

Synthetic Triterpenes Composition, Method Of Production And Applications Thereof**Cross-Reference to Related Applications**

This application claims priority to U.S. Provisional Application No. 63/537,012, filed on September 7, 2023, and U.S. Provisional Application No. 63/653,454, filed on May 30, 2024, the contents of which are herein incorporated by reference in their entirety.

Technical Field

The invention generally relates to a synthetic triterpene composition such as squalene and methods for producing the composition by novel chemical synthetic routes. The squalene produced by the synthetic method covered in this invention formulation is suitable to use in making vaccine adjuvants, antioxidant formulations, and drug delivery agents.

Background

Squalene is a naturally occurring polyunsaturated hydrocarbon lipid having molecular formula $C_{30}H_{50}$ with a chemical name 2,6,10,15,19,23-hexamethyltetracos-2,6,10,14,18,22-hexaene and belongs to a triterpene class (containing three terpene units) of compounds. As described above squalene is mostly sourced from animals (Sharks) or plants, which are deemed to be unsustainable sources. Squalene and some of the derivatives of squalene serve as precursors for a variety of triterpenes classes of compounds. Squalene has been used mainly in the pharmaceutical industry as part of formulations (as an excipient) to design drug delivery systems and in the cosmetic industry. In vaccine formulations, squalene has been used as an adjuvant. In preclinical and clinical settings, squalene-based emulsions have been proven to help improve immune responses against several diseases. The anti-inflammatory effect of squalene has been demonstrated in the Zebrafish Model. Reactive oxygen species (ROS) and free radicals are known to cause damage to proteins, lipids, and DNA. Several studies have been reported in the literature to demonstrate the antioxidant properties of squalene. The antioxidant activity of squalene has been assessed in vitro by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay. The

activity was found to be dose dependent and at 1% squalene concentration the radical scavenging activity was highest. Therefore, squalene can also be used to improve the anti-inflammatory and antioxidant capacity of lipid nanoparticle (LNP) formulations.

The technological processes for isolating squalene from various sources have been well documented in the literature. However, the squalene isolation processes from the plant or animal sources are lengthy and economically inefficient and may not always yield squalene of adequate purity necessary especially for pharmaceutical applications. Due to a growing demand of squalene in the future it is reasonable to state that the natural or biosynthetic sources of squalene will fall well short of supply. There is, therefore, a need for the development of new synthetic routes which not only will be sustainable but also yield high purity squalene. The present invention addresses this issue by providing a novel and totally synthetic route to produce squalene. Fisher et al., US Pat appl No. 16/972,291 uses farnesene, which is typically obtained as a mixture of alkene geometrical isomers that would be reflected as impurities in squalene. The present invention is related to produce homogeneous squalene, free of unwanted geometrical and positional isomers by using pure farnesol as the starting material. Pure farnesol is superior to the mixture of 'farnesenes' as a starting material for a synthesis of squalene. This will help overcome the drawback of any mixture of alkene isomers in the starting material. Moreover, the route from pure farnesol requires relatively few steps.

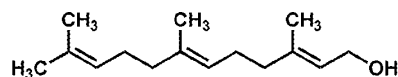
Summary

The invention covers a high purity composition of synthetic squalene. The high purity composition refers to the squalene that has purity greater than 97% and that is free of isomers, that is clear, colorless, or light yellow, oily liquid. Typically, squalene is obtained from animal (shark liver) & plant (olive oil) sources. However, these sources are deemed not the best when squalene is intended for pharmaceutical applications because (1) the isolation, extraction and fractional crystallization processes used for manufacturing the squalene are cumbersome; (2) they do not yield the squalene of high purity required for pharmaceutical applications; and (3) there is risk of pathogen, allergens. It is therefore important and desirable to have a viable alternate, synthetic route for high purity squalene particularly intended for pharmaceutical applications. In the present invention, the novel chemical synthetic process is disclosed to

