

CRYSTALLINE AND LIQUID CRYSTALLINE 25-HYDROXY-CHOLEST-5-EN-3-SULFATE SODIUM AND METHODS FOR PREPARING SAME

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to United States Provisional Patent Application Serial No. 62/954,279 filed on December 27, 2019, the disclosure of which application is herein incorporated by reference.

INTRODUCTION

[0002] It has been shown previously that cholesterol metabolite, 5-cholesten-3 β -25-diol-3-sulphate (“25HC3S”), decreases lipid biosynthesis and increases cholesterol secretion and degradation, and may be useful for the treatment and prevention of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation (e.g., non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, and atherosclerosis).

[0003] Cholesterol is used by the body for the manufacture and repair of cell membranes, and the synthesis of steroid hormones and vitamin D, and is transformed to bile acids in the liver. There are both exogenous and endogenous sources of cholesterol. The average American consumes about 450 mg of cholesterol each day and produces an additional 500 mg to 1,000 mg in the liver and other tissues. Another source is the 500 mg to 1,000 mg of biliary cholesterol that is secreted into the intestine daily, and about 50 percent is reabsorbed (enterohepatic circulation).

[0004] High serum lipid levels (hypercholesterolemia and hypertriglyceridemia) are associated with the accumulation of cholesterol in arterial walls, and can result in NAFLD and atherosclerosis. The plaques that characterize atherosclerosis inhibit blood flow and promote clot formation, and can ultimately cause death or severe disability via heart attacks and/or stroke. A number of therapeutic agents for the treatment of hyperlipidemia have been developed and are widely prescribed by physicians. Unfortunately, only about 35% of patients are responsive to the currently available therapies.

[0005] Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the United States. This condition is associated with obesity, type-II adult onset diabetes, sedentary lifestyle, and diets high in fat. The earlier stage of NAFLD, fatty liver, is potentially reversible when proper treatment steps are taken. However, left unchecked, it can progress to inflammation of liver cells (non-alcoholic steatohepatitis, or NASH) which is much more difficult to treat. Without treatment, NASH can result in irreversible scarring of liver tissue (steatonecrosis), with the potential to cause cirrhosis, liver failure, and liver cancer.

[0006] 25HC3S has been disclosed as a pharmaceutically acceptable salt, such as a sodium salt, for example (e.g., U.S. Patent 10,144,759 and Ogawa et al., Steroids, 74, 81-87 (2009)). Crystalline solids tend to be more favorable for processing, storage, and stability than non-crystalline solids. However, energetics may not favor the ready formation of suitable crystalline solids and polymorphism may make creating stable crystalline solids of a particular active pharmaceutical ingredient impractical. Herein, the inventors disclose crystalline 25HC3S sodium including stable anhydrides, solvates, and hydrates as well as liquid crystal 25HC3S sodium. Methods for preparing 25HC3S are also provided.

SUMMARY

[0007] In some aspects of the present disclosure, crystalline 25HC3S sodium is provided.

[0008] In some aspects of the present disclosure, stable crystalline 25HC3S sodium is provided.

[0009] In further aspects of the present disclosure, hydrates of crystalline 25HC3S sodium are provided.

[0010] In yet further aspects of the present disclosure, monohydrates of crystalline 25HC3S sodium are provided.

[0011] In still further aspects of the present disclosure, dihydrates of crystalline 25HC3S sodium are provided.

[0012] In additional aspects of the present disclosure, variable hydrates of crystalline 25HC3S sodium are provided.

[0013] In some aspects of the present disclosure, anhydrous crystalline 25HC3S sodium is provided.

[0014] In additional aspects of the present disclosure, Form I, Form II, Form III, Form IX, Form XI, and Form XIII of crystalline 25HC3S sodium are provided.

[0015] In additional aspects of the present disclosure, liquid crystal 25HC3S sodium is provided.

[0016] In some aspects of the present disclosure, mesophases of 25HC3S sodium are provided.

[0017] In further aspects of the present disclosure, Form V 25HC3S sodium is provided.

[0018] In yet additional aspects of the present disclosure, mixtures of two or more of Form I of 25HC3S sodium, Form II of 25HC3S sodium, Form III of 25HC3S sodium, Form V of 25HC3S sodium, Form IX of 25HC3S sodium, Form XI of 25HC3S sodium, or Form XIII of 25HC3S sodium are provided.

[0019] In some aspects of the present disclosure, methods of treating one or more of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation, for example, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, or atherosclerosis, comprising administering to a patient in need thereof an effective amount of a compound or pharmaceutical composition thereof of crystalline or liquid crystal mesophase 25HC3S sodium are provided.

[0020] In further aspects of the present disclosure, pharmaceutical compositions comprising crystalline 25HC3S sodium or liquid crystal 25HC3S sodium or both and at least one pharmaceutically acceptable excipient are provided.

[0021] In further aspects of the present disclosure, pharmaceutical compositions comprising mixtures of two or more of crystalline 25HC3S sodium Form I, crystalline 25HC3S sodium Form II, crystalline 25HC3S sodium Form III, liquid crystal 25HC3S sodium Form V, crystalline 25HC3S sodium Form IX, crystalline 25HC3S sodium Form XI, or crystalline 25HC3S sodium Form XIII and at least one pharmaceutically acceptable excipient are provided.

[0022] Aspects of the present disclosure also include methods for preparing 25HC3S. In some cases, methods include contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfating agent to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt.

[0023] In some cases, the sulfating agent is chosen from sulfur trioxide complexes, sulfuric acid compounds, sulfonic acid compounds, and sulfonate compounds. In some cases, the sulfating agent is a sulfur trioxide-pyridine complex. In some cases, the sulfating agent is chosen from sulfur trioxide dimethyl formamide, sulfur trioxide triethylamine, and sulfur trioxide trimethylamine. In some cases, the sulfating reagent is sulfuric acid and acetic anhydride and pyridine. In some cases, the sulfating reagent is sulfur trioxide triethylamine and pyridine. In some cases, the sulfating reagent is chosen from chlorosulfonic acid and pyridine and chlorosulfonic acid and 2,6-lutidine. In some cases, the sulfating reagent is ethyl chlorosulfonate. In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt. In some cases, the sulfating reagent is contacted with an anhydride prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some cases, the anhydride is chosen from acetic anhydride, trifluoroacetic anhydride and triflic anhydride. In some cases, the sulfating reagent is contacted with 25-hydroxy-(3 β)-cholest-5-en-3-ol in the presence of a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is present as particles (e.g., seed crystals produced in a previous reaction or purified reaction batch).

[0024] In certain cases, the sulfating reagent is characterized prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In certain cases, the sulfating reagent is characterized by ¹H-NMR. In some cases, characterizing the sulfating reagent includes determining the extent of degradation of the sulfating reagent prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In certain cases, determining the extent of degradation of the sulfating reagent includes determining the amount of impurity in the sulfating reagent prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[0025] In some cases, methods include quenching unreacted sulfating agent after producing the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In some cases, quenching the unreacted sulfating agent includes adding water to the reaction mixture after producing the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, quenching the unreacted sulfating agent includes adding water to the reaction mixture followed by the addition of at least one base pyridine to the reaction mixture. In some cases, the at least one base is selected from a trialkylamine such as triethylamine or trimethylamine. In some cases, the at least one base is selected from 2,6-lutidine or pyridine. In certain cases the base is pyridine. In

some cases, the unreacted sulfating agent in the reaction mixture is quenched under slow agitation.

[0026] In some cases, methods include purifying the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt prior to contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with the at least one metal salt. In some cases, the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has a purity of 70% or greater, such as 80% or greater, 90% or greater, 95% or greater, 98% or greater, or 99% or greater.

[0027] In certain cases, the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has one or more by-products of sulfation (e.g., by-products from sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol where the one or more by-products is present in an amount of 5% w/w or less, 4% w/w or less, 3% w/w or less, 2% w/w or less, or 1% w/w or less, relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In some cases, the bis-sulfated product (i.e., 5-cholesten-3 β -25-diol-disulfate) is present in the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt composition in an amount of 5% w/w or less, 4% w/w or less, 3% w/w or less, 2% w/w or less, or 1% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[0028] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is purified by liquid chromatography. In some cases, purifying the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt includes liquid chromatography employing a silica gel stationary phase and a mobile phase that includes at least one base. In some case, the at least one base is pyridine.

[0029] In some cases, one or more fractions collected from liquid chromatography may be combined. In some cases, the combined fractions may be concentrated. In certain cases, the combined fractions are concentrated by distillation. In certain cases, the combined fractions are concentrated under vacuum. In certain cases, the combined fractions are concentrated by distillation under vacuum. In some cases, the combined fractions are contacted with a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, the combined fractions are contacted with one or more particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (e.g., particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt) is contacted with the combined fractions by adding the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic

cationic salt during distillation of the combined fractions. In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is contacted with the combined fractions by adding the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt after distillation of the combined fractions. In some cases, the combined fractions are concentrated and contacted with a composition containing particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt and at least one solvent.

[0030] In some cases, methods to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt includes contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one sodium salt. In some cases, the at least one sodium salt is chosen from sodium acetate, sodium iodide, sodium chloride, sodium hydroxide and sodium methoxide. In certain cases, methods include contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with sodium iodide to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate sodium salt.

[0031] In some cases, methods for preparing 25-hydroxy-3 β -cholesten-5-en-3-sulfate include contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfur trioxide-pyridine complex to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with at least one sodium salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate sodium salt.

[0032] In some cases, methods for preparing 25-hydroxy-3 β -cholesten-5-en-3-sulfate include contacting (3 β)-cholest-5-en-3-ol with a sulfating agent to produce a first (3 β)-cholest-5-en-3-sulfate organic cationic salt; contacting the first (3 β)-cholest-5-en-3-sulfate organic cationic salt with an organic base to produce a second (3 β)-cholest-5-en-3-sulfate organic cationic salt; oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt in the presence of at least one surfactant to produce a 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt; generating a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt by deoxygenation; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt.

[0033] In some cases, the sulfating agent is chosen from sulfur trioxide complexes, sulfuric acid compounds, sulfonic acid compounds, and sulfonate compounds. In some cases, the sulfating agent is a sulfur trioxide-pyridine complex. In some cases, the sulfating agent is chosen from sulfur trioxide dimethyl formamide, sulfur trioxide triethylamine, and sulfur trioxide

trimethylamine. In some cases, the sulfating agent is sulfuric acid and acetic anhydride and pyridine. In some cases, the sulfating agent is chosen from chlorosulfonic acid and pyridine. In some cases, the sulfating agent is chosen from chlorosulfonic acid and 2,6-lutidine. In some cases, the sulfating agent is chosen from ethyl chlorosulfonate. In some cases, the first (3 β)-cholest-5-en-3-sulfate organic cationic salt is a (3 β)-cholest-5-en-3-sulfate pyridinium salt.

[0034] In some cases, the organic base contacted with the first (3 β)-cholest-5-en-3-sulfate organic cationic salt is chosen from a hydroxide base. In some cases, the hydroxide base is chosen from tetraethylammonium hydroxide, tetrabutylammonium hydroxide, tetrapropylammonium hydroxide and tetramethylammonium hydroxide. In some cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is chosen from a tetraethylammonium cationic salt, a tetrabutylammonium cationic salt, a tetrapropylammonium cationic salt and a tetramethylammonium cationic salt.

[0035] In cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized in the presence of at least one surfactant. In some cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with a composition having an oxidizing agent and at least one surfactant. In certain cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt to produce a 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with a composition having potassium peroxymonosulfate in the presence of at least one surfactant. In some cases, the at least one surfactant is chosen from non-ionic surfactants, anionic surfactants, cationic surfactants and zwitterionic surfactants. Non-ionic surfactants may be chosen from polyoxyethylene glycol ether surfactants (e.g., polyoxyethylene glycol octylphenol ether), polyoxyethylene glycol sorbitan alkyl esters, sorbitan alkyl esters, block copolymers of polyethylene glycol and polypropylene glycol, among other non-ionic surfactants. Anionic surfactants may be chosen from surfactants having an anionic functional head group, such as a sulfonate, phosphate, sulfate or carboxylate head group-containing surfactant. For example, anionic surfactants may be chosen from alkyl sulfates such as ammonium lauryl sulfate, dioctyl sodium sulfosuccinate, perfluorooctanesulfonate, perfluorononanoate, perfluorooctanoate, a linear alkylbenzene sulfonate, an alkyl-aryl ether phosphate, sodium lauryl ether sulfate, lignosulfonate or sodium stearate, among other anionic surfactants. Cationic surfactants may be chosen from surfactants

having a cationic functional head group, such as a pyridinium or a quaternary ammonium head group. For example, cationic surfactants may be chosen from cetyltrimethylammonium hydrogen sulfate, tetrabutylammonium hydrogen sulfate, cetyltrimethylammonium bromide, tetrabutylammonium bromide, tetrabutylammonium iodide, tetrabutylphosphonium bromide, tetraoctylammonium bromide, tetraoctylammonium iodide, benzyltriethylammonium chloride, benzyltriethylammonium bromide, benzylcetyldimethylammonium chloride and benzylcetyldimethylammonium bromide. Zwitterionic surfactants include both cationic and anionic centers, such as a sultaine (e.g., 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or a betaine (e.g., cocamidopropyl betaine). In certain cases, the surfactant is an Extran laboratory soap, La Parisienne soap or DL- α -tocopherol methoxypolyethylene glycol succinate (e.g., TPGS-750-M-2).

[0036] In some cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is contacted with an oxidizing agent and at least one ketone in the presence of at least one surfactant. In some cases, methods include contacting the oxidizing agent (e.g., potassium peroxymonosulfate) with the at least one ketone in the presence of the at least one surfactant in a separate oxidative reaction mixture and contacting the oxidative reactive mixture with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, methods include oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt in the presence of water. In some instances, the at least one ketone is chosen from tetrahydrothiopyran-4-one 1,1-dioxide and halogenated ketones. In some cases, the halogenated ketones are chosen from 1,1,1-trifluoro-2-butanone, 4,4-difluorocyclohexanone, 2,2,2,4'-tetrafluoroacetophenone, and 1,1,1-trifluoroacetone. In certain cases, the at least one ketone is 1,1,1-trifluoro-2-butanone.

[0037] In certain cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one oxidative species. In some instances, the at least one oxidative species is chosen from dioxiranes. In some instances, the dioxiranes are generated in situ in a composition having the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In some instances, the dioxiranes are generated separately (e.g., in a separate reaction container, e.g., flask) and then contacted with the composition having the second (3 β)-cholest-5-en-3-sulfate organic cationic salt.

[0038] In certain cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized in the presence of at least one base. In certain cases, the at least one base is chosen

from weak bases. In some cases, the at least one base is chosen from potassium hydrogen carbonate, sodium hydrogen carbonate, potassium phenoxide, sodium citrate buffer, sodium phosphate buffer, potassium formate and potassium acetate. In certain cases, the at least one base is potassium hydrogen carbonate.

[0039] In some cases, generating the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt includes deoxygenation by contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc. In certain instances, the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt is contacted with zinc in the presence of at least one halide and at least one acid. In some cases, the at least one halide is chosen from iodine and metal halides. In some cases, the metal halide is chosen from sodium iodide and lithium iodide. In some cases, the at least one acid is chosen from weak acids. In some cases, the at least one acid is chosen from acetic acid, hydrochloric acid, citric acid, para-toluene sulfonic acid, formic acid, and methane sulfonic acid. In some cases, methods include contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc in the presence of acetic acid to generate the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[0040] In some cases, methods to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt includes contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one sodium salt. In some cases, the at least one sodium salt is chosen from sodium acetate, sodium iodide, sodium chloride, sodium hydroxide and sodium methoxide. In certain cases, methods include contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with sodium iodide to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate sodium salt.

BRIEF DESCRIPTION OF THE FIGURES

[0041] Figure 1 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.

[0042] Figure 2 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.

[0043] Figure 3 is an XRPD Diffractogram of liquid crystal 25HC3S sodium Form V.

[0044] Figure 4 is an XRPD Diffractogram of liquid crystal 25HC3S sodium Form V.

[0045] Figure 5 is an XRPD Diffractogram of crystalline 25HC3S sodium Form IX.

[0046] Figure 6 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XI.

[0047] Figure 7 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XI.

- [0048] Figure 8 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XIII.
- [0049] Figure 9 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XIII.
- [0050] Figure 10 is indexing results for crystalline 25HC3S sodium Form I.
- [0051] Figure 11 is thermograms for crystalline 25HC3S sodium Form I.
- [0052] Figure 12 is a variable temperature XRPD experiment for crystalline 25HC3S sodium Form I.
- [0053] Figure 13 is a variable humidity XRPD experiment for crystalline 25HC3S sodium Form I and crystalline 25HC3S sodium Form XIII.
- [0054] Figure 14 is XRPD Diffractograms from the dehydration of crystalline 25HC3S sodium Form I.
- [0055] Figure 15 is XRPD Diffractograms from the dehydration of crystalline 25HC3S sodium Form I (expanded).
- [0056] Figure 16 is a DVS Isotherm for crystalline 25HC3S sodium Form II.
- [0057] Figure 17 is indexing results for crystalline 25HC3S sodium Form II (99:01 acetonitrile (ACN)/H₂O slurry, 0.21 a_w, 3 days).
- [0058] Figure 18 is indexing results for crystalline 25HC3S sodium Form II (95:05 EtOH/H₂O slurry, 0.30 a_w, 10 days).
- [0059] Figure 19 is indexing results for crystalline 25HC3S sodium Form II (Acetone slurry, 55 °C, 1 day).
- [0060] Figure 20 is an XRPD peak position variability for crystalline 25HC3S sodium Form II for slurries indexed in Figures 17-19, shown from approximately 4°2θ to approximately 11°2θ.
- [0061] Figure 21 is an XRPD peak position variability for crystalline 25HC3S sodium Form II for slurries indexed in Figures 17-19, shown from approximately 13°2θ to approximately 21.5°2θ.
- [0062] Figure 22 is a variable humidity XRPD experiment for crystalline 25HC3S sodium Form II.
- [0063] Figure 23 is an XRPD peak position variability for crystalline 25HC3S sodium Form II observed by variable humidity XRPD, shown from approximately 7.5°2θ to approximately 10.2°2θ.
- [0064] Figure 24 is TGA thermograms for crystalline 25HC3S sodium Form II.
- [0065] Figure 25 is DSC thermograms for crystalline 25HC3S sodium Form II.

- [0066] Figure 26 is a cycling DSC experiment for crystalline 25HC3S sodium Form II.
- [0067] Figure 27 is a variable temperature XRPD experiment for crystalline 25HC3S sodium Form II.
- [0068] Figure 28 is a TGA thermogram for liquid crystal 25HC3S sodium Form V.
- [0069] Figure 29 is a DSC thermogram for liquid crystal 25HC3S sodium Form V.
- [0070] Figure 30 is indexing results for crystalline 25HC3S sodium Form XIII (generated from a mixture of crystalline 25HC3S sodium Form I and Form XIII exposed to 70°C vacuum for 2 days).
- [0071] Figure 31 is XRPD Diffractograms of crystalline 25HC3S sodium Form III and Form IX.
- [0072] Figure 32 is indexing results for crystalline 25HC3S sodium Form III.
- [0073] Figure 33 is a ¹H-NMR spectrum of crystalline 25HC3S sodium Form IX in solution.
- [0074] Figure 34 is a ¹H-NMR spectrum of 25HC3S sodium in solution.
- [0075] Figure 35 is an XRPD Diffractogram of crystalline 25HC3S sodium Form III.
- [0076] Figure 36 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.
- [0077] Figure 37 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.
- [0078] Figure 38 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.
- [0079] Figure 39 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I and Form XIII.
- [0080] Figure 40 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XIII.
- [0081] Figure 41 is an XRPD Diffractogram of crystalline 25HC3S sodium Form XIII.
- [0082] Figure 42 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.
- [0083] Figure 43 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.
- [0084] Figure 44 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.
- [0085] Figure 45 is an XRPD Diffractogram of crystalline 25HC3S sodium Form III and Form IX Mixture.
- [0086] Figure 46 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.
- [0087] Figure 47 is an XRPD Diffractogram of liquid crystalline 25HC3S sodium Form V.
- [0088] Figure 48 is an XRPD Diffractogram of liquid crystalline 25HC3S sodium Form V.
- [0089] Figure 49 is an XRPD Diffractogram of crystalline 25HC3S sodium Form III.
- [0090] Figure 50 is an XRPD Diffractogram of crystalline 25HC3S sodium Form III.

- [0091] Figure 51 is an XRPD Diffractogram of crystalline 25HC3S sodium Form IX.
- [0092] Figure 52 is an XRPD Diffractogram of crystalline 25HC3S sodium Form III and Expanded.
- [0093] Figure 53 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.
- [0094] Figure 54 is an XRPD Diffractogram of crystalline 25HC3S sodium Form II.
- [0095] Figure 55 is an XRPD Diffractogram of crystalline 25HC3S sodium Form I.
- [0096] Figure 56 is an XRPD Diffractogram of liquid crystalline 25HC3S sodium Form V.
- [0097] Figure 57 is a ^1H -NMR spectrum of three different samples of sulfur trioxide pyridine in deuterated acetone.
- [0098] Figure 58A is an enhancement of the region between 8.1 and 9.3 ppm of a ^1H -NMR spectrum of a sample of sulfur trioxide-pyridine with 21% impurity in deuterated acetone. Figure 58B is an enhancement of the region between 8.1 and 9.3 ppm of a ^1H -NMR spectrum of a sample of sulfur trioxide-pyridine with 33% impurity in deuterated acetone. Figure 58C is an enhancement of the region between 8.1 and 9.3 ppm of a ^1H -NMR spectrum of a sample of sulfur trioxide-pyridine with 36% impurity in deuterated acetone.

DETAILED DESCRIPTION

Crystalline and Liquid Crystalline 25-hydroxy-3 β -cholesten-5-en-3-sulfate (25HC3S)

[0099] Crystalline 25HC3S sodium and liquid crystalline 25HC3S are readily analyzed by x-ray powder diffraction. An x-ray powder diffraction pattern is an x-y graph with $^{\circ}2\theta$ (diffraction angle) on the x-axis and intensity on the y-axis. The x-axis can also be in the form of d-spacings which is related to the diffraction angle via the Bragg's law whereby $2d\sin\theta = n\lambda$ where d is the d-spacing and λ is the wavelength of the incident x-ray wave. The pattern contains peaks which may be used to characterize crystalline 25HC3S sodium. Unless otherwise specified, peaks are referred to by their position on the x-axis and not their y-axis intensity. It can also occur that due to sample orientation, a peak that is present in one sample on one instrument may not be present in another sample taken on a different instrument due to the orientation of the sample with respect to the instrument.

[00100] The data from x-ray powder diffraction may be used in multiple ways to characterize crystalline forms. For example, the entire x-ray powder diffraction pattern output from a

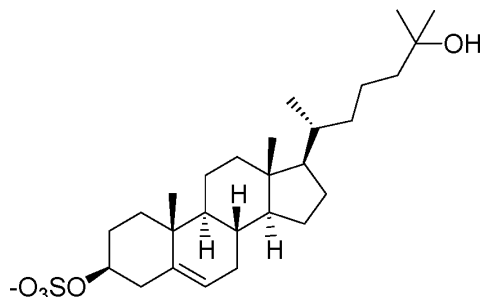
diffractometer may be used to characterize 25HC3S sodium. A smaller subset of such data, however, may also be, and typically is, suitable for characterizing 25HC3S sodium. For example, a collection of one or more peaks from such a pattern may be used to characterize crystalline 25HC3S sodium. In the present application, all reported peak values are in $^{\circ}2\theta$ with Cu-K α radiation as set forth in Example 16 and Example 17. Indeed, often even a single x-ray powder diffraction peak may be used to characterize such a crystalline form. When crystalline 25HC3S sodium herein is characterized by “one or more peaks” of an x-ray powder diffraction pattern and such peaks are listed, what is generally meant is that any combination of the peaks listed may be used to characterize crystalline 25HC3S sodium. Further, the fact that other peaks are present in the x-ray powder diffraction pattern, generally does not negate or otherwise limit that characterization.

[00101] In addition to the variability in peak intensity, there may also be variability in the position of peaks on the x-axis. This variability can, however, typically be accounted for when reporting the positions of peaks for purposes of characterization. Such variability in the position of peaks along the x-axis may derive from several sources (e.g., sample preparation, particle size, moisture content, solvent content, instrument parameters, data analysis software, and sample orientation). For example, samples of the same crystalline material prepared under different conditions may yield slightly different diffractograms, and different x-ray instruments may operate using different parameters, and these may lead to slightly different diffraction patterns from the same crystalline solid.

[00102] Due to such sources of variability, it is common to recite x-ray diffraction peaks using the word “about” prior to the peak value in $^{\circ}2\theta$. For purposes of data reported herein, that value is generally $\pm 0.1^{\circ}2\theta$. This generally means that on a well-maintained instrument one would expect the variability in peak measurement to be $\pm 0.1^{\circ}2\theta$. Unless specified otherwise, x-ray powder diffraction peaks cited herein are generally reported with this variability of $\pm 0.1^{\circ}2\theta$ and are generally intended to be reported with such a variability whenever disclosed herein whether the word “about” is present or not, however, variability may, in some instances, be as high as $\pm 0.2^{\circ}2\theta$ or even higher depending on instrumentation conditions.

[00103] As described herein, the compound 25-hydroxy-3 β -cholesten-5-en-3-sulfate (25HC3S) refers to [(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-

dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate, the compound of Formula I:



Formula I

[00104] The present disclosure uses the term “Form” to identify different crystalline forms of crystalline 25HC3S sodium or liquid crystalline forms. The differences in the forms can be seen by structure, such as x-ray powder diffraction; properties such as hygroscopicity or thermal behaviors; and/or both. The use of the term “Form I” means crystalline 25HC3S sodium of Form I. Likewise, “Form II” means crystalline 25HC3S sodium of Form II. Similarly, Form III, Form IX, Form XI, and Form XIII mean crystalline 25HC3S sodium of Form III, Form IX, Form XI, and Form XIII, respectively. Form V means a form of 25HC3S sodium that is in a liquid crystalline phase.

[00105] The generated solids were observed by one or more of polarized light microscopy and x-ray powder diffraction. Materials exhibiting unique crystalline x-ray powder diffraction patterns, based on visual inspection of peaks associated with these materials, were given designations set forth in the present disclosure.

[00106] Table 1 summarizes some of the experiments performed to obtain the reported forms of 25HC3S sodium in the present disclosure, namely those experiments associated with Examples 1-36.

[00107] Cells in Table 1 with an asterisk (*) indicate a solid was identified which is not one of Form I, Form II, Form III, Form V, Form IX, Form XI, or Form XIII.

[00108] The times, temperatures, and humidity in Table 1 are approximate. The term “B” means birefringent and “NB” means non-birefringent when a sample is viewed by polarized light microscopy with cross polars. Solvent-based methods were used to screen for crystalline 25HC3S sodium using a diversity of solvents and conditions as set forth in Table 1. Methods using solvents or solvent mixtures included, for example, cooling a solution, evaporation,

antisolvent addition, and suspensions (slurries). Variations on these methods can include changes in solvent, solvent mixtures, antisolvent, temperature, cooling rate, concentration, rate of addition, and order of mixing, to name a few possibilities.

Table 1 – A Screen of 25HC3S sodium

Solvent		Method	Observation	Result
acetone	(i)	slurry, ambient, 14 days, dried under N ₂ for <20 seconds	opaque fines, NB	II
	(ii)	(i) sub sample analyzed wet	-	II
	(iii)	slurry, 55 °C, 1 day	opaque fines	II
ACN	(i)	slurry, ambient, 14 days, dried under N ₂ for <20 seconds	opaque fines, NB	II
	(ii)	(i) sub sample analyzed wet	-	II
	(iii)	slurry, 55 °C, 1 day, analyzed wet	plug formed at top isolated by tweezers very fine particles, wispy, B	II
	(iv)	slurry, 55 °C, 1 day, dried under N ₂	-	II
	(v)	slurry, 55 °C, 1 day, analyzed wet	-	II
	(vi)	slurry, 60 °C, 1 day, N ₂ dried	-	II
DCM	(i)	heated to reflux, filtered 1. fast evaporation 2. treated with heptane	1. oily residue, fines, limited, B 2. hazy	-
	(ii)	slurry, ambient, 14 days	opaque fines, NB	I + II
diethyl ether		slurry, ambient, 14 days 1. filtered 2. N ₂ dried	1. thick filter cake 2. collapsed, fines, B	XI
1,4-dioxane	(i)	slurry, ambient, 14 days	opaque fines, NB limited recovery	*
	(ii)	slurry, ambient, 5 days	gel/plug translucent, B striations in solids	* + II
	(iii)	(ii) slurried ambient, 10 days	-	*
DMSO	(i)	solvent/anti-solvent 1. added to EtOAc 2. ambient, 3 days 3. decanted, chopped	1. hazy followed by precipitates, NB, formed gel plug in suspension	limited reflections

Solvent		Method	Observation	Result
		plug	2. gel plug remained 3. solvent displaced, slight opaqueness	
	(ii)	(i) dried w/ N ₂	$\frac{3}{4}$ reduction in size gel/film with fines, B	*
DMSO	(iii)	(i) post XRPD 1. seeded with (ii) 2. EtOAc added 3. fast evaporation 4. DMSO drops added stored ambient	1. gel + fines 2. gel "broke" no bulk crystallization 3. film, limited fines 4. bulk nucleated, dendritic, blades B	*
EtOH	(i)	fast evaporation partial analyzed wet	wispy, fine aciculars, B	II
	(ii)	(iii) bulk slurry fast evaporation, N ₂	-	III + peaks
	(iii)	slow evaporation partial 1. singles isolated 2. sub sample analyzed wet	1. rosettes of blades and aciculars, B wouldn't elucidate 2. -	III
	(iv)	1. 12 mg/mL suspension 2. capped, 5 min. 3. sub sampled, solvent flushed 4. sub sample analyzed wet	1. faint haze 2. rosettes of fine aciculars 3. crystals turn opaque 4. -	III
	(v)	1. solvent added 2. heated on 75 °C plate 3. N ₂ dried, 75 °C plate for 15 minutes	1. - 2. solvent absorbed 3. cake collapsed	III + IX
	(vi)	1. solvent added 2. heated on 75 °C plate 3. filtered, EtOH rinse and dried under N ₂	1. - 2. thick slurry 3. fine blades and aciculars, B	III + II
	(vii)	slurry, ambient, 5 days	large blades and fine aciculars, B	III
EtOAc		slurry, ambient, 5 days	paste/gel, decanted	II
IPA	(i)	slurry, ambient, 21 days	irregular fines, B	II
	(ii)	1. slow cool from reflux 2. warmed to ambient	1. solid gel plug, NB 2. solution	-
	(iii)	(ii) solution 1. fast evaporation, $\frac{1}{2}$ 2. sonicated 3. fast evaporation	1. gel plug, striations of opaque regions, NB, white wispy B material in upper regions	V

Solvent		Method	Observation	Result
			2. slurry, hazy 3. white fines, B	
MeOH	(i)	1. contacted with MeOH 2. additional MeOH 3. fast evaporation	1. faint haze 2. no changes 3. thin film, cracked, striations, B	V
	(ii)	(i) reanalyzed after 7 days	appeared visually to be more crystalline	V
	(iii)	slow cool to ambient analyzed wet	irregular fines, B	* + V
	(iv)	cooling of solution 1. solvent added 2. filtered 3. freezer overnight 4. warmed ambient	1. faint haze 2. difficult/slow to filter 3. limited fines, B 4. clear solution	-
	(v)	slurry, ambient, 4 days	very fine aciculars, B	* + V
	(vi)	solvent/anti-solvent 1. heated MeOH solution 2. filtered into Et ₂ O 3. filtered, N ₂ dried	1. clear 2. difficult to filter, precipitation, NB 3. filter cake curled as dried	V
	(vii)	solvent/anti-solvent 1. heated MeOH solution 2. filtered into DCM 3. filtered	1. clear 2. precipitation, gel opaque, NB 3. gel cake isolated 4. gel crystallized after isolating, B	I
	(viii)	(vii) sub sample gel pressed between filter paper	solvent expelled, film, irregular solids, B	V
toluene	(i)	fast evaporation	limited material, oil fines, B	-
	(ii)	slurry, ambient, 21 days	irregular masses, zones/particles B	I
water	(i)	slurry, ambient, 14 days dried briefly under N ₂	very slow to filter opaque masses when pressed expelled water, gel like clumps, when pressed further between slide film exhibited B, mesophase like	V
	(ii)	(i) sub sample analyzed wet	-	V
acetone/H ₂ O 92:08 v/v	(i)	1. slurry, acetone, heated 2. removed, water added	1. slurry 2. majority dissolved	I

Solvent		Method	Observation	Result
0.98 a_w		3. left at ambient, 4 days 4. filtered, N ₂ dried for 10 minutes	3. limited flocculent, gel deposits on vial converted to flocculent, then dendritic 4. fines, B	
	(ii)	(i) filtrate fast evaporation	dendritic and fine wispy aciculars	V
ACN/H ₂ O 97:03 v/v 0.50 a_w	(i)	slurry, ambient, 4 days analyzed wet cake	-	I + peak @ 9.0°
	(ii)	(i) sub sampled 1.5 hrs after initial solvent contact	-	I + peak @ 9.0°
	(iii)	(i) bulk dried under N ₂ for 0.5 hrs	-	I + XIII
heptane/ DMSO 100:1 v/v		slurry, ambient, 5 days	white fines, B	II

[00109] The effect of water activity (a_w) on the hydration state can be investigated through competitive water activity trituration experiments (slurries) in various aqueous solvent mixtures. The resulting solid phase can be characterized by x-ray powder diffraction. The experiments can establish the physically stable form at various a_w . Water activity can also be related to relative humidity in that $RH\% = a_w \times 100$. Therefore, it is possible to directly relate the stability of an anhydrous/hydrate system in slurry experiments to solid-state stability. The slurry technique at controlled water activities can provide an accurate method of rapidly predicting the physically stable form in anhydrous/hydrate systems. The results from water activity experiments are provided in Table 2 and were done at approximately room temperature. The times, temperatures, and humidity are approximate. As with Table 1, the term “B” means birefringent when viewed by polarized light microscopy with cross polars.

