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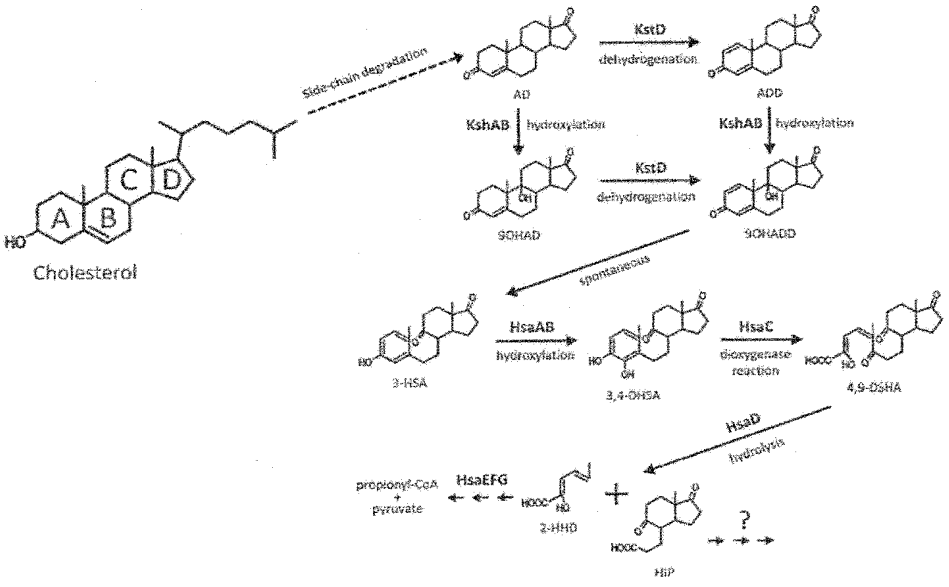

Figure 1

(57) **Abstract:** A genetically-modified bacterium, for example of the class *Actinobacteria*, and the use of such a bacterium in the bioconversion of a steroidal substrate into a steroidal product of interest. A method of converting a steroidal substrate into a steroidal product of interest, wherein the method comprises: inoculating culture medium with genetically-modified bacteria according to any of Claims 1 to 28 and growing the bacterial culture until a target OD_{600} is reached; adding a steroidal substrate to the bacterial culture when the target OD_{600} is reached; culturing the bacterial culture so that the steroidal substrate is converted to the steroidal product of interest; and extracting and/or purifying the steroidal product of interest from the bacterial culture.

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GENETICALLY-MODIFIED BACTERIA AND USES THEREOF

The present invention relates to genetically-modified bacteria and the use of such bacteria in the bioconversion of steroidal substrates into steroidal compounds of interest. The genetically-modified bacteria may be from the genera *Rhodococcus* or *Mycobacterium*.

Steroids are a large and diverse class of organic compounds, with many essential functions in eukaryotic organisms. For example, naturally occurring steroids are involved in maintaining cell membrane fluidity, controlling functions of the male and female reproductive systems and modulating inflammation.

As signalling through steroid controlled pathways is important in a wide variety of processes, the ability to modulate these pathways using synthetically produced steroid drugs means they are an important class of pharmaceuticals. For example, corticosteroids are used as anti-inflammatories for the treatment of conditions such as asthma and rheumatoid arthritis, synthetic steroid hormones are widely used as hormonal contraceptives and anabolic steroids can be used to increase muscle mass and athletic performance.

The synthesis of steroids for use as pharmaceuticals involves either semi-synthesis from natural sterol precursors or total synthesis from simpler organic molecules. Semi-synthesis from sterol precursors such as cholesterol often involves the use of bacteria. The advantages of using bacteria to carry out these bioconversions are that the synthesis involves less steps and the reactions performed by the enzymes are stereospecific, resulting in the production of the desired isomers without the need for protection and deprotection used in traditional chemical synthesis. The products of bacterial bioconversions can then be used as pharmaceuticals or as precursors for further chemical modification to produce the compound of interest.