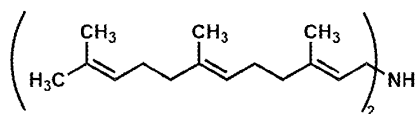
produce this high purity composition. In certain embodiments, the invention provides a novel method to synthesize squalene of high purity by chemical reactions. In particular embodiments, the key starting material is bis-farnesylamine. Certain embodiments of this method further include conversion of *trans, trans*-farnesol to bis-farnesylamine. Other embodiments of this synthetic route further include conversion of bis-farnesylamine to squalene using Levin's anomeric amide reagent. The novelty of this process is the use of Levin's reagent makes it possible to create highly reactive carbon-centered radicals from bis-farnesylamine by a deamination process. The rapid coupling of two solvent-caged radicals (i.e., a radical-radical coupling) then produces the squalene. In this approach, the coupling of radicals occurs spontaneously and does not require the presence of any transition metal catalyst. The exclusion of transition metal catalyst enables production of high purity composition.

In one embodiment, a high purity squalene composition containing squalene with a purity of at least 98% by weight and less than 1ppm heavy metals is claimed.

In another embodiment, a method of preparing squalene is claimed including (1) reacting *trans, trans*-farnesol

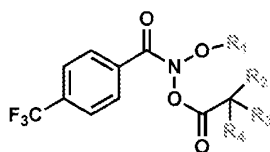


with reagents to form bis-farnesylamine

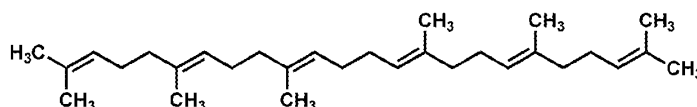


; and

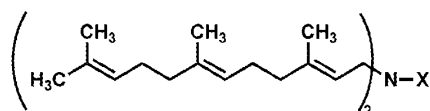
(2) reacting bis-farnesylamine with



wherein **R1** is benzyl, and **R2**, **R3**, and **R4** are independently C₁₋₁₀ alkyl, to form squalene

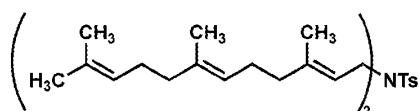


Preferably, **R2**, **R3**, and **R4** are methyl. The reagents in step (1) & (2) are preferably p-toluene sulfonamide, cyanomethylenetriethylphosphorane (CMBP) and sodium naphthalene. Preferably, in step (1) *trans*, *trans*-farnesol is reacted with reagents to form an intermediate



wherein **X** is tosylate, trityl, or nosyl, and the intermediate is reacted with sodium naphthalene to form bis-farnesylamine.

In a preferred embodiment, in step (1) *trans*, *trans*-farnesol is reacted with p-toluene sulfonamide and (Tributylphosphoranylidene)acetonitrile solution or cyanomethylenetriethylphosphorane (CMBP) to form bis-farnesyl-tosylamine



and then bis-farnesyl-tosylamine is reacted with sodium naphthalene to form bis-farnesylamine.

Preferably, the synthesis occurs in the absence of a transition metal catalyst.

In another preferred embodiment, a composition including substantially pure squalene is prepared using the method of claim 2, wherein the substantially pure squalene comprises greater than 98% squalene by weight.

In one embodiment, a method of making a vaccine formulation including adding the substantially pure squalene of claim 8 into an oil-in-water emulsion to form a vaccine formulation is claimed.

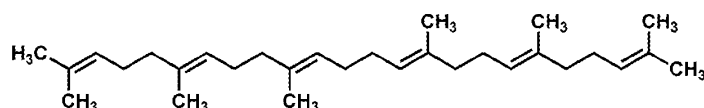
In another embodiment, method of making a drug delivery formulation including adding the substantially pure squalene of claim 8 into an oil-in-water emulsion to form a drug delivery formulation is claimed.

In another embodiment, a method of making a cosmetic formulation including adding the substantially pure squalene of claim 8 into a cosmetic formulation is claimed.

In another embodiment, a method of improving anti-inflammatory and antioxidant properties of lipid nanoparticle (LNP) formulations including adding the substantially pure squalene of claim 8 into a lipid nanoparticle formulation is claimed.