Table 2 – Water Activity Slurries of 25HC3S Sodium

Water Activity (a_w) Solvent System (v:v)¹	Duration	Observation	Result
0.98 a_w 92:08 H ₂ O/acetone	10 days	irregular fines, B	V
0.82 a_w 92:08 ACN/H ₂ O	10 days	irregular fines, B	V
0.73 a_w 94:06 ACN/H ₂ O	8 days	fines, B	I + peak @ 9.0°
0.70 a_w 89:11 IPA/H ₂ O	10 days	fine aciculars, B	I
0.64 a_w 92:08 acetone/H ₂ O	10 days	irregular fines, B	I
0.50 a_w 97:03 ACN/H ₂ O	3 days	irregular fines, B	I
0.44 a_w 97:03 acetone/H ₂ O	7 days	irregular fines, B	I
0.38 a_w 98:02 ACN/ H ₂ O	3 days	irregular fines, B	I
0.30 a_w 95:05 EtOH/ H ₂ O	10 days	irregular fines, B	II
0.21 a_w 99:01 ACN/ H ₂ O	3 days	irregular fines, B	II
Combination of samples from 0.5 a_w and 0.38 a_w	56 days	combined slurries filtered and dried under N ₂ for 30 min.	I + XIII

[00110] Several x-ray powder diffraction patterns were indexed. “Indexing,” as used herein, generally refers to the process of determining the size and shape of the crystallographic unit cell given the peak positions in a diffraction pattern. The term gets its name from the assignment of Miller index labels to individual peaks. For example, if all of the peaks in a pattern are indexed

¹ Choy, B.; Reible, D. (1996). UNIFAC Activity Coefficient Calculator (Version 3.0) [Software]. University of Sydney, Australia and Louisiana State University, USA.

by a single unit cell, this can be strong evidence that the sample contains a single crystalline phase. Given the indexing solution, the unit cell volume may be calculated directly and can be useful to determine their solvation states. Indexing may also be a description of a crystalline form and provides a concise summary of all available peak positions for that phase at a particular thermodynamic state point.

[00111] Multiple stable crystalline forms of crystalline 25HC3S sodium are herein reported – Forms I, II, IX, XI, and XIII, and one liquid crystal form, Form V. In the present disclosure, “stable” means that the Form does not readily interconvert to another Form under a given set of conditions. A metastable form, can, however, so readily convert when exposed to certain conditions. Thus, a form that is stable under one set of conditions (e.g., humidity) may not be stable under another set of conditions. Not all forms were able to be indexed herein. In addition, Form III is a metastable form which formed and can be a starting material for Form IX. Although metastable, Form III was sufficiently stable for isolation for XRPD.

[00112] In many cases, crystalline 25HC3S sodium is provided including stable crystalline 25HC3S sodium. Examples of crystalline 25HC3S sodium include anhydrous crystalline 25HC3S sodium, hydrates of crystalline 25HC3S sodium, and solvates of crystalline 25HC3S sodium.

[00113] The hydrates of crystalline 25HC3S sodium include monohydrates, dihydrates, and variable hydrates. Liquid crystal hydrates of 25HC3S sodium are also provided herein.

[00114] In some cases, hydrates of crystalline 25HC3S sodium may be characterized by an x-ray powder diffraction pattern comprising one or more of (i) a peak at less than about $2.8^{\circ}2\theta$ such as between about $2.1^{\circ}2\theta$ and about $2.6^{\circ}2\theta$, (ii) a peak between about $4.3^{\circ}2\theta$ and about $4.6^{\circ}2\theta$, (iii) a peak between about $5.0^{\circ}2\theta$ and about $5.5^{\circ}2\theta$, (iv) a peak between about $8.6^{\circ}2\theta$ and about $9.1^{\circ}2\theta$ and (v) a peak between about $15.0^{\circ}2\theta$ and about $15.3^{\circ}2\theta$. In these and other cases, hydrates of crystalline 25HC3S sodium may be characterized by an x-ray powder diffraction pattern comprising one or more of (i) a peak between about $2.1^{\circ}2\theta$ and about $2.3^{\circ}2\theta$ and (ii) a peak between about $9.9^{\circ}2\theta$ and about $10.0^{\circ}2\theta$.

[00115] In some cases, anhydrous crystalline 25HC3S sodium may be characterized by an x-ray powder diffraction pattern comprising one or more of (i) a peak at between about $4.5^{\circ}2\theta$ and about $4.8^{\circ}2\theta$, (ii) a peak between about $9.8^{\circ}2\theta$ and about $9.9^{\circ}2\theta$, (iii) a peak at between about $14.1^{\circ}2\theta$ and about $14.3^{\circ}2\theta$, and (iv) a peak at about $16.1^{\circ}2\theta$.

[00116] In many cases, crystalline 25HC3S sodium Form I is provided for in the present disclosure. Form I is a dihydrate of crystalline 25HC3S sodium. Form I may be prepared as set forth in Table 1. For example, slurrying 25HC3S sodium in an acetone/H₂O solution, heating, removing the solution, adding water, and leaving at ambient results in Form I. Form I may also be prepared from methanol. Preparations of Form I are further described in Examples 20-23, and in a mixture with Form XIII in Example 24. The following Table 3 correlates the Examples producing Form I with the corresponding XRPD diffractogram figure.

Table 3 – Form I XRPD Examples / Figures

Example #	Figure
20	36
21	37
22	38
23	55
24	39

[00117] Table 4 shows peaks found in Figure 55, whereas peaks for the other figures in Table 3 are presented on the figures themselves.

Table 4 – Figure 55 Peak List

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
4.482064	1692.179000	0.255840	19.71534	14.68
5.511474	425.857900	0.204672	16.03508	3.69
6.629832	1796.915000	0.204672	13.33249	15.59
8.788433	6365.006000	0.204672	10.06204	55.22
10.006760	2252.715000	0.179088	8.83955	19.54
10.919930	1279.297000	0.179088	8.10231	11.10
13.149270	330.143300	0.255840	6.73323	2.86
14.526540	1649.401000	0.230256	6.09780	14.31
15.105460	11525.940000	0.179088	5.86538	100.00
15.722710	5185.476000	0.204672	5.63648	44.99
16.343610	3132.548000	0.179088	5.42372	27.18
17.302730	2804.740000	0.179088	5.12517	24.33
17.632490	3663.537000	0.153504	5.03006	31.79
18.491410	2629.055000	0.153504	4.79831	22.81
19.006600	1076.854000	0.204672	4.66939	9.34
20.279930	1715.928000	0.409344	4.37900	14.89
21.076530	1403.260000	0.409344	4.21525	12.17

21.521580	1343.477000	0.204672	4.12908	11.66
22.042530	1077.881000	0.153504	4.03266	9.35
22.663660	768.489500	0.409344	3.92353	6.67
23.901110	634.608200	0.255840	3.72312	5.51
24.707990	481.575700	0.204672	3.60333	4.18
26.801360	243.341400	0.409344	3.32645	2.11
29.289260	693.492100	0.204672	3.04931	6.02
30.747140	292.114300	0.255840	2.90797	2.53
33.013470	203.217300	0.307008	2.71334	1.76
38.783180	201.201500	0.460512	2.32194	1.75

[00118] Form I of crystalline 25HC3S sodium may be characterized by various analytical techniques, including by x-ray powder diffraction. The x-ray powder diffraction pattern of Form I, or portions thereof, may be used to identify Form I. Form I contains various x-ray powder diffraction peaks which alone or together may help identify the presence of Form I. For example, in many cases, Form I may be characterized by an x-ray powder diffraction pattern comprising a peak at about $2.1^{\circ}2\theta$ (not all diffractograms herein display such low angle peaks). In addition to the peak at about $2.1^{\circ}2\theta$, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about $5.4^{\circ}2\theta$, about $6.5^{\circ}2\theta$, about $10.8^{\circ}2\theta$, and about $15.0^{\circ}2\theta$.

[00119] In many cases, Form I may be characterized by an x-ray powder diffraction pattern comprising peaks at about $2.1^{\circ}2\theta$, about $6.5^{\circ}2\theta$, and about $10.8^{\circ}2\theta$. The x-ray powder diffraction pattern may, in some cases, further comprise one or more peaks at about $9.9^{\circ}2\theta$, about $15.0^{\circ}2\theta$, and about $15.6^{\circ}2\theta$.

[00120] Figure 1 is an x-ray powder diffraction pattern of a representative sample of Form I where the x-axis begins at $0^{\circ}2\theta$ and goes to $40^{\circ}2\theta$, whereas Figure 53 shows the same pattern but between about $3^{\circ}2\theta$ and about $39^{\circ}2\theta$. Either of Figure 1 or Figure 53 may, for example, be used to characterize Form I.

[00121] Form I appears to be stable at relative humidities at and between about 38% and about 70% RH. As such, Form I resulted from slurries in aqueous solvent mixtures with water activities at and between about 0.38 and about 0.70 a_w . Form I dehydrates to Form XIII when exposed to elevated temperatures or to humidity conditions near 0% RH. Form I is hygroscopic and is believed to form a liquid crystal called Form V at water activity conditions above 0.73 a_w .

[00122] The x-ray powder diffraction pattern of Form I was successfully indexed, indicating the pattern represents a single crystalline phase (Figure 10). The indexing result has a monoclinic

unit cell containing six molecules of 25HC3S sodium. The unit cell volume of $4544.7 \text{ \AA}^3 (\pm 5\%)$ results in a formula unit volume of $757 \text{ \AA}^3 (\pm 5\%)$ (6 molecules/cell) which would be consistent with a hydrate. The formula unit volume of Form I is larger than the formula unit for the anhydrous form, Form XIII, by approximately $52 \text{ \AA}^3$. The difference in volume provides sufficient room to accommodate up to 2 mol/mol of water. For reference, one water molecule occupies about $22 \text{ \AA}^3$.

[00123] Thermograms for Form I are shown in Figure 11. The TGA thermogram provides an 8% weight loss up to 130°C which occurs concurrently with broad dehydration endotherms in the DSC thermogram (Figure 11). Assuming the loss is due to the volatilization water, the weight loss corresponds to approximately 2.4 mol/mol water. The DSC curve in Figure 11 also shows endotherms near 168°C and 182°C . These events are associated with decomposition.

[00124] The dehydration of Form I results in form conversion to Form XIII. The conversion was confirmed with a variable temperature x-ray powder diffraction experiment (Figure 12) which shows Form XIII when heating to 170°C and then dropping to 135°C (Pattern 5 of Figure 12). During the same experiment, a decomposition product identified as trisodium hydrogen disulfate, $\text{Na}_3\text{H}(\text{SO}_4)_2$, was evident by 170°C . These results are consistent with the thermograms for Form I in Figure 11.

[00125] A variable humidity x-ray powder diffraction experiment on a mixture composed predominately of Form I and a minor amount of Form XIII is presented in Figure 13. The laboratory humidity in which the sample was prepared for analysis was 15% relative humidity (“RH”). The material was exposed to increasing and then decreasing humidity during x-ray powder diffraction analysis. The material was identified as Form I at humidity conditions spanning 25% to 85% RH. However, the material partially dehydrated back to Form XIII immediately upon exposure to 0% RH. Complete dehydration to Form XIII at 0% RH was evident after 20 minutes. Exposure to either 0% RH or 70°C under vacuum dehydrated Form I to Form XIII. Figure 14 and Figure 15 compare the x-ray powder diffraction patterns of the material as Form I dehydrates to Form XIII.

[00126] Substantially pure Form I is further disclosed. “Substantially pure,” as described herein, generally refers to a form herein that is present without any appreciable amounts, other than potentially trace levels of other forms of crystalline 25HC3S sodium, liquid crystalline form of 25HC3S sodium, or non-crystalline 25HC3S such as amorphous 25HC3S sodium. Examples

of trace levels include not more than about 10%, 5%, 2%, 1.5%, 1%, 0.5%, 0.25%, 0.1%, or less in total relative to the total amount (based on weight) of 25HC3S sodium present.

[00127] Form II crystalline 25HC3S sodium, including substantially pure Form II crystalline 25HC3S sodium, is provided for in many cases of the present disclosure. Form II is a variable hydrate of crystalline 25HC3S sodium. The water content is typically present at no greater than about 3 moles of water per mole of 25HC3S sodium. In some cases, the molar content of water to 25HC3S sodium is from about 1 to 3, including, for example, from about 2 to 3. Form II may be prepared as set forth in Table 1. For example, slurring 25HC3S sodium in acetone and drying under nitrogen results in Form II. Form II may also be prepared as set forth in Examples 27-30. The following Table 5 correlates the Examples producing Form II with the corresponding XRPD diffractogram Figure.

Table 5 – Form II XRPD Examples / Figures

Example #	Figure
27	42
28	43
29	44
30	46

[00128] Table 6 show peaks found in Figure 46, whereas peaks for the other figures in Table 5 are presented on the figures themselves.

Table 6 – Figure 46 Peak List

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
4.568637	2176.180000	0.076752	19.34194	52.97
5.093186	1019.875000	0.089544	17.35102	24.83
6.024972	222.111200	0.102336	14.66954	5.41
6.833070	1157.846000	0.089544	12.93639	28.19
7.500985	52.534870	0.307008	11.78592	1.28
9.102928	4107.982000	0.089544	9.71510	100.00
9.924992	200.682800	0.102336	8.91219	4.89
10.444960	185.467200	0.204672	8.46967	4.51
12.706730	118.823500	0.102336	6.96672	2.89
14.530520	182.929600	0.102336	6.09614	4.45
14.887640	1538.703000	0.127920	5.95070	37.46
15.254160	464.882600	0.127920	5.80854	11.32
15.526900	453.899600	0.115128	5.70711	11.05

15.920180	454.991900	0.089544	5.56700	11.08
17.796940	219.076700	0.127920	4.98395	5.33
18.224860	795.017100	0.076752	4.86788	19.35
18.779150	200.652400	0.102336	4.72543	4.88
19.831570	259.643200	0.102336	4.47697	6.32
20.525570	726.830800	0.063960	4.32714	17.69
21.123060	313.674400	0.089544	4.20607	7.64
22.241580	301.102600	0.102336	3.99702	7.33
22.828300	485.040800	0.127920	3.89560	11.81
25.218110	42.303550	0.818688	3.53159	1.03
25.606150	71.888400	0.204672	3.47895	1.75
26.769420	121.605300	0.153504	3.33035	2.96
27.429070	64.946410	0.153504	3.25174	1.58
29.066220	74.634100	0.153504	3.07220	1.82
31.423480	44.097820	0.255840	2.84691	1.07
36.048690	33.706900	0.204672	2.49155	0.82
38.425090	58.863040	0.204672	2.34275	1.43

[00129] Form II may be characterized by various analytical techniques, including by x-ray powder diffraction. The x-ray powder diffraction pattern of Form II, or portions thereof, may be used to identify Form II. Form II contains various x-ray powder diffraction peaks which alone or together may help identify the presence of Form II. In many cases, Form II may be characterized by an x-ray powder diffraction pattern comprising a peak at about $2.3^{\circ}2\theta$. In addition to the peak at about $2.3^{\circ}2\theta$, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about $4.5^{\circ}2\theta$, a peak at and between about $5.0^{\circ}2\theta$ and about $5.1^{\circ}2\theta$, a peak at and between about $5.9^{\circ}2\theta$ and about $6.1^{\circ}2\theta$, and at least one peak at and between about $14.8^{\circ}2\theta$ and about $15.1^{\circ}2\theta$.

[00130] In many cases, Form II may be characterized by an x-ray powder diffraction pattern comprising peaks at about $2.3^{\circ}2\theta$ and about $5.0^{\circ}2\theta$. The x-ray powder diffraction pattern may, in some cases, comprise one or more peaks at about $4.5^{\circ}2\theta$, about $5.9^{\circ}2\theta$, about $9.1^{\circ}2\theta$, and about $15.1^{\circ}2\theta$.

[00131] Figure 2 is an x-ray powder diffraction pattern of Form II where the x-axis begins at about $0^{\circ}2\theta$ and goes to about $40^{\circ}2\theta$, whereas Figure 54 shows the same pattern but between about $3^{\circ}2\theta$ and about $39^{\circ}2\theta$. Either of Figure 2 or Figure 54 may be used to characterize Form II.

[00132] Form II is stable under various conditions of temperature and humidity. Both Form II and substantially pure Form II are described herein. Form II is stable at relative humidities

ranging from about 21% to about 30% RH. As such, Form II was generated from slurries in aqueous solvent mixtures with water activities at and between 0.21 and 0.30 a_w . Variable hydrates so prepared appear kinetically stable. Form II has been seen to dehydrate to an anhydrous state without form conversion when exposed to elevated temperatures or low humidity conditions. In a fully hydrated state, Form II appears to accommodate approximately 3 mol/mol of water (Figure 16 shows the DVS sorption isotherm at 85% RH of Form I). Upon extended exposure to an RH of 75% or greater, Form II converts to liquid crystalline Form V of 25HC3S sodium.

[00133] Multiple x-ray powder diffraction patterns of Form II obtained from various experiments were successfully indexed (Figure 17, Figure 18, and Figure 19). These indices show that the unit cell volume of Form II changes to accommodate varying amounts of water and thus Form II is a variable hydrate. Differences in these patterns, attributed to variation in the positions of peaks, suggest that a range exists for the observed peaks (Figures 18 and 19). Figures 20 and 21 are expanded regions of the x-ray powder diffraction patterns of the slurries. Given that x-ray powder diffraction peak positions are a direct result of the unit cell parameters, one x-ray powder diffraction pattern is not necessarily representative of the crystal form under all conditions due to the variable hydration and its effect on lattice parameters. The x-ray powder diffraction patterns should be considered discrete states of the same crystalline phase of Form II. The general trend is for peaks to shift toward smaller scattering angles with increased cell volume. Anisotropic strain can cause some peaks to shift toward higher scattering angles with increased cell volume. Some peak positions are more sensitive than others to changes in cell volume. Peaks that are sensitive to changes in cell volume can be useful for determining the cell volume for a particular specimen. Peaks that move result in x-ray powder diffraction patterns that appear to be qualitatively different by visual inspection, but do not indicate a change in crystalline phase.

[00134] Multiple x-ray powder diffraction patterns were acquired for Form II at ambient temperature and different RH conditions spanning 0% to 85% RH through an in situ variable humidity x-ray powder diffraction experiment performed in general accordance with Example 18. The experiment provides a systematic approach to determine the variation in unit cell volume dictated by water sorption, where the cell expands/contracts to accommodate water. As expected, a form change did not occur; however, peak shifts between the patterns are observed,

due to unit cell volume differences (Figure 22 and Figure 23). The peaks shift toward smaller scattering angles at higher humidity, suggesting that the unit cell volume is directly proportional to relative humidity and likely increases to incorporate water.

[00135] The DVS isotherm, provided in Figure 16, shows water content variability for Form II at the same relative humidity conditions in which kinetic form stability was demonstrated by variable humidity x-ray powder diffraction. Form II gained approximately 3.5 wt% from 5 to 55% RH (1 mol/mol water), 7 wt% from 55 to 85% RH (2 mol/mol water), and another 7 wt% from 85 to 95% RH. The instrument timed-out above 85% RH, suggesting that additional water sorption may be possible if left longer at this condition. Hysteresis was observed upon desorption. The material was recovered and identified as a mixture of Form II and Form V. Form II remained unchanged, by x-ray powder diffraction, under dry nitrogen for up to 4 days or stored at 0% RH for up to 35 days. Conversion to Form V occurred within 35 days upon exposure to 75% RH.

[00136] Of the three indexing solutions obtained, the largest unit cell volume observed from an indexing result for Form II is provided in Figure 17. Assuming the orthorhombic unit cell contains 12 molecules of 25HC3S sodium, the formula unit volume of $756 \text{ \AA}^3$ would be larger than the formula unit for the anhydrous form, Form XIII, by approximately $50 \text{ \AA}^3$. This corresponds to a unit cell volume of $9066.5 \text{ \AA}^3$ ($\pm 5\%$). The difference in volume provides sufficient room to accommodate up to 2 mol/mol of water.

[00137] Thermally, up to 150°C , weight losses of 5 and 6% are observed by TGA (See Figure 24). Assuming the losses are due to the volatilization water, the weight losses correspond to approximately 1.5 and 1.8 mol/mol water, respectively. The losses occur concurrently with the broad dehydration endotherms in the DSC (Figure 25). The DSC curves also show endotherms near 167°C and 189°C . These events are associated with decomposition.

[00138] A cycling DSC experiment for Form II is shown in Figure 26. Broad desolvation endotherms are observed during the first heating cycle up to 130°C (trace 1), as expected for the hydrate. However, no events are observed upon cooling (trace 2) or upon the second heating (trace 3) up to 130°C . This suggests that a form change did not occur upon dehydration and, once heated again to 130°C , the material does not contain water. The sample then proceeds through decomposition as evidenced by the endotherms near 167°C and 188°C . The kinetic stability of Form II through dehydration at elevated temperatures was confirmed with a variable

temperature x-ray powder diffraction experiment done in general accordance with Example 19 (Figure 27). Form II is still observed by x-ray powder diffraction at 135 °C. Therefore, the results from both variable temperature x-ray powder diffraction and cycling DSC experiments show that Form II can be maintained through complete dehydration. During the same variable temperature x-ray powder diffraction experiment, a decomposition product identified as trisodium hydrogen disulfate, $\text{Na}_3\text{H}(\text{SO}_4)_2$, was evident by 170 °C.

[00139] In some cases, Form XI, including substantially pure Form XI, is provided of crystalline 25HC3S sodium. Form XI is a hydrate of crystalline 25HC3S sodium which may be prepared as set forth in Table 1. For example, Form XI may be prepared by slurrying in diethyl ether and exposing to ambient conditions for 14 days, filtering, and drying under nitrogen. It may thus be prepared in a stable form. In addition, Example 37 shows a preparation of Form XI. Form XI may be characterized by various analytical techniques, including by x-ray powder diffraction. The x-ray powder diffraction pattern of Form XI, or portions thereof, may be used to identify Form XI. Form XI contains various x-ray powder diffraction peaks which alone or together may help identify the presence of Form XI.

[00140] In many cases, Form XI may be characterized by an x-ray powder diffraction pattern comprising a peak at about $2.6^\circ 2\theta$. In addition to the peak at about $2.6^\circ 2\theta$, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about $3.1^\circ 2\theta$, about $3.5^\circ 2\theta$, and about $14.5^\circ 2\theta$. The x-ray powder diffraction patterns for Form XI are at Figure 6 and Figure 7. Either Figure 6 or Figure 7 may be used to characterize Form XI.

[00141] In some cases, Form V, a lyotropic mesophase (also known as a liquid crystal) of 25HC3S sodium, including substantially pure Form V, is provided herein. Lyotropic mesophases are induced by the presence of solvent. In addition, concentration and temperature are determining factors in their phase transitions. Form V may be prepared as set forth in Table 1. For example, transparent and viscous gels were formed from water, aqueous solvent mixtures with a water activity above 0.73 a_w , methanol, and DMSO. Form V formed as gels specifically from water, aqueous solvent systems with water activity above 0.73 a_w , and methanol exhibited disordered x-ray powder diffraction patterns. Form V also formed when either Forms I or II were exposed to relative humidity at or above 75% RH. These gels did not typically exhibit birefringence under polarized light microscopy until a shear force, such as sliding between two glass slides, was applied. Optical birefringence is suggestive of an ordered phase having at least

one-dimension of order. Flow of the material was also observed. In addition, Form V may be prepared as set forth, for example, in Examples 34, 35, and 36.

[00142] A mesophase is characterized by its birefringence, which is absent in amorphous solids or isotropic liquids and present in almost all cases for crystalline solids. Further, the material flows like a liquid when pressed upon, rather than break and fracture like a solid. As typical for mesophases, the x-ray powder diffraction patterns of Form V exhibit few peaks superimposed on a diffuse background (Figure 3).

[00143] In many cases, Form V may be characterized by an x-ray powder diffraction pattern comprising a peak at about $2.2^{\circ}2\theta$. In addition to the peak at about $2.2^{\circ}2\theta$, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about $4.4^{\circ}2\theta$, about $6.6^{\circ}2\theta$, and about $8.8^{\circ}2\theta$.

[00144] In many cases, Form V may be characterized by an x-ray powder diffraction pattern comprising peaks at about $2.2^{\circ}2\theta$, about $6.6^{\circ}2\theta$, and a single peak at and between about $4.0^{\circ}2\theta$ and about $6.0^{\circ}2\theta$. The x-ray powder diffraction, in some cases, may further comprise one or more peaks at about $8.8^{\circ}2\theta$, about $9.9^{\circ}2\theta$, and about $14.9^{\circ}2\theta$. The x-ray powder diffraction patterns for Form V are at Figure 3 and Figure 4. Either of Figure 3 or Figure 4 may be used to characterize Form V.

[00145] Form V exhibits a 7% weight loss up to 147°C by TGA (Figure 28) which occurs concurrently with a broad dehydration endotherm in the DSC (Figure 29). Events associated with decomposition are evident above 148°C .

[00146] The physical stability of Form V showed no changes observed by x-ray powder diffraction on storage at ambient conditions for 25 days. In addition, the material remained Form V upon exposure to 55°C and vacuum for 1 day. Thus, disclosed herein is also stable Form V. The following Table 7 correlates the Examples producing Form V with the corresponding XRPD diffractogram Figure.

Table 7 – Form V XRPD Examples / Figures

Example #	Figure
34	47
35	48
36	56

[00147] Table 8 shows peaks found in Figure 56, whereas peaks for the other figures in Table 8 are presented on the figures themselves.

Table 8 – Figure 56 Peak List

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
4.456570	1011.928000	0.102336	19.82806	30.00
6.670990	515.763400	0.102336	13.25033	15.29
8.883880	3372.782000	0.140712	9.95415	100.00
11.079420	116.481300	0.153504	7.98604	3.45
15.392700	171.999000	0.255840	5.75657	5.10
17.756100	662.088400	0.153504	4.99532	19.63
19.986240	896.322600	0.127920	4.44267	26.58
22.239050	304.758100	0.153504	3.99747	9.04
24.583640	42.981330	0.307008	3.62128	1.27
26.762670	66.577970	0.204672	3.33117	1.97
29.038120	41.789920	0.307008	3.07511	1.24

[00148] In many cases, Form XIII crystalline 25HC3S sodium is provided, including substantially pure Form XIII crystalline 25HC3S sodium. Form XIII is anhydrous and was observed through the dehydration of Form I upon exposure to 130 °C, 70 °C and vacuum, or to humidity conditions near 0% RH. Under ambient conditions, Form XIII quickly hydrates to Form I. Form XIII was also seen by dissolving in an acetonitrile/H₂O solution and drying as set forth in Table 1. Form XIII may also, for example, be prepared as set forth in Examples 25 and 26.

[00149] The x-ray powder diffraction pattern of Form XIII was successfully indexed, indicating the pattern represents a single crystalline phase (Figure 30). The indexing result has a monoclinic unit cell containing six molecules of 25HC3S sodium. Consequently, the formula unit volume of 705 Å³ (±5%) would be consistent with an anhydrous form and is not capable of accommodating any additional solvent or water molecules, with a unit cell volume of 4230.8 Å³ (±5%).

[00150] In many cases, Form XIII may be characterized by an x-ray powder diffraction pattern comprising a peak at about 2.3°2θ. In addition to the peak at about 2.3°2θ, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about 4.6°2θ, about 9.3°2θ, about 14.3°2θ, and about 15.0°2θ (Figure 8 and Figure 9).

[00151] In many cases, Form XIII may be characterized by an x-ray powder diffraction pattern comprising peaks at about $2.3^{\circ}2\theta$, about $5.4^{\circ}2\theta$, about $9.3^{\circ}2\theta$, and about $11.6^{\circ}2\theta$. The x-ray powder diffraction pattern, in some cases, may further comprise one or more peaks at about $4.6^{\circ}2\theta$ and about $15.0^{\circ}2\theta$. Either Figure 8 or Figure 9 may be used to characterize Form XIII.

[00152] A variable humidity x-ray powder diffraction experiment on a mixture composed predominately of Form I and a minor amount of Form XIII is presented in Figure 13. The material was exposed to increasing and then decreasing humidity during x-ray powder diffraction analysis. These results provide evidence that Forms I and XIII quickly interconvert. A mixture of Forms I and XIII was evident at 14% RH. Form XIII was shown to hydrate to Form I before reaching 25% RH. Conversely, Form I was shown to dehydrate within minutes to Form XIII upon exposure to 0% RH, remaining stable under those conditions.

[00153] In many cases of the present disclosure, solvates of crystalline 25HC3S sodium are provided, including substantially pure crystalline solvates of 25HC3S sodium.

[00154] In many cases, Form III crystalline 25HC3S sodium is provided, including substantially pure Form III crystalline 25HC3S sodium. Form III is an ethanol solvate of crystalline 25HC3S sodium. X-ray powder diffraction patterns for Form III are set forth in Figure 35, Figure 49, and Figure 50. Form III crystallized only from experiments involving ethanol. Form III is metastable and desolvates to Form II at ambient conditions. When exposed to 58°C and vacuum overnight, or a temperature of 130°C , it desolvates to another anhydrous form, Form IX (Figure 31). Preparations of Form III are further described in Examples 38-39.

[00155] Table 9 shows peaks found in Figure 50, whereas peaks for Figure 35 and 49 are presented on the figure itself.

Table 9 – Figure 50 Peak List (Form III)

Pos. [$^{\circ}2\theta$.]	Height [cts]	FWHM Left [$^{\circ}2\theta$.]	d-spacing [$\text{\AA}$]	Rel. Int. [%]
6.387487	4693.291000	0.051168	13.83778	17.97
7.814675	26117.580000	0.051168	11.31351	100.00
9.872075	811.450200	0.076752	8.95984	3.11
11.401980	342.370700	0.076752	7.76083	1.31
12.764300	213.323200	0.076752	6.93542	0.82
13.379850	14526.280000	0.063960	6.61771	55.62
15.621830	15172.540000	0.089544	5.67265	58.09
19.173970	227.941600	0.102336	4.62901	0.87
19.474080	1176.151000	0.102336	4.55834	4.50
21.008570	719.226400	0.089544	4.22874	2.75

25.810620	93.194700	0.204672	3.45185	0.36
26.862540	34.265340	0.614016	3.31902	0.13
28.882870	63.145900	0.153504	3.09129	0.24
29.799090	42.999270	0.153504	2.99829	0.16
30.535120	65.048610	0.153504	2.92768	0.25
31.510870	84.185980	0.153504	2.83921	0.32
32.225590	48.569530	0.204672	2.77786	0.19
33.137800	47.258860	0.153504	2.70345	0.18
34.683980	171.839700	0.102336	2.58639	0.66

[00156] The x-ray powder diffraction pattern of Form III was successfully indexed, indicating the pattern represents a single crystalline phase as seen in Figure 32. The indexing result has a triclinic unit cell containing two molecules of 25HC3S sodium. Consequently, the formula unit volume of 768 Å³ is consistent with a monoethanolate. The volume is larger than the formula unit for the anhydrous form, Form XIII, by approximately 63 Å³. The difference in volume provides sufficient room to accommodate up to 1 mol/mol of ethanol which further suggests Form III is a monoethanolate.

[00157] In many cases, Form III may be characterized by an x-ray powder diffraction pattern comprising a peak at about 4.9°2θ. Although no peak at about 4.9°2θ appears in Table 9, an expanded view of that diffractogram in Figure 52 shows the presence of a peak at about 4.9°2θ. In addition to the peak at about 4.9°2θ, the x-ray powder diffraction pattern may comprise, for example, one or more peaks at about 6.3°2θ, about 7.8°2θ, and about 9.8°2θ.

[00158] In many cases, Form III may be characterized by peaks at about 4.9°2θ and about 6.3°2θ. The x-ray powder diffraction pattern, in some cases, may further comprise one or more peaks at about 7.8°2θ, about 9.8°2θ, about 13.3°2θ, and about 15.5°2θ. Form III may be characterized by the x-ray powder diffraction pattern of Figure 35.

[00159] With respect to Form IX, in many cases, Form IX may be characterized by an x-ray powder diffraction pattern comprising a peak at about 4.9°2θ. The x-ray powder diffraction pattern may comprise for example, one or more peaks at about 7.9°2θ, about 11.2°2θ, about 14.1°2θ, about 16.1°2θ, and about 16.6°2θ.

[00160] In many cases, Form IX may be characterized by an x-ray powder diffraction pattern comprising peaks at about 2.2°2θ, about 4.9°2θ, and about 7.9°2θ. The x-ray diffraction pattern may, in some cases, may further comprise one or more peaks at about 14.1°2θ, about 16.1°2θ, and about 16.6°2θ. Form IX may further be characterized by Figure 5.

[00161] Residual organic solvent, such as ethanol, was not observed. A 1.6% weight loss observed up to 145 °C by TGA is likely due to the volatilization of water. Congruent dehydration endotherms are evident by DSC, and events above 150 °C are associated with decomposition. Form IX may be prepared as set forth in Example 32 and Example 33. Figure 5 corresponds to Example 32 and Figure 51 to Example 33.

[00162] Table 10 shows peaks found in Figure 51, whereas the peaks of Figure 5 are presented on that Figure.