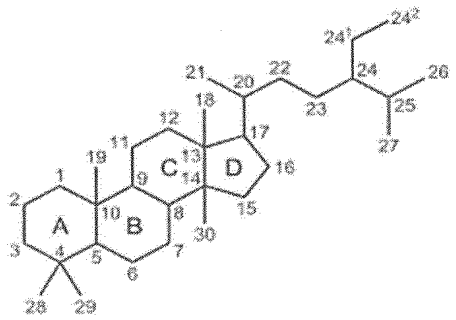
Steroids naturally occur in both plant, animal and fungal species, and are produced by certain species of bacteria. Despite them only occurring naturally in only a few bacterial species, several bacterial species are able to metabolise sterol compounds as growth substrates. Examples of bacteria that can degrade sterol compounds include those from the genera *Rhodococcus* and *Mycobacterium*.

The bacterial sterol metabolism pathway involves progressive oxidation of the sterol side-chain, and breakdown of the polycyclic ring system. The pathway of sterol side-chain

degradation in *Rhodococcus* has been previously investigated using mutant strains (Wilbrink *et al*, 2011. *Applied and Environmental Microbiology*, 77(13):4455-4464) and an overview of the cholesterol catabolic pathway is shown in Figure 1. It has now been found that bacterial species may be used for steroid compound production by genetic modification to block the degradation pathway prior to breakdown of the polycyclic ring system and at various points in side-chain oxidation to allow accumulation of the steroidal compounds of interest in order to improve the yields obtained.

In a first aspect, the invention provides a genetically-modified bacterium blocked in the steroid metabolism pathway prior to degradation of the polycyclic steroid ring system, wherein the bacterium is disrupted in the steroid side-chain degradation pathway, and wherein the bacterium converts a steroidal substrate into a steroidal product of interest.

By "steroid" or "steroidal" compounds we include the meaning of a class of natural or synthetic organic compounds derived from the steroid core structure represented below (with IUPAC-approved ring lettering and atom numbering):



Steroidal compounds generally comprise four fused rings (three six-member cyclohexane rings (rings A, B and C above) and one five-member cyclopentane ring (ring D above)) but vary by the functional groups attached to that four-ring core and by the oxidation state of the rings. For example, sterols are a sub-group of steroidal compounds where one of the defining features is the presence of a hydroxy group (OH) at position 3 or the structure shown above. The structure formed by the atoms labelled 20 to 27 (including positions 24^1 and 24^2) in the above diagram is referred to as the steroid side-chain. Non-limiting examples of steroids include: sterols, 3-oxo-4-cholenic acid, 3-oxo-chola-4,22-dien-24-oic acid, 3-oxo-7-hydroxy-4-cholenic acid, 3-oxo-9-hydroxy-4-cholenic acid, 3-oxo-7,9-dihydroxy-4-cholenic acid, 3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC), 3-oxo-23,24-bisnor-1,4-choladiene-22-oic acid (1,4-BNC), 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD), sex steroids (e.g. progesterone, testosterone, estradiol),

corticosteroids (e.g. cortisol), neurosteroids (e.g. DHEA and allopregnanolone), and secosteroids (e.g. ergocalciferol, cholecalciferol, and calcitriol). Non-limiting examples of steroidal compounds are also shown in Figure 4.

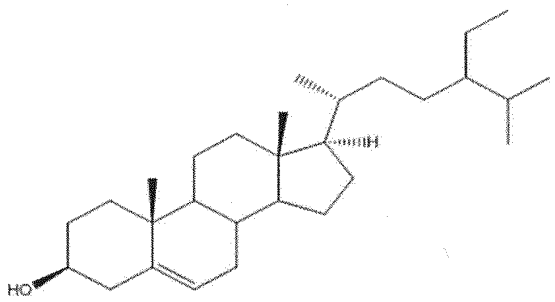
By "disrupted in the steroid side-chain degradation pathway" we include the meaning of a bacterium in which the normal degradation of the steroid side-chain is impaired. Normally, degradation of the steroid side-chain involves the initial cycle of side-chain activation followed by three successive cycles of β-oxidation (i.e. first, second, and third cycles of β-oxidation). In an unimpaired side-chain degradation pathway, the final product of the side-chain degradation steps is usually 4-androstene-3,17-dione (AD). Thus, a bacterium disrupted in the steroid side-chain degradation pathway will accumulate steroidal products that are upstream of the production of AD. The suggested side-chain degradation pathways of the sterols cholesterol and β-sitosterol are shown in Figure 2 and Figure 3 respectively (Wilbrink, 2011. *Microbial sterol side chain degradation in Actinobacteria.* Thesis).

By "polycyclic steroid ring system" we include the meaning of the ABCD system of rings found in the core steroidal structure shown above in the definition of steroidal.