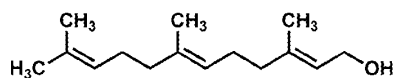
Detailed Description

The present invention generally relates to a method of preparing a triterpene class of compound having the structural formula **1** (squalene).



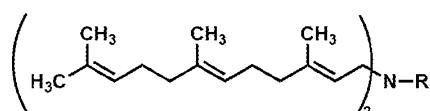
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A suitable starting material for synthesizing squalene mentioned in this invention can be from a class of compounds having structural formula **2** (*trans, trans*-farnesol) which has a significantly higher purity and is free from isomeric impurities.



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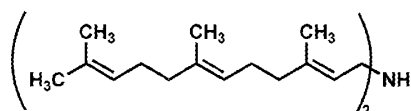
In certain embodiments, the invention provides a method of preparing class of compound having structural formula 3 from *trans, trans*-farnesol.



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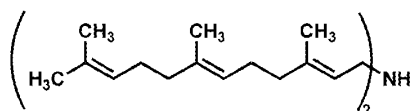
The **R** in formula 3 may be a tosylate group or a suitable good leaving group such as trityl or nosyl groups. Compound in formula 3 can be prepared by tosylation reaction in the presence cyanomethylenetriethylphosphorane (CMBP) or Tsunoda reagent following the procedures and methods disclosed in the literature by Tsunoda and coworkers (*Tetrahedron Letters*, 1996, vol. 37, 2457-2458).

The key intermediate having formula structure 4 (bis-farnesylamine) can then be prepared by reaction with sodium naphthalenide in a dry organic solvent such THF following similar procedures as disclosed by Tsunoda *et al.* in *Tetrahedron Letters*, 1996, vol. 37, 2457-2458).



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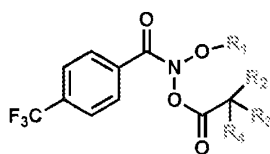
In certain embodiments, the invention provides a method of preparing class of compound having structural formula 4 from *trans, trans*-farnesol. The key intermediate namely bis-farnesylamine having formula structure 4 can be prepared by reaction of *trans, trans* farnesol with 2-Nitrobenzenesulfonamide



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The nitrobenzenesulfonamides (Ns-amides) serves as a protecting as well as activating group. The Ns group can easily be cleaved off or deprotected under mild conditions.

In certain embodiments, the method further comprises of preparation and use of Levin's anomeric amide class of compounds having formula structure **5** where **R1** can be benzyl and **R2**, **R3**, and **R4** can be a suitable alkyl group such as C₁₋₁₀, most preferably methyl. It is critical to shield the reaction from light at all times, due to the light sensitivity of the N-chloro intermediate used in its preparation.



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The inventors discovered conditions where, bis-farnesylamine, which is a secondary amine, forms farnesyl radicals in presence of Levin's anomeric amide, which then couple under suitable solvent and reaction conditions to form triterpene types of compounds such as squalene. A reaction mixture is formed by contacting equimolar quantities of the bis-farnesylamine and the Levin's anomeric amide in a suitable solvent. Suitable solvents include anhydrous tetrahydrofuran (THF). The anomeric amide reagent was dissolved separately in THF and transferred via syringe to a homogenous and soluble reaction mixture.

The squalene of the invention is of high purity (*i.e.*, substantially pure), meaning it has a purity of greater than 97%, 97.5%, and most preferably 98% by weight, and the squalene contains no more than 1 ppm heavy metals.

The high purity squalene maybe used to prepare vaccine formulations, drug delivery formulations, cosmetic formulations, and to improve anti-inflammatory and antioxidant properties of lipid nanoparticle (LNP) formulations.

High purity squalene made by organic synthesis route can be used as an adjuvant for a variety of vaccine emulsions including the common seasonal flu vaccine formulations. Per literature or example, oil-in-water emulsion composition that contains Span85, Tween80 and squalene as the oil phase is the best-known squalene-based vaccine adjuvant formulation

employed in influenza vaccine emulsion formulation MF59[®]. (O'Hagan, D.T.; Singh, M. MF59: a safe and potent oil-in-water emulsion adjuvant. In *Vaccine Adjuvants and Delivery Systems*; Singh, M., Ed.; John Wiley & Sons: Hoboken, NJ, USA, 2007, pp. 115-129.)