Table 10 – Figure 51 Peak List

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
4.811323	45.357440	0.614016	18.36685	3.59
6.984234	453.736100	0.089544	12.65673	35.92
8.010692	655.509800	0.102336	11.03711	51.90
9.860124	152.477100	0.102336	8.97068	12.07
11.274460	472.185700	0.127920	7.84832	37.39
14.158540	1263.031000	0.127920	6.25545	100.00
16.117560	95.677850	0.204672	5.49927	7.58
16.601830	183.637300	0.127920	5.33994	14.54
18.391860	51.683560	0.409344	4.82405	4.09
20.142210	46.117470	0.307008	4.40862	3.65
20.881010	103.616700	0.153504	4.25428	8.20
21.966780	142.983300	0.153504	4.04639	11.32
24.041400	57.026530	0.307008	3.70171	4.52

[00163] The present disclosure also relates to pharmaceutical compositions containing crystalline 25HC3S sodium as disclosed herein. Such pharmaceutical compositions are comprised of one or more pharmaceutically acceptable excipients and crystalline 25HC3S sodium as set forth in the present disclosure. Such pharmaceutical compositions may be administered orally or configured to be delivered as any effective conventional dosage unit forms, including, for example, immediate, slow and timed-release oral preparations, parenterally, topically, nasally, ophthalmically, optically, sublingually, rectally, vaginally, and the like.

[00164] The present disclosure further includes mixtures of forms of 25HC3S sodium such as crystalline 25HC3S sodium forms with each other and/or with lyotropic forms of 25HC3S sodium. For examples, mixtures of two or more of crystalline 25HC3S sodium Form I, crystalline 25HC3S sodium Form II, liquid crystal 25HC3S sodium Form V, crystalline 25HC3S sodium Form IX, crystalline 25HC3S sodium Form XI, or crystalline 25HC3S sodium Form XIII

are provided. The amount of each form present in such mixtures ranges from, for example, about 0.01% to about 99.9% by weight. Other ranges include about 0.1% to about 95%, about 0.1% to about 90%, about 0.1% to about 85%, about 0.1% to about 80%, about 0.1% to about 75%, about 0.1% to about 70%, about 0.1% to about 65%, about 0.1% to about 60%, about 0.1% to about 55%, about 0.1% to about 50%, about 0.1% to about 45%, about 0.1% to about 40%, about 0.1% to about 35%, about 0.1% to about 30%, about 0.1% to about 25%, about 0.1% to about 20%, about 0.1% to about 15%, about and 0.1% to about 10% by weight. Other ranges include about 0.1% to about 9%, about 0.1% to about 8%, about 0.1% to about 7%, about 0.1% to about 6%, about 0.1% to about 5%, about 0.1% to about 4%, about 0.1% to about 3%, about 0.1% to about 2%, about 0.1% to about 1% by weight. Additional ranges include about 0.1% to about 0.9%, about 0.1% to about 0.8%, about 0.1% to about 0.7%, about 0.1% to about 0.6%, about 0.1% to about 0.5%, about 0.1% to about 0.4%, about 0.1% to about 0.3%, and about 0.1% to about 0.2% by weight. Further ranges include between about 0.01% and about 0.1% by weight. Additional ranges include between about 0.01% and about 0.09%, about 0.01% to about 0.08%, about 0.01% to about 0.07%, about 0.01% to about 0.06%, about 0.01% to about 0.05%, about 0.01% to about 0.04%, about 0.01% to about 0.03% and about 0.01% to about 0.02% by weight. Such mixtures may also be present in pharmaceutical compositions comprising one or more pharmaceutically acceptable excipients.

[00165] The present disclosure further includes methods and uses for treating diseases in humans such as one or more of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation (e.g., non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, and atherosclerosis) with effective amounts crystalline 25HC3S sodium and/or pharmaceutical compositions comprising crystalline 25HC3S sodium of the present disclosure.

Methods for preparing 25-hydroxy-cholesten-5-en-3-sulfate (25HC3S)

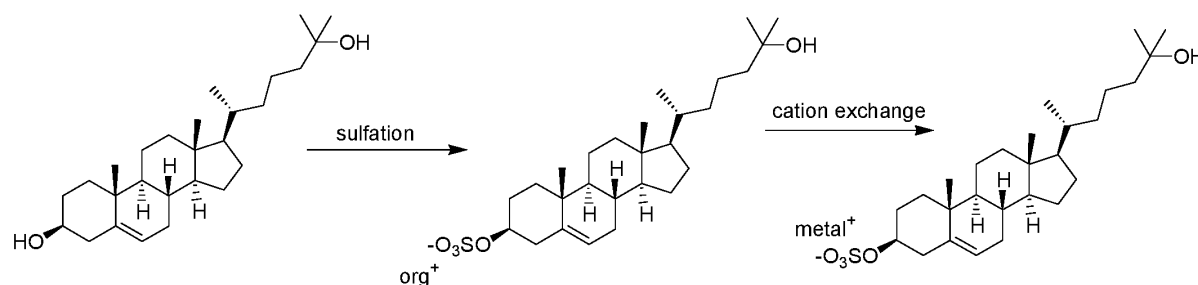
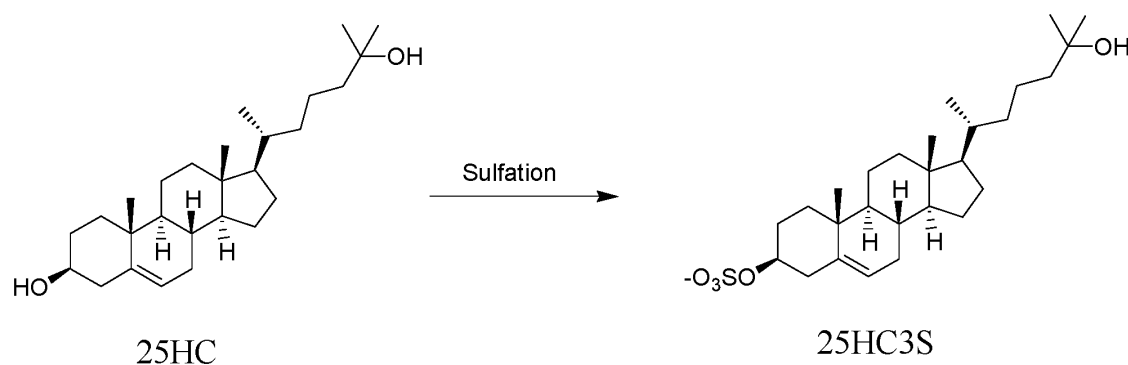
[00166] As summarized above, the present disclosure also provides methods for preparing 25-hydroxy-cholesten-5-en-3-sulfate, such as 25-hydroxy-3 β -cholesten-5-en-3-sulfate (25HC3S). Although many of the teachings herein involve a sulfate in the 3 β position, the teachings of the present disclosure are also generally applicable to a sulfate in the 3 α position. The components used in each step of the subject methods for preparing 25-hydroxy-3 β -cholesten-5-en-3-sulfate

described herein may be a purified composition or a crude composition as desired. The term “purified” is used in its conventional sense to refer to a composition where at least some isolation or purification process has been conducted, such as for example, filtration or aqueous workup of a reaction mixture. In certain instances, purification includes at least one of liquid chromatography, recrystallization, distillation (e.g., azeotropic distillation) and other type of compound purification. For example, compounds as described herein may be purified by chromatographic means, such as high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), thin layer chromatography, flash column chromatography and ion exchange chromatography. Any suitable stationary phase can be used, including normal and reversed phases as well as ionic resins. Mobile phases may be chosen from polar solvents and non-polar solvents. In some cases, the mobile phase includes a polar solvent. In some cases, the polar solvent is chosen from chloroform, dichloromethane, tetrahydrofuran, dichloroethane, acetone, dioxane, ethyl acetate, dimethylsulfoxide, aniline, diethylamine, nitromethane, acetonitrile, pyridine, isopropanol, ethanol, methanol, ethylene glycol, acetic acid and water. In some cases, the mobile phase includes a non-polar solvent. In some cases, the non-polar solvent is chosen from diethyl ether, toluene, benzene, pentane, hexanes, cyclohexane, petroleum ether and carbon tetrachloride. See, e.g., *Introduction to Modern Liquid Chromatography*, 2nd Edition, ed. L. R. Snyder and J. J. Kirkland, John Wiley and Sons, 1979; and *Thin Layer Chromatography*, ed. E. Stahl, Springer-Verlag, New York, 1969.

[00167] In some cases, a reaction mixture is used in a subsequent step in the methods described herein as a crude mixture where no purification or other workup of the reaction mixture has been conducted. In certain instances, the crude mixture includes the compound of interest in sufficient purity such as where the reaction mixture includes the compound of interest in a purity of 70% or greater, such as 75% or greater, such as 80% or greater, such as 85% or greater, such as 90% or greater, such as 95% or greater, such as 97% or greater, such as 99% or greater, such as 99.5% or greater, such as 99.9% or greater, such as 99.99% or greater and including 99.999% or greater, relative to the crude reaction mixture (apart from solvent when present), as determined by chromatography (e.g., HPLC or SFC), nuclear magnetic resonance spectroscopy (e.g., ^1H NMR or ^{13}C NMR) or a combination thereof. In some cases, the compound of interest is present in the reaction mixture in an amount that is 30% by weight or greater relative to the crude reaction mixture (apart from solvent when present), such as 40% by

weight or greater, such as 50% by weight or greater, such as 60% by weight or greater, such as 70% by weight or greater, such as 75% by weight or greater, such as 80% by weight or greater, such as 85% by weight or greater, such as 90% by weight or greater, such as 95% by weight or greater, such as 97% by weight or greater, such as 99% by weight or greater, such as 99.5% by weight or greater, such as 99.9% by weight or greater, such as 99.99% by weight or greater and including 99.999% by weight or greater relative to the crude reaction mixture, and may range from 5% by weight to 99.999% by weight, such as 30% by weight to 99.99% by weight, 40% by weight to 99.9% by weight, 50% by weight to 99% by weight, 70% by weight to 95% by weight, 75% by weight to 90% by weight, 80% by weight to 99% by weight, or 80% by weight to 95% by weight. In some cases, the compound of interest is present at 30 mol% or greater in the crude reaction mixture (apart from solvent when present), such as 40 mol% or greater, such as 50 mol% or greater, such as 60 mol% or greater, such as 70 mol% or greater, such as 75 mol% or greater, such as 80 mol% or greater, such as 85 mol% or greater, such as 90 mol% or greater, such as 95 mol% or greater, such as 97 mol% or greater, such as 99 mol% or greater, such as 99.5 mol% or greater, such as 99.9 mol% or greater, such as 99.99 mol% or greater and including 99.999 mol% or greater relative to the crude reaction mixture, and may range from 30 mol% to 99.999 mol%, such as 50 mol% to 99 mol%, 70 mol% to 95 mol%, 75 mol% to 90 mol%, 80 mol% to 99 mol%, or 80 mol% to 95 mol%.

[00168] Methods for preparing a metal salt of 25-hydroxy-3 β -cholesten-5-en-3-sulfate ([(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate metal salt) according to the present disclosure include contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfating agent to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt (**Scheme 1a**).

Scheme Ia**Scheme IA1**

[00169] The 25-hydroxy-(3β)-cholest-5-en-3-ol is sulfated by contacting with a sulfating agent (**Scheme IA1**). In some cases, the sulfating reagent is chosen from sulfur trioxide complexes, sulfuric acid compounds, sulfonic acid compounds, and sulfonate compounds. In some cases, the sulfating reagent is chosen from sulfur trioxide dimethyl formamide, sulfur trioxide triethylamine, and sulfur trioxide trimethylamine. In some cases, the sulfating reagent includes from sulfuric acid and acetic anhydride and pyridine. In some cases, the sulfating reagent includes sulfur trioxide triethylamine and pyridine. In some cases, the sulfating reagent is chosen from 1) chlorosulfonic acid and pyridine and 2) chlorosulfonic acid and 2,6-lutidine. In some cases, the sulfating reagent is ethyl chlorosulfonate.

[00170] The 25-hydroxy-(3β)-cholest-5-en-3-ol may be sulfated at a temperature that ranges from -10 °C to 50 °C, such as from -5 °C to 45 °C, such as from -4 °C to 40 °C, such as from -3 °C to 35 °C, such as from -2 °C to 30 °C, such as from -1 °C to 25 °C, and including from 0 °C to 20 °C. The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours,

such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours and including from 1 hour to 15 hours. The amount of sulfating agent used relative to the 25-hydroxy-(3 β)-cholest-5-en-3-ol may vary and may be 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 5 equivalents, 0.5 equivalents to 10 equivalents, 0.5 equivalents to 8 equivalents, 0.5 equivalents to 5 equivalents, 0.9 equivalents to 10 equivalents, 0.9 equivalents to 8 equivalents, 0.9 equivalents to 5 equivalents, 1.3 equivalents to 10 equivalents, 1.3 equivalents to 8 equivalents, 1.3 equivalents to 5 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 5 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 5 equivalents, or 1 equivalent to 2 equivalents, 1 equivalents to 1.5 equivalents, or 1.1 to 1.2 equivalents, relative to the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

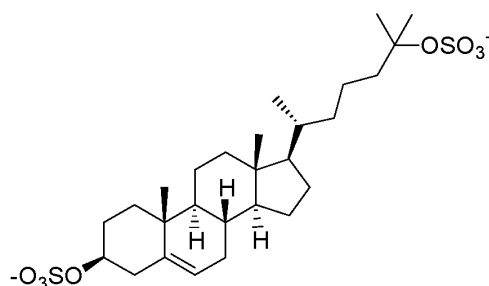
[00171] In some cases, methods include sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol in at least one solvent where the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product exhibits low solubility. In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-ol is sulfated in at least one solvent where the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product exhibits a solubility of 100 mmol/L or less, such as 90 mmol/L or less, such as 80 mmol/L or less, such as 70 mmol/L or less, such as 60 mmol/L or less, such as 50 mmol/L or less, such as 40 mmol/L or less, such as 30 mmol/L or less, such as 20 mmol/L or less, such as 10 mmol/L or less, and including sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol in at least one solvent where the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product exhibits a solubility of 5 mmol/L or less. In some cases, the 25-hydroxy-

(3 β)-cholest-5-en-3-ol is sulfated in at least one solvent where 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product precipitates after formation. In some cases, the at least one solvent is chosen from chloroform, methylene chloride, acetone, acetonitrile, toluene, tetrahydrofuran, and methyltetrahydrofuran.

[00172] In some cases, methods include sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol in a manner sufficient to reduce or eliminate bis-sulfation of the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some instances, the 25-hydroxy-(3 β)-cholest-5-en-3-ol is sulfated and a bis-sulfate product (i.e., 5-cholesten-3 β -25-diol-disulfate, Structure IA) is formed in an amount that is 10% by weight or less of the reaction product formed by contacting the 25-hydroxy-(3 β)-cholest-5-en-3-ol with the sulfating agent, such as 9% by weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including where the 25-hydroxy-(3 β)-cholest-5-en-3-ol is sulfated and the bis-sulfate product is formed in an amount that is 0.0001% by weight or less, and may range from 10% by weight to 0.001% by weight, such as 10% by weight to 0.1% by weight, 10% by weight to 1% by weight, 10% by weight to 2% by weight, 8% by weight to 0.001% by weight, 8% by weight to 0.1% by weight, 8% by weight to 1% by weight, 8% by weight to 2% by weight, 6% by weight to 0.001% by weight, 6% by weight to 0.1% by weight, 6% by weight to 1% by weight, 6% by weight to 2% by weight, 4% by weight to 0.001% by weight, 4% by weight to 0.1% by weight, 4% by weight to 1% by weight, 4% by weight to 2% by weight, 3% by weight to 0.001% by weight, 3% by weight to 0.1% by weight, 3% by weight to 1% by weight, 2% by weight to 0.001% by weight, 2% by weight to 0.1% by weight, or 2% by weight to 1% by weight.

[00173] In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate formed is 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate formed is

10⁹:1 or more, and may range from a ratio by weight of 10:1 to a ratio by weight of 10⁹:1, such as a ratio of weight of 10:1 to a ratio of weight of 10⁶:1, a ratio of weight of 10:1 to a ratio of weight of 10³:1, a ratio of weight of 10:1 to a ratio of weight of 100:1, a ratio of weight of 100:1 to a ratio of weight of 10⁹:1, a ratio of weight of 100:1 to a ratio of weight of 10⁶:1, a ratio of weight of 100:1 to a ratio of weight of 10³:1, a ratio of weight of 250:1 to a ratio of weight of 10⁹:1, a ratio of weight of 250:1 to a ratio of weight of 10⁶:1, a ratio of weight of 250:1 to a ratio of weight of 10³:1, a ratio of weight of 500:1 to a ratio of weight of 10⁹:1, a ratio of weight of 500:1 to a ratio of weight of 10⁶:1, a ratio of weight of 500:1 to a ratio of weight of 10³:1, a ratio of weight of 10³:1 to a ratio of weight of 10⁹:1, a ratio of weight of 10³:1 to a ratio of weight of 10⁶:1, or a ratio of weight of 250:1 to a ratio of weight of 10³:1.



IA

[00174] In some cases, the 5-cholesten-3β-25-diol-disulfate formed when sulfating 25-hydroxy-(3β)-cholest-5-en-3-ol remains solubilized in the at least one solvent. In some cases, the 5-cholesten-3β-25-diol-disulfate has high solubility in the at least one solvent. In some instances, the 5-cholesten-3β-25-diol-disulfate exhibits a solubility of 500 mmol/L or more in the at least one solvent, such as 600 mmol/L or more, such as 700 mmol/L or more, such as 800 mmol/L or more, such as 900 mmol/L, or more and including a solubility of 1 mol/L or more in the at least one solvent.

[00175] In certain cases, methods further include separating the 25-hydroxy-(3β)-cholest-5-en-3-sulfate product from the bis-sulfate product (i.e., 5-cholesten-3β-25-diol-disulfate). In some cases, the 25-hydroxy-(3β)-cholest-5-en-3-sulfate product is separated from the bis-sulfate product by vacuum filtration. In some cases, the 25-hydroxy-(3β)-cholest-5-en-3-sulfate product is separated from the bis-sulfate product by recrystallization of the 25-hydroxy-(3β)-cholest-5-en-3-sulfate product. In some cases, the 25-hydroxy-(3β)-cholest-5-en-3-sulfate product is separated from the bis-sulfate product by chromatography (e.g., silica column).

[00176] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-ol is sulfated in a reaction mixture having a pH that ranges from 5.0 to 8.0, such as a pH from 5.1 to 7.9, such as a pH from 5.2 to 7.8, such as a pH from 5.3 to 7.7, such as a pH from 5.4 to 7.6, such as a pH from 5.5 to 7.5, such as a pH from 5.6 to 7.4, such as a pH from 5.7 to 7.3, such as a pH from 5.8 to 7.2, such as a pH from 5.9 to 7.1, and including sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol in a reaction mixture having a pH of from 6.0 to 7.0.

[00177] In some cases, 25-hydroxy-(3 β)-cholest-5-en-3-ol is sulfated in the presence of a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is present as particles (e.g., seed crystals of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt produced in a previous reaction or purified reaction batch). In some cases, sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol in the presence of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (e.g., as particles) is sufficient to reduce the solubility of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt produced by reaction of the sulfating agent with 25-hydroxy-(3 β)-cholest-5-en-3-ol as compared to the solubility when the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is not present. In certain cases, the solubility of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt produced in the reaction mixture is reduced as compared to the solubility when the added 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is not present by 5% or more, such as by 10% or more, such as by 25% or more, such as by 50% or more, such as by 75% or more, such as by 90% or more and including by reducing the solubility of the produced 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt by 99% or more. The size of the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt added to the reaction mixture may vary and may have a dimension (e.g., length, width or diameter) of 0.01 mm or more, such as 0.025 mm or more, such as 0.05 mm or more, such as 0.075 mm or more, such as 0.1 mm or more, such as 0.25 mm or more, such as 0.5 mm or more, such as 0.75 mm or more, such as 1 mm or more, such as 2 mm or more, such as 3 mm or more, such as 4 mm or more and including 5 mm or more. In some cases, the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt are added to the reaction mixture immediately after contacting the sulfating agent with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some cases, the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt are added to the reaction mixture 1 minute or more after contacting the sulfating agent with the 25-hydroxy-(3 β)-cholest-5-en-3-ol, such as 5

minutes or more, such as 10 minutes or more, such as 15 minutes or more, such as 20 minutes or more, such as 30 minutes or more, such as 40 minutes or more, such as 50 minutes or more and including adding the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt to the reaction mixture 60 minutes or more after contacting the sulfating agent with the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[00178] In certain cases, the sulfating agent is characterized prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some cases, characterizing the sulfating agent includes determining the extent of degradation of the sulfating agent prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In certain cases, determining the extent of degradation of the sulfating reagent includes determining the amount of impurity in the sulfating reagent prior to contactin with the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[00179] In some instances, the degradation of the sulfating agent is determined by proton nuclear magnetic resonance spectroscopy (^1H -NMR). Proton NMR spectroscopy of the sulfating agent may be conducted in at least one deuterated solvent. In certain cases, the at least one deuterated solvent is deuterated acetone ($(\text{CD}_3)_2\text{CO}$). In certain cases, the at least one deuterated solvent is not deuterated benzene (C_6D_6). In certain cases, the at least one deuterated solvent is not deuterated acetonitrile (CD_3CN). In certain cases, the at least one deuterated solvent is not deuterated chloroform (CD_3Cl).

[00180] In some instances, methods for determining the extent of degradation include integrating one or more peaks in the ^1H -NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm and calculating the impurity level of the sulfating agent based on the integrated peaks. In certain instances, methods for determining the extent of degradation include integrating one or more peaks in the ^1H -NMR spectrum at a chemical shift of about 9.25 ppm and calculating the impurity level of the sulfating agent based on the integrated peaks. In some cases, the sulfating agent is contacted with the 25-hydroxy-(3 β)-cholest-5-en-3-ol when the impurity level of the sulfating agent is below a predetermined threshold, such as where the impurity level is 25% or less as determined by integrating one or more peaks in the proton NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm, such as 24% or less, such as 23% or less, such as 22% or less, such as 21% or less, such as 20% or less, such as 19% or less, such as 18% or less, such as 17% or less, such as 16% or less, such as 15% or less, such as 14% or less such as 13% or less, such as 12% or less, such as 11% or less, such as 10% or less, such as 9% or less, such as 8% or less,

such as 7% or less, such as 6% or less, such as 5% or less, such as 4% or less, such as 3% or less such as 2% or less and including where the impurity level is 1% or less as determined by integrating one or more peaks in the proton NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm. In some cases, the sulfating agent is not contacted with the 25-hydroxy-(3 β)-cholest-5-en-3-ol when the impurity level is above a predetermined threshold, such as where the impurity level is 25% or more as determined by integrating one or more peaks in the proton NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm, such as 26% or more, such as 27% or more, such as 28% or more, such as 29% or more, such as 30% or more, such as 31% or more, such as 32% or more, such as 33% or more, such as 34% or more and including where the impurity level is 35% or more as determined by integrating one or more peaks in the proton NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm.

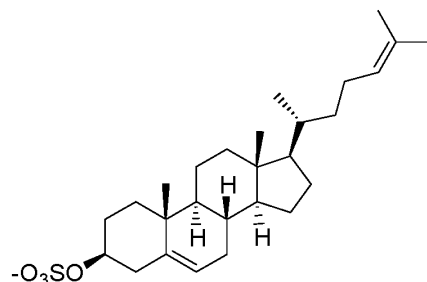
[00181] In certain cases, the generated 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product includes one or more byproducts. In some cases, the byproduct is 5-cholesten-3 β -25-diol-disulfate. In some cases, 5-cholesten-3 β -25-diol-disulfate byproduct is present in the composition produced by sulfation of 25-hydroxy-(3 β)-cholest-5-en-3-ol in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 10% by weight or less, such as 9% by weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including where 5-cholesten-3 β -25-diol-disulfate byproduct is present in the composition produced by sulfation of 25-hydroxy-(3 β)-cholest-5-en-3-ol in an amount of 0.001% by weight or less, and may range from 0.1% by weight to 50% by weight, such as 0.5% by weight to 20% by weight or 1% by weight to 12% by weight. In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate byproduct formed is 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate formed is 10⁹:1 or more. In some cases, the ratio by weight of the

25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the 5-cholesten-3 β -25-diol-disulfate formed ranges from 10:1 to 10⁹:1, such as from 100:1 to 10⁸:1, such as from 1000:1 to 10⁷:1, and including from 10000:1 to 10⁶:1.

[00182] Aspects of the present disclosure also include compositions having 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and 5-cholesten-3 β -25-diol-disulfate that is present in the composition in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 10% by weight or less, such as 9% by weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including 0.001% by weight or less, and may range from 10% by weight to 0.001% by weight, such as 10% by weight to 0.1% by weight, 10% by weight to 1% by weight, 10% by weight to 2% by weight, 8% by weight to 0.001% by weight, 8% by weight to 0.1% by weight, 8% by weight to 1% by weight, 8% by weight to 2% by weight, 6% by weight to 0.001% by weight, 6% by weight to 0.1% by weight, 6% by weight to 1% by weight, 6% by weight to 2% by weight, 4% by weight to 0.001% by weight, 4% by weight to 0.1% by weight, 4% by weight to 1% by weight, 4% by weight to 2% by weight, 3% by weight to 0.001% by weight, 3% by weight to 0.1% by weight, 3% by weight to 1% by weight, 2% by weight to 0.001% by weight, 2% by weight to 0.1% by weight, or 2% by weight to 1% by weight.

[00183] In some cases, compositions include a ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the 5-cholesten-3 β -25-diol-disulfate of 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate in the composition is 10⁹:1 or more. In some cases, compositions include a ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the 5-cholesten-3 β -25-diol-disulfate that ranges from 10:1 to 10⁹:1, such as from 100:1 to 10⁸:1, such as from 1000:1 to 10⁷:1, and including from 10000:1 to 10⁶:1.

[00184] In some cases, the byproduct is sulfated desmosterol (Structure IB).



IB

[00185] In some cases, sulfated desmosterol ([[(3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhex-4-enyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate) is present in the composition produced by sulfation of 25-hydroxy-(3 β)-cholest-5-en-3-ol in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 10% by weight or less, such as 9% by weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including where sulfated desmosterol is present in the composition produced by sulfation of 25-hydroxy-(3 β)-cholest-5-en-3-ol in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 0.001% by weight or less, and may range from 0.1% by weight to 10% by weight, such as 0.2% by weight to 5% by weight or 0.3% by weight to 3% by weight. In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the sulfated desmosterol formed is 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the sulfated desmosterol formed is 10⁹:1 or more. In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the sulfated desmosterol formed ranges from 10:1 to 10⁹:1, such as from 100:1 to 10⁸:1, such as from 1000:1 to 10⁷:1 and including from 10000:1 to 10⁶:1.

[00186] Aspects of the present disclosure also include compositions having 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and sulfated desmosterol that is present in the composition in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 10% by weight or less, such as 9% by weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including 0.001% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate, and may range from 10% by weight to 0.001% by weight, such as 10% by weight to 0.1% by weight, 10% by weight to 1% by weight, 10% by weight to 2% by weight, 8% by weight to 0.001% by weight, 8% by weight to 0.1% by weight, 8% by weight to 1% by weight, 8% by weight to 2% by weight, 6% by weight to 0.001% by weight, 6% by weight to 0.1% by weight, 6% by weight to 1% by weight, 6% by weight to 2% by weight, 4% by weight to 0.001% by weight, 4% by weight to 0.1% by weight, 4% by weight to 1% by weight, 4% by weight to 2% by weight, 3% by weight to 0.001% by weight, 3% by weight to 0.1% by weight, 3% by weight to 1% by weight, 2% by weight to 0.001% by weight, 2% by weight to 0.1% by weight, or 2% by weight to 1% by weight.

[00187] In some cases, compositions include a ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the sulfated desmosterol of 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the sulfated desmosterol in the composition is 10⁹:1 or more. In some cases, compositions include a ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the sulfated desmosterol that ranges from 10:1 to 10⁹:1, such as from 100:1 to 10⁸:1, such as from 1000:1 to 10⁷:1 and including from 10000:1 to 10⁶:1.

[00188] In some cases, the byproduct of sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol that is present in the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate composition is a thermal degradation product. In some cases, the byproduct is identified by relative retention time when the components of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate composition are separated by liquid

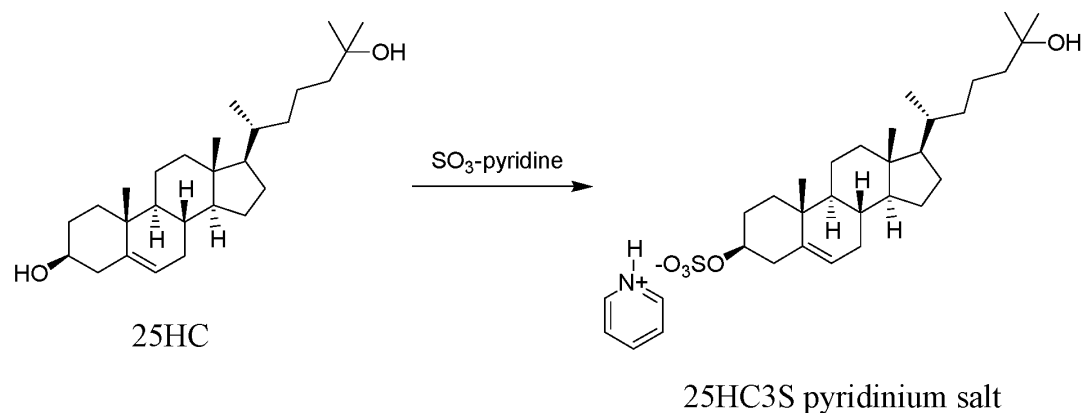
chromatography (e.g., HPLC). In certain cases, the byproduct is sulfated desmosterol, a compound having a retention time of about 18.3 minutes when the components of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate composition are separated by HPLC operating at about 45 °C with a C8 stationary phase and separates the components of the composition with a first mobile phase comprising a buffer (e.g., an aqueous buffer of sodium phosphate) and a second mobile phase comprising one or more organic solvents (see e.g., Tables 13 and 14 below). In some cases, the first mobile phase is an aqueous buffer. In certain cases, the first mobile phase includes sodium phosphate. In some cases, the second mobile phase is chosen from one or more of methoxypropyl acetate, acetonitrile and methanol. In some cases, the flow rate of the first mobile phase is about 1.0 mL/minute. In some cases, the flow rate of the second mobile phase is about 1.0 mL/minute or more. In some cases, 25-hydroxy-(3 β)-cholest-5-en-3-sulfate has a retention time of about 7.7 minutes under the same HPLC conditions. In some cases, the byproduct is a compound having a retention time of about 37.7 minutes when the components of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate composition are separated by HPLC operating at about 45 °C with a C8 stationary phase and separates the components of the composition with a first mobile phase comprising a buffer (e.g., an aqueous buffer of sodium phosphate) and a second mobile phase comprising one or more organic solvents (see e.g., Tables 13 and 14 below). While not wishing to be bound by theory, it is believed that the compound having a retention time of about 37.7 minutes is desmosterol. In some cases, the first mobile phase is an aqueous buffer. In certain cases, the first mobile phase includes sodium phosphate. In some cases, the second mobile phase is chosen from one or more of methoxypropyl acetate, acetonitrile and methanol. In some cases, the flow rate of the first mobile phase is about 1.0 mL/minute. In some cases, the flow rate of the second mobile phase is about 1.0 mL/minute or more. In some cases, and 25-hydroxy-(3 β)-cholest-5-en-3-sulfate has a retention time of about 7.7 minutes under the same HPLC conditions such that sulfated desmosterol has a relative retention time of about 2.4 ($= 18.3 / 7.7$) and the compound believed to be desmosterol has a relative retention time of about 4.9 ($= 37.7 / 7.7$).

[00189] Aspects of the present disclosure also include compositions having 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and one or more byproducts of sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some cases, the one or more byproducts are present in the composition in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate of 10% by weight or less, such as 9% by

weight or less, such as 8% by weight or less, such as 7% by weight or less, such as 6% by weight or less, such as 5% by weight or less, such as 4% by weight or less, such as 3% by weight or less, such as 2% by weight or less, such as 1% by weight or less, such as 0.5% by weight or less, such as 0.1% by weight or less, such as 0.01% by weight or less, such as 0.001% by weight or less, and including 0.001% by weight or less, and may range from 0.1% by weight to 5% by weight, such as 0.2% by weight to 10% by weight or 0.3% by weight to 15% by weight. In some cases, compositions include 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the one or more byproducts in an amount relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate that ranges from 0.0001% by weight to 10% by weight, such as from 0.005% by weight to 9.5% by weight, such as from 0.001% to 9.0% by weight, such as from 0.05% by weight to 8.5% by weight, such as from 0.1% by weight to 8.0% by weight, such as from 0.5% by weight to 7.5% by weight, such as from 1% by weight to 7% by weight, such as from 1.5% by weight to 6.5% by weight, and including from 2% by weight to 6% by weight.

[00190] In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the one or more byproducts formed is 10:1 or more, such as 25:1 or more, such as 50:1 or more, such as 100:1 or more, such as 250:1 or more, such as 500:1 or more, such as 1000:1 or more, such as 2500:1 or more, such as 5000:1 or more, such as 10,000:1 or more, such as 25,000:1 or more, such as 50,000:1 or more, such as 100,000:1 or more, such as 10⁶:1 or more, such as 10⁷:1 or more, such as 10⁸:1 or more, and including where the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the one or more byproducts formed is 10⁹:1 or more. In some cases, the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate and the one or more byproducts formed ranges from 10:1 to 10⁹:1, such as from 100:1 to 10⁸:1, such as from 1000:1 to 10⁷:1, and including from 10000:1 to 10⁶:1.

[00191] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt (**Scheme IA2**).

