solvent by vacuum evaporation.

In one embodiment the alcohol is tert-BuOH.

- 20 In one embodiment cooling is performed in two steps, in the first step the solution is cooled to 0 to 10 °C, preferably 2 to 8 °C, thereafter, in the second step the solution is cooled to -15 to -25 °C, preferably -18 to -20 °C.

In one embodiment the pressure in the vacuum evaporation is reduced to 1 mbar.

- 25 In one embodiment thereafter the vessel is flushed with inert gas, preferably nitrogen or argon, more preferably argon.

Examples

Optimization reaction

5 A properly sized reaction vessel was flushed with nitrogen and the respective temperature was adjusted. The base and 3-(dimethylamino)propane-1,2-diol were added and mixed with the solvent at the given temperature. Thereafter, the tosylate was added and stirring was continued for the given time. The conversion was checked by sampling the reaction mixture and analyze it via HPLC-CAD.

In the beginning stearyl tosylate was used as a substitute for oleyl tosylate, because of its higher purity and missing homologous. Once oleyl tosylate was available with the desired purity it was used for optimization reactions.

Table 1: Optimization of reaction conditions

| Entry | Base (eq)      | Solvent  | Tosylate reagent (eq) | IPC   C18:X-Tos (area%) | IPC   DODMA (area%) |
|-------|----------------|----------|-----------------------|-------------------------|---------------------|
| 1     | HexLi (2)      | THF      | Stearyl tosylate (2)  | 89.9 (X=0)              | 0.3 (DSDMA)         |
| 2     | NaH (2)        | THF      | Stearyl tosylate (2)  | 81.9 (X=0)              | 0.7 (DSDMA)         |
| 3     | NaOtBu (2)     | THF      | Stearyl tosylate (2)  | 65.6 (X=0)              | 11.0 (DSDMA)        |
| 4     | NaHMDS (2)     | THF      | Stearyl tosylate (2)  | 72.5 (X=0)              | 9.4 (DSDMA)         |
| 5     | KOtBu (2)      | THF      | Stearyl tosylate (2)  | 6.0 (X=0)               | 38.7 (DSDMA)        |
| 6     | KHMDS (2)      | THF      | Stearyl tosylate (2)  | 14.5 (X=0)              | 52.3 (DSDMA)        |
| 7     | KOtBu (2)      | 2-MeTHF  | Stearyl tosylate (2)  | 12.0 (X=0)              | 42.9 (DSDMA)        |
| 8     | KOH powder (6) | n-Hexane | Stearyl mesylate (2)  | n.d. (X=0)              | 62.6 (DSDMA)        |
| 9     | KOtBu (2.5)    | 2-MeTHF  | Oleyl tosylate (2)    | 16.3 (X=1)              | 22.6                |
| 10    | KHMDS (2)      | 2-MeTHF  | Oleyl tosylate (2.1)  | 1.1 (X=1)               | 59.5                |
| 11    | KHMDS (2.2)    | 2-MeTHF  | Oleyl tosylate (2)    | n.d. (X=1)              | 40.7                |

For the initial optimization reactions (entry 1-7) stearyl tosylate has been used as a substitute for oleyl tosylate and the 3-(dimethylamino)propane-1,2-diol has been used as a racemic mixture yielding DSDMA as the final product.

15 The reagents were mixed at 25 °C and the reactions were performed with the given solvent at 50 °C for 24 hours (entry 1-10). The reaction conversion was analyzed via HPLC-CAD.

Stearyl mesylate was used as in EP 3 231 790 A1 in entry 8 (comparative example). Although the conversion to DSDMA was satisfying, the isolation of the product in sufficient quality was not possible.

Addition of KHMDS in entry 11 was (in THF solution) within 5 minutes.

20

Description of the R-DODMA manufacturing process

Preparation of Starting Materials

Bottle 1: KHMDS (about 882 g, 884 mmol, 2.3 eq, about 20% in THF, the exact amount will be calculated according to the assay of KHMDS) is weighted in a glass bottle.

25 Bottle 2: 2-MeTHF (2290 mL, 50 mL/g R-DMAP-Diol) is weighted in a glass bottle.

Bottle 3: R-DMAP-Diol (45.8 g, 384 mmol, 1.0 eq) was melted in a tempered water bath at 40 °C, transferred into a glass bottle and 2-MeTHF (275 mL, 6 mL/g R-DMAP-Diol) was added.

Bottle 4: 2-MeTHF (229 mL, 5 mL/g R-DMAP-Diol) is weighted in a glass bottle.