In another embodiment, squalene emulsions have been used for drug delivery applications. For example, squalene-based emulsions were successfully applied to deliver lipophilic ester prodrugs of nalbuphine (Wang, J. J., Sung, K., Hu, O. Y. P., Yeh, C. H., and Fang, J. Y. (2006). *J. Control. Release* 115, 140–149.).

The anti-inflammatory effect of squalene has been demonstrated in the Zebrafish Model. Reactive oxygen species (ROS) and free radicals are known to cause damage to proteins, lipids, and DNA. Several studies have been reported in the literature to demonstrate antioxidant properties of squalene (Zhang, P. et al., Anti-Inflammatory and Antioxidant Properties of Squalene in Copper Sulfate-Induced Inflammation in Zebrafish (*Danio rerio*). *Int. J. Mol. Sci.* **2023**, 24, 8518.

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains valuable information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

Examples

This section incorporates examples which will help understand the novelty of the invention. The examples are used for illustrative purposes only.

Example 1: Synthesis of bis-farnesyl-tosylamine

Tosylamine (20 - 40 mmol) was added under air to a 1L round bottom flask. The flask was then flushed with nitrogen or argon to create an inert atmosphere. Appropriate quantity of a suitable anhydrous solvent such as benzene, toluene or xylene was added, and the contents were agitated until the tosylamine had fully dissolved. Farnesol usually the double the amount of amine was added via syringe. Reagent cyanomethylenetriethylphosphorane (CMBP, 10 - 25 g) was added, and the contents were agitated for 15 - 30 h at 20 - 30° C. Upon completion of the reaction, the solvent was removed via evaporation under reduced pressure at 30 - 50° C. The crude product was mixed with silica gel using a suitable solvent. The solvent was removed under reduced pressure to render a free-flowing powder in the flask. The crude mixture was then column chromatographed using ethyl acetate-hexanes solvent system. Fractions were collected and the target product with UV activity was isolated by monitoring with TLC.

¹H NMR (500 MHz, CDCl₃) δ 7.69(d), 7.27(d), 5.04(m), 3.8(d), 2.4(s), 1.99(ddt), 1.58 (dd), 1.68(d)

Example 2: Synthesis of bis-farnesylamine

Bis-farnesyl-tosylamine (1 - 5 g) was added to a suitable flask. The flask was then sealed and flushed with nitrogen or argon to create an inert atmosphere. An anhydrous solvent such as THF was added, and the contents were mixed & cooled to -20 to -40 °C. 1M solution of sodium naphthalenide solution was introduced dropwise while maintaining the temperature of the contents between -20 to -40 °C. The quantity of this reagent was varied between 2 to 5 molar equivalents. Upon completion, the reaction was quenched with water. solvents were distilled off under reduced pressure to yield a waxy yellow crude mixture. The crude product was mixed with silica gel using a suitable solvent. The solvent was removed under reduced pressure to render a free-flowing powder in the flask. The crude mixture was then column chromatographed using ethyl acetate solvent. Fractions were collected and the target product with UV activity was isolated by monitoring with TLC. The product was confirmed by staining with potassium

permanganate (KMnO₄) as the material with a brown stained color. Fractions with the target material were collected and concentrated under reduced pressure.

¹H NMR (500 MHz, CDCl₃) δ 5.28 (t), 5.10 (dt), 3.23 (d), 2.03(m), 1.67(s), 1.64(s), 1.59(d)

Example 3: Synthesis of *N*-(benzyloxy)-*N*-(pivaloyloxy)-4-(trifluoromethyl)benzamide

An amber flask was charged with *N*-(benzyloxy)-4-(trifluoromethyl) benzamide (25 - 40 mmol) and 60 - 100 mL anhydrous dichloromethane (DCM). ^tBuOCl (20 - 60 mmol) was introduced via syringe. The reaction mixture was agitated for 1 - 4 h under ambient conditions. The crude mixture containing the desired *N*-chloro compound was concentrated and re-dissolved in 30 - 90 mL anhydrous acetonitrile. In a separate vial a suspension of sodium pivalate (35 - 55 mmol) was made in 15 - 35 mL anhydrous acetonitrile. The crude *N*-chloro mixture was removed by inversion and extraction of the suspension into a syringe. The contents were then added to the flask containing the sodium pivalate and stirred for 1 - 5 h at ambient conditions. The crude mixture was concentrated and purified by chromatography using a suitable solvent such as THF or diethyl ether.