Scheme IA2

[00192] In certain cases, the sulfating agent is contacted with an anhydride prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol. In some cases, the anhydride is chosen from acetic anhydride, trifluoroacetic anhydride and triflic anhydride. The amount of anhydride relative to the 25-hydroxy-(3 β)-cholest-5-en-3-ol may vary and may be 0.001 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 5 equivalents, 0.5 equivalents to 10 equivalents, 0.5 equivalents to 8 equivalents, 0.5 equivalents to 5 equivalents, 0.9 equivalents to 10 equivalents, 0.9 equivalents to 8 equivalents, 0.9 equivalents to 5 equivalents, 1.3 equivalents to 10 equivalents, 1.3 equivalents to 8 equivalents, 1.3 equivalents to 5 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 5 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 5 equivalents, 0.1 equivalent to 1.5 equivalents, 0.5 equivalents to 1.1 equivalents, or 0.1 equivalent to 1 equivalent relative to the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[00193] In some cases, methods include quenching (i.e., deactivating) unreacted sulfating agent after producing the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In some cases, quenching the the sulfating agent includes adding water to the reaction mixture. The amount of water added to the reaction mixture relative to the amount of sulfating agent contacted with the 25-hydroxy-(3 β)-cholest-5-en-3-ol may vary and may be 1 equivalent or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, such as 6 equivalents or more, such as 7 equivalents or more, such as 8 equivalents or more, such as 9 equivalents or more, such as 10 equivalents or more, such as 15 equivalents or more, such as 20 equivalents or more and including 25 equivalents or more.

[00194] In certain cases, quenching the reactivity of unreacted sulfating agent includes adding water to the reaction mixture followed by the addition of at least one base. In some cases, the at least one base is a trialkylamine, such as trimethylamine or triethylamine. In some cases, the at least one base is 2,6-lutidine. In certain cases, the at least one base is pyridine. The pyridine may be added to the reaction mixture 1 minute or more after adding the water, such as 5 minutes or more, such as 10 minutes or more, such as 15 minutes or more, such as 30 minutes or more, such as 45 minutes or more, such as 60 minutes or more, such as 90 minutes or more, such as 120 minutes or more, such as 150 minutes or more, such as 180 minutes or more, such as 210 minutes or more and including 240 minutes or more after adding the water to the reaction mixture. In certain cases, pyridine is added to the reaction mixture 60 minutes after adding the water. The amount of pyridine added to the reaction mixture relative to the amount of sulfating agent may vary and may be 0.001 equivalents or more, such as 0.005 equivalents or more, such as 0.01 equivalents or more, such as 0.05 equivalents or more, such as 0.1 equivalents or more, such as 0.5 equivalents or more, such as 1 equivalent or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, such as 6 equivalents or more and including 10 equivalents or more.

[00195] In some cases, the unreacted sulfating agent in the reaction mixture is quenched under slow agitation. In certain cases, quenching the unreacted sulfating agent under slow agitation includes stirring the reaction mixture in a manner sufficient to maintain agglomerates of the unreacted sulfating agent in the reaction mixture. In some cases, slow agitation of the reaction mixture is sufficient such that agglomerates of unreacted sulfating agent reduce in size during quenching by 10% or less, such as by 9% or less, such as by 8% or less, such as by 7% or less,

such as by 6% or less, such as by 5% or less, such as by 4% or less, such as by 3% or less, such as by 2% or less, such as by 1% or less and including where the reaction mixture is slowly agitated such that agglomerates of unreacted sulfating agent reduce in size during quenching by 0.1% or less. In certain cases, slow agitation of the reaction mixture is sufficient such that agglomerates of unreacted sulfating agent remain at the bottom of the reaction flask during quenching. In certain cases, slow agitation of the reaction mixture is sufficient such that little to no agglomerates of unreacted sulfating agent is present in the stirring vortex of the agitated reaction mixture.

[00196] In some cases, methods include purifying the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt prior to contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with the at least one metal salt. In some cases, the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has a purity of 97% or greater, such as a purity of 98% or greater, such as a purity of 99% or greater, such as purity of 99.5% or greater, such as purity of 99.7% or greater, such as a purity of 99.9% or greater and including a purity of 99.99% or greater. In certain cases, the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has one or more by-products of sulfation (e.g., by-products from sulfating the 25-hydroxy-(3 β)-cholest-5-en-3-ol) where the one or more by-products is present in an amount of 5% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt, such as 4% w/w or less, such as 3% w/w or less, such as 2% w/w or less, such as 1% w/w or less, such as in an amount of 0.9% w/w or less, such as 0.8% w/w or less, such as 0.7% w/w or less, such as 0.6% w/w or less, such as 0.5% w/w or less, such as 0.4% w/w or less, such as 0.3% w/w or less, such as 0.2% w/w or less, such as 0.1% w/w or less, such as 0.05% w/w or less, such as 0.01% w/w or less and including being present in an amount of 0.001% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In some cases, the bis-sulfated product (i.e., 5-cholesten-3 β -25-diol-disulfate) is present in the purified 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt composition in an amount of 1% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt, such as in an amount of 0.9% w/w or less, such as 0.8% w/w or less, such as 0.7% w/w or less, such as 0.6% w/w or less, such as 0.5% w/w or less, such as 0.4% w/w or less, such as 0.3% w/w or less, such as 0.2% w/w or less, such as 0.1% w/w or less, such as 0.05% w/w or less, such as 0.01% w/w or less and including being present

in an amount of 0.001% w/w or less relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00197] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is purified by liquid chromatography. In some cases, purifying the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt includes liquid chromatography using a silica gel stationary phase (e.g., a silica gel plug column, ≥ 5 mass equivalents). In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is purified using the silica gel stationary phase and a mobile phase that includes pyridine. In certain cases, the mobile phase includes methylene chloride, methanol, and pyridine. In certain cases, the mobile phase includes a mixture of methylene chloride-methanol (85:15) and pyridine (1%).

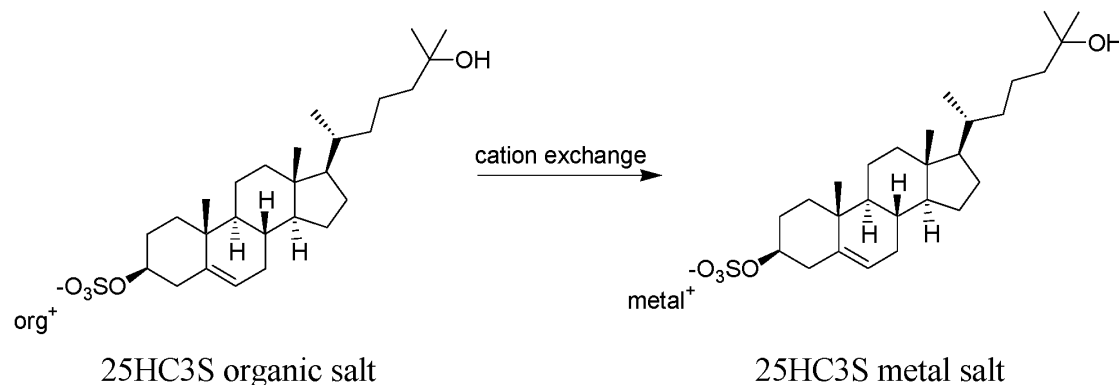
[00198] In some cases, one or more fractions collected from the stationary phase may be combined. In some cases, the combined fractions may be concentrated. In certain cases, the combined fractions are concentrated by distillation. In certain cases, the combined fractions are concentrated under vacuum. In certain cases, the combined fractions are concentrated by distillation under vacuum.

[00199] In some cases, the combined fractions are contacted with one or more particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (e.g., particles from a previously purified sample of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt). In some cases, contacting the particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with the combined fractions is sufficient to precipitate 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt in the combined fractions. In some cases, contacting particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with the combined fractions includes adding the particles during distillation of the combined fractions. In some cases, the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt are added to the combined fractions before distilling the combined fractions. In some cases, the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt are added to the combined fractions while distilling the combined fractions, such as 1 minute or more after beginning the distillation, such as 5 minutes or more, such as 10 minutes or more, such as 15 minutes or more, such as 20 minutes or more, such as 30 minutes or more, such as 40 minutes or more, such as 50 minutes or more and including adding the particles of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt to the combined fractions 60 minutes or more after beginning the distillation of the combined

fractions. In certain cases, the combined fractions are distilled under constant pressure, such as where the pressure changes by 10% or less, such as by 9% or less, such as by 8% or less, such as by 7% or less, such as by 6% or less, such as by 5% or less, such as by 4% or less, such as by 3% or less, such as by 2% or less, such as by 1% or less and including by 0.1% or less. In some cases, the pressure during distillation changes by 10 inHg or less, such as by 9 inHg or less, such as by 8 inHg or less, such as by 7 inHg or less, such as by 6 inHg or less, such as by 5 inHg or less, such as by 4 inHg or less, such as by 3 inHg or less, such as by 2 inHg or less, such as by 1 inHg or less, such as by 0.5 inHg or less, such as by 0.1 inHg or less, such as by 0.05 inHg or less and including by 0.01 inHg or less. In some cases, the combined fractions are distilled under a reduced pressure wherein the pressure is maintained between 15 inHg to 30 inHg, such as from 17.5 inHg to 27.5 inHg, such as from 20 inHg to 25 inHg, such as from 21 inHg and 24 inHg and including maintained at a pressure of from 22 inHg to 23 inHg.

[00200] In some cases, the combined fractions are concentrated under vacuum and the concentrated combined fractions are contacted with a composition containing particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, the concentrated combined fractions are contacted with a composition containing particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt and at least one solvent. In certain cases, the at least one solvent is chosen from tetrahydrofurans, such as 2-methyltetrahydrofuran. The concentrated combined fractions may be contacted with the composition containing the particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt over a duration of 0.001 minutes or more, such as over 0.005 minutes or more, such as over 0.01 minutes or more, such as over 0.05 minutes or more, such as over 0.1 minutes or more, such as over 0.5 minutes or more, such as over 1 minute or more, such as over 2 minutes or more, such as over 3 minutes or more, such as over 4 minutes or more, such as over 5 minutes or more, such as over 10 minutes or more, such as over 15 minutes or more, such as over 30 minutes or more, such as over 45 minutes or more and including over 60 minutes or more. In certain cases, the combined fractions are added dropwise to a composition containing 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt in 2-methyltetrahydrofuran.

[00201] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is contacted with a metal salt to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt (**Scheme IB1**).

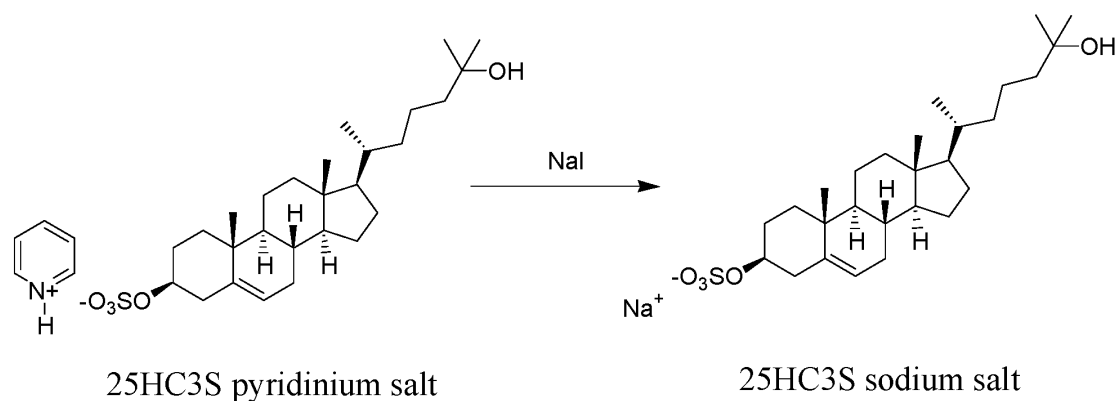
Scheme IB1

[00202] In some cases, methods to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt includes contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one sodium salt. In some cases, the at least one sodium salt is chosen from sodium acetate, sodium iodide, sodium chloride, sodium hydroxide and sodium methoxide. The 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt may be contacted with the metal salt at a temperature that ranges from -10 °C to 75 °C, such as from -5 °C to 70 °C, such as from -4 °C to 65 °C, such as from -3 °C to 60 °C, such as from -2 °C to 55 °C, such as from -1 °C to 50 °C, such as from 0 °C to 45 °C, such as from 5 °C to 40 °C, and including from 10 °C to 35 °C.

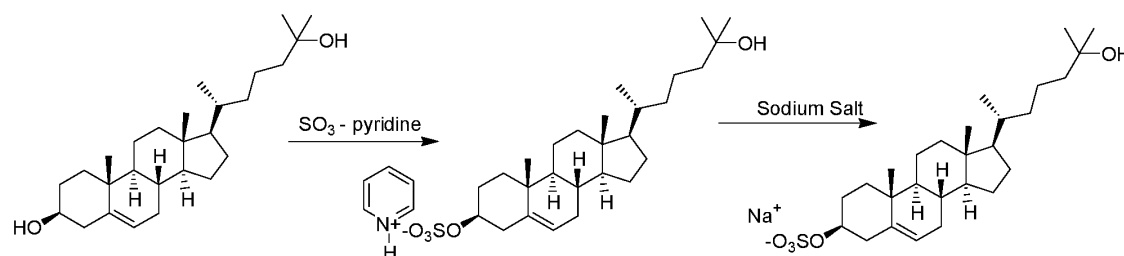
[00203] The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours, and including from 1 hours to 15 hours. The amount of metal salt used relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt may vary and may be 0.0001 equivalents or more, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5

equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to 3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, or 2 equivalents to 3 equivalents, 1 equivalent to 100 equivalents, 1 equivalent to 5 equivalents, 1 equivalent to 2 equivalents.

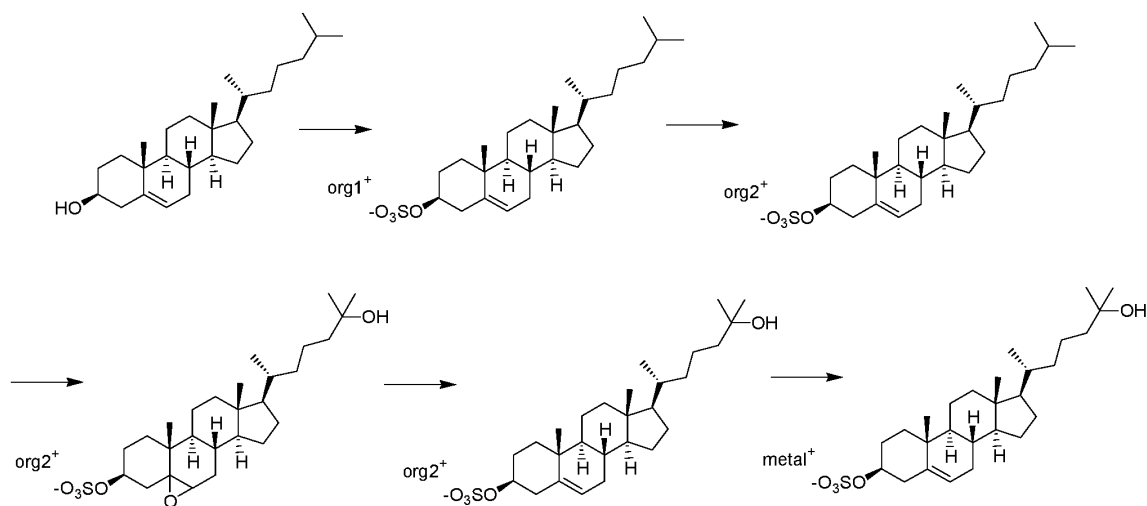
[00204] In some cases, methods include contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with sodium iodide to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate sodium salt (**Scheme IB2**).



[00205] In some cases, methods for preparing 25-hydroxy-3 β -cholesten-5-en-3-sulfate include contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfur trioxide-pyridine complex to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with a sodium salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate sodium salt (**Scheme Ib**).

Scheme Ib

[00206] In some cases, methods for preparing 25-hydroxy-3β-cholest-5-en-3-sulfate include contacting (3β)-cholest-5-en-3-ol with a sulfating agent to produce a first (3β)-cholest-5-en-3-sulfate organic cationic salt; contacting the first (3β)-cholest-5-en-3-sulfate organic cationic salt with an organic base to produce a second (3β)-cholest-5-en-3-sulfate organic cationic salt; oxidizing the second (3β)-cholest-5-en-3-sulfate organic cationic salt in the presence of at least one surfactant to produce a 25-hydroxy-(3β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt; generating a 25-hydroxy-(3β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt by deoxygenation; and contacting the 25-hydroxy-(3β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholest-3β,25-diol 3-sulfate metal salt (**Scheme IIa**).

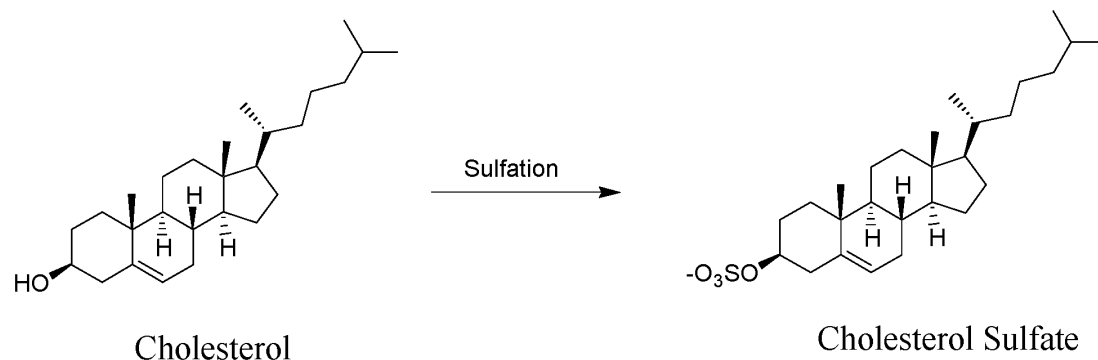
Scheme IIa

[00207] In some cases, cholesterol is sulfated with a sulfating agent (**Scheme IIA1**). In some cases, the sulfating agent is chosen from sulfur trioxide complexes, sulfuric acid compounds, sulfonic acid compounds, and sulfonate compounds. In some cases, the sulfating agent is a sulfur trioxide-pyridine complex. In some cases, the sulfating agent is chosen from sulfur trioxide dimethyl formamide, sulfur trioxide triethylamine, and sulfur trioxide trimethylamine. In some cases, the sulfating agent is sulfuric acid and acetic anhydride and pyridine. In some cases, the sulfating agent is chosen from chlorosulfonic acid and pyridine. In some cases, the sulfating agent is chosen from chlorosulfonic acid and 2,6-lutidine. In some cases, the sulfating agent is chosen from ethyl chlorosulfonate.

[00208] Cholesterol may be sulfated at a temperature that ranges from 0 °C to 100 °C, such as from 5 °C to 95 °C, such as from 10 °C to 90 °C, such as from 15 °C to 85 °C, such as from 20 °C to 80 °C, such as from 25 °C to 75 °C, and including from 30 °C to 70 °C. The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, and including from 0.7 hours to 18 hours. The amount of sulfating agent used relative to cholesterol may vary and may be 0.0001 equivalents or more, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to

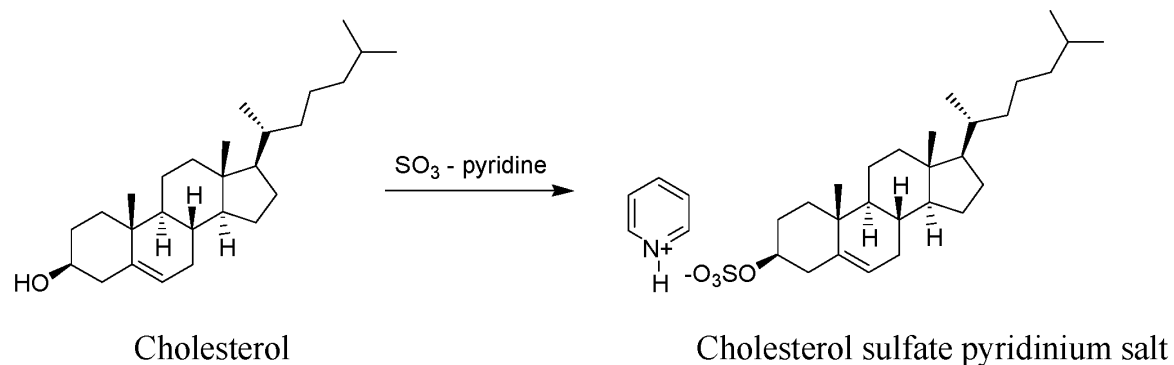
3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, 2 equivalents to 3 equivalents, 1 equivalent to 30 equivalents, 1 equivalent to 5 equivalents, or 1 equivalent to 2 equivalents.

Scheme IIA1

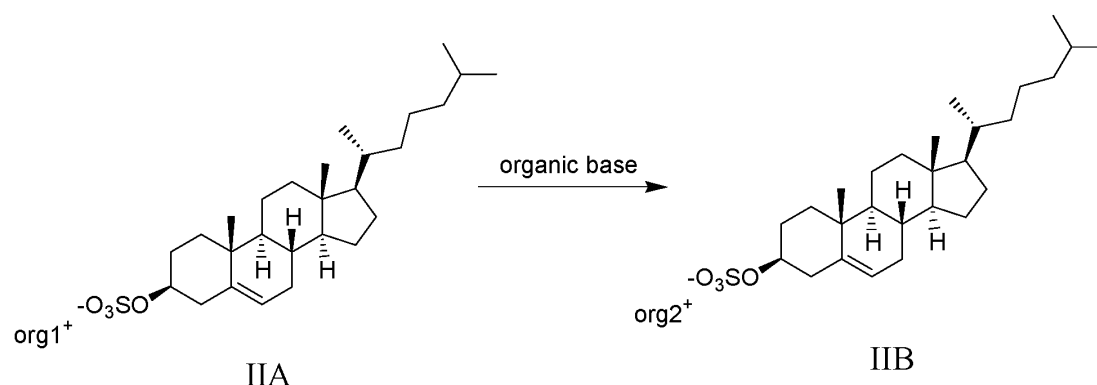


[00209] In some cases, the first (3 β)-cholest-5-en-3-sulfate organic cationic salt is a (3 β)-cholest-5-en-3-sulfate pyridinium salt (**Scheme IIA2**).

Scheme IIA2



[00210] In some cases, the first (3 β)-cholest-5-en-3-sulfate organic cationic salt (Structure IIA) is contacted with an organic base to produce a second (3 β)-cholest-5-en-3-sulfate organic cationic salt (Structure IIB) (**Scheme IIB1**).

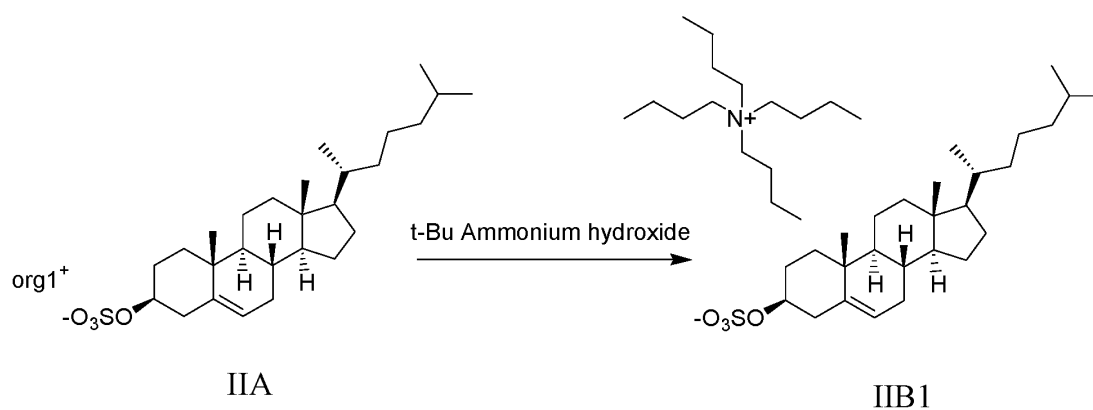
Scheme VB1

[00211] In some cases, the organic base contacted with the first (3 β)-cholest-5-en-3-sulfate organic cationic salt is chosen from a hydroxide base. In some cases, the hydroxide base is chosen from tetraethylammonium hydroxide, tetrabutylammonium hydroxide, tetrapropylammonium hydroxide and tetramethylammonium hydroxide. In some cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is chosen from a tetraethylammonium cationic salt, a tetrabutylammonium cationic salt, a tetrapropylammonium cationic salt and a tetramethylammonium cationic salt. In some cases, the organic base is contacted with the first (3 β)-cholest-5-en-3-sulfate organic cationic salt at a temperature that ranges from -10 °C to 75 °C, such as from -5 °C to 70 °C, such as from -4 °C to 65 °C, such as from -3 °C to 60 °C, such as from -2 °C to 55 °C, such as from -1 °C to 50 °C and including from 0 °C to 15 °C. The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours, and including from 1 hour to 15 hours. The amount of the organic base used relative to the first (3 β)-cholest-5-en-3-sulfate organic cationic salt may vary and may be 0.0001 equivalents or more, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3

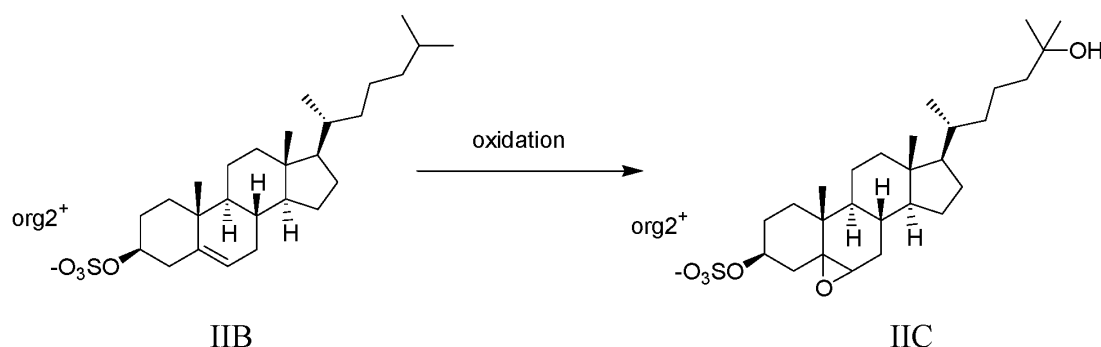
equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to 3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, 2 equivalents to 3 equivalents, 1 equivalent to 10 equivalents, 1 equivalent to 5 equivalents, or 1 equivalent to 2 equivalents.

[00212] In certain cases, methods include contacting the first (3 β)-cholest-5-en-3-sulfate organic cationic salt with tetrabutylammonium hydroxide to generate a (3 β)-cholest-5-en-3-sulfate tetrabutylammonium cationic salt (Structure IIB1) (**Scheme IIB2**).

Scheme VB1



[00213] In some cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized to produce a 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt (Structure IIC) (**Scheme IIC1**).

Scheme IIC1

[00214] In some cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with a composition having an oxidizing agent and at least one surfactant.

[00215] In some cases, the at least one surfactant is chosen from non-ionic surfactants, anionic surfactants, cationic surfactants and zwitterionic surfactants. Non-ionic surfactants may be chosen from polyoxyethylene glycol ethers (e.g., polyoxyethylene glycol octylphenol ether), polyoxyethylene glycol sorbitan alkyl esters, sorbitan alkyl esters, block copolymers of polyethylene glycol and polypropylene glycol, among other non-ionic surfactants. Anionic surfactants may be chosen from surfactants having an anionic functional head group, such as a sulfonate, phosphate, sulfate or carboxylate head group-containing surfactant. For example, anionic surfactants may be chosen from alkyl sulfates such as ammonium lauryl sulfate, dioctyl sodium sulfosuccinate, perfluorooctanesulfonate, perfluorononanoate, perfluorooctanoate, a linear alkylbenzene sulfonate, an alkyl-aryl ether phosphate, sodium lauryl ether sulfate, lignosulfonate or sodium stearate, among other anionic surfactants. Cationic surfactants may be chosen from surfactants having a cationic functional head group, such as a pyridinium or a quarternary ammonium head group. For example, cationic surfactants may be chosen from cetyltrimethylammonium hydrogen sulfate, tetrabutylammonium hydrogen sulfate, cetyltrimethylammonium bromide, tetrabutylammonium bromide, tetrabutylammonium iodide, tetrabutylphosphonium bromide, tetraoctylammonium bromide, tetraoctylammonium iodide, benzyltriethylammonium chloride, benzyltriethylammonium bromide, benzylcetyldimethylammonium chloride or benzylcetyldimethylammonium bromide.

Zwitterionic surfactants include both cationic and anionic centers, such as a sultaine (e.g., 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) or a betaine (e.g., cocamidopropyl betaine). In certain cases, the at least one surfactant is an Extran laboratory soap, La Parisienne soap or DL- α -tocopherol methoxypolyethylene glycol succinate (e.g., TPGS-750-M-2).

[00216] The amount of surfactant used relative to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt may vary, where in some instances, 0.0001 equivalents or more of the surfactant is used, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more of the surfactant, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to 3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, 2 equivalents to 3 equivalents, 0.1 equivalent to 5 equivalents, 0.15 equivalents to 1 equivalent, or 0.2 equivalents to 0.3 equivalents.

[00217] In some cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with an oxidizing agent and at least one ketone in the presence of at least one surfactant.

[00218] In some instances, the at least one ketone is chosen from tetrahydrothiopyran-4-one 1,1-dioxide and halogenated ketones. In some cases, the halogenated ketones are chosen from 1,1,1-trifluoro-2-butanone, 4,4-difluorocyclohexanone, 2,2,2,4'-tetrafluoroacetophenone, and

1,1,1-trifluoroacetone. In certain cases, the at least one ketone is 1,1,1-trifluoro-2-butanone. The amount of ketone used relative to the oxidizing agent in the subject reaction may vary, and may be 1 equivalent or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, such as 6 equivalents or more, such as 7 equivalents or more, such as 8 equivalents or more, such as 9 equivalents or more, such as 10 equivalents or more, such as 15 equivalents or more, such as 20 equivalents or more, such as 25 equivalents or more, such as 30 equivalents or more, such as 35 equivalents or more, and including 50 equivalents or more of the ketone, and may range from 1 equivalent to 50 equivalents, such as 1 equivalent to 35 equivalents, 1 equivalent to 25 equivalents, 1 equivalent to 15 equivalents, 1 equivalent to 10 equivalents, 1 equivalent to 8 equivalents, 1 equivalent to 5 equivalents, 2 equivalent to 50 equivalents, 2 equivalent to 35 equivalents, 2 equivalent to 25 equivalents, 2 equivalent to 15 equivalents, 2 equivalent to 10 equivalents, 2 equivalent to 8 equivalents, 2 equivalent to 5 equivalents, 4 equivalent to 50 equivalents, 4 equivalent to 35 equivalents, 4 equivalent to 25 equivalents, 4 equivalent to 15 equivalents, 4 equivalent to 10 equivalents, 4 equivalent to 8 equivalents, 1 equivalent to 50 equivalents, 2 equivalent to 25 equivalents, or 5 equivalents to 10 equivalents.

[00219] In certain cases, the ketone is further purified before use. For example, the ketone may be purified by distillation prior to use. In some instances, the reactivity of the ketone is tested (e.g., tested for impurities by ¹H-NMR) in order to determine whether purification may be required.

[00220] In certain cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with an oxidizing agent and at least one ketone in the presence of at least one surfactant and water. The amount of water present may vary, ranging from 0.0000001% w/v or more of the reaction mixture, such as 0.000001% w/v or more, such as 0.00001% w/v or more, such as 0.0001% w/v or more, such as 0.001% w/v, such as 0.01% w/v or more, such as 0.1% w/v, such as 0.05% w/v or more, such as 0.1% w/v or more, such as 0.5% w/v or more, such as 1% w/v or more, such as 5% w/v or more, such as 10% w/v or more, such as 15% w/v or more, and including 25% w/v or more of the reaction mixture, and may range from 0.0000001% w/v to 25% w/v, such as 0.0000001% w/v to 15% w/v, 0.0000001% w/v to 10% w/v, 0.0000001% w/v to 5% w/v, 0.0000001% w/v to 1% w/v, 0.001% w/v to 25% w/v, 0.001% w/v to 15% w/v, 0.001% w/v to 10% w/v, 0.001% w/v to

5% w/v, 0.001% w/v to 1% w/v, 0.1% w/v to 25% w/v, 0.1% w/v to 15% w/v, 0.1% w/v to 10% w/v, 0.1% w/v to 5% w/v, 0.1% w/v to 1% w/v, 1% w/v to 25% w/v, 1% w/v to 15% w/v, 1% w/v to 10% w/v, 1% w/v to 5% w/v, 0.1% w/v to 50% w/v, 0.1% w/v to 10% w/v, or 0.5% w/v to 1% w/v.

[00221] The second (3 β)-cholest-5-en-3-sulfate organic cationic salt may be oxidized at a temperature that ranges from -25 °C to 50 °C, such as from -20 °C to 45 °C, such as from -15 °C to 40 °C, such as from -10 °C to 35 °C, such as from -5 °C to 30 °C, such as from -1 °C to 25 °C, and including from 0 °C to 15 °C. In certain cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized at a temperature of from 0 °C to 5 °C. Where the reaction mixture includes an amount of water, the reaction may be conducted at a temperature that is from -10 °C to 50 °C, such as from -5 °C to 45 °C, such as from 0 °C to 40 °C, such as from 0 °C to 35 °C, such as from 0 °C to 30 °C, such as from 0 °C to 25 °C, such as from 0 °C to 20 °C, such as from 0 °C to 15 °C, and including from 0 °C to 10 °C.

[00222] The second (3 β)-cholest-5-en-3-sulfate organic cationic salt may be oxidized at a pH that ranges from 5 to 7.5, such as a pH of from 5.5 to 7.0 and including a pH of from 5.5 to 6.5. In some cases, where the reaction mixture contains water (e.g., in a biphasic solvent system), the pH ranges from 5.0 to 6.0, such as a pH of from 5.0 to 5.9, such as a pH of from 5.0 to 5.8, such as a pH of from 5.0 to 5.7, such as a pH from 5.0 to 5.6, and including a pH of from 5.0 to 5.5.

[00223] The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours, and including from 1 hours to 15 hours.