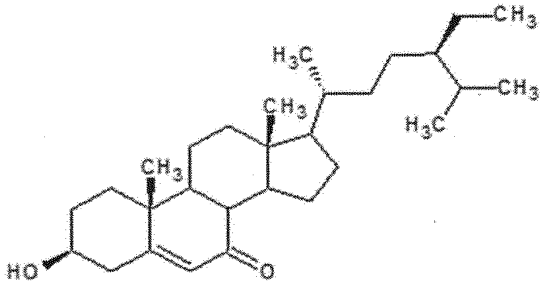
In some embodiments, the disruption in the steroid side-chain degradation pathway occurs after the first cycle of β-oxidation.

By "first cycle of β-oxidation" we include the meaning of the first cycle of β-oxidation in the steroid side-chain degradation pathway (Wipperman *et al*, 2014. *Crit. Rev. Biochem. Mol. Biol.*, 49(4):269-293). Specifically, the first cycle of β-oxidation is the process immediately following the side-chain activation cycle step, resulting in the shortening of the side-chain and the production of a C_{24} steroidal compound.

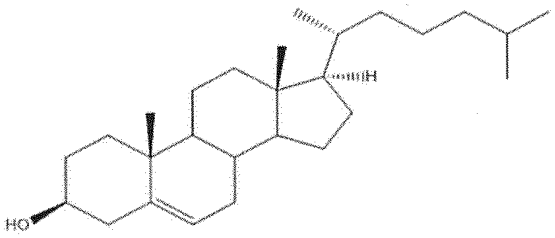
In some embodiments, the steroidal substrate may be a sterol substrate. In certain embodiments, the sterol substrate may comprise:



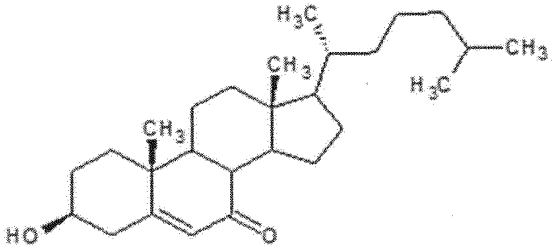
β-sitosterol;



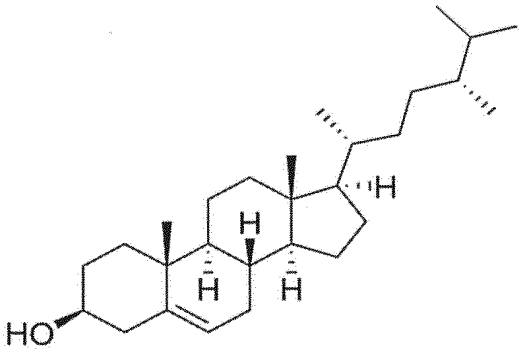
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



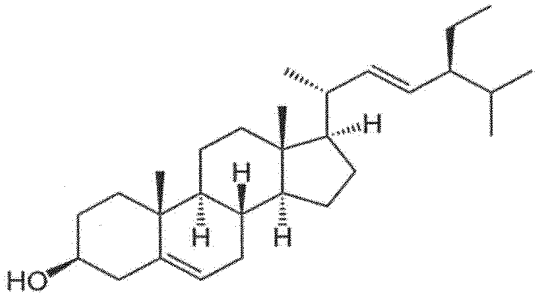
cholesterol;



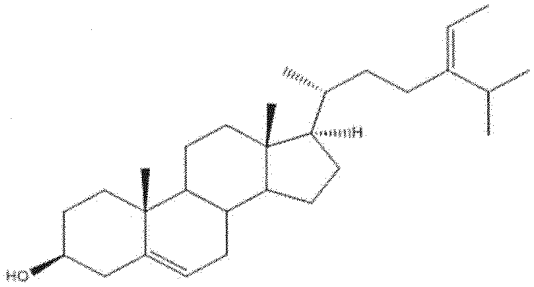
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



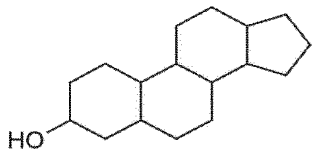
stigmasterol;



fucosterol;

7-oxo-phytosterol; or a combination thereof.