¹H NMR (500 MHz, CDCl₃) δ 7.76(d), 7.64(d), 7.36(m), 5.13(s), 1.09(s)

Example 4: Synthesis of Squalene

To a 100 mL amber flask was added bis-farnesylamine (8 - 16 mmol). Anhydrous THF was introduced via syringe and the solution temperature was raised to 40 - 50 °C. In a separate container a solution of anomeric amide reagent was prepared in THF and transferred via syringe to the reaction vessel. The contents were stirred for 10 - 20 h. The solvents were removed under reduced pressure and the crude mixture was then column chromatographed over silica gel using pure hexanes as eluent. The fractions were collected and third fraction to come of the silica gel column was identified as the target compound squalene by NMR analysis. The third collected fraction was a colorless, odorless oil which was a pure sample of squalene as confirmed by ¹H NMR.

¹H NMR (500 MHz, CDCl₃) δ 5.15(m), 2.06(m), 1.62(s), 1.71(d)

Example 5: Synthesis of 2-nitro-*N,N*-bis((2*E*, 6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)benzenesulfonamide

2-Nitrobenzenesulfonamide (5 - 15 mmol) was added to an oven-dried round bottom flask. To this was added (*E,E*)-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (10 - 30 mmol). The anhydrous toluene (20 - 40 mL) was added, and the mixture was cooled to -5 to +5 °C. To the cooled solution was added tri-*n*-butylphosphine (10 - 30 mmol,) followed by 1,1'-azobis(*N,N*-dimethylformamide) (10 - 30 mmol). The contents were mixed for 10 min and allowed to warm to 20 - 30 °C while continuing the mixing. The mixing continued for 15 - 30 h. After concentrating and extraction with hexanes subsequent washing of hexane layers with water and saturated aqueous sodium chloride solution the hexane layer was concentrated under vacuum to get an oily residue which was further purified by a silica gel plug to get a viscous oil.

¹H NMR (500 MHz, CDCl₃) δ 8.03 (dd, *J* = 7.2, 1.9 Hz, 1H), 7.70 – 7.59 (m, 3H), 5.14 – 5.01 (m, 6H), 3.90 (d, *J* = 6.8 Hz, 4H), 2.10 – 1.92 (m, 16H), 1.68 (d, *J* = 1.7 Hz, 6H), 1.61 – 1.54 (m, 18H).

¹³C NMR (126 MHz, CDCl₃) δ 140.97, 135.64, 134.60, 133.24, 131.58, 131.53, 131.10, 124.42, 124.24, 123.73, 118.96, 44.52, 39.85, 39.74, 26.88, 26.41, 25.85, 17.84, 16.30, 16.17.

Example 6: Synthesis of (*2E, 6E*)-bis((*2E, 6E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)amine (Bis-farnesylamine)

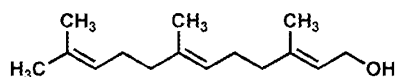
To a solution of 2-nitro-*N,N*-bis((*2E,6E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl) benzenesulfonamide prepared in Example 5 above (0.5 - 1.5 mmol) in *N,N*-dimethylformamide (1 - 5 mL) was added lithium hydroxide (1 - 5 mmol) followed by thioglycolic acid (0.5 - 3 mmol). They were mixed for 12 - 30 h at ambient temperature. The reaction mixture was diluted with saturated aqueous sodium bicarbonate solution and H₂O (5 - 50 mL). After extraction with hexane washing the hexane layer with water and saturated aqueous sodium chloride solution and drying with anhydrous sodium sulfate it was concentrated at 40 °C. The resultant oily residue was further purified by solvent extraction with hexane and acetonitrile to get bis-farnesylamine as an orange oil.