[00224] In some instances, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is contacted *in situ* with a composition having potassium peroxymonosulfate and at least one ketone in the presence of at least one surfactant. In some cases, methods include contacting the potassium peroxymonosulfate with at least one ketone in the presence of at least one surfactant to form a separate oxidative reactive mixture and adding the oxidative reactive mixture to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In these cases, the potassium peroxymonosulfate may be contacted with the at least one ketone in the presence of the at least one surfactant for a duration of 0.1 minute or more before contacting the oxidative reactive

mixture with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt, such as 1 minute or more, such as 2 minutes or more, such as 3 minutes or more, such as 5 minutes or more, and including 10 minutes or more, and the time may range from 2 minutes to 180 minutes, such as 3 minutes to 120 minutes or 4 minutes to 60 minutes. In certain instances, the potassium peroxymonosulfate may be contacted with the at least one ketone in the presence of the at least one surfactant to form a separate oxidative reactive mixture and immediately contacting the oxidative reactive mixture with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. The oxidative reactive mixture may be formed at a temperature that ranges from -10 °C to 50 °C, such as from -5 °C to 45 °C, such as from -4 °C to 40 °C, such as from -3 °C to 35 °C, such as from -2 °C to 30 °C, such as from -1 °C to 25 °C and including from 0 °C to 15 °C. Where the oxidative reactive mixture is not immediately contacted with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt, the oxidative reactive mixture may be maintained at a temperature that ranges from -10 °C to 50 °C, such as from -5 °C to 45 °C, such as from -4 °C to 40 °C, such as from -3 °C to 35 °C, such as from -2 °C to 30 °C, such as from -1 °C to 25 °C, and including from 0 °C to 15 °C.

[00225] In some cases, methods further include adding the oxidative reactive mixture to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In some instances, methods include adding dropwise the oxidative reactive mixture to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In some instances, the oxidative reactive mixture is added to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt in metered amounts. The metered amounts may be added continuously or at predetermined time intervals (e.g., every 30 seconds, 1 minute, 2 minutes, 3 minutes, 5 minutes, or some other interval). In some instances, the oxidative reactive mixture is added to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt by controlled addition, such as with a mechanically or computer controlled pump, e.g., syringe pump. In some cases, methods include generating the oxidative reactive mixture and adding a composition containing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt to the oxidative reactive mixture. In some instances, methods include adding dropwise the second (3 β)-cholest-5-en-3-sulfate organic cationic salt to the oxidative reactive mixture. In some instances, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is added to the oxidative reactive mixture in metered amounts. The metered amounts may be added continuously or at predetermined time intervals (e.g., every 30 seconds, 1 minute, 2 minutes, 3 minutes, 5 minutes, or some other

interval). In some instances, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is added to the oxidative reactive mixture by controlled addition, such as with a mechanically or computer-controlled pump, e.g., syringe pump.

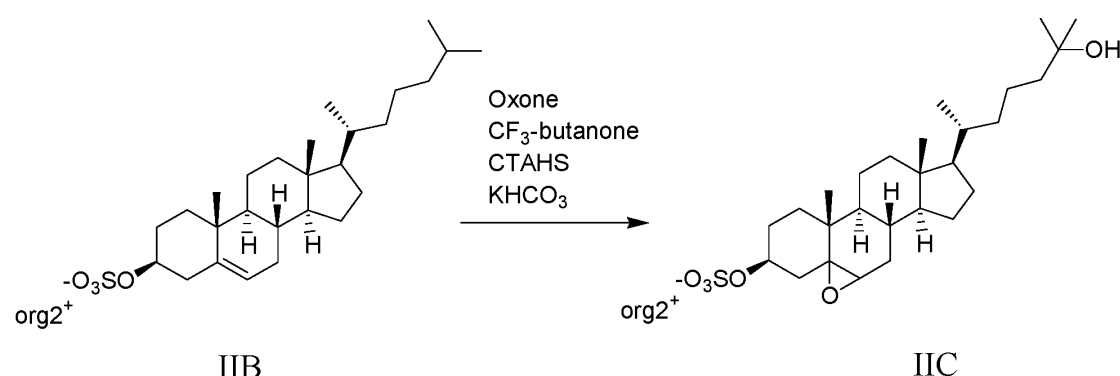
[00226] In certain cases, oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one oxidative species. In some instances, the at least one oxidative species is chosen from dioxiranes. In some instances, the dioxiranes are generated *in situ* in a composition having the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In some instances, the dioxiranes are generated separately (e.g., in a separate reaction container, e.g., flask) and added to the composition having the second (3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00227] In certain cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized in the presence of at least one base. In certain cases, the at least one base is chosen from weak bases. In some cases, the at least one base is chosen from potassium hydrogen carbonate, sodium hydrogen carbonate, potassium phenoxide, sodium citrate buffer, sodium phosphate buffer, potassium formate and potassium acetate. In certain cases, the at least one base is potassium hydrogen carbonate. In some cases, the at least one base may be added to the reaction mixture over time, such as in metered amounts where the base is added at predetermined time intervals (e.g., every 30 seconds, 1 minute, 2 minutes, 3 minutes, 5 minutes, or some other interval). In some cases, the at least one base may be a composition having water where the base present in the composition may be 0.0000001% w/v or more of the composition, such as 0.000001% w/v or more, such as 0.00001% w/v or more, such as 0.0001% w/v or more, such as 0.001% w/v or more, such as 0.01% w/v or more, such as 0.05% w/v or more, such as 0.1% w/v or more, such as 0.5% w/v or more, such as 1% w/v or more, such as 5% w/v or more, such as 10% w/v or more, such as 15% w/v or more, and including 25% w/v or more of the composition, and may range from 0.0000001% w/v to 25% w/v, such as 0.0000001% w/v to 15% w/v, 0.0000001% w/v to 10% w/v, 0.0000001% w/v to 5% w/v, 0.0000001% w/v to 1% w/v, 0.001% w/v to 25% w/v, 0.001% w/v to 15% w/v, 0.001% w/v to 10% w/v, 0.001% w/v to 5% w/v, 0.001% w/v to 1% w/v, 0.1% w/v to 25% w/v, 0.1% w/v to 15% w/v, 0.1% w/v to 10% w/v, 0.1% w/v to 5% w/v, 0.1% w/v to 1% w/v, 1% w/v to 25% w/v, 1% w/v to 15% w/v, 1% w/v to 10% w/v, 1% w/v to 5% w/v, 0.1% w/v to 20% w/v, 0.2% w/v to 15% w/v, or 0.3% w/v to 10%

w/v. In certain cases, the at least one base may be an aqueous potassium hydrogen carbonate composition.

[00228] In certain cases, the second (3 β)-cholest-5-en-3-sulfate organic cationic salt is oxidized by contacting with oxone in the presence of cetyltrimethylammonium hydrogen sulfate (CTAHS) followed by adding trifluorobutanone and potassium hydrogen sulfate to form 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt (**Scheme IIC2**).

Scheme IIC2



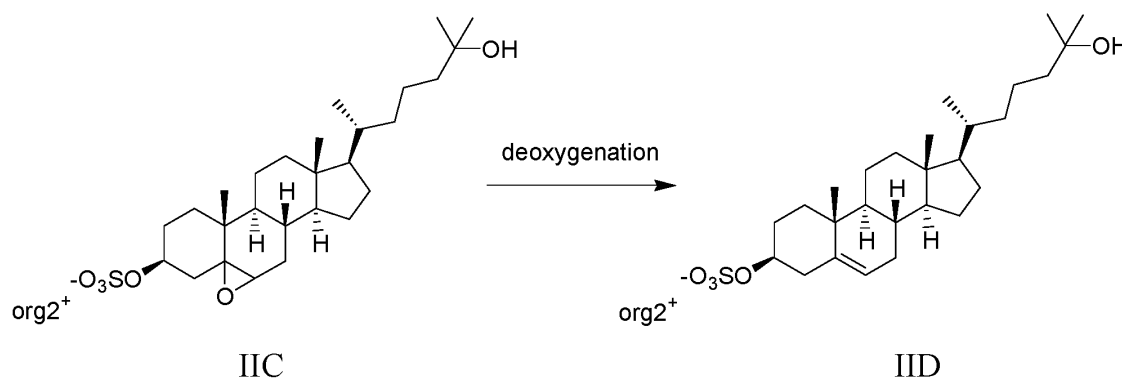
[00229] In certain instances, methods include forming an oxidative species *in situ* with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt, such as by contacting potassium peroxymonosulfate and trifluorobutanone in the presence of cetyltrimethylammonium hydrogen sulfate (CTAHS) in a reaction mixture with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In certain cases, forming an oxidative species *in situ* with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt includes forming a dioxirane *in situ* with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00230] In certain cases, methods include forming a dioxirane in a separate reaction and adding the dioxirane to the second (3 β)-cholest-5-en-3-sulfate organic cationic salt. In these cases, the potassium peroxymonosulfate may be contacted with the trifluorobutanone in the presence of cetyltrimethylammonium hydrogen sulfate (CTAHS) for a duration of 0.1 minute or more before contacting the reactive composition with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt, such as 1 minute or more, such as 2 minutes or more, such as 3 minutes or more, such as 5 minutes or more, and including 10 minutes or more), and the time may range from 0.01 minutes to 120 minutes, such as 0.1 minutes to 90 minutes or 0.5 minutes to 60

minutes. In certain instances, the potassium peroxymonosulfate may be contacted with trifluorobutanone in the presence of cetyltrimethylammonium hydrogen sulfate (CTAHS) to form the oxidative reactive composition, which is immediately contacted with the second (3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00231] The 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt is deoxygenated to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (Structure IID) (**Scheme IID1**).

Scheme IID1



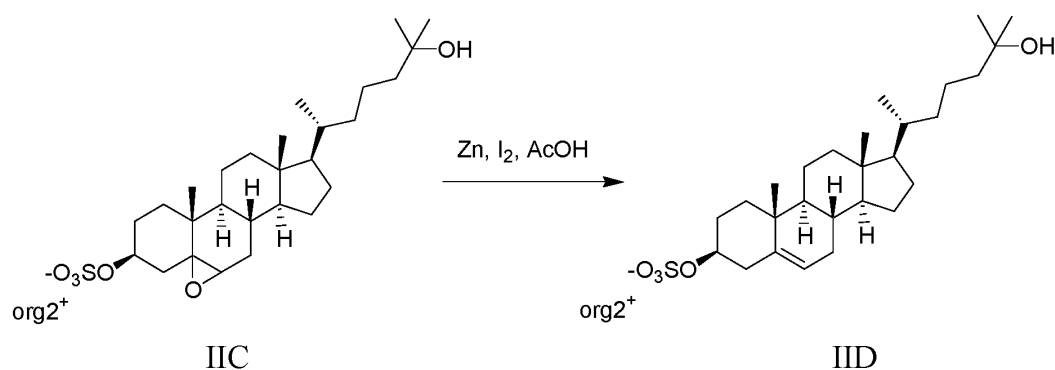
[00232] In some cases, generating 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt includes deoxygenation by contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc. In certain instances, the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt is contacted with zinc in the presence of at least one halide and at least one acid. In some cases, the at least one halide is chosen from iodine and metal halides. In some cases, the metal halide is chosen from sodium iodide and lithium iodide. In some cases, the at least one acid is chosen from weak acids. In some cases, the at least one acid is chosen from acetic acid, hydrochloric acid, citric acid, para-toluene sulfonic acid, formic acid and methane sulfonic acid.

[00233] The amount of reagent used to deoxygenate the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt may vary, where in some instances, 0.0001 equivalents or more of reagent relative to the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt is

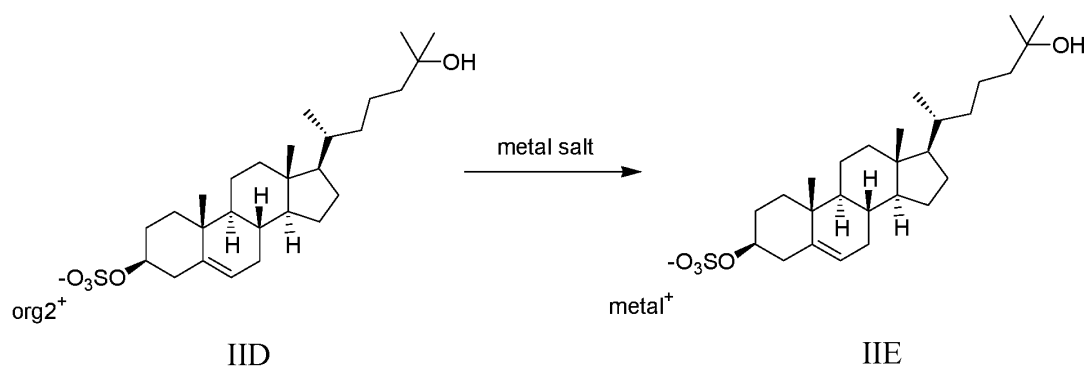
used, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to 3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, 2 equivalents to 3 equivalents, 1 equivalent to 20 equivalents, 1 equivalent to 10 equivalents, or 4 equivalents to 6 equivalents.

[00234] The 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt may be deoxygenated at a temperature that ranges from -10 °C to 75 °C, such as from -5 °C to 70 °C, such as from -4 °C to 65 °C, such as from -3 °C to 60 °C, such as from -2 °C to 55 °C, such as from -1 °C to 50 °C and including from 0 °C to 25 °C. The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours, and including from 1 hours to 15 hours.

[00235] In certain instances, methods include contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc in the presence of iodine and acetic acid to generate the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (**Scheme IID2**).

Scheme IID2

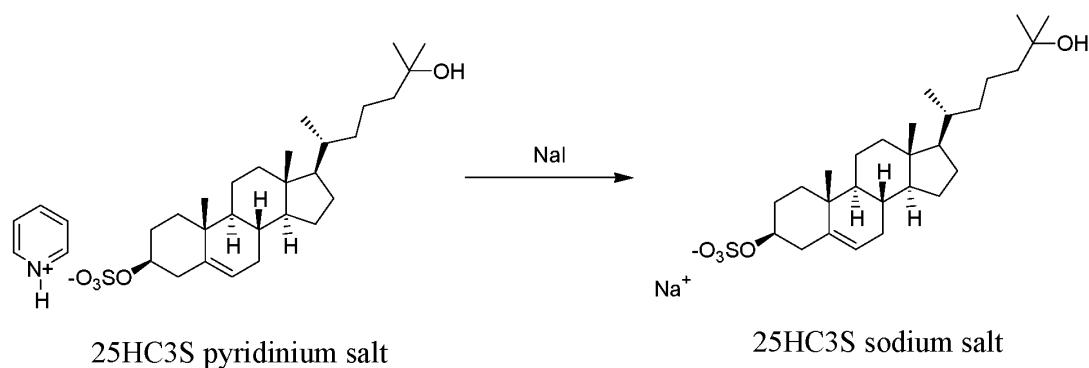
[00236] In some cases, the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt (Structure IID) is contacted with a metal salt to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt (Structure IIE) (**Scheme IIE1**).

Scheme IIE1

[00237] In some cases, methods to produce the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate metal salt include contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one sodium salt. In some cases, the at least one sodium salt is chosen from sodium acetate, sodium iodide, sodium chloride, sodium hydroxide and sodium methoxide. The 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt may be contacted with the metal salt at a temperature that ranges from -10 °C to 75 °C, such as from -5 °C to 70 °C, such as from -4 °C to 65 °C, such as from -3 °C to 60 °C, such as from -2 °C to 55 °C, such as from -1 °C to 50 °C, such as from 0 °C to 45 °C, such as from 5 °C to 40 °C, and including from 10 °C to 35 °C.

[00238] The reaction may be carried out for a duration that ranges from 0.1 hours to 72 hours, such as from 0.2 hours to 48 hours, such as from 0.3 hours to 24 hours, such as from 0.4 hours to 21 hours, such as from 0.5 hours to 20 hours, such as from 0.6 hours to 19 hours, such as from 0.7 hours to 18 hours, such as from 0.8 hours to 17 hours, such as from 0.9 hours to 16 hours, and including from 1 hours to 15 hours. The amount of metal salt used relative to the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt may vary and may be 0.0001 equivalents or more, such as 0.001 equivalents or more, such as 0.01 equivalents or more, such as 0.1 equivalents or more, such as 0.2 equivalents or more, such as 0.3 equivalents or more, such as 0.4 equivalents or more, such as 0.5 equivalents or more, such as 0.6 equivalents or more, such as 0.7 equivalents or more, such as 0.8 equivalents or more, such as 0.9 equivalents or more, such as 1 equivalent or more, such as 1.1 equivalents or more, such as 1.2 equivalents or more, such as 1.3 equivalents or more, such as 1.4 equivalents or more, such as 1.5 equivalents or more, such as 1.6 equivalents or more, such as 1.7 equivalents or more, such as 1.8 equivalents or more, such as 1.9 equivalents or more, such as 2 equivalents or more, such as 3 equivalents or more, such as 4 equivalents or more, such as 5 equivalents or more, and including 10 equivalents or more, and may range from 0.001 equivalents to 10 equivalents, such as 0.1 equivalents to 10 equivalents, 0.1 equivalents to 8 equivalents, 0.1 equivalents to 6 equivalents, 0.1 equivalents to 4 equivalents, 0.1 equivalents to 3 equivalents, 1 equivalents to 10 equivalents, 1 equivalents to 8 equivalents, 1 equivalents to 6 equivalents, 1 equivalents to 4 equivalents, 1 equivalents to 3 equivalents, 1.5 equivalents to 10 equivalents, 1.5 equivalents to 8 equivalents, 1.5 equivalents to 6 equivalents, 1.5 equivalents to 4 equivalents, 1.5 equivalents to 3 equivalents, 2 equivalents to 10 equivalents, 2 equivalents to 8 equivalents, 2 equivalents to 6 equivalents, 2 equivalents to 4 equivalents, 2 equivalents to 3 equivalents, 1 equivalent to 20 equivalents, 1 equivalent to 10 equivalents, or 1 equivalent to 7 equivalents.

[00239] In some cases, methods include contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with sodium iodide to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate sodium salt (**Scheme IIE2**).

Scheme IIE2**CLAUSES**

[00240] The present disclosure may be further described by one or more of the non-limiting clauses that follow.

[00241] Clause 1. Crystalline 25HC3S sodium.

[00242] Clause 1. Crystalline 25HC3S sodium.

[00243] Clause 2. A hydrate of crystalline 25HC3S sodium.

[00244] Clause 3. A monohydrate of crystalline 25HC3S sodium.

[00245] Clause 4. A dihydrate of crystalline 25HC3S sodium.

[00246] Clause 5. A variable hydrate of crystalline 25HC3S sodium.

[00247] Clause 6. Form I of crystalline 25HC3S sodium.

[00248] Clause 7. The crystalline hydrate of any one of clauses 2-6, having an x-ray powder diffraction pattern comprising a peak at about $2.1^{\circ}2\theta$.

[00249] Clause 8. The crystalline hydrate of clause 1 or 7, further comprising one or more peaks at about $5.4^{\circ}2\theta$, about $6.5^{\circ}2\theta$, about $10.8^{\circ}2\theta$, and about $15.0^{\circ}2\theta$.

[00250] Clause 9. The crystalline hydrate of any one of clauses 7-8 and 85-86, wherein the crystalline hydrate has a weight loss of about 8% when heated from ambient to 130°C at a rate of $10^{\circ}\text{C}/\text{min}$.

[00251] Clause 10. The substantially pure crystalline hydrate of 25HC3S sodium of any one of clauses 2-9 and clauses 85-86.

- [00252] Clause 11. The crystalline hydrate of any one of clauses 2-10 and clauses 85-86, having a unit cell volume of about $4544.7 \text{ \AA}^3 \pm 5\%$.
- [00253] Clause 12. The crystalline hydrate of any one of clauses 2-11 and clauses 85-86, having a monoclinic unit cell.
- [00254] Clause 13. A stable crystalline hydrate of any one of clauses 2-12 and clauses 85-86.
- [00255] Clause 14. The stable crystalline hydrate of clause 13, wherein the stable crystalline hydrate is stable at a relative humidity from about 38% to about 70%.
- [00256] Clause 15. Form II of crystalline 25HC3S sodium.
- [00257] Clause 16. The crystalline hydrate of clause 2 or 5, wherein the water content is not greater than about 3 moles of water per mole of 25HC3S sodium.
- [00258] Clause 17. The crystalline hydrate of clause 2 or 5, wherein the water content is from about 2 moles of water to about 3 moles of water per mole of 25HC3S sodium.
- [00259] Clause 18. The crystalline hydrate of clause 2 or 5, wherein the water content is from about 1 mole of water to about 3 moles of water per one mole of 25HC3S sodium.
- [00260] Clause 19. The crystalline hydrate of any one of clauses 15-18, having an x-ray pattern comprising a peak at about $2.3^\circ 2\theta$.
- [00261] Clause 20. The crystalline hydrate of clause 2 or 19, further comprising an x-ray pattern comprising one or more peaks at about $4.5^\circ 2\theta$, a peak at and between about $5.0^\circ 2\theta$ and about $5.1^\circ 2\theta$, a peak at and between about $5.9^\circ 2\theta$ and about $6.1^\circ 2\theta$, and a peak at and between about $14.8^\circ 2\theta$ and about $15.1^\circ 2\theta$.
- [00262] Clause 21. The substantially pure crystalline hydrate of 25HC3S sodium of any one of clauses 15-20 and clauses 87-88.
- [00263] Clause 22. A stable crystalline hydrate of any one of clauses 15-21 and clauses 87-88.
- [00264] Clause 23. The stable crystalline hydrate of clause 22, wherein the stable crystalline hydrate is stable at a relative humidity ranging from about 21% to about 30%.
- [00265] Clause 24. Form XI of crystalline 25HC3S sodium.
- [00266] Clause 25. The crystalline hydrate of 25HC3S sodium of any one of clauses 2 and 24 having an x-ray powder diffraction pattern comprising a peak at about $2.6^\circ 2\theta$.
- [00267] Clause 26. The crystalline hydrate of clause 2 or 25, having an x-ray powder diffraction pattern further comprising one or more peaks at about $3.1^\circ 2\theta$, about $3.5^\circ 2\theta$, and about

14.5°2 θ .

[00268] Clause 27. The substantially pure crystalline hydrate of 25HC3S sodium of any one of clauses 24-26.

[00269] Clause 28. A stable crystalline hydrate of any one of clauses 24-27.

[00270] Clause 29. Anhydrous crystalline 25HC3S sodium.

[00271] Clause 30. Form XIII crystalline 25HC3S sodium.

[00272] Clause 31. The crystalline 25HC3S sodium of clause 1, 29, or 30 having an x-ray powder diffraction pattern comprising a peak at about 2.3°2 θ .

[00273] Clause 32. The crystalline 25HC3S sodium of clause 1 or 31, having an x-ray powder diffraction pattern comprising one or more peaks at about 4.6°2 θ , about 9.3°2 θ , about 14.3°2 θ , and about 15.0°2 θ .

[00274] Clause 33. The crystalline 25HC3S sodium of clause 1, 29, or 30, wherein the unit cell has a volume of about 4230.8 Å³ ±5%.

[00275] Clause 34. Stable crystalline 25HC3S sodium of any one of clauses 29-33 and clauses 91-92.

[00276] Clause 35. The crystalline 25HC3S sodium of any one of clauses 29-34 and clauses 91-92, wherein the crystalline 25HC3S is stable at a relative humidity ranging from about 0% to about 14% RH.

[00277] Clause 36. The crystalline 25HC3S sodium of any one of clauses 29-35 and clauses 91-92, wherein the crystalline 25HC3S is stable at a temperature of 70 °C or greater.

[00278] Clause 37. The crystalline 25HC3S sodium of any one of clauses 29-36 and clauses 91-92, wherein the unit cell is monoclinic.

[00279] Clause 38. The substantially pure crystalline 25HC3S sodium of any one of clauses 30-37 and clauses 91-92.

[00280] Clause 39. Form IX crystalline 25HC3S sodium.

[00281] Clause 40. The crystalline 25HC3S sodium of clause 1 or 39, having an x-ray powder diffraction pattern comprising a peak at about 4.9°2 θ .

[00282] Clause 41. The crystalline 25HC3S sodium of clause 1 or 39, further comprising one or more peaks at about 7.9°2 θ , about 11.2°2 θ , about 14.1°2 θ , about 16.1°2 θ , and about 16.6°2 θ .

[00283] Clause 42. A liquid crystal of 25HC3S sodium.

[00284] Clause 43. A mesophase of 25HC3S sodium.

- [00285]** Clause 44. Form V of 25HC3S sodium.
- [00286]** Clause 45. The liquid crystalline material of clause 1 or 44, having an x-ray powder diffraction pattern comprising a peak at about $2.2^{\circ}2\theta$.
- [00287]** Clause 46. The crystalline material of clause 1 or 45, further comprising one or more peaks at about $4.4^{\circ}2\theta$, about $6.6^{\circ}2\theta$, and about $8.8^{\circ}2\theta$.
- [00288]** Clause 47. The substantially pure crystalline 25HC3S sodium of any one of clauses 42-46 and clauses 89-90.
- [00289]** Clause 48. Stable crystalline 25HC3S sodium of any one of clauses 42-46 and clauses 89-90.
- [00290]** Clause 49. The crystalline 25HC3S sodium of clause 1 or 6, having at least one of substantially the same x-ray powder diffraction pattern as Figure 1 and a TGA thermogram substantially the same as shown in Figure 11.
- [00291]** Clause 50. The crystalline 25HC3S sodium of clause 1 or 6, having at least one of substantially the same x-ray powder diffraction pattern as Figure 53 and a TGA thermogram substantially the same as shown in Figure 11.
- [00292]** Clause 51. The crystalline 25HC3S sodium of clause 1 or 15, having at least one of substantially the same x-ray powder diffraction pattern as Figure 2 and a TGA thermogram substantially the same as shown in Figure 24.
- [00293]** Clause 52. The crystalline 25HC3S sodium of clause 1 or 15, having at least one of substantially the same x-ray powder diffraction pattern as Figure 54 and a TGA thermogram substantially the same as shown in Figure 24.
- [00294]** Clause 53. The crystalline 25HC3S sodium of clause 1 or 44, having at least one of substantially the same x-ray powder diffraction pattern as Figure 3 and a TGA thermogram substantially the same as shown in Figure 28.
- [00295]** Clause 54. The crystalline 25HC3S sodium of clause 1 or 44, having at least one of substantially the same x-ray powder diffraction pattern as Figure 4 and a TGA thermogram substantially the same as shown in Figure 28.
- [00296]** Clause 55. The crystalline 25HC3S sodium of clause 1 or 39, having substantially the same x-ray powder diffraction pattern as Figure 5.
- [00297]** Clause 56. The crystalline 25HC3S sodium of clause 1 or 24, having substantially the same x-ray powder diffraction pattern as Figure 6.

[00298] Clause 57. The crystalline 25HC3S sodium of clause 1 or 24, having substantially the same x-ray powder diffraction pattern as Figure 7.

[00299] Clause 58. The crystalline 25HC3S sodium of clause 1 or 30, having substantially the same x-ray powder diffraction pattern as Figure 8.

[00300] Clause 59. The crystalline 25HC3S sodium of clause 1 or 35, having substantially the same x-ray powder diffraction pattern as Figure 9.

[00301] Clause 60. A method of treating one or more of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation comprising administering to a patient in need thereof an effective amount of a compound of 25HC3S sodium of any one of clauses 1-59 or 64.

[00302] Clause 61. A pharmaceutical composition comprising 25HC3S sodium of any one of clauses 1-59, 64, 68-83, and 85-96, and at least one pharmaceutically acceptable excipient.

[00303] Clause 62. A method of treating one or more of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation, comprising administering to a patient in need thereof an effective amount of a pharmaceutical composition of 25HC3S sodium of clause 61.

[00304] Clause 63. The method of clause 60 or 62 wherein the condition related to fat accumulation and inflammation comprises nonalcoholic fatty liver disease (NAFLD).

[00305] Clause 64. Stable crystalline 25HC3S sodium.

[00306] Clause 65. A method of clause 60 for treating nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, and atherosclerosis, comprising administering to a patient in need thereof an effective amount of a compound of 25HC3S sodium of any one of clauses 1-59, 64, 68-83, and 85-96.

[00307] Clause 66. The method of clause 65 for treating nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, and atherosclerosis, comprising administering to a patient in need thereof an effective amount of a pharmaceutical composition of 25HC3S sodium of clause 61.

[00308] Clause 67. A composition comprising two or more of Form I 25HC3S sodium, Form II 25HC3S sodium, Form III 25HC3S sodium, Form V 25HC3S sodium, Form IX 25HC3S sodium, Form XI 25HC3S sodium, and Form XIII 25HC3S sodium.

- [00309] Clause 68. Form I of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 1.
- [00310] Clause 69. Form I of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 53.
- [00311] Clause 70. Form II of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 2.
- [00312] Clause 71. Form II of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 54.
- [00313] Clause 72. Form XI of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 6 or Figure 7.
- [00314] Clause 73. Form V of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 3.
- [00315] Clause 74. Form V of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 4.
- [00316] Clause 75. Form XIII of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 8.
- [00317] Clause 76. Form XIII of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 9.
- [00318] Clause 77. Form III of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 35.
- [00319] Clause 78. Form IX of 25HC3S sodium having substantially the same x-ray powder diffraction pattern as Figure 5.
- [00320] Clause 79. Form III of crystalline 25HC3S sodium.
- [00321] Clause 80. A solvate of crystalline 25HC3S sodium.
- [00322] Clause 81. An ethanol solvate of crystalline 25HC3S sodium.
- [00323] Clause 82. The crystalline solvate of clause 1 or 79 having an x-ray powder diffraction pattern comprising a peak at about $4.9^{\circ}2\theta$.
- [00324] Clause 83. The crystalline material of clause 1 or 82, further comprising one or more peaks at about $6.3^{\circ}2\theta$, about $7.8^{\circ}2\theta$, and about $9.8^{\circ}2\theta$.
- [00325] Clause 84. A pharmaceutical composition comprising two or more of Form I 25HC3S sodium, Form II 25HC3S sodium, Form III 25HC3S sodium, Form V 25HC3S sodium, Form IX

25HC3S sodium, Form XI 25HC3S sodium, and Form XIII 25HC3S sodium and at least one pharmaceutically acceptable excipient.

[00326] Clause 85. Form I of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $2.1^{\circ}2\theta$, about $6.5^{\circ}2\theta$, and about $10.8^{\circ}2\theta$.

[00327] Clause 86. Form I of crystalline 25HC3S sodium of clause 85 further comprising one or more peaks at about $9.9^{\circ}2\theta$, about $15.0^{\circ}2\theta$, and about $15.6^{\circ}2\theta$.

[00328] Clause 87. Form II of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $2.3^{\circ}2\theta$ and about $5.0^{\circ}2\theta$.

[00329] Clause 88. Form II of crystalline 25HC3S sodium of clause 87 further comprising one or more peaks at about $4.5^{\circ}2\theta$, about $5.9^{\circ}2\theta$, about $9.1^{\circ}2\theta$, and about $15.1^{\circ}2\theta$.

[00330] Clause 89. Form V of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $2.2^{\circ}2\theta$, about $6.6^{\circ}2\theta$, and a single peak at and between about $4.0^{\circ}2\theta$ and about $6.0^{\circ}2\theta$.

[00331] Clause 90. Form V of crystalline 25HC3S sodium of clause 89 further comprising one or more peaks at about $8.8^{\circ}2\theta$, about $9.9^{\circ}2\theta$, and about $14.9^{\circ}2\theta$.

[00332] Clause 91. Form XIII of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $2.3^{\circ}2\theta$, about $5.4^{\circ}2\theta$, about $9.3^{\circ}2\theta$, and about $11.6^{\circ}2\theta$.

[00333] Clause 92. Form XIII of crystalline 25HC3S sodium of clause 91 further comprising one or more peaks at about $4.6^{\circ}2\theta$ and about $15.0^{\circ}2\theta$.

[00334] Clause 93. Form III of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $4.9^{\circ}2\theta$ and about $6.3^{\circ}2\theta$.

[00335] Clause 94. Form III of crystalline 25HC3S sodium of clause 93 further comprising one or more peaks at $7.8^{\circ}2\theta$, about $9.8^{\circ}2\theta$, about $13.3^{\circ}2\theta$, and about $15.5^{\circ}2\theta$.

[00336] Clause 95. Form IX of crystalline 25HC3S sodium having an x-ray powder diffraction pattern comprising peaks at about $2.2^{\circ}2\theta$, about $4.9^{\circ}2\theta$, and about $7.9^{\circ}2\theta$.

[00337] Clause 96. Form IX of crystalline 25HC3S sodium of clause 95 further comprising one or more peaks at about $14.1^{\circ}2\theta$, about $16.1^{\circ}2\theta$, and about $16.6^{\circ}2\theta$.

[00338] Clause 97. A pharmaceutical composition comprising crystalline 25HC3S sodium or liquid crystal 25HC3S sodium or both and at least one pharmaceutically acceptable excipient.

[00339] Clause 98. The pharmaceutical composition of clause 97, the crystalline 25HC3S sodium or liquid crystal 25HC3S sodium comprising any one of clauses 1-59, 64, 68-83, and 85-96.

[00340] Clause 99. The pharmaceutical composition of clause 97, wherein the crystalline 25HC3S sodium or liquid crystal 25HC3S sodium comprises a mixture of Form I of 25HC3S sodium and Form II of 25HC3S sodium.

[00341] Clause 100. A method of producing a 5-cholesten-3 β ,25-diol-3-sulfate metal salt, the method comprising: contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with at least one sulfating agent in at least one solvent to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt, wherein the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has low solubility in the at least one solvent; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt.

[00342] Clause 101. The method according to clause 100, wherein the at least one sulfating agent is a sulfur trioxide complex.

[00343] Clause 102. The method according to clause 101, wherein the at least one sulfating agent is a sulfur trioxide-pyridine complex.

[00344] Clause 103. The method according to any one of clauses 100-102, wherein the at least one metal salt is sodium acetate.

[00345] Clause 104. The method according to any one of clauses 100-102, wherein the at least one metal salt is sodium iodide.

[00346] Clause 105. The method according to any one of clauses 100-104, wherein the at least one sulfating agent is contacted with acetic anhydride prior to contacting with 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[00347] Clause 106. A method of producing a 5-cholesten-3 β ,25-diol 3-sulfate sodium salt, the method comprising: contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfur trioxide-pyridine complex in at least one solvent to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt, wherein the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt has low solubility in the at least one solvent; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate pyridinium salt with a sodium salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate sodium salt.