By "sterol" we include the meaning of molecules belonging to a class of lipids which are a sub-group of steroids with a hydroxyl group at the 3-position of the A-ring. Sterols have the general structure:



Sterols may also be referred to as steroid alcohols, and occur naturally in plants (phytosterols), animals (zoosterols), and fungi, and can be also produced by some bacteria. Non-limiting examples of sterols include: β-sitosterol, 7-oxo-β-sitosterol, 7-hydroxy-β-sitosterol, cholesterol, 7-oxo-cholesterol, 7-hydroxy-β-cholesterol, campesterol, stigmasterol, fucosterol, 7-oxo-phytosterol, adosterol, atheronals, avenasterol, azacosterol, cerevisterol, colestolone, cycloartenol, 7-dehydrocholesterol, 5-dehydroepisterol, 7-dehydrositosterol, 20α,22R-dihydroxycholesterol, dinosterol, epibrassicasterol, episterol, ergosterol, ergosterol peroxide, fecosterol, fucosterol, fungisterol, ganoderiol, ganodermadiol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 27-hydroxycholesterol, inotodiol, lanosterol, lathosterol, lichesterol, lucidadiol, lumisterol, oxycholesterol, oxysterol, parkeol, spinasterol, trametenolic acid, and zymosterol. Non-limiting examples of sterols are also shown in Figure 5.

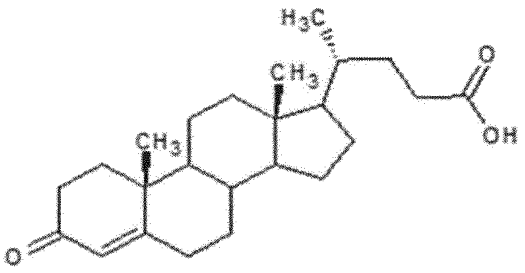
In some embodiments, the steroidal product of interest comprises an intact polycyclic ring system.

By "intact polycyclic ring system" we include the meaning of a steroidal molecule in which the ABCD ring system of the core steroid structure is still present, i.e. the ABCD ring system has not undergone degradation and/or oxidation such that any of the rings have been opened or removed.

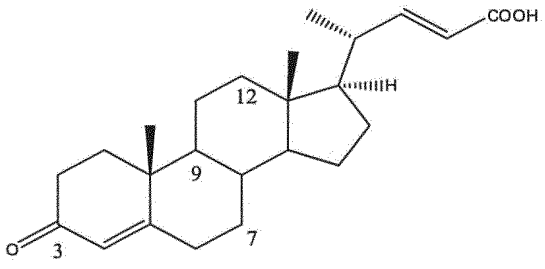
In some embodiments, the steroidal product of interest is a steroidal compound with a side-chain having a backbone of five carbons.

By "backbone" we include the meaning of the longest consecutive chain of carbon atoms in the steroid side-chain being five carbon atoms in length. Generally, the five carbons in the backbone are those at positions 20, 21, 22, 23, and 24, as shown in the diagram of the steroid core structure in the definition of the term "steroidal" above.

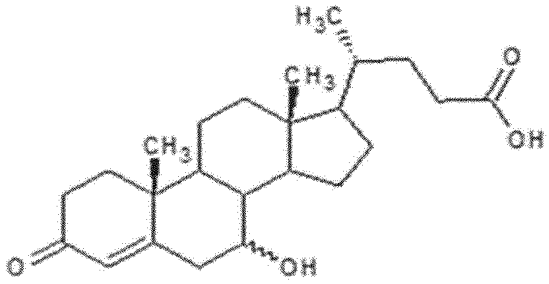
In certain embodiments, the steroidal product of interest may be:



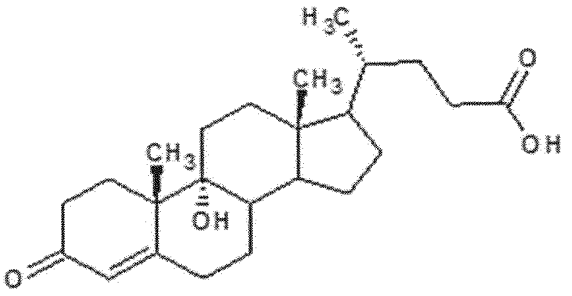
3-oxo-4-cholenic acid;



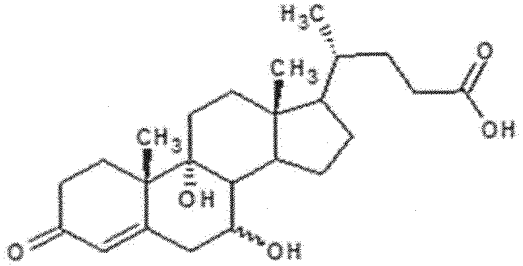
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