Bottle 5: Oleyl tosylate (325 g, 769 mmol, 2.0 eq) is weighted in a glass bottle.

5 Bottle 6: 2-MeTHF (229 mL, 5 mL/g R-DMAP-Diol) is weighted in a glass bottle.

### Raw Material Charging and Substitution Reaction

A 5 L HWS reactor is flushed with nitrogen and tempered to 28 °C (jacket). Bottle 1 is poured through a funnel into the HWS reactor. Then, the agitation rate is set to 350-400 rpm. Bottle 1 and funnel are  
10 washed with 2-MeTHF from Bottle 2. The remaining 2-MeTHF in Bottle 2 is poured into the reactor. Bottle 3 is poured through a funnel to the reaction mixture and washed with 2-MeTHF from Bottle 4. The mixture is stirred for 30 minutes under the given conditions. Then, Bottle 5 is poured through a funnel to the reaction mixture and washed with 2-MeTHF from Bottle 6. The reaction is stirred for 16 hours.

15

### Aqueous Workup

The jacket temperature is reduced to 25 °C and water (1000 g) is added through a funnel into the reactor. If the material still sticks on the reactors glass wall, the agitation rate can be increased temporarily. Stirring is continued for at least 10 minutes, then the stirrer is stopped. Wait at least 10  
20 minutes until a clearly defined phase separation is achieved – an upper yellow, turbid, organic product phase and a lower brown/orange, turbid water phase. The lower water phase is drained from the reactor and discarded.

A pH-meter is installed. Water (1000 g) is added through a funnel into the reactor. A dropping funnel is filled with aqueous HCl solution (180 g, about 250 mL, 25% in water) and weighted together with  
25 the funnel, then placed onto the reactor. The pH-value is adjusted to 1.6 (target range 1.2 – 2.0). If the pH drops too deep, it can be readjusted with aqueous NaOH solution (5N in water). Stirring is continued for at least 10 minutes until the pH is on a constant level and the product temperature drops below 28 °C, then the stirrer is stopped.

The amount of used HCl is back weighted. Wait at least 10 minutes until a clearly defined phase  
30 separation is achieved – an upper yellow, turbid, organic product phase and a lower colorless, turbid water phase. The lower water phase is drained from the reactor and discarded. Water (1000 g) is added through a funnel into the reactor.

A dropping funnel is filled with aqueous NaOH solution (69.0 g, about 100 mL, 5 M in water) and weighted together with the funnel, then placed onto the reactor. The pH-value is adjusted to 8.4

- (target range 8.2 – 9.0). If the pH soars too high, it can be readjusted with aqueous HCl solution (25% in water). Stirring is continued for minimum 10 minutes until the pH is on a constant level, then the stirrer is stopped. The amount of used NaOH is back weighted. It takes at least 10 minutes until a clearly defined phase separation is achieved – an upper yellow, turbid, organic product phase and
- 5 a lower colorless, clear water phase. The lower water phase is drained from the reactor and discarded. The organic product phase is transferred into a 5 L glass bottle.

### Column Chromatography and Isolation

- The organic phase is transferred from the bottle through a hose into a 2 L evaporating flask, which
- 10 is connected to the rotary evaporator and concentrated at 60 °C water bath temperature and a pressure of 250 mbar. When the distillation stops at the given pressure the same is reduced gradually to 20 mbar. The crude product is weighted, dissolved in cyclohexane and filtered through a glass frit to obtain 1500 mL of a 20% R-DODMA crude solution.

- Half of the crude R-DODMA solution (750 mL) is injected into the chromatography system and
- 15 purified using a cyclohexane/ethyl acetate gradient. After a specific period of time several fractions are collected.

Fractions, which meet the specification, are concentrated on a rotary evaporator at 60 °C water bath temperature and a pressure of 250 mbar. When the distillation stops at the given pressure the same is reduced gradually to 20 mbar. Pure R-DODMA is obtained as a colorless to yellow liquid.

20

### Description of the R-DOTMA-Cl manufacturing process

#### Preparation of Starting Materials

Bottle 1: R-DODMA (350 g, 564 mmol, 1.0 eq) is weighted in a glass bottle.

Bottle 2: MeOH (2772 g, 3500 mL, 10 mL/g R-DODMA) is weighted in a glass bottle.

- 25 Bottle 3: MeI (120.2 g, 52.7 mL, 847 mmol, 1.5 eq) is weighted in a glass bottle.

Bottle 4: MeOH (3150 g, 3977 mL, 9 g/g R-DODMA) is weighted in a glass bottle.