[00348] Clause 107. The method according to clause 106, wherein the sodium salt is sodium acetate.

[00349] Clause 108. The method according to clause 106, wherein the sodium salt is sodium iodide.

[00350] Clause 109. A method of producing a 5-cholesten-3 β ,25-diol 3-sulfate metal salt, the method comprising: contacting (3 β)-cholest-5-en-3-ol with at least one sulfating agent to produce a first (3 β)-cholest-5-en-3-sulfate organic cationic salt; contacting the first (3 β)-cholest-5-en-3-sulfate organic cationic salt with an organic base to produce a second (3 β)-cholest-5-en-3-sulfate organic cationic salt; oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt in the presence of at least one surfactant to produce a 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt; generating a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt by deoxygenation; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt.

[00351] Clause 110. The method according to clause 109, wherein the at least one sulfating agent is a sulfur trioxide complex.

[00352] Clause 111. The method according to clause 110, wherein the at least one sulfating agent is a sulfur trioxide-pyridine complex.

[00353] Clause 112. The method according to any one of clauses 109-111, wherein the organic base is tetraethylammonium hydroxide.

[00354] Clause 113. The method according to any one of clauses 109-112, wherein oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt comprises contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with a composition comprising an oxidizing agent in the presence of at least one surfactant.

[00355] Clause 114. The method according to clause 113, wherein the method comprises contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with a composition comprising the oxidizing agent and at least one ketone in the presence of the at least one surfactant.

[00356] Clause 115. The method according to clause 114, wherein the at least one ketone is 1,1,1-trifluoro-2-butanone.

[00357] Clause 116. The method according to any one of clauses 114-115, wherein the composition comprising the oxidizing agent and the at least one ketone further comprises the second (3 β)-cholest-5-en-3-sulfate organic cationic salt and the at least one surfactant.

[00358] Clause 117. The method according to any one of clauses 114-116, wherein oxidizing the second (3 β)-cholest-5-en-3-sulfate organic cationic salt comprises contacting the composition comprising the oxidizing agent and the at least one ketone with a second composition comprising the second (3 β)-cholest-5-en-3-sulfate organic cationic salt in the presence of the at least one surfactant.

[00359] Clause 118. The method according to any one of clauses 114-117, wherein the method comprises contacting the second (3 β)-cholest-5-en-3-sulfate organic cationic salt with the oxidizing agent in the presence of at least one base.

[00360] Clause 119. The method according to clause 118, wherein the at least one base is potassium hydrogen carbonate.

[00361] Clause 120. The method according to any one of clauses 113-119, wherein the oxidizing agent is potassium peroxymonosulfate.

[00362] Clause 121. The method according to any one of clauses 109-120, wherein the at least one surfactant is cetyltrimethylammonium hydrogen sulfate.

[00363] Clause 122. The method according to any one of clauses 109-121, wherein generating the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt from the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt comprises contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc.

[00364] Clause 123. The method according to clause 122, wherein the method comprises contacting the 25-hydroxy-(3 β)-cholest-(5,6-epoxy)-3-sulfate organic cationic salt with zinc in the presence of at least one halide and at least one acid.

[00365] Clause 124. The method according to clause 123, wherein the at least one acid is acetic acid.

[00366] Clause 125. The method according to any one of clauses 123-124, wherein the at least one halide is iodine.

[00367] Clause 126. The method according to any one of clauses 109-125, wherein the at least one metal salt is sodium acetate.

[00368] Clause 127. The method according to any one of clauses 109-125, wherein the at least one metal salt is sodium iodide.

[00369] Clause 128. A composition comprising: 25-hydroxy-(3 β)-cholest-5-en-3-sulfate; and sulfated desmosterol.

[00370] Clause 129. The composition according to clause 128, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the sulfated desmosterol is from 100:1 to 10000:1.

[00371] Clause 130. The composition according to clause 128, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the sulfated desmosterol is from 100:1 to 1000:1.

[00372] Clause 131. A composition comprising: 25-hydroxy-(3 β)-cholest-5-en-3-sulfate; and 5-cholesten-3 β -25-diol-disulfate.

[00373] Clause 132. The composition according to clause 131, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate is from 100:1 to 10000:1.

[00374] Clause 133. The composition according to clause 131, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate is from 100:1 to 1000:1.

[00375] Clause 134. The composition according to clause 133, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the 5-cholesten-3 β -25-diol-disulfate is from 250:1 to 500:1.

[00376] Clause 135. A method comprising: sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol to produce 25-hydroxy-(3 β)-cholest-5-en-3-sulfate in at least one solvent, wherein 25-hydroxy-(3 β)-cholest-5-en-3-sulfate has low solubility in the at least one solvent; and precipitating 25-hydroxy-(3 β)-cholest-5-en-3-sulfate in the solvent.

[00377] Clause 136. The method according to clause 135, wherein 25-hydroxy-(3 β)-cholest-5-en-3-sulfate has a solubility of 100 mmol/L or less in the at least one solvent.

[00378] Clause 137. The method according to clause 136, wherein 25-hydroxy-(3 β)-cholest-5-en-3-sulfate has a solubility of 10 mmol/L or less in the at least one solvent.

[00379] Clause 138. The method according to any one of clauses 135-137, wherein the at least one solvent is chosen from chloroform, methylene chloride, acetone, acetonitrile, toluene, tetrahydrofuran and methyltetrahydrofuran.

[00380] Clause 139. The method according to any one of clauses 135-138, wherein sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol generates 5-cholesten-3 β -25-diol-disulfate that remains solubilized in the at least one solvent.

[00381] Clause 140. The method according to clause 139, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to 5-cholesten-3 β -25-diol-disulfate generated is from 100:1 to 10000:1.

[00382] Clause 141. The method according to clause 140, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to 5-cholesten-3 β -25-diol-disulfate generated is from 100:1 to 1000:1.

[00383] Clause 142. The method according to any one of clauses 139-141, wherein 5-cholesten-3 β -25-diol-disulfate has high solubility in the at least one solvent.

[00384] Clause 143. The method according to clause 142, wherein 5-cholesten-3 β -25-diol-disulfate has a solubility of 500 mmol/L or more in the at least one solvent.

[00385] Clause 144. A composition comprising: 25-hydroxy-(3 β)-cholest-5-en-3-sulfate; and byproducts from sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol comprising a first compound comprising a high-pressure liquid chromatography (HPLC) retention time of about 18.3 minutes, a second compound comprising a HPLC retention time of about 37.7 minutes, or any combination thereof, and the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate comprising a HPLC retention time of about 7.7 minutes, wherein the first compound, the second compound and the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate are capable of being separated by HPLC comprising a C8 stationary phase operating at about 45 °C with a first mobile phase comprising a buffer and a second mobile phase comprising one or more organic solvents.

[00386] Clause 145. The composition according to clause 144, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the byproducts is from 100:1 to 10000:1.

[00387] Clause 146. The composition according to clause 144, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the byproducts is from 100:1 to 1000:1.

[00388] Clause 147. The composition according to any one of clauses 144-146, wherein the flow rate of the first mobile phase is about 1.0 mL/minute

[00389] Clause 148. The composition according to any one of clauses 144-147, wherein the flow rate of the second mobile phase is about 1.0 mL/minute.

[00390] Clause 149. The composition according to any one of clauses 144-148, wherein the first mobile phase comprises sodium phosphate.

[00391] Clause 150. The composition according to any one of clauses 144-149, wherein the second mobile phase is chosen from one or more of methoxypropyl acetate, acetonitrile and methanol.

[00392] Clause 151. A composition comprising: 25-hydroxy-(3 β)-cholest-5-en-3-sulfate; and byproducts from sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol comprising sulfated desmosterol, a second compound comprising a HPLC retention time of about 37.7 minutes, or any combination thereof, and the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate comprising a HPLC retention time of about 7.7 minutes, wherein the sulfated desmosterol, the second compound and the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate are capable of being separated by HPLC comprising a C8 stationary phase operating at about 45 °C with a first mobile phase comprising a buffer and a second mobile phase comprising one or more organic solvents.

[00393] Clause 152. The composition according to clause 151, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the byproducts is from 100:1 to 10000:1.

[00394] Clause 153. The composition according to clause 151, wherein the ratio by weight of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate to the byproducts is from 100:1 to 1000:1.

[00395] Clause 154. The composition according to any one of clauses 151-152, wherein the flow rate of the first mobile phase is about 1.0 mL/minute

[00396] Clause 155. The composition according to any one of clauses 151-154, wherein the flow rate of the second mobile phase is about 1.0 mL/minute.

[00397] Clause 156. The composition according to any one of clauses 151-155, wherein the first mobile phase comprises sodium phosphate.

[00398] Clause 157. The composition according to any one of clauses 151-156, wherein the second mobile phase is chosen from one or more of methoxypropyl acetate, acetonitrile and methanol.

[00399] Clause 158. The composition according to any one of clauses 151-157, wherein the second compound is desmosterol.

[00400] Clause 159. A method of producing a 5-cholesten-3 β ,25-diol-3-sulfate metal salt, the method comprising: contacting 25-hydroxy-(3 β)-cholest-5-en-3-ol with at least one sulfating agent in at least one solvent to produce a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic

salt, wherein the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt has low solubility in the at least one solvent; and contacting the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt with at least one metal salt to produce the 5-cholesten-3 β ,25-diol 3-sulfate metal salt.

[00401] Clause 160. The method according to clause 159, wherein the sulfating reagent is characterized by ¹H-NMR (proton nuclear magnetic resonance) spectroscopy prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol.

[00402] Clause 161. The method according to clause 160, wherein the method comprises determining the extent of degradation of the sulfating reagent prior to contacting with the 25-hydroxy-(3 β)-cholest-5-en-3-ol by determining the amount of impurity present in the sulfating reagent.

[00403] Clause 162. The method according to clause 161, wherein the extent of degradation of the sulfating reagent is determined by proton nuclear magnetic resonance spectroscopy by integrating one or more peaks in the ¹H-NMR spectrum at a chemical shift indicative of the presence of impurities in the sulfating reagent.

[00404] Clause 163. The method according to clause 162, wherein proton nuclear magnetic resonance spectroscopy of the sulfating agent is conducted in at least one deuterated solvent.

[00405] Clause 164. The method according to clause 163, wherein the at least one deuterated solvent is deuterated acetone.

[00406] Clause 165. The method according to clause 164, wherein the at least one deuterated solvent is not deuterated benzene, deuterated acetonitrile, or deuterated chloroform.

[00407] Clause 166. The method according to any one of clauses 162-165, wherein the method comprises integrating one or more peaks in the proton NMR spectrum at a chemical shift of from 9.2 ppm to 9.3 ppm and calculating the impurity level of the sulfating agent based on the integrated peaks.

[00408] Clause 167. The method according to clause 166, wherein the method comprises integrating one or more peaks in the proton NMR spectrum at a chemical shift of about 9.25 ppm.

[00409] Clause 168. The method according to any one of clauses 159-167, wherein 25-hydroxy-(3 β)-cholest-5-en-3-ol is contacted with the sulfating agent in the presence of particles of a 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00410] Clause 169. The method according to clause 168, wherein the particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt have an average particle width of 0.1 mm or greater.

[00411] Clause 170. The method according to any one of clauses 159-169, wherein the method further comprises quenching unreacted sulfating agent.

[00412] Clause 171. The method according to clause 170, wherein quenching unreacted sulfating agent comprises contacting the reaction mixture with water.

[00413] Clause 172. The method according to clause 171, wherein 1 equivalent or more of water is contacted with the reaction mixture.

[00414] Clause 173. The method according to any one of clauses 171-172, wherein quenching unreacted sulfating agent comprises contacting the reaction mixture with water and pyridine.

[00415] Clause 174. The method according to clause 173, wherein the pyridine is contacted with the reaction mixture after a predetermined period of time after contacting the reaction mixture with the water.

[00416] Clause 175. The method according to any one of clauses 170-174, wherein the reactivity of the unreacted sulfating agent is quenched under slow agitation.

[00417] Clause 176. The method according to any one of clauses 159-175, wherein the method further comprises purifying the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00418] Clause 177. The method according to clause 176, wherein the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is purified by liquid chromatography.

[00419] Clause 178. The method according to clause 177, wherein the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt is purified by liquid chromatography comprising a silica gel stationary phase and a mobile phase comprising pyridine.

[00420] Clause 179. The method according to clause 178, wherein the mobile phase comprises methylene chloride, methanol, and pyridine.

[00421] Clause 180. The method according to any one of clauses 178-179, wherein fractions collected from the liquid chromatography are combined and concentrated by distillation.

[00422] Clause 181. The method according to any one of clauses 178-179, wherein fractions collected from the liquid chromatograph are combined and concentrated under vacuum.

[00423] Clause 182. The method according to any one of clauses 180-181, wherein the concentrated fractions are contacted with one or more particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt.

[00424] Clause 183. The method according to clause 182, wherein the particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt are contacted during distillation of the combined fractions.

[00425] Clause 184. The method according to clause 182, wherein the method comprises:

concentrating the combined fractions under vacuum; and

contacting the concentrated fractions with particles of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt and at least one solvent.

[00426] Clause 185. The method according to clause 184, wherein the at least one solvent is chosen from 2-methyl tetrahydrofuran, heptane, or a combination thereof.

[00427] Clause 186. The method according to any one of clauses 184-185, wherein the at least one solvent is 2-methyl tetrahydrofuran.

[00428] Clause 187. The hydrate of crystalline 25HC3S sodium of clause 2, having an x-ray powder diffraction pattern comprising a peak at less than about 2.8°2 θ .

[00429] Clause 188. The hydrate of crystalline 25HC3S sodium of clause 187, having a diffraction pattern comprising a peak at between about 2.1°2 θ and about 2.6°2 θ .

[00430] Clause 189. The hydrate of crystalline 25HC3S sodium of clause 188, having a diffraction pattern comprising a peak between about 2.1°2 θ and about 2.3°2 θ .

[00431] Clause 190. The hydrate of crystalline 25HC3S sodium of any one of clauses 2, 187, or 188, having an x-powder diffraction pattern comprising a peak between about 4.3°2 θ and about 4.6°2 θ .

[00432] Clause 191. The hydrate of crystalline 25HC3S sodium of any one of clauses 2, 187, 188, 189, or 190 having an x-ray powder diffraction pattern comprising a peak between about 5.0°2 θ and about 5.5°2 θ .

[00433] Clause 192. The hydrate of crystalline 25HC3S sodium of any one of clauses 2, 187-191, having an x-ray diffraction pattern comprising a peak between about 8.6°2 θ and about 9.1°2 θ .

[00434] Clause 193. The hydrate of crystalline 25HC3S sodium of any one of clauses 2, 187-192, having an x-ray powder diffraction pattern comprising a peak between about $15.0^{\circ}2\theta$ and about $15.3^{\circ}2\theta$.

[00435] Clause 194. The hydrate of crystalline 25HC3S sodium of any one of clauses 2 or 188 having an x-ray powder diffraction pattern comprising a peak at between about $9.9^{\circ}2\theta$ and about $10.0^{\circ}2\theta$.

[00436] Clause 195. The anhydrous crystalline 25HC3S sodium of any one of clauses 29, 30, or 39, having an x-ray powder diffraction pattern comprising a peak at between about $4.5^{\circ}2\theta$ and about $4.8^{\circ}2\theta$.

[00437] Clause 196. The anhydrous crystalline 25HC3S sodium of any one of clauses 29, 30, 39, or 195 having an x-ray powder diffraction pattern comprising a peak between about $9.8^{\circ}2\theta$ and about $9.9^{\circ}2\theta$.

[00438] Clause 197. The anhydrous crystalline 25HC3S sodium of any one of clauses 29, 30, 39, 195, or 196 having an x-ray powder diffraction pattern comprising a peak at between about $14.1^{\circ}2\theta$ and about $14.3^{\circ}2\theta$.

[00439] Clause 198. The anhydrous crystalline 25HC3S sodium of any one of clauses 29, 30, 39, 195, 197, or 197 having an x-ray powder diffraction pattern comprising a peak at about $16.1^{\circ}2\theta$.

EXPERIMENTAL

[00440] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for.

[00441] Examples 1-19 are associated with samples prepared in accordance with Table 1 and Examples 20, 21, 22, 24, 25, 26, 27, 28, 29, 31, 32, 34, 35, 37, and 38. Examples 23, 30, 33, 36 and 39 were prepared as written; XRPD patterns were collected in accordance with Example 40, and no other analytical data are reported in the disclosure for these examples.

Example 1 – Analysis of 25HC3S sodium used in the screen

[00442] Approximately 10 grams of 25HC3S sodium were used for screening. X-ray powder diffraction analysis identifies the material as a physical mixture of hydrates (Form I and Form II). The solution ¹H-NMR spectrum is consistent with the chemical structure (See Figure 34 as an example of a ¹H-NMR structure of 25HC3S). The mixture contained 6.1 wt% water by Karl Fischer titration. This corresponds to approximately 1.8 mol/mol of water. The DVS isotherm suggests that the material is hygroscopic. The mixture gained approximately 5 wt% from 5 to 25% RH (1.5 mol/mol water), 6 wt% from 25 to 85% RH (1.8 mol/mol water), and another 6 wt% from 85 to 95% RH. The material was recovered and identified as predominantly Form I with an additional peak near 8.8° (2θ) by x-ray powder diffraction. The peak is believed to be due to Form V.

[00443] TGA provided a 5.4% weight loss up to 128 °C which occurred concurrently with broad dehydration endotherms in the DSC. The DSC curve also showed endotherms near 167 °C and 184 °C. These events were associated with decomposition. Hot stage microscopy was consistent with dehydration between 58 °C and 112 °C, followed by decomposition near 163 °C.

[00444] Briefly exposing the mixture to 130°C resulted in a mixture of Forms I, II, and XIII. In addition, the peaks associated with Form II were shifted. Exposure to 75% RH for 10 days resulted in predominately Form I and two additional peaks near 4.4°2θ and 8.8°2θ by x-ray powder diffraction, and the peaks were believed to be due to Form V.

Example 2 – General screening methods

[00445] Methods using solvents or solvent mixtures include cooling a solution, evaporation, antisolvent addition, and suspensions (slurries). Variations on these methods can include changes in solvent, solvent mixtures, antisolvent, temperature, cooling rate, concentration, rate of addition, and order of mixing, to name a few possibilities. The generated solids were observed by Polarized light microscopy and/or analyzed by x-ray powder diffraction.

[00446] Specific methods of screening are set forth in Examples 3-7. Methods and results are summarized, e.g., in Table 1 and the Figures of the present disclosure.

Example 3 – Anti-Solvent Additions

[00447] Solutions were contacted with anti-solvents. These anti-solvent additions were added to help lower the solubility of the solvent system and induce crystallization.

Example 4 – Cooling and Slow Cools

[00448] Solutions were prepared in the selected solvent or solvent/anti-solvent system. These solutions were chilled below room temperature within a refrigerator for varying lengths of time in an attempt to induce nucleation. The presence or absence of solids was noted. Upon observation of solids, in quantities sufficient for analysis, isolation of material was conducted. If insufficient quantities were present, further cooling was performed in a freezer. Samples were isolated for analysis either wet or as dry powders.

Example 5 – Fast Evaporation

[00449] Solutions were prepared in selected solvents and agitated between aliquot additions to assist in dissolution. Once a mixture reached complete dissolution, as judged by visual observation, the solution was filtered through a 0.2- μ m nylon filter and allowed to evaporate at ambient conditions in an uncapped vial or at ambient temperature under nitrogen. The solids that formed were isolated for evaluation.

Example 6 – Slow Evaporation

[00450] Solutions were prepared in selected solvents and agitated between aliquot additions to assist in dissolution. Once a mixture reached complete dissolution, as judged by visual observation, the solution was filtered through a 0.2- μ m nylon filter into a sample vial. The vial opening was covered with foil and pierced 3x and allowed to evaporate at ambient conditions. The solids that formed were isolated for evaluation.

Example 7 – Slurry

[00451] Solutions were prepared by adding enough solids to a given solvent so that excess solids were present. The mixture was then agitated in a sealed vial at either ambient or elevated temperature. After a given amount of time, the solids were isolated for analysis.

Example 8 – Differential Scanning Calorimetry (DSC)

[00452] Differential scanning calorimetry analyses were performed using a Mettler-Toledo DSC3+ differential scanning calorimeter. Temperature calibration was performed using octane, phenyl salicylate, indium, tin, and zinc. The sample was placed into a hermetically sealed or an open aluminum DSC pan, and the weight was recorded. A weighed aluminum pan configured as the sample pan was placed on the reference side of the cell. The samples were analyzed from -30 to 250 °C at a ramp rate of 10 °C/min. Although thermograms were plotted by reference temperature (x-axis), results were reported according to sample temperatures.

Example 9 – Dynamic Vapor Sorption/Desorption (DVS)

[00453] Moisture sorption/desorption data were collected on a VTI SGA-100 Vapor Sorption Analyzer. NaCl and PVP were used as calibration standards. Samples were not dried prior to analysis. Sorption and desorption data were collected over a range from 5% to 95% RH at 10% RH increments under a nitrogen purge. The equilibrium criterion used for analysis was less than 0.0100% weight change in 5 minutes with a maximum equilibration time of 3 hours. Data were not corrected for the initial moisture content of the samples.

Example 10 – Hot Stage Microscopy

[00454] Hot stage microscopy was performed using a Linkam hot stage (FTIR 600) mounted on a Leica DM LP microscope equipped with a SPOT Insight™ color digital camera. Temperature calibrations were performed using USP melting point standards. Samples were placed on a cover glass, prepared either neat or with mineral oil, and a second cover glass was placed on top of the sample. As the stage was heated, each sample was visually observed using a 10x 0.22 or 20x 0.40 numerical aperture objective with crossed polarizers and a first order red compensator. Images were captured using SPOT software (v. 4.5.9).

Example 11 – Karl Fischer

[00455] Coulometric Karl Fischer (KF) analysis for water determination was performed using a Mettler Toledo DL39 KF titrator. A NIST-traceable water standard (Hydranal Water Standard 1.0) was analyzed to check the operation of the coulometer. A blank titration was carried out prior to sample analyses. The sample was prepared at ambient conditions, where a weighed

amount of sample was dissolved in approximately 1 mL Hydranal-Coulomat AD in a pre-dried vial. The entire solution was added to the KF coulometer through a septum and mixed for 10 seconds. The sample was then titrated by means of a generator electrode, which produces iodine by electrochemical oxidation: $2 \text{I}^- \rightarrow \text{I}_2 + 2\text{e}^-$. Two replicates were obtained to ensure reproducibility.

Example 12 – Polarized Light Microscopy

[00456] Polarized light microscopy was performed using a Motic SMZ-168. Each sample was observed using a 10x objective at 0.75 up to 5.0x magnification with crossed polarizers.

Example 13 – ^1H NMR Spectroscopy

[00457] The solution ^1H NMR spectra were obtained at Spectral Data Solutions.

Example 14 – Thermogravimetric Analysis (TGA)

[00458] Thermogravimetric analyses were performed using a Mettler Toledo TGA/DSC3+ analyzer. Temperature calibration was performed using phenyl salicylate, indium, tin, and zinc. The sample was placed in an aluminum pan. The open pan was inserted into the TG furnace. The furnace was heated under nitrogen. Each sample was heated from ambient temperature to 350 °C at 10°C/min. Although thermograms were plotted by reference temperature (x-axis), results were reported according to sample temperatures.

Example 15 – X-ray Powder Diffraction (XRPD)

[00459] XRPD patterns (also called diffractograms) were collected in either transmission or reflection mode.

Example 16 – Transmission

[00460] X-ray powder diffraction patterns were collected with a PANalytical X'Pert PRO MPD or PANalytical Empyrean diffractometer using an incident beam of Cu radiation produced using a long, fine-focus source. An elliptically graded multilayer mirror was used to focus Cu $K\alpha$ x-rays through the specimen and onto the detector. Prior to the analysis, a silicon specimen (NIST SRM 640e) was analyzed to verify the observed position of the Si 111 peak was consistent with

the NIST-certified position. A specimen of the sample was sandwiched between 3- μ m-thick films and analyzed in transmission geometry. A beam-stop, short anti-scatter extension, and anti-scatter knife edge were used to minimize the background generated by air. Soller slits for the incident and diffracted beams were used to minimize broadening and asymmetry from axial divergence. Diffraction patterns were collected using a scanning position-sensitive detector (X'Celerator) located 240 mm from the specimen and Data Collector software v. 2.2b or 5.5. All images were produced with the instrument labeled as X'Pert PRO MPD regardless of the instrument used.

Example 17 – Reflection

[00461] X-ray powder diffraction patterns were collected with a PANalytical X'Pert PRO MPD diffractometer using an incident beam of Cu K α radiation produced using a long, fine-focus source and a nickel filter. The diffractometer was configured using the symmetric Bragg - Brentano geometry. Prior to the analysis, a silicon specimen (NIST SRM 640e) was analyzed to verify that the observed position of the Si 111 peak was consistent with the NIST-certified position. A specimen of the sample was packed in a well. Anti-scatter slits were used to minimize the background generated by air. Soller slits for the incident and diffracted beams were used to minimize broadening from axial divergence. Diffraction patterns were collected using a scanning position-sensitive detector (X'Celerator) located 240 mm from the sample and Data Collector software v. 2.2b.

Example 18 – Variable Humidity (VH-XRPD)

[00462] X-ray powder diffraction patterns were collected with a PANalytical X'Pert PRO MPD diffractometer using an incident beam of Cu K α radiation produced using a long, fine-focus source and a nickel filter. The diffractometer was configured using the symmetric Bragg - Brentano geometry. Data were collected and analyzed using Data Collector software v. 2.2b. Prior to the analysis, a silicon specimen (NIST SRM 640e) was analyzed to verify that the observed position of the Si 111 peak was consistent with the NIST-certified position. A specimen of the sample was packed in a nickel-coated copper well. Antiscatter slits were used to minimize the background generated by air. Soller slits for the incident and diffracted beams were used to minimize broadening from axial divergence. Diffraction patterns were collected

using a scanning position-sensitive detector (X'Celerator) located 240 mm from the sample and Data Collector software v. 2.2b.

[00463] An Anton Paar temperature-humidity chamber (THC) was used to collect *in-situ* x-ray powder diffraction patterns as a function of humidity. The temperature of the specimen was controlled with a Peltier thermoelectric device located directly under the specimen holder and monitored with a platinum-100 resistance sensor located in the specimen holder. The thermoelectric device was powered and controlled by an Anton Paar TCU 50 interfaced with Data Collector software.

[00464] The humidity was generated with an RH-200 manufactured by VTI Inc. and carried by a flow of nitrogen gas. The humidity and temperature were monitored by a Rotronic HygroClip sensor located next to the specimen inside the THC.

Example 19 – Variable Temperature (VT-XRPD)

[00465] X-ray powder diffraction patterns were collected with a PANalytical X'Pert PRO MPD diffractometer using an incident beam of Cu K α radiation produced using a long, fine-focus source and a nickel filter. The diffractometer was configured using the symmetric Bragg - Brentano geometry. Data were collected and analyzed using Data Collector software v. 2.2b. Prior to the analysis, a silicon specimen (NIST SRM 640e) was analyzed to verify that the observed position of the Si 111 peak was consistent with the NIST-certified position. A specimen of the sample was packed in a nickel-coated copper well. Antiscatter slits were used to minimize the background generated by air scattering. Soller slits for the incident and diffracted beams were used to minimize broadening from axial divergence. Diffraction patterns were collected using a scanning position-sensitive detector (X'Celerator) located 240 mm from the sample.

[00466] An Anton Paar TTK 450 stage was used to collect *in-situ* x-ray powder diffraction patterns as a function of temperature. The sample was heated with a resistance heater located directly under the sample holder, and the temperature was monitored with a platinum-100 resistance sensor located in the specimen holder. The heater was powered and controlled by an Anton Paar TCU 100 interfaced with Data Collector software.

Example 20 – Form I – Preparation 1

[00467] 25HC3S sodium (164.6 mg) and methanol (2 mL) were heated on a hot plate set at 75 °C until a clear solution was achieved. The solution was filtered through a 0.2-µm nylon filter into 20 mL of dichloromethane. This resulted in immediate precipitation of a gel, which was harvested by water-aspirated vacuum filtration. The gel crystallized to Form I after isolation. Figure 36 is an XRPD diffractogram of this preparation.

Example 21 – Form I – Preparation 2

[00468] A slurry was obtained with 64.5 mg of 25HC3S sodium and 1 mL of toluene. The slurry was magnetically stirred at approximately 400 RPM at room temperature for 21 days. Form I was obtained by allowing the suspended solids to settle and decanting the excess solution from the solids. Figure 37 is an XRPD diffractogram of this preparation.

Example 22 – Form I – Preparation 3

[00469] A slurry was obtained with 41.9 mg of 25HC3S sodium and 12 mL of acetone. The slurry was briefly heated on a hot plate set at 85 °C and magnetically stirred at approximately 200 RPM. The slurry was removed from the heat source, and 1 mL of water was added while magnetically stirring at approximately 400 RPM. Nearly complete dissolution was followed by the formation of a flocculent suspension which then nucleated to fine dendrites. The slurry was left to stir for 4 days. Form I was harvested by water-aspirated vacuum filtration and dried under nitrogen for approximately 10 minutes. Figure 38 is an XRPD diffractogram of this preparation.

Example 23 – Form I – Preparation 4

[00470] Form I was obtained by performing the following Dynamic Vapor Sorption Test:

[00471] Dynamic Vapor Sorption (DVS) was measured using an SMS (Surface Measurement Systems) DVS Intrinsic. Parameters for the DVS test are listed in Table 11.

Table 11 Parameters for DVS

Parameters	Values
Temperature	25 °C
Sample size	10-20 mg
Gas and flow rate	N ₂ , 200 mL/min
dm/dt	0.002%/min
Min. dm/dt stability duration	10 min
Max. equilibrium time	360 min
RH range	40% RH - 95%RH-0%RH-95%RH
RH step size	10% (0%RH-90%RH) 5% (90%RH-95%RH)

[00472] From the above DVS test, the compound was found to be hygroscopic, and the starting material reversibly absorbed 7.0% moisture below 30% RH and 8.5% water by 95% RH. Figure 355 is an XRPD diffractogram of this preparation.

Example 24 – Form I + XIII

[00473] A slurry was obtained with 586.6 mg of 25HC3S sodium and 10 mL of 97:03 v/v acetonitrile/water. The slurry was magnetically stirred at approximately 250 RPM for 4 days at room temperature. A mixture of Forms I+XIII was harvested by water-aspirated vacuum filtration and dried under nitrogen for approximately 30 minutes. Figure 39 is an XRPD diffractogram of this mixture.

Example 25 – Form XIII – Preparation 1

[00474] Form XIII was obtained by exposing a mixture of Forms I+XIII (obtained from Example 24) to 70 °C under vacuum for 2 days. Figure 40 is an XRPD diffractogram of this preparation.

Example 26 – Form XIII – Preparation 2

[00475] A mixture of Forms I+XIII (obtained from Example 24) isolated at 15% RH was exposed to 25%, 55%, 75%, 85%, 75%, 25%, and 0% RH, successively. The material was held at each RH condition for a minimum of one hour before proceeding to the next condition. The mixture converted to Form I once exposed to 25% RH and remained Form I at 55%, 75%, 85%,

75%, and 25% RH. Form I converted to a mixture of Form I+XIII upon reaching 0% RH. The mixture of Form I+XIII converted to Form XIII within 20 minutes of continued exposure to 0% RH. Figure 41 is an XRPD diffractogram of this preparation.

Example 27 – Form II – Preparation 1

[00476] A slurry was obtained with 75.4 mg of 25HC3S sodium and 20 mL of acetone. The slurry was magnetically stirred at approximately 400 RPM for 14 days at room temperature. Form II was harvested by water-aspirated vacuum filtration and briefly dried under nitrogen. Figure 42 is an XRPD diffractogram of this preparation.

Example 28 – Form II – Preparation 2

[00477] A slurry was obtained with 66.9 mg of 25HC3S sodium and 19 mL of acetonitrile. The slurry was magnetically stirred at approximately 400 RPM for 14 days at room temperature. Form II was harvested by water-aspirated vacuum filtration and briefly dried under nitrogen. Figure 43 is an XRPD diffractogram of this preparation.

Example 29 – Form II – Preparation 3

[00478] A slurry was obtained with 75.4 mg of 25HC3S sodium and 8 mL of ethanol. The slurry was filtered through a 0.2- μ m nylon filter resulting in a clear solution. A 4-mL aliquot of the clear solution was allowed to evaporate at ambient conditions until solids were evident and approximately 0.1 mL of the mother liquor remained. Form II was harvested by decantation. Figure 44 is an XRPD diffractogram of this preparation.

Example 30 - Form II – Preparation 4

[00479] Form II was obtained from anti-solvent addition (dimethylacetamide (DMA) solvent and methyl isobutyl ketone (MIBK) antisolvent). The anti-solvent method was as follows: a concentrated stock of starting material was prepared in DMA. The solution was stirred and MIBK added quickly to induce precipitation. Solids isolated for XRPD analysis after centrifugation and filtration yielded Form II. Figure 46 is an XRPD diffractogram of this preparation.

Example 31 – Form III + Form IX

[00480] A slurry was obtained with 46.6 mg of 25HC3S sodium and 0.5 mL of ethanol. The slurry was heated briefly on a hot plate set at 75 °C, resulting in sorption of solvent. The mixture of Forms III+IX was removed from the heat source and dried under nitrogen for approximately 15 minutes. Figure 45 is an XRPD diffractogram of a mixture of Form III and Form IX.

Example 32 – Form IX – Preparation 1

[00481] Form IX was obtained by exposing a mixture of Forms III+IX (obtained from Example 31) to 58 °C under vacuum for 1 day. Figure 5 is an XRPD diffractogram of this preparation.