¹H NMR (500 MHz, CDCl₃) δ 5.27 (t, *J* = 6.9 Hz, 2H), 5.10 (dt, *J* = 13.3, 7.0 Hz, 4H), 3.22 (d, *J* = 6.8 Hz, 4H), 2.04 (ddq, *J* = 38.7, 23.0, 7.4 Hz, 16H), 1.68 (s, 6H), 1.64 (s, 6H), 1.60 (s, 12H).

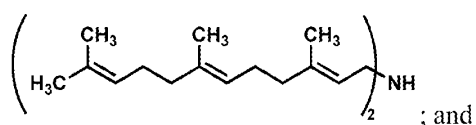
¹³C NMR (126 MHz, CDCl₃) δ 137.93, 135.30, 131.44, 124.51, 124.17, 123.02, 46.80, 39.86, 39.80, 29.85, 26.90, 26.63, 25.84, 17.83, 16.46, 16.15.

Claims

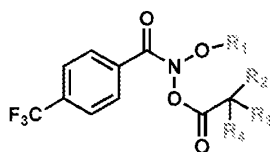
1. A high purity squalene composition comprising squalene with a purity of at least 98% by weight and less than 1ppm heavy metals.
2. A method of preparing squalene comprising (1) reacting *trans, trans*-farnesol



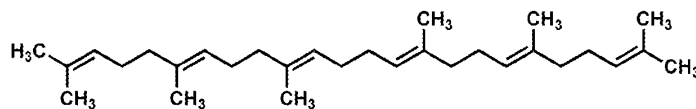
with reagents to form bis-farnesylamine



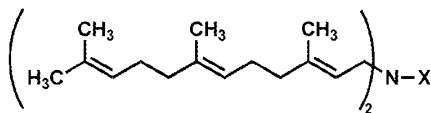
(2) reacting bis-farnesylamine with



wherein **R1** is benzyl, and **R2**, **R3**, and **R4** are independently C₁₋₁₀ alkyl, to form squalene

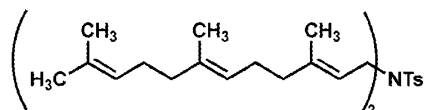


3. The method of claim 2, wherein **R2**, **R3**, and **R4** are methyl.
4. The method of claim 2, wherein the reagents in step (1) & (2) are p-toluene sulfonamide, cyanomethylenetriethylphosphorane (CMBP) and sodium naphthalene.
5. The method of claim 2, wherein in step (1) *trans, trans*-farnesol is reacted with reagents to form an intermediate



wherein **X** is tosylate and the intermediate is reacted with sodium naphthalene to form bis-farnesylamine., may be trityl which is reacted with any acid, or may be nosyl which is reacted with thioglycolic acid and a base to form bis-farnesylamine.,

6. The method of claim 4, wherein in step (1) *trans, trans*-farnesol is reacted with p-toluenesulfonamide and (Tributylphosphoranylidene)acetonitrile solution or cyanomethylenetriethylphosphorane (CMBP) to form bis-farnesyl-tosylamine



and then bis-farnesyl-tosylamine is reacted with sodium naphthalene to form bis-farnesylamine.

7. The method of claim 2, wherein the synthesis occurs in the absence of a transition metal catalyst.

8. A composition comprising substantially pure squalene prepared using the method of claim 2, wherein the substantially pure squalene comprises greater than 98% squalene by weight.

9. A method of making a vaccine formulation comprising adding the substantially pure squalene of claim 8 into an oil-in-water emulsion to form a vaccine formulation.

10. A method of making a drug delivery formulation comprising adding the substantially pure squalene of claim 8 into an oil-in-water emulsion to form a drug delivery formulation.

11. A method of making a cosmetic formulation comprising adding the substantially pure squalene of claim 8 into a cosmetic formulation.

12. A method of improving anti-inflammatory and antioxidant properties of lipid nanoparticle (LNP) formulations comprising adding the substantially pure squalene of claim 8 into a lipid nanoparticle formulation.

13. The method of claim 2, wherein the reagent in step (1) is 2-Nitrobenzenesulfonamide.