#### Raw Material Charging and Methylation Reaction

- A 5 L HWS reactor is purged with nitrogen and tempered to 25 °C. Bottle 1 is poured through a funnel
- 30 into the reactor. Bottle 1 and funnel are washed with a partial amount of MeOH from bottle 2. The remaining MeOH in bottle 2 is poured into the reactor, then the stirring speed is set to 300-400 rpm. Thereafter, bottle 3 is poured into the reaction mixture through a funnel. The reaction is stirred for min. 8 hours.

### Anion exchange chromatography

The reaction mixture is transferred into a 5 L glass bottle. The mixture is then transferred through a hose into the rotary evaporator and concentrated at a water bath temperature of 60 °C and a pressure of 325 mbar until the solvent condensation drops drastically. Then the crude product (R-DOTMA-I) is transferred into a 5 L glass bottle. The flask is washed with MeOH from bottle 4 and the remaining MeOH is poured into the bottle.

The PFA 500Plus anion exchange material (1332 g) is subjected to a washing procedure. First the resin is washed with NaOH (aqueous 5N), then water (WFI), NaCl (10% in water), water (WFI) and MeOH are applied successively. Once the resin is conditioned with MeOH, the methanolic solution of R-DOTMA-I is injected into the chromatography system and rinsed through the anion exchange column with MeOH (4600 mL).

The collected product fractions are concentrated on a rotary evaporator at a water bath temperature of 60 °C and a pressure of 325 mbar until roughly a 10% methanolic solution is obtained (missing MeOH can be added to obtain the 10% solution).

### C18 Flash Column Chromatography

The C18 flash column is conditioned with MeOH (~15.2 L). The methanolic crude product solution is injected into the chromatography system and rinsed through the reversed-phase column using MeOH (approximately 15 L) and is collected in several fractions.

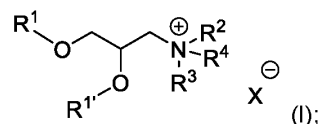
The methanolic solution is filtered through a glass frit (10-20 µm pore size) into a flask and concentrated on a rotary evaporator at a water bath temperature of 60 °C and a pressure of 325 mbar until the solvent condensation drops drastically. Then the pressure is reduced to 60 mbar. Thereafter, t-BuOH (1400 g) is added and distilled at 60 °C water bath temperature and a pressure of 130 mbar until the solvent condensation drops drastically. Then the pressure is reduced to 60 mbar. This t-BuOH distillation is conducted twice.

### Freeze-drying and insulation

The pure product is diluted in t-BuOH (in total 3150 mL) and split into twelve flasks. The flasks are cooled in the fridge at 2-8 °C and thereafter in the freezer at -18 to -20 °C until they are completely frozen. The freeze dryers' pressure is set to 1.0 mbar. The flasks are attached to the freeze dryer, dried until the flasks reach room temperature. Argon is used to flush the system. The product is obtained as a colorless powder and transferred into glass bottles.

## Claims

1. Method of manufacturing a compound of formula (I)



- 5 wherein R<sup>1</sup> and R<sup>1'</sup> are independently selected from saturated or unsaturated, with up to three

-C=C- bonds, C<sub>6-22</sub> hydrocarbon groups;

R<sup>2</sup> and R<sup>3</sup> are independently selected from -H and C<sub>1-6</sub> alkyl groups;

R<sup>4</sup> is a C<sub>1-6</sub> alkyl group; and

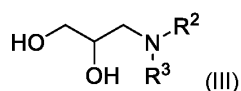
- 10 X = F, Cl, or Br,

comprising or consisting of the steps:

- i) allowing to react
- (IIa)

(IIb);

wherein R<sup>1</sup> and R<sup>1'</sup> are the same as defined in formula (I) with



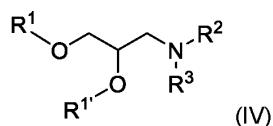
- 15 wherein R<sup>2</sup> and R<sup>3</sup> are as defined in formula (I);

in an organic solvent;

in the presence of a potassium base having a pK<sub>a</sub> value in DMSO of 20 to 35 measured by spectrophotometry;

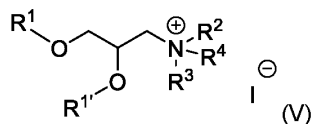
- ii) thereafter performing an aqueous work-up at a pH value of 1.2 to 2.0;

- 20 to obtain a compound of formula (IV)



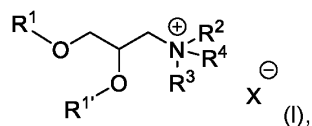
in the organic phase

- iii) optionally performing a concentration step;
- iv) optionally performing a chromatography step;
- v) allowing to react the compound of formula (IV) obtained after step ii), step iii), or step iv) with  $R^4I$  in order to obtain a compound of formula (V)



wherein  $R^1$  to  $R^4$  and  $R^{1'}$  are defined as in formula (I);

- vi) performing ion exchange to obtain a compound of formula (I)



- 10      wherein  $X = F, Cl, \text{ or } Br$

- vii) optionally performing chromatography;
- viii) optionally performing distillation; and
- ix) optionally performing freeze drying.