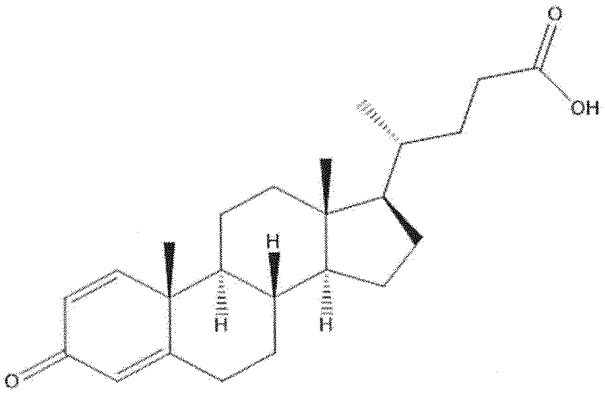
3-oxo-7-hydroxy-4-cholenic acid;



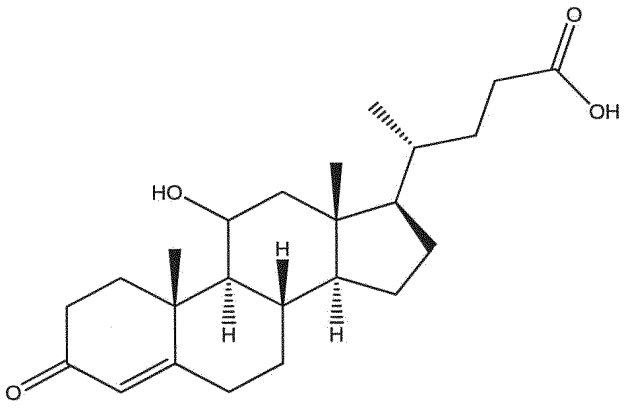
3-oxo-9-hydroxy-4-cholenic acid;



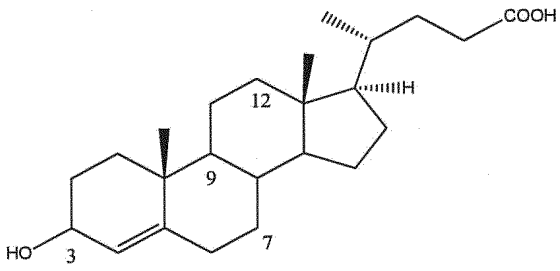
3-oxo-7,9-dihydroxy-4-cholenic acid;



3-oxo-1,4-choladienoic acid;



3-oxo-11-hydroxy-4-cholenic acid;



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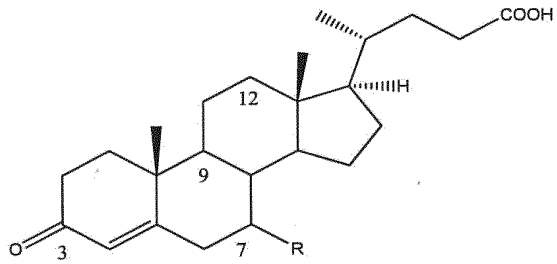
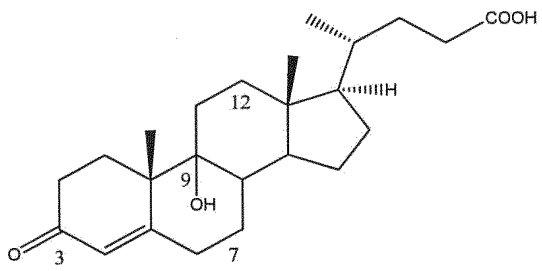


wherein R can be hydroxyl, oxo, or a halogen;



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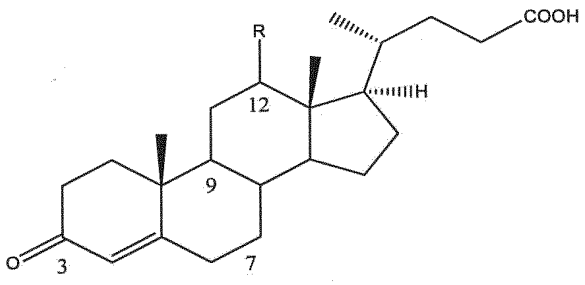