Example 33 – Form IX – Preparation 2

[00482] 20.3 mg of 25HC3S sodium was weighed into a 3-mL vial. This 3-mL vial was placed inside a 20 mL vial containing 3 to 4 mL of ethanol, and the outer vial was sealed. The system was kept at room temperature for 12 hours, and the isolated solids were analyzed by XRPD. Figure 51 is an XRPD diffractogram of this preparation.

Example 34 – Form V – Preparation 1

[00483] A turbid suspension was obtained with 85.1 mg of 25HC3S sodium and 7 mL of methanol. The suspension was filtered through a 0.2-µm nylon filter to provide a clear solution. Form V was provided by evaporating the solution until visually dry at ambient conditions. Figure 47 is an XRPD diffractogram of this preparation.

Example 35 – Form V – Preparation 2

[00484] A slurry was obtained with 70.4 mg of 25HC3S sodium and 20 mL of water. The slurry was magnetically stirred at approximately 400 RPM for 14 days. An opaque gel was harvested by water-aspirated vacuum filtration. The gel transformed to Form V after isolation. Figure 48 is an XRPD diffractogram of this preparation.

Example 36 – Form V – Preparation 3

[00485] Form V was obtained by anti-solvent addition (MeOH solvent and EtOAc antisolvent). The anti-solvent method was as follows: a concentrated stock of starting material was prepared

in MeOH. The solution was stirred and EtOAc added quickly to induce precipitation. Solids isolated for XRPD analysis after centrifugation and filtration yielded Form V. Figure 56 is an XRPD diffractogram of this preparation.

Example 37 – Form XI

[00486] A slurry was obtained with 97.5 mg of 25HC3S sodium and 20 mL of diethyl ether. The slurry was magnetically stirred at approximately 400 RPM for 14 days. Form XI was harvested by water-aspirated vacuum filtration and dried under nitrogen. Figures 6 and 7 are XRPD diffractograms of this preparation.

Example 38 – Form III – Preparation I

[00487] A turbid suspension was obtained with 97 mg of 25HC3S sodium and 8 mL of ethanol. The suspension was left to settle at ambient temperature for ~1 day. The suspension was centrifuged, and the wet solids were harvested by decantation. Figure 49 is an XRPD diffractogram of this preparation.

Example 39 – Form III – Preparation 2

[00488] 25HC3S sodium (1.98 mg) was weighed into a 3-mL glass vial. EtOH (3 mL) was added into the glass vial to produce a suspension at room temperature and heated to 50 °C. The solution was filtered at 50 °C with a 0.45-µm PTFE filter, and the filtrates were collected into clean vials. The solutions were cooled to room temperature and then stored at -20 °C. Solids were isolated by centrifuging at 14,000 RPM for 5 minutes, then collected and analyzed by XRPD. Figure 50 is an XRPD diffraction of this preparation.

Example 40 – XRPD Analyses for Examples 23, 30, 33, 35, and 36.

[00489] XRPD was performed with Panalytical X'Pert³ Powder XRPD on a Si zero-background holder. The 2θ position was calibrated against a Panalytical 640 Si powder standard. The 4-min method was used for most samples. Spray-dried dispersion stability samples were analyzed using the 80-min method. Details of XRPD used in the experiments are listed in Table 12 below.

Table 12 XRPD parameters

	Parameters for Reflection Mode
X-Ray wavelength	Cu, $k\alpha$, $K\alpha_1$ (Å): 1.540598, $K\alpha_2$ (Å): 1.544426 $K\alpha_2/K\alpha_1$ intensity ratio: 0.50
X-Ray tube setting	45 kV, 40 mA
Fixed slit	1/4° (0.38 mm height)
Divergence slit	1/8° (0.19 mm height)
Scan mode	Continuous
Scan range (°2 θ)	3° - 40°
Step size (°2 θ)	0.0131 (4-min method), 0.0033 (80-min method)
Scan speed (°/s)	0.16 (4-min method), 0.0075 (80-min method)

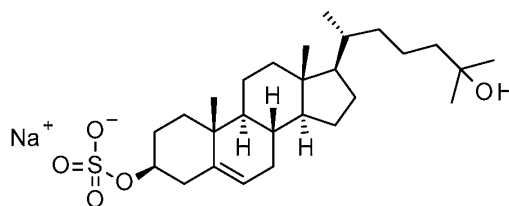
General Synthetic Procedures for Preparing 25-hydroxy-(3 β)-cholest-5-en-3-sulfate

[00490] 25HC3S may be prepared by various methods. Enclosed herein are exemplary methods of making 25HC3S.

[00491] All temperatures are in degrees Celsius (°C) and are uncorrected. Reagent grade chemicals and anhydrous solvents were purchased from commercial sources and, unless otherwise mentioned, used without further purification. The names of the products were determined using the naming software included in Biovia electronic lab notebook. Silica gel chromatography was performed on Teledyne Isco instruments using pre-packaged disposable SiO₂ stationary phase columns with eluent flow rates of 15 to 200 mL/min. The analytical HPLC chromatograms were performed using an Agilent 1100 series instrument with DAD detector (190 nm to 300 nm). The mass spectra were recorded with a Waters Micromass ZQ detector at 130 °C. The mass spectrometer was equipped with an electrospray ion source (ESI) operated in a positive ion mode and was set to scan between m/z 150-750 with a scan time of 0.3 s. Products and intermediates were analyzed by HPLC/MS on a Gemini-NX (5 μ M, 2.0 x 30 mm) using a high pH buffer gradient of 5% to 100% of MeCN in H₂O (0.03% (NH₄)₂CO₃/0.375% NH₄OH) over 2.5 min at 1.8 mL/min for a 3.5 min run (B05), and EVO C18 (5 μ M, 3.0 x 50 mm) using a low pH buffer gradient of 5% to 100% of MeCN in H₂O (0.1% HCOOH) over

2.5 min at 2.2 mL/min for a 3.5 min run (A05). The ^1H NMR spectra were recorded on a Bruker UltraShield 500 MHz/54 mm instrument (BZH 43/500/70B, D221/54-3209). The chemical shifts are referenced to solvent peaks which, in ^1H NMR, appear at 7.26 ppm for CDCl_3 , 2.50 ppm for $\text{DMSO}-d_6$, and 3.31 ppm for CD_3OD .

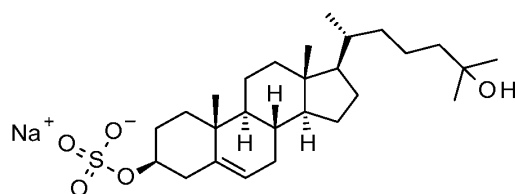
Synthesis of Sodium [(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00492] A dry 3-necked flask was charged with pyridine sulfur trioxide complex (12.45 g, 78 mmol), and the solid was suspended in toluene (1.5 L) and acetic anhydride (7.2 mL, 74.5 mmol). The mixture was stirred at 20 °C for 40 min, and pyridine (60 mL, 745 mmol) was added. The mixture was stirred at 20 °C for 20 min. (3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (30 g, 74.5 mmol) was added in a single portion as a solid. The mixture was stirred at 20 °C for 23 h. Aqueous sodium acetate solution (10 wt %, 123 mL, 149 mmol) was added dropwise with vigorous stirring over 5 min. The resultant mixture was stirred at 20 °C for 1 h. The solvent was pumped out of the reactor, collecting any solids onto a glass frit. ACN (700 mL) was added, and the slurry was stirred vigorously for 3 h. The slurry was pumped out of the reactor onto the same frit, and the remaining solids in the reactor were again suspended in ACN (700 mL) and stirred for 1 h before pumping out of the reactor to the glass frit. The solids in the frit were rinsed with diethyl ether (750 mL) and then suspended in DMF (800 mL). The mixture was stirred for 1 h at 20 °C. The suspension was filtered, and the filtrate collected. To the filtrate, with stirring, was added diethyl ether (3.2 L). The resulting solids were collected by vacuum filtration, and the filter cake rinsed with diethyl ether (1 L). The solids were dried under reduced pressure to provide the title compound as a solid (15 g, 40%). ^1H NMR (500 MHz, MeOD) δ 5.56 – 5.32 (m, 1H), 4.17 (tt, J = 11.5, 4.8 Hz, 1H), 2.55 (dd, J =

4.9, 2.2 Hz, 1H), 2.47 – 2.29 (m, 1H), 2.14 – 2.06 (m, 2H), 2.01 (ddd, $J = 12.4, 7.7, 5.1$ Hz, 1H), 1.97 – 1.85 (m, 2H), 1.73 – 1.22 (m, 15H), 1.20 (s, 6H), 1.19 – 1.08 (m, 4H), 1.07 (s, 3H), 1.04 – 0.95 (m, 1H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.76 (s, 3H); m/z : ES- $[M]^+$ 481.3; LCMS (B05); $t_R = 1.18$ m.

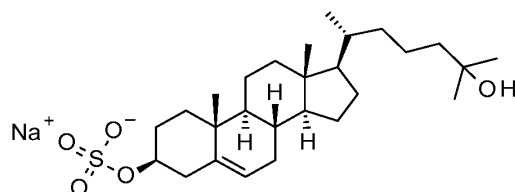
Synthesis of Sodium [(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00493] A dry 3-necked flask was charged with sulfur trioxide pyridine complex (4.74 g, 29.8 mmol). The solid was suspended in toluene (500 mL), and acetic anhydride (2.61 mL, 27.67 mmol) was added in a single portion. The resultant mixture stirred at 23 °C for 1 h. Pyridine (20 mL, 248.4 mmol) was added, and the mixture was stirred at 23 °C for 5 min. (3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (10 g, 24.83 mmol) was added in a single portion as a solid. The mixture stirred at 23 °C for 23 h. The reaction was diluted with MeOH (2.01 mL, 49.7 mmol) and stirred at 23 °C for 1 h. The suspension was filtered, and the solids washed with toluene (2 x 200 mL). The solids were collected and dried under high vacuum to provide a solid. The solids were partially dissolved in ACN (600 mL), and sodium iodide (14.9 g, 99.3 mmol) was added. The mixture was stirred at 23 °C for 10 min before being cooled to 0 °C with an ice-bath and stirred for 1.5 h. The suspension was filtered, and the solids washed with cold ACN (2 x 275 mL) and acetone (2 x 200 mL). The solids were collected and dried under high vacuum to provide the title compound as a solid (7.24 g, 57 %). ^1H NMR (500 MHz, MeOD) δ 5.56 – 5.32 (m, 1H), 4.17 (tt, $J = 11.5, 4.8$ Hz, 1H), 2.55 (dd, $J = 4.9, 2.2$ Hz, 1H), 2.47 – 2.29 (m, 1H), 2.14 – 2.06 (m, 2H), 2.01 (ddd, $J = 12.4, 7.7, 5.1$ Hz, 1H), 1.97 – 1.85 (m, 2H), 1.73 – 1.22 (m, 15H), 1.20 (s, 6H), 1.19 – 1.08

(m, 4H), 1.07 (s, 3H), 1.04 – 0.95 (m, 1H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.76 (s, 3H); m/z : ES- $[M]^+$ 481.3; LCMS (B05); $t_R = 1.18$ m.

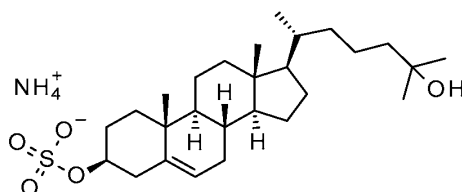
Synthesis of Sodium [(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00494] A 15 L jacketed reactor was heated to 60 °C and purged with nitrogen for 1.5 h. The jacket temperature was set to 30 °C and 2-MeTHF (7 L) was charged. (3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (495 g, 1.23 mol) was charged, and the manway/glassware was rinsed with 2-MeTHF (6 L). The solution was cooled to 25 °C, additional 2-MeTHF (1 L) was added, and sulfur trioxide pyridine complex (234.8 g, 1.47 mol) was added. The mixture was stirred at 28 °C for 24 h. 2-MeTHF (2 L) was added, the mixture was stirred for a further 16 h, cooled to 20 °C and filtered. The solids were rinsed with 2-MeTHF (3.5 L). The solids were taken up in a solution of NaOH (118 g, 2.95 mmol) in MeOH (6 L). The mixture was stirred at 25 °C for 1 h and then filtered on a plug of Celite. The filtrate was concentrated to 3.5 L and diluted with diethyl ether (8 L). The suspension was chilled to 15 °C and filtered to provide the title compound as a solid (146.8 g, 24 %). The filtrate was concentrated to 1 L and again mixed with diethyl ether (4 L). The solids were collected by vacuum filtration to provide the title compound as a solid (68.5 g, 11 %). The Celite was extracted with MeOH (2 L), which was concentrated to 500 mL and diluted with diethyl ether (3 L) and the solids were collected by vacuum filtration to provide the title compound as a solid (53.3 g, 8.6 %). A fourth crop was isolated from the filtrates (11.88 g, 2 %). Total yield: 280.5 g, 45 %. ^1H NMR (500 MHz, MeOD) δ 5.56–5.32 (m, 1H), 4.17 (tt, $J = 11.5, 4.8$ Hz, 1H), 2.55 (dd, $J = 4.9, 2.2$ Hz, 1H), 2.47 – 2.29 (m, 1H), 2.14–2.06 (m, 2H), 2.01 (ddd, $J = 12.4, 7.7, 5.1$ Hz, 1H), 1.97–1.85 (m, 2H), 1.73–1.22 (m, 15H), 1.20 (s, 6H), 1.19–1.08 (m, 4H), 1.07 (s, 3H),

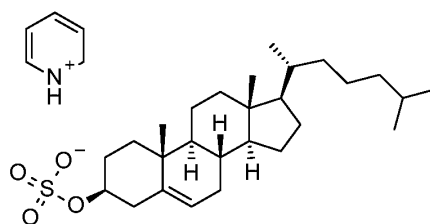
1.04–0.95 (m, 1H), 1.00 (d, $J = 6.5$ Hz, 3H), 0.76 (s, 3H); m/z : ES- $[M]^+$ 481.3; LCMS (B05); t_r = 1.18 m.

Synthesis of Ammonium [(3S,10R,13R,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00495] Sulfur trioxide dimethyl formamide complex (42 mg, 0.273 mmol) was added to a stirred solution of (3S,10R,13R,17R)-17-(5-hydroxy-1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (100 mg, 0.25 mmol) in anhydrous DCM (20 mL) at 0 °C. The mixture was stirred at 0 °C for 5 h, and then the reaction was warmed to 20 °C. The mixture was concentrated under reduced pressure to afford a crude solid which was purified by column chromatography on silica gel (12 g cartridge) eluting with mixtures of DCM and MeOH (0 - 20 %) to afford impure title compound. m/z : ES- $[M-H]^+$ 481.

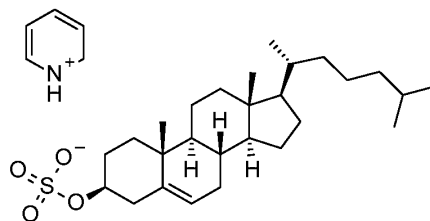
Synthesis of Pyridin-1-ium [(3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00496] In an oven-dried round-bottom flask, sulfur trioxide pyridine complex (4.53 g, 28.5 mmol) was suspended in toluene (240 mL). Acetic anhydride (2.44 mL) was added,

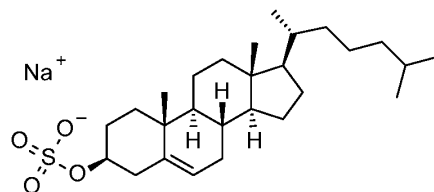
followed by pyridine (20.8 mL). The reaction was stirred at 23 °C for 1 h, and cholesterol (10 g, 25.9 mmol) was added in a single portion as a solid. The suspension was stirred at 23 °C for 18 h and, filtered on a glass frit, and the solids rinsed with toluene (100 mL) followed by hexanes (100 mL). The solids were suspended in chloroform (400 mL) and filtered on the same frit. The frit was rinsed with chloroform (200 mL) and the filtrate collected. The filtrate was diluted to 1.8 L with hexanes and refrigerated for 1 h. The suspension was filtered; the solids were rinsed with diethyl ether (100 mL) and dried under high vacuum to provide the title compound as a solid (10.06 g, 71 %). ¹H NMR (500 MHz, MeOD) δ 8.89 (dd, *J* = 6.6, 1.4 Hz, 2H), 8.79–8.61 (m, 1H), 8.27–8.05 (m, 2H), 5.38 (d, *J* = 5.3 Hz, 1H), 4.13 (tt, *J* = 11.5, 4.7 Hz, 1H), 2.53 (ddd, *J* = 13.3, 5.0, 2.3 Hz, 1H), 2.43–2.28 (m, 1H), 2.12–2.02 (m, 2H), 2.01–1.94 (m, 1H), 1.94–1.80 (m, 2H), 1.70–0.83 (m, 20H), 1.03 (s, 3H), 0.95 (d, *J* = 6.6 Hz, 3H), 0.88 (dd, *J* = 6.6, 1.9 Hz, 6H), 0.72 (s, 3H); *m/z*: ES- [M]⁺ 465.3; LCMS (B05); *t*_R = 1.40 m.

Synthesis of Pyridin-1-ium [(3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



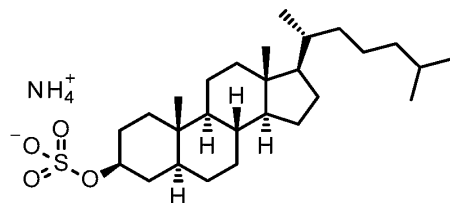
[00497] Cholesterol sulfate pyridinium salt was prepared by adding sulfur trioxide pyridine complex (4.53 g, 28.5 mmol) to a solution of cholesterol (10 g, 25.9 mmol) in 2-MeTHF (250 mL) at 30 °C and stirring the mixture for 16 h. The suspension was then filtered, and the solids rinsed with 2-MeTHF (50 mL) to afford the title compound.

Synthesis of Sodium [(3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00498] Chlorosulfonic acid (0.03 mL, 0.45 mmol) was added to a solution of 2,6-lutidine (0.08 mL, 0.69 mmol) in acetone (2.5 mL) over molecular sieves. The solution was stirred at 20 °C for 2 min before being cooled to 0 °C. A solution of cholesterol (100 mg, 0.26 mmol) in acetone (5 mL), which was previously dried over molecular sieves, was added dropwise. The mixture stirred at 0 °C for 2 h before warming to 20 °C over 16 h. The mixture was filtered and the solid was collected. The solid was then suspended in acetone (10 mL) and aqueous sodium bicarbonate was added until bubbling subsided. The suspension was filtered and the solid triturated with MeOH (10 mL) and DCM (10 mL). The solvent was removed under reduced pressure to afford a solid. The solid was triturated with ACN (30 mL), filtered, and the filtrate was lyophilized to afford the title compound as a solid (7.3 mg, 5.8 %). ¹H NMR (500 MHz, DMSO) δ 5.31–5.19 (m, 1H), 4.10 (s, 1H), 3.87 – 3.78 (m, 1H), 2.42–2.31 (m, 1H), 2.13 (dd, *J* = 14.5, 7.6 Hz, 1H), 2.02 – 1.69 (m, 5H), 1.62–0.95 (m, 20H), 0.94 (s, 3H), 0.89 (d, *J* = 6.5 Hz, 4H), 0.84 (dd, *J* = 6.6, 2.5 Hz, 7H), 0.65 (s, 3H).

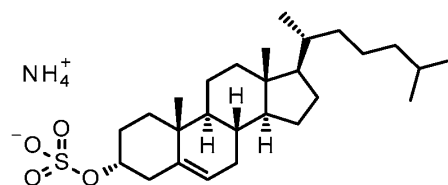
Synthesis of Ammonium [(3S,5S,8R,9S,10S,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00499] Sulfur trioxide pyridine complex (300 mg, 1.88 mmol) was added to a solution of cholestanol (300 mg, 0.772 mmol) in pyridine (5.00 mL), and the suspension was stirred at 20 °C

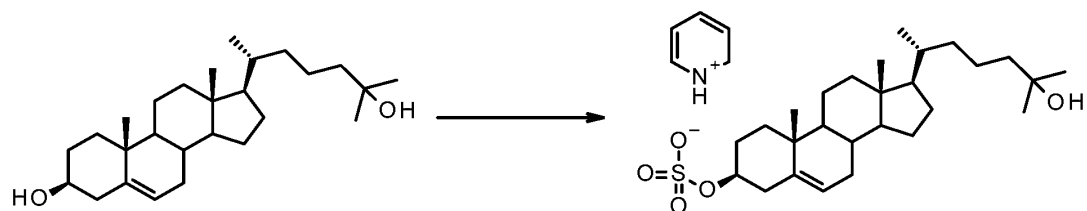
for 16 h. The residue was purified by silica gel chromatography (24 g cartridge) with MeOH (5% NH₄OH) in DCM eluting with mixtures of DCM and MeOH (0-30 %) to afford the title compound as a solid (314 mg, 84%). ¹H NMR (500 MHz, DMSO-d₆) δ 7.08 (s, 4H), 3.97–3.86 (m, 1H), 1.91 (dd, *J* = 12.5, 3.5 Hz, 1H), 1.86 – 1.71 (m, 2H), 1.69–1.55 (m, 3H), 1.55–1.41 (m, 3H), 1.38–1.25 (m, 5H), 1.25–0.90 (m, 15H), 0.88 (d, *J* = 6.6 Hz, 4H), 0.84 (dd, *J* = 6.6, 2.4 Hz, 7H), 0.74 (s, 3H), 0.62 (s, 3H); *m/z*: ES [M-NH₄]⁺ 467.3; HPLC (BEH Ambicarb/ACN 5- 100%) *t_R* = 7.48 min.

Synthesis of Ammonium [(3R,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



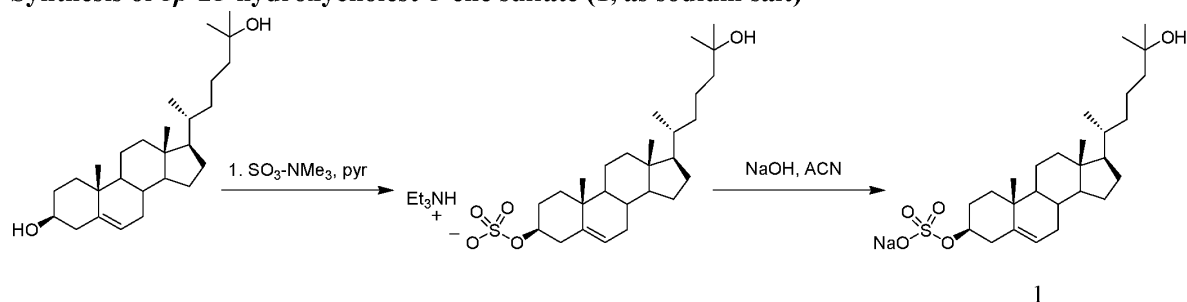
[00500] Sulfur trioxide pyridine complex (206 mg, 1.29 mmol) was added to a solution of (3R,8S,9S,10R,13R,14S,17R)-17-[(1R)-1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (200 mg, 0.517 mmol) in pyridine (5.00 mL). The suspension was stirred at 20 °C for 16 h, then concentrated under reduced pressure. The residue was purified by silica gel chromatography (24.0 g cartridge) eluting with mixtures of DCM and 5 % NH₄OH in MeOH (0- 30 %) to afford the title compound as a solid (160 mg, 64%). ¹H NMR (500 MHz, DMSO) δ 7.07 (s, 4H), 5.18 – 5.14 (m, 1H), 4.32–4.27 (m, 1H), 2.40–2.29 (m, 1H), 2.16 (dt, *J* = 14.9, 2.4 Hz, 1H), 2.01 – 1.72 (m, 4H), 1.60–0.96 (m, 22H), 0.94 (s, 3H), 0.90 (d, *J* = 6.5 Hz, 3H), 0.84 (dd, *J* = 6.6, 2.4 Hz, 6H), 0.65 (s, 3H); *m/z*: ES [M-NH₄]⁺ 465.6; HPLC (BEH AmForm/ACN 5-100%) *t_R* = 2.76 min.

Synthesis of Pyridinium [(3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl] sulfate



[00501] Acetic anhydride (0.0704 mL, 0.745 mmol) was added to a suspension of sulfur trioxide pyridine complex (125 mg, 0.782 mmol) in anhydrous toluene (15.0 mL). The suspension was stirred at 20 °C for 40 min, and pyridine (0.600 mL) was added. The suspension was stirred at 20 °C for 20 min. (3S,8S,9S,10R,13R,14S,17R)-17-[(1R)-5-hydroxy-1,5-dimethyl-hexyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (300 mg, 0.745 mmol) was added in a single portion as a solid. The suspension was stirred at 20 °C for 20 h. The mixture was filtered on a glass frit to afford title compound as a solid (329 mg, 92% purity, 72% yield). ¹H NMR (500 MHz, DMSO-d₆) δ 8.99–8.88 (m, 2H), 8.65–8.53 (m, 1H), 8.13–7.97 (m, 2H), 5.30–5.20 (m, 1H), 3.93–3.71 (m, 1H), 2.41–2.32 (m, 1H), 2.18–2.08 (m, 1H), 2.02–1.71 (m, 5H), 1.59–0.95 (m, 20H), 1.05 (s, 6H), 0.94 (s, 3H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.65 (s, 3H); *m/z* ES⁺ [M+H]⁺ 481.32; HPLC (DUR B) *t*_R = 1.36 min.

Synthesis of 3β-25-hydroxycholest-5-ene sulfate (1, as sodium salt)



Preparation of 3 β -25-hydroxycholest-5-ene sulfate – Route 1

[00502] 3 β , 25-dihydroxycholest-5-ene (4.6 g, 0.011 mol) and triethylamine (1.7 ml, 0.023 mol) were suspended in pyridine (57 ml) and heated to 50 °C. The sulfur trioxide trimethylamine complex (3.2 g, 0.023 mol) was added and the mixture agitated for 24 hours. A further charge of sulfur trioxide trimethylamine complex (0.77 g, 0.006 mol) was made and the mixture agitated for an additional 4 hours. With jacket at 50 °C, the reaction mixture was distilled to -20% of initial volume. The residue was purified by silica gel chromatography (110 g), eluting with an ethyl acetate/methanol/triethylamine (90/9/1 v/v) mixture; fractions were analyzed by TLC (4:1 methylene chloride:methanol) using a phosphomolybdic acid stain. Fractions containing the 3- and 25-sulfate regioisomers were combined and evaporated (bath temp <35 °C). The residue (4.2 g, 0.0072 mol) was slurried in acetonitrile (25 g), treated with 1 N sodium hydroxide (7.2 ml, diluted from 30% sodium hydroxide solution) for 1 hour, and then filtered. Solids were rinsed through with acetonitrile (25 g) and dried to a constant weight (2.77 g). The solids, containing a mixture of 3- and 25-sodium sulfate salts (2.77 g), were triturated with ethanol (27.7 g, 10S) at 50 °C for 1 hour and then filtered at 5 °C. The isolated solids were dried to a constant weight (1.2 g). The solids (1.2 g) were suspended in 6:1 acetonitrile/water (10 S) at 30 °C for 30 minutes and then filtered. Filtration required about 40 minutes. Solids were dried to a constant weight (0.86 g) and analyzed.

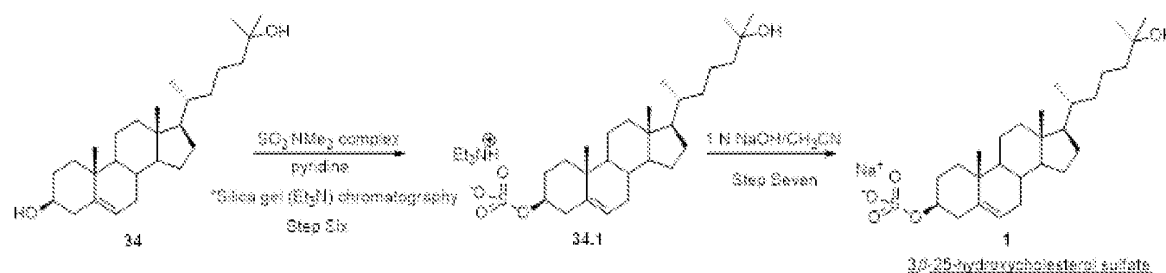
Preparation of 3 β -25-hydroxycholest-5-ene sulfate – Route 2 – Excess sulfating agent

[00503] An excess of sulfur trioxide trimethylamine complex was used to drive the reaction toward formation of the disulfate. The 3-hydroxy group of 3 β , 25-dihydroxycholest-5-ene is about 6 times more reactive towards sulfation than the 25-hydroxyl. Providing excess sulfating agent and allowing the reaction to proceed to high conversion will provide monosulfate of higher regioisomeric purity. This result was observed during this synthesis. A solution of 3 β , 25-dihydroxycholest-5-ene (4.1 g) in pyridine (75 ml) was vacuum distilled to reduce the volume to 50 ml. This was performed to remove isopropanol (from the recrystallization of the diol) and any moisture present. Triethylamine (2 equivalents) and then a total of 1.75 equivalents of sulfur trioxide trimethylamine complex was added in portions (1.0, 0.5 and 0.25 equivalents) to the reaction at 50 °C over a period of 18 hours and the reaction was allowed to run for a total of 43 hours. The reaction mixture was concentrated by vacuum distillation and the residue was

absorbed onto SiO₂ (10 g). The loaded SiO₂ was placed on a SiO₂ column and eluted with 2-50% methanol/ethyl acetate/1 % triethylamine. The appropriate fractions from the column were combined and evaporated to yield the disulfate (3.1 g, 39.7%) and the monosulfate (2.6 g, 44.7%). The monosulfate was obtained as a 22:1 mixture of the 3-sulfate and 25-sulfate. The solids were suspended in acetonitrile (25 g), treated with 1 N sodium hydroxide (4.44 ml), and then filtered. A thick gel formed, which was difficult to manipulate and was not filtered. The product was a suspension in acetonitrile/water. The solvents were removed by rotary evaporation at 40 °C and the residue was dried in a vacuum oven at 40 °C. Trituration of the solid with acetone yielded a white solid: 1.27 g, 24.9%. This product showed only the 3 β -sulfated product, but was contaminated with peaks at RRT 8.18 (unknown, 2.0%), RRT 15.17 (diol, 2.2%) and RRT 16.70 (unknown, 1.8%).

Preparatory Scale Synthesis

Synthesis of 3 β -25-hydroxycholest-5-ene sulfate (1, as sodium salt)



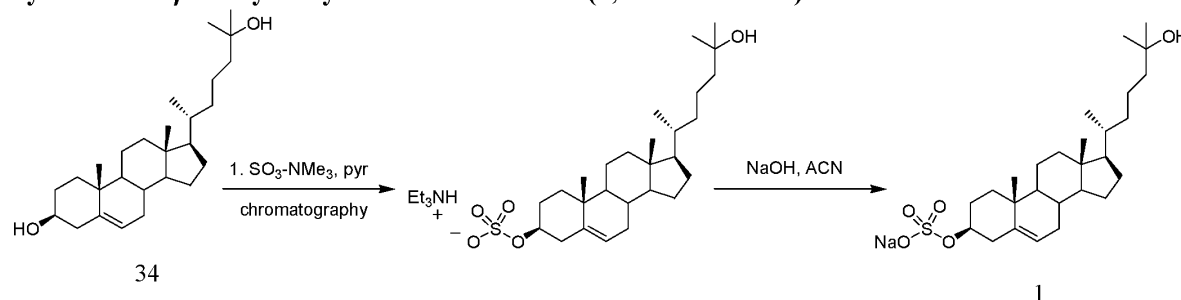
[00504] A 2 L, three-necked, round-bottomed flask with an overhead stirrer was charged with 3 β , 25-dihydroxycholest-5-ene (**34**) (30 g, 74.5 mmol) and dry pyridine (500 mL, Sigma-Aldrich, cat. #270970-1L, lot #SHBC6287V). Sulfur trioxide-trimethylamine complex (12.2 g, 89.4 mmol, Sigma-Aldrich, cat. #135879-100G, lot # MKBH5585V) was added in one portion. The suspension was stirred at room temperature overnight. The reaction mixture was concentrated and the residue was purified by column chromatography to give 25.9 g (59%) of white solid as the triethylamine salt (HPLC: 98.6% purity). To a suspension of triethylamine salt **34.1** (64 g, 110.1 mmol) in ACN (1 L) was added 1 N NaOH (110 mL, 110.1 mmol, NaOH, Fisher, cat. #S318-3, lot #034906), and the mixture was stirred for 1 h at room temperature. The

solid was filtered, washed with ACN (1 L), and dried under vacuum (P_2O_5) overnight, yield: 51.5 g, 93% (HPLC: 98.6% purity).

[00505] After overnight stirring, the reaction was a gel-like mixture. TLC showed the expected product as the major spot (TLC: 20% MeOH in DCM, $R_f = 0.4$), with both the starting material ($R_f > 0.9$) and 3*b*-25-hydroxycholesterol disulfate ($R_f < 0.1$) as minor spots. Silica gel (1kg, Sorbent Technologies, cat. #40930-2.5kg) was packed to form a column of dimension 10 cm x 42 cm. The column equilibration was accomplished with 1% triethylamine (Et_3N , Fisher, cat #04885-4, lot #062833) in DCM (2.8 L). The crude residue was dissolved in DCM (200 mL) and Et_3N (20 mL), which was directly loaded into the column. Triethylamine was used at this stage to avoid decomposition of the product and of the disulfate (which forms an olefin that is then very difficult to remove from the product). Initial elution was DCM (1% Et_3N) (2 L), followed by 1% MeOH in DCM (1% Et_3N) (1 L), 2% MeOH in DCM (1% Et_3N) (3 L), 5% MeOH in DCM (1% Et_3N) (1 L). The product began to elute in 2% MeOH in CH_2Cl_2 (1% Et_3N). The collected fractions were concentrated via rotary evaporation below 36 °C (if the temperature is higher than 45 °C, decomposition of the product in the presence of MeOH is observed). Both TLC and NMR were checked for the selected fractions. HPLC (Zorbax SB-18, 4.6 x 150 mm, 5 μ m, 202 nm, flow rate 0.8 mL/min): Solvent A: MeOH/5%ACN/7.4 mM NH_4OAc ; Solvent B: H_2O /5%ACN/7.4 mM NH_4OAc . Gradient 75% A and 25% B to 100% A. Product: 98.6% purity; 1.4% (starting material **34**). HPLC: Durashell C18 (Agela Technologies, 4.6x50 mm, 3 mm, 100 Å); Solvent A: MeOH/5%ACN/7.4 mM NH_4OAc ; Solvent B: H_2O /5%ACN/7.4 mM NH_4OAc . Product: 98.6% purity; 1.4% (starting material **34**).