- 15      2. Method according to claim 1, wherein in formula (I)

$R^1$  and  $R^{1'}$  are the same and selected from saturated or unsaturated, with one  $-C=C-$  bond,  $C_{16-18}$  hydrocarbon groups;

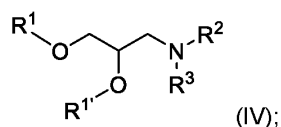
$R^2$  and  $R^3$  are the same and selected from  $C_{1-3}$  alkyl groups;

$R^4$  is selected from  $C_{1-3}$  alkyl groups; and

- 20       $X = Cl$ .

3. The method of claim 1 or 2, wherein the compound of formula (I) is 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt).

4. Method of manufacturing a compound of formula (IV)



wherein R<sup>1</sup> and R<sup>1'</sup> are independently selected from saturated or unsaturated, with up to three

- 5 -C=C- bonds, C<sub>6-22</sub> hydrocarbon groups;

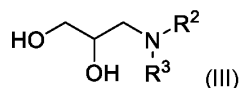
R<sup>2</sup> and R<sup>3</sup> are independently selected from -H and C<sub>1-6</sub> alkyl groups;

comprising or consisting of the steps:

- i) allowing to react
- (IIa) and

(IIb);

- 10 wherein R<sup>1</sup> and R<sup>1'</sup> are the same as defined in formula (IV) with

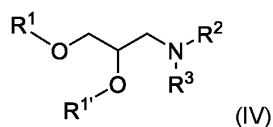


wherein R<sup>2</sup> and R<sup>3</sup> are as defined in formula (IV);

in an organic solvent;

- 15 in the presence of a potassium base having a pK<sub>a</sub> value in DMSO of 20 to 35 measured by spectrophotometry;

- ii) thereafter performing an aqueous work-up at an pH value of 1.2 to 2.0;  
to obtain a compound of formula (IV)



in the organic phase;

- 20 iii) optionally performing a concentration step;  
iv) optionally performing a chromatography step.

5. The method according to claim 4, wherein in formula (IV)
 

R<sup>1</sup> and R<sup>1'</sup> are the same and selected from saturated or unsaturated, with one -C=C- bond, C<sub>16-18</sub> hydrocarbon groups;

R<sup>2</sup> and R<sup>3</sup> are the same and selected from C<sub>1-3</sub> alkyl groups;

5 R<sup>4</sup> is selected from C<sub>1-3</sub> alkyl groups; and

X = Cl.
6. The method according to claim 4 or 5, wherein the compound of formula (IV) is 1,2-dioleyloxy-3-dimethylaminopropane.
- 10 7. The method according to any of claims 1 to 6, wherein
 

the molar ratio of compounds (IIa) and (IIb) to compound (III) is 10 to 1.
- 15 8. The method according to any of claims 1 to 7, wherein in step i) the organic solvent is selected from tetrahydrofuran or a tetrahydrofuran based solvent or mixtures thereof.
9. The method according to any of claims 1 to 8, wherein in step i)
 

the potassium base is selected from potassium tert-butoxide (KOt-Bu), potassium bis(trimethylsilyl)amide (KHMDs), KNH<sub>2</sub>, KOH, and KH, or mixtures thereof.
- 20 10. A method of freeze drying a lipid comprising or consisting of the steps:
  - i) providing the lipid to be freeze-dried in an alcohol solution and
  - ii) cooling the solution to a temperature of -25 to 5 °C;
  - iii) thereafter stopping the cooling and removing the solvent by vacuum evaporation.
- 25 11. The method of freeze drying according to claim 10, wherein the alcohol solution comprises or consists of tert-BuOH.

12. The method of freeze drying according to claim 10 or 11, wherein  
cooling is performed in two steps, in the first step the solution is cooled to 0 to 10 °C, thereafter,  
in the second step the solution is cooled to -15 to -25 °C.