Large Scale Synthesis

Synthesis of 3*β*-25-hydroxycholest-5-ene sulfate (**1**, as sodium salt)



Summary of Kilogram-scale Preparation

[00506] 3 β , 25-dihydroxycholest-5-ene (**34**) (2.6 kg) and pyridine (39.2 kg) was combined and the mixture heated to 40 °C with agitation in two 50L reactors. Sulfur trioxide-trimethylamine (1.1 kg) was added to the mixture and stirred at 40 °C for 6-12 hours until the reaction was complete. The mixture was concentrated to minimum stir volume under vacuum distillation and then diluted with methylene chloride and triethylamine.

[00507] The crude reaction mixture in methylene chloride was loaded onto a 2.33 ft³ stainless steel column (C-105) packed with silican gel and eluted with methylene chloride (containing 1% methanol and 1% triethylamine). Fractions containing undesired product were collected in waste drums. Fractions containing desired product are collected and concentrated in the reactor.

[00508] Acetonitrile, water and sodium hydroxide were added to the reactor containing the desired product and the mixture was agitated until the reaction was deemed complete. The resulting slurry was cooled to 10-15 °C and filtered to isolate compound 1. The cake of isolated compound 1 was washed with acetonitrile and then dried at 40 °C under vacuum until a constant weight was achieved.

[00509] The solid was filtered, washed with acetonitrile (1 L), and dried under vacuum (P₂O₅) overnight, yield: 51.5 g, 93% (HPLC: 98.6% purity).

Discussion

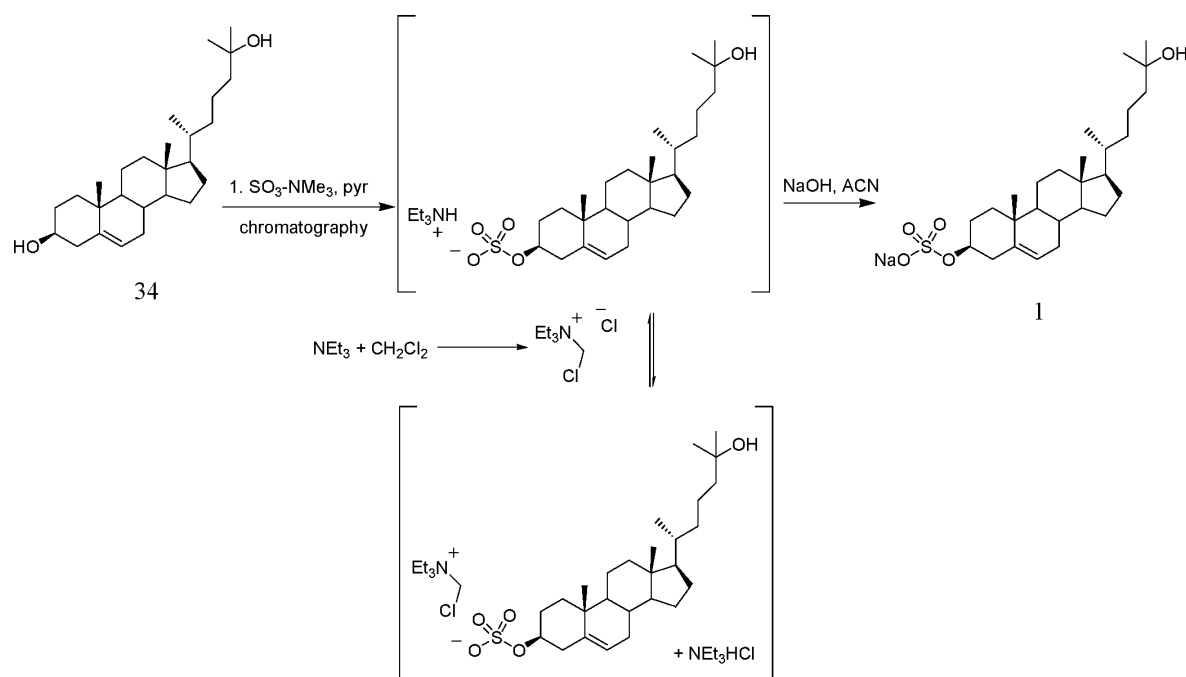
[00510] Analysis of the reaction mixture by HPLC after 6 h showed 44.1% remaining starting material. The reaction was considered complete and distilled under vacuum to a minimum stir volume (Step 5.3). To the resulting thick residue were added methylene chloride and triethylamine, and the solution was transferred to a clean 5 gallon glass carboy. Thick solids precipitated in the glass carboy after holding the solution overnight. The solids were filtered away using the benchtop filter. Approximately 1/3 of the clear filtrate was charged to the top of a C-105 column. The silica in the C-105 column was previously flushed with ethyl acetate and methanol, and then equilibrated with 1% triethylamine in methylene chloride eluent.

[00511] Once the crude solution was loaded to the top of the column, eluent was charged to maintain a pressure of ~10 psi. Eluent was sampled as it exited the column every 10-15 minutes. Pyridine and 3 β , 25-dihydroxycholest-5-ene were present in the first two samples, but the 3 β -triethylamine salt and 25-sulfate regioisomer were detected in the third sample in addition

to pyridine and 3 β , 25-dihydroxycholest-5-ene. Since minimal separation occurred, all remaining material was eluted from the column using the polar eluent (1% MeOH, 1% NEt₃, and 98% DCM). The filtrate was concentrated and combined with the remaining two-thirds of the crude solution from the carboy. After distillation, the crude solution was transferred to a clean carboy. The eluent exiting the column was analyzed and contained 1.7% methanol (¹H NMR area%). The column was equilibrated with eluent (1% triethylamine in methylene chloride) and analyzed for methanol (0.25% methanol, ¹H NMR area%). Solids began to form in the carboy during this time. The slurry was filtered, and the filtrate was collected in a clean carboy.

[00512] Approximately one-third of the crude solution was loaded onto a second C-105 column. Eluent was charged to the column to maintain <5 psi. Analysis of the eluent leaving the column by thin layer chromatography (TLC) showed that separation was taking place. Once 3 β , 25-dihydroxycholest-5-ene was no longer detected by TLC, the eluent was analyzed by ¹H NMR to ensure that the 3 β -triethylamine salt was separated from the 25-sulfate regioisomer. A sample was removed from the eluent containing drum, and the purity of the 3 β -triethylamine salt was 85% with 15% 25-sulfate regioisomer present (¹H NMR). An HPLC weight percentage assay showed that 127 g of 3 β -triethylamine salt /25-sulfate regioisomer was collected in the drum (85% 3 β -triethylamine salt). The purified material was set aside and the remaining two-thirds of the crude solution was purified by chromatography. The silica in the C-105 column was flushed with methanol and then equilibrated with 1% triethylamine in methylene chloride (0.2% methanol by ¹H NMR area % in the eluent after regeneration).

[00513] The solids that precipitated from the carboy were analyzed by ¹H NMR and identified as the quaternary ammonium salt produced from the reaction of methylene chloride with triethylamine (from the SO₃NMe₃ reagent) and methylene chloride with triethylamine. The methylene chloride-triethylamine complex was separated by filtration, while methylene chloride-triethylamine complex was formed in the chromatography. Formation of the salt occurred under ambient conditions and was rapid in certain instances in a pressurized environment. The establishment of an equilibrium in which the triethylammonium moiety of the 3 β -triethylamine salt may be exchanging for the quaternary ammonium salt to give the quaternary ammonium complex and the triethylamine hydrochloride. Equilibrium favors the formation of the quaternary ammonium complex since there is more methylene chloride-triethylamine complex present. Triethylamine hydrochloride was isolated and characterized.



[00514] Of the remaining two-thirds of the crude mixture in the carboy, one-third was subjected to chromatography on a fourth column. Pressure was maintained at 0-1 psi during the entire purification. Separation of 3β -triethylamine salt from the 25-sulfate regioisomer was successful. The purity of the 3β -triethylamine salt in the drum was 99.79% by HPLC. Approximately 0.050 kg of 34.1 was isolated from the column (HPLC weight percent assay). The silica gel was cleaned with methanol and regenerated with 1% triethylamine in methylene chloride. The amount of methanol present after regeneration was 0.44% (^1H NMR area %). No separation occurred for this column. No further purifications were done with the material from the fourth column. Eluent from the third column (~50 g of the 3β -triethylamine salt) was subjected to the cation exchange beginning with a solvent swap to acetonitrile. After the addition of acetonitrile, water, and 30% sodium hydroxide, the slurry was agitated and then held overnight. Solids were present in the reactor after the post-stir. The mixture was cooled and filtered using a new 8.5" benchtop filter. The cake was washed with fresh acetonitrile and dried. A sample was analyzed by ^1H NMR and peaks consistent with a quaternary ammonium salt were present in the spectrum. The 25- sulfate regioisomer was present by HPLC.

[00515] Eluent from the second column was concentrated under vacuum and dried to a constant weight. Analysis of the yellow powder (540 g) by ^1H NMR showed a ratio (3:1) of the methylene chloride-triethylamine quaternary ammonium salt to the monosulfate compound. All of the crude material (540 g) was charged to a 3 L jacketed reactor. Acetonitrile (1400 g) was charged, and the slurry was heated to 50 ± 5 °C for 30 min. The slurry was cooled to 26 °C and then filtered. The wet cake was analyzed and the ratio of 3 β -triethylamine salt /25- sulfate regioisomer to quaternary ammonium salt was ~1:1. The purified solids and fresh acetonitrile (1400 g) were charged back to the reactor. Water (200 g) was charged after 45 min, agitated for 15 min, and then filtered. The granular powder was dried in a vacuum oven at 40 °C overnight. The filtrate was concentrated to dryness, and the residue was combined with the dried material and both were charged to a 3 L reactor. Acetonitrile (1500 g), 1 N sodium hydroxide (600 g), and 30% sodium hydroxide (40 g) were sequentially charged to the reactor. The slurry was agitated for 48 hours and then filtered at ambient temperature. The cake was dried to a constant weight (173 g) and analyzed by HPLC.

Purification to separate the 25- sulfate regioisomer from Compound 1

[00516] Several solvents were explored to purge the 25-sulfate sodium salt from compound 1. No solids were recovered after dissolving impure compound 1 in polar solvents and then charging anti-solvents (Entries 1 and 2, Table 13). Minimal solids were formed after dissolving the material in methanol and then adding acetonitrile (Entry 3, Table 13). Using 2-propanol (Entry 4, Table 13) and a mixture of methanol and water resulted in a form change, which caused the material to become a thick paste that would not transfer or filter. Trituration of impure compound 1 with ethanol at 40-50 °C was sufficient to purge the majority of the 25-sulfate regioisomer (Entry 6, Table 13). A mixture of compound 1 (1 g) and ethanol (10 mL) was heated to reflux, cooled, and filtered. The isolated material (55% recovery) was 99.6% pure with the 25-sulfate and 3 β , 25-dihydroxycholest-5-ene products reduced to 0.1% and 0.3%, respectively.

Table 13 – Trituration/recrystallization of Compound 1

Sample	Solvent	Unpurified Compound 1 (g)	Compound 1 Purity	25-sulfate regioisomer	3 β , 25-dihydroxycholest-5-ene	Comment
1	Ethyl acetate/methanol	0.5	N/A	N/A	N/A	No Solids
2	Dimethyl sulfoxide/isopropanol	0.5	N/A	N/A	N/A	No Solid
3	Methanol/acetonitrile	0.1	N/A	N/A	N/A	Minimal Solids
4	Isopropanol	0.1	N/A	N/A	N/A	Gel/paste
5	Methanol/water	0.5	96.3	1.8	1.9	Gel/paste
6	Ethanol	0.1	97.0	0.4	2.6	Trituration
7	ethanol	1.0	99.6	0.1	0.3	55% recovery

Purification of the 3 β -triethylamine salt

[00517] The 3 β -triethylamine salt was purified to eliminate methylene chloride due to reactivity with trimethylamine and triethylamine. Purification was achieved using an isocratic solvent system that includes 90% ethyl acetate, 9% methanol and 1% triethylamine.

Optimization of SO₃NMe₃ equivalents

[00518] The amount of added SO₃NMe₃ complex needed to either completely consume 3 β , 25-dihydroxycholest-5-ene or arrive at a point at which the bis-sulfate and unreacted starting material byproducts were minimal was determined. A solution of 3 β , 25-dihydroxycholest-5-ene (0.5 g, 1.0S) in pyridine (18.6S) containing triethyl amine (0.5S) was heated to 50 °C. A sample was removed from the reaction every 30 min, which was followed by the addition of the SO₃NMe₃ complex. Following the final charge of the SO₃NMe₃ complex, the vial was allowed to stir at 50 °C for a total of 24 h. (Table 14). Approximately 1.75 equivalents of SO₃NMe₃ complex were sufficient to consume 86.6% of starting material 3 β , 25-dihydroxycholest-5-ene

(Sample 7, Table 14). 3β , 25-dihydroxycholest-5-ene was completely consumed after 2.5 equivalents of SO_3NMe_3 complex were added. Formation of the bis-sulfate will out-compete mono-sulfation of 3β , 25-dihydroxycholest-5-ene as the reaction progresses. The 3β -triethylamine salt was completely converted to the bis-sulfate after 24 h.

Table 14 – Equivalents of sulfur trioxide-trimethylamine complex

Sample	Equivalents of SO_3NMe_3	3β -triethylamine salt	3β , 25-dihydroxycholest-5-ene	Bis-sulfate
1	0.25	18.0	75.9	6.1
2	0.5	28.6	71.4	0.0
3	0.75	39.8	60.2	0.0
4	1.00	48.6	46.7	4.7
5	1.25	53.1	39.4	7.4
6	1.50	58.4	28.6	12.9
7	1.75	68.1	13.4	18.5
8	2.00	60.9	13.1	26.0
9	2.30	58.3	7.2	34.4
10	2.60	49.6	1.0	49.4
11	2.90	46.6	0.0	53.4
12	3.20	37.7	0.0	62.3
13	3.50	32.3	0.0	67.7

One-hundred gram scale synthesis of 3β -25-hydroxycholest-5-ene sulfate (1, as sodium salt)

[00519] A slurry of 3β , 25-dihydroxycholest-5-ene (100 g, 1.0S) and triethylamine (0.5S) in pyridine (15.6S) was heated to 50 °C. The SO_3NMe_3 complex (1.75 equivalents, 0.6S) was charged in one portion. The mixture was agitated for 5 hours and then analyzed for reaction completion by HPLC (Sample 1 - 3β -triethylamine salt /25- sulfate regioisomer (67.1%); 3β , 25-dihydroxycholest-5-ene (12.2%); bis-sulfate (20.8%)). The jacket was set to 70 °C and the reaction was concentrated to <20% of the initial volume. A sample was removed and analyzed by HPLC for stability (Sample 2- 3β -triethylamine salt /25- sulfate regioisomer (60.5%); 3β , 25-dihydroxycholest-5-ene (10.0%); bis-sulfate (29.5%)). The amount of monosulfate decreased

from 67.1% to 60.5% during the distillation, while the amount of bisulfate increased ~9%. The amount of 3 β , 25-dihydroxycholest-5-ene did not decrease much during the distillation.

[00520] Solids were present in the reactor following a 48 h post stir, and the addition of methanol (0.5S) did not dissolve the solids. The crude material (300 g) was subjected to purification by silica gel chromatography eluting with 90% ethyl acetate, 9% methanol, and 1% triethylamine. Silica gel (2.4 kg) was slurried in the eluent and packed to form a 5.25" x 28" column. The crude mixture was transferred to the column, and the purification was carried on over three days. The eluent was collected in 1 L fractions. Fractions 1-7 contained no material detected by TLC; Fractions 8-11 contained pyridine and 3 β , 25-dihydroxycholest-5-ene; Fractions 12-20 contained no material detected by TLC; Fractions 21-22 contained an undetermined compound and Fractions 23-59 contained 3 β -triethylamine salt /25- sulfate regioisomer.

[00521] Approximately 82 g of 3 β -triethylamine salt /25- sulfate regioisomer (56.5% yield) was isolated after the column determined by weight percentage analysis. After chromatography, the eluent containing a mixture of 3 β -triethylamine salt /25- sulfate regioisomer was concentrated to a slurry and transferred to a 2 liter reactor. The solvent was swapped to acetonitrile, the slurry was cooled to 10 °C, and 1 N sodium hydroxide (1.8S, 1 equivalent based on 82 g of 3 β -triethylamine salt /25- sulfate regioisomer) was charged over 10 minutes. The slurry was agitated for 1 hour and then filtered. The filtration was very fast, requiring <5 minutes. The solids were dried at 40 °C under vacuum to a constant weight (70 g, 99% yield for the cation exchange). A sample was analyzed by HPLC (Sample 1, Table 15) which indicated that the 25- sulfate regioisomer was present at 5.1%. The white powder (70 g) was transferred to a 2 liter reactor and slurried with ethanol (700 g) at 50 °C for 1 hour. A form change was observed after 30 minutes of stirring by the thickening of the slurry mixture. The slurry was cooled to 10 °C, stirred for 1 hour, and then filtered at 10 °C. The reactor was rinsed with ethanol (170 g), cooled to 10 °C and then transferred to the filter as a cake wash. The solids were dried to a constant weight (64.6 g, 92.3% recovery) and analyzed by HPLC (Sample 2, Table 15). After trituration, the purity of compound 1 improved to 97.4%, but the 25-sulfate regioisomer was 1.6%. Impure compound 1 (64.6 g, 1.0S) was slurried in ethanol (581 g, 9S) at 55 °C for 1.5 hours. The slurry was cooled to 10 °C and then filtered. The reactor and cake

were rinsed with ethanol (84 g) at 10 °C, and the resulting solid was dried at 40 °C under vacuum to a constant weight (60.4 g, ethanol present at 5.9%, 87.9% recovery).

[00522] A sample of compound 1 following ethanol trituration was analyzed by HPLC (Sample 3, Table 15). The 25-sulfate regioisomer was purged, but the amount of unknown 1 increased to 0.9%. The purified material (56.8 g) was slurried in acetonitrile (5S) and water (0.9S) at 30 °C for 30 minutes in a 1 liter reactor. The slurry formed stiff peaks during this time, but the paste was easily transferred to the filtration setup using an FMI pump. The reactor and cake were rinsed with fresh acetonitrile (30 g), and the material was dried to a constant weight (54.5 g, 90.2% recovery). Analysis by ¹H NMR showed that ethanol was absent, but water was present at 1.2% by weight. The purity of the final material improved to >99% (Sample 4, Table 15). The unknown impurities at RRT 1.68 and 1.85 were present at 0.6% and 0.2%, respectively. Taking into account the residual water, the final isolated yield of compound 1 in the 100 g demonstration run was 43.2%.

Table 15 – Purification of Crude Compound 1

Sample	25-sulfate regioisomer (%) RRT 0.78	3 β -triethylamine salt (%) RRT 1.00	Unknown 1 (%) RRT 1.68	Unknown 2 (%) RRT 1.85	Unknown 3 (%) RRT 1.99
1	5.1	93.1	0.9	0.6	0.3
2	1.6	97.4	0.6	0.4	ND
3	ND	98.9	0.9	0.2	ND
4	ND	99.2	0.6	0.2	ND

Azeotropic Removal of Water from 3 β , 25-dihydroxycholest-5-ene

[00523] A slurry of 3 β , 25-dihydroxycholest-5-ene (5 g, 1.0S) and pyridine (15.6S, 0.016% water, Entry 1, Table 16) was heated to 50 °C. A sample of the reaction was removed for water content analysis (0.29%, Entry 2, Table 16). The reaction volume was reduced 50% and sampled for water content (0.042%, Entry 3, Table 16). The amount of pyridine that was collected in the distillate (39 g) was replaced with fresh pyridine in the reactor and sampled again for water (0.027%, Entry 4, Table 16). Once the internal temperature reached 50°C,

triethylamine (0.5S) and SO_3NMe_3 (0.6S) were charged to the reactor. The thin white slurry became a clear solution within 15 minutes, and the reaction was agitated at 50 °C. A sample was removed at 2 hours and 3 hours for IPC analysis (Entries 1 and 2, Table 17). Only 7.1% 3β , 25-dihydroxycholest-5-ene remained after 2 h. Azeotropically removing the water prior to the addition of SO_3NMe_3 improves the consumption of starting material.

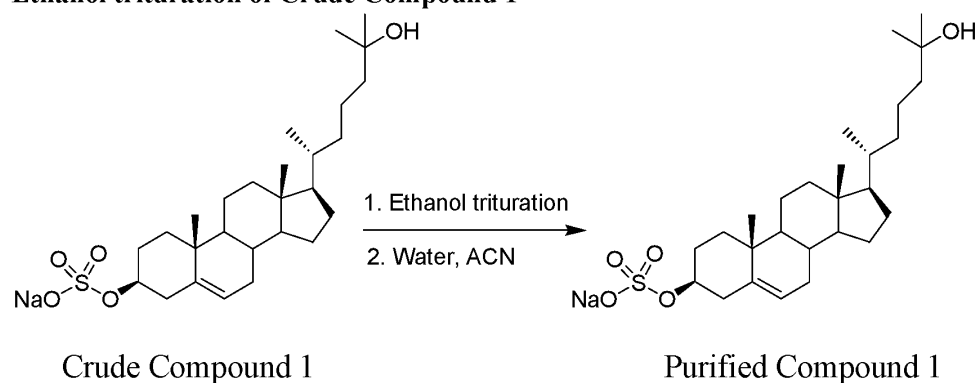
Table 16 – Water content analysis

Sample	Comment	% Water
1	Pyridine solvent	0.016
2	Reaction solution before pyridine distillation	0.285
3	Reaction solution after pyridine distillation	0.042
4	Reaction solution after addition of pyridine	0.027

Table 17 – Reaction completion profile by HPLC

Sample	25-sulfate regioisomer (%)	3β -triethylamine salt/ 25-sulfate regioisomer (%)	3β , 25-dihydroxycholest-5-ene (%)
1	30.8	62.2	7.1
2	38.9	55.6	5.6

Ethanol trituration of Crude Compound 1



[00524] Crude compound 1 was suspended in ethanol and heated to 55 °C and stirred for 1 hour. The slurry mixture is cooled, filtered and washed with ethanol. The resulting cake is dried overnight at 50 °C. The cake was charged back into the reactor and suspended in acetonitrile and

water. The mixture is heated to 30 °C and stirred for 1 hour. The mixture is then cooled to 15 °C, filtered and washed with acetonitrile and water (90:10). The resulting cake is dried for not longer than 24 hours at 50 °C until a constant weight is achieved. Impurity content in purified compound 1 was determined by HPLC. (RRT 0.67 <0.05%; RRT 0.77 <0.05%; RRT 0.79 <0.05%; RRT 0.95 <0.05%; RRT 1.13 <0.05%; RRT 1.22 <0.05%; RRT 1.31 <0.05%; RRT 1.95 = 0.09%; RRT 2.09 <0.05%; RRT 2.67 <0.05%; RRT 2.75 = 0.05%; RRT 3.04 <0.05%; RRT 3.23 = 0.09%; RRT 3.64 = 0.3%; RRT 5.00 <0.05%; Total impurities = 1.1%.

Identification of Byproducts from Sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol to produce 25-hydroxy-(3 β)-cholest-5-en-3-sulfate

[00525] A composition of 25-hydroxy-(3 β)-cholest-5-en-3-ol was sulfated with a sulfur-trioxide pyridine complex in toluene at 23 °C for 1 h to produce 25-hydroxy-(3 β)-cholest-5-en-3-sulfate. Compounds formed in a reaction mixture when preparing the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate product were analyzed by high performance liquid chromatography. Tables 13 and 14 provide the HPLC chromatography conditions. Table 15 lists retention times of compounds identified as being formed in the reaction mixture when sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol with a sulfur-trioxide pyridine complex.

Table 17 – Chromatographic Conditions

Column	Aglient Zorbax Eclipse XDB-C8, 3.5 μ m (4.6 x 150) mm
Mobile Phase A	2.5 mM sodium phosphate, pH 2.1 with 0.2% SDS
Mobile Phase B	10:85:5 MPA: ACN: MeOH
Detection	Impurities and calculated purity: 205 nm; Assay: 220 nm
Column Temperature	45 °C
Injection Volume	25.0 μ L
Flow Rate	1.0 mL/min
Acquisition Time	50.0 minutes (including 5.0 min. re-equilibration)

Table 18 – Chromatographic Conditions - Gradient

<u>Time (minutes)</u>	<u>%A</u>	<u>%B</u>
0.0	62	38

35	0	100
45	0	100
45.1	62	38
50.0	62	38

Table 19 –Retention Times

<u>Compound</u>	<u>Retention Time (min)</u>
Pyridine	3.2
25-sulfated cholesterol	6.6
25-hydroxy-(3 β)-cholest-5-en-3-sulfate	7.7
Unknown Byproduct #1	18.3
25-hydroxy-(3 β)-cholest-5-en-3-ol	26.5
Unknown Byproduct #2	37.7

Determining purity of sulfur trioxide pyridine sulfating agent

[00526] Proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$) was conducted on samples of sulfur trioxide pyridine in a deuterated solvent. Sulfur trioxide pyridine is a colorless solid that can degrade due to the presence of moisture, which can impact the overall yield and reproducibility of sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol. A sample of sulfur trioxide pyridine from three lots (A-C) was dissolved in deuterated acetone ($(\text{CD}_3)_2\text{CO}$) and proton NMR spectra were recorded using a 500 MHz Bruker spectrometer (Figure 57). An enhancement of the region between 8.1 and 9.3 ppm in the NMR spectra is shown in Figures 58A-58C. The NMR spectrum of lot A in Figure 58A exhibits a smaller set of peaks at 9.25 ppm than the NMR spectrum of lots B and C in Figures 58B and 58C, respectively. Based on the integrated peak at 9.25 ppm in each spectrum, an impurity level of 21% was calculated for the sulfating agent of lot A (Figure 58A), an impurity level of 33% was calculated for the sulfating agent of lot B (Figure 58B) and an impurity level of 36% was calculated for the sulfating agent of lot C (Figure 58C).

Process Parameters for Sulfating 25-hydroxy-(3 β)-cholest-5-en-3-ol

[00527] A sulfation reaction study to minimize and control the formation of *bis*-sulfated product 5-cholesten-3 β -25-diol-disulfate was conducted.

Sulfation with particles of 25-hydroxy-(3 β)-cholest-5-en-3-ol in reaction mixture

[00528] During the sulfation reaction, it was observed that the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt precipitates as a gel-like solid during the reaction. Some of this colloidal material may be solubilized in the reaction mixture due to its particle size. To minimize this solubility effect, the addition of seed crystals of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt to the reaction to modify the product crystal shape was tested. As the reaction proceeds by charging the sulfur trioxide-pyridine complex, the gel-like solids of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt produced during the sulfation reaction turned into an amorphous slurry with a larger particle size. This allowed for control of the solubility of the generated 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt in the reaction mixture. This also resulted in minimizing the formation of *bis*-sulfated product 5-cholesten-3 β -25-diol-disulfate in the reaction mixture.

[00529] 25-hydroxy-(3 β)-cholest-5-en-3-ol was dissolved with 2-methyl tetrahydrofuran (30V); and heated to about 35-40°C. The solution was cooled to about 20 $\pm$ 5 °C and seed crystals of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt were added. The sulfating agent sulfur-trioxide-pyridine complex was added in four portions held 2 hours apart from each other. Water (2 equivalents) was added to the slurry and held for 1 hour. At this point, agitation was reduced to a minimum vortex deep. Pyridine (2 equivalents) in 2-methyl tetrahydrofuran was added and the slurry was held for 12 hours or longer. Crude 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product was collected by filtration and washed with 2-methyl tetrahydrofuran - pyridine (5%). The presence of *bis*-sulfated product 5-cholesten-3 β -25-diol-disulfate was estimated to be about 2-5% in the crude product.

Quenching of unreacted Sulfur Trioxide-Pyridine Sulfation Reagent

[00530] Quenching excess unreacted sulfur-trioxide pyridine sulfation reagent was evaluated using two equivalents of water and pyridine to keep basic conditions and to avoid hydrolysis of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product. In Items 1-3 of Table 20, water and pyridine were added simultaneously and held for one hour; then, the product was isolated by vacuum filtration. In item 4 of Table 20, the holding time was extended to mimic time expansion. To control the competition reactions between reagent hydrolysis and

bis-sulfation, reagent hydrolysis was evaluated by adding water and holding it for one hour. This approach maximized excess hydrolysis. Pyridine was then added to minimize product hydrolysis (item5, Table 20). As summarized in Table 20, the addition of water for 1 hour followed by mixing with pyridine overnight afforded the highest yield of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product and the lowest amount of *bis*-sulfated product and desmosterol impurity.

[00531] During quenching of excess unreacted sulfation reagent, it was determined that the agitation speed can play a role in competition between formation of *bis*-sulfated product 5-cholesten-3 β -25-diol-disulfate and reagent quench. At high agitation speed, the unquenched sulfur trioxide-pyridine complex agglomerates brake apart, allowing further reaction with the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product. At slow agitation speed agglomerated complex remains at the bottom of the reactor minimizing this side reaction. *Bis*-sulfated product 5-cholesten-3 β -25-diol-disulfate formation is observed under these reaction conditions in a range of 2-5%. Isolated crude 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product was stable enough for further purification.

Table 20 – Quenching of unreacted Sulfur Trioxide-Pyridine Sulfation Reagent

Item	HPLC area (%)				Stage	Time
	<i>Bis</i> -sulfated product 5-cholesten-3 β -25-diol-disulfate	25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt	Desmosterol sulfate	25-hydroxy-(3 β)-cholest-5-en-3-ol		
1	4.4	92.9	1.329	1.26	IPC	1h
	5.0	92.0	1.46	1.54	Solid	
2	1.4	97.3	0.58	0.67	IPC	1h
	2.8	94.3	0.68	2.16	Solid	
3	1.9	96.6	0.79	0.61	IPC	1h
	2.6	95.8	0.46	1.11	Solid	
4	1.1	96.4	0.77	1.67	IPC	overnight
	3.5	95.4	0.27	0.86	Solid	

5	1.8	97.0	0.69	0.57	IPC	Water 1h, pyridine overnight
	2.6	96.1	0.62	0.59	Solid	

*IPC – in process control

Liquid Chromatography and Recrystallization of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product

[00532] The 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product was purified using a plug column employing a silica gel ($\geq$ mass equivalent) stationary phase and a mixture of methylene chloride-methanol (85:15) and pyridine (1%) mobile phase. The chromatographic column was prepared with silica gel (5mass-eq)/DCM-Pyridine (1%), with a 1:2 ratio diameter-silica gel. The column was carefully prepared to avoid disturbing the silica gel top layer. Crude 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product was dissolved in methylene chloride-methanol (1:1) -pyridine (1%) (2.4V), the solution charged to the column, and rinsed with methylene chloride-methanol (15%)-Pyridine (1%) (2V). The column was eluted with methylene chloride-methanol (15%)-Pyridine (1%) (~75V). Samples of about 10V were taken and monitored by thin layer chromatography (mobile phase methylene chloride -methanol 7:3 one drop pyridine and CAM stain). Fractions containing the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product were combined and the fractions containing *bis*-sulfated product were excluded.

[00533] The 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product was isolated and purified from collected fractions by two different processes:

[00534] Isolation and Recrystallization Process (IP)-A. Fractions with the product from the plug column were concentrated under the constant volume technique. The 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product solution was added to the initial constant volume (28V) mixture of 2-methyl tetrahydrofuran-heptane (1:2) – Particle seeds of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product were added while synchronizing distillation and addition. Pressure was maintained between 20-25 in. Hg. Under these conditions, the product precipitated out immediately and remains as a slurry during the

distillation. The slurry temperature is adjusted to 20-25°C and held for a minimum of 1 hour. The product is collected by filtration and rinsed with 2-methyl tetrahydrofuran-heptane (1:2) followed by heptane. The collected material was dried at 30-35 °C under vacuum for 24 hours.

[00535] Isolation Process (IP)-B. Fractions with the product from the plug column are concentrated under vacuum to ~7V. If the solution remained or turned cloudy or solids were observed, methylene chloride was added until a clear solution was obtained. This concentrated 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product solution was added dropwise to a mixture of 2-methyl tetrahydrofuran-heptane (1:3) containing seeds of the 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt for about 1 hour to 1.5 hours. The product was rinsed in the container with methylene chloride-methanol (1:1) (0.5V) for 1 hour at 20-25 °C. After aging the slurry, the product was collected by filtration and rinsed with 2-methyl tetrahydrofuran-heptane (1:3), followed by heptane. The solids were dried at 30-35 °C under vacuum for 24 hours.

[00536] The purity of 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product obtained by each isolation and recrystallization process is summarized in Table 21.

Table 21 – Purity 25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt product after Isolation and recrystallization

Step	Scale (g)	HPLC area (%)				Isolation Process	Yield
		<i>Bis-sulfated product 5-cholesten-3β-25-diol-disulfate</i>	25-hydroxy-(3 β)-cholest-5-en-3-sulfate organic cationic salt	Desmosterol sulfate	25-hydroxy-(3 β)-cholest-5-en-3-ol		
Sulfation	430	3.89	95.23	0.40	0.43	--	--
Purified	540	ND	99.88	0.12	ND	(IP)-A	75.1
Sulfation	40	2.28	96.83	0.75	0.14	--	--
Purified	45	ND	99.45	0.51	ND	(IP)-B	84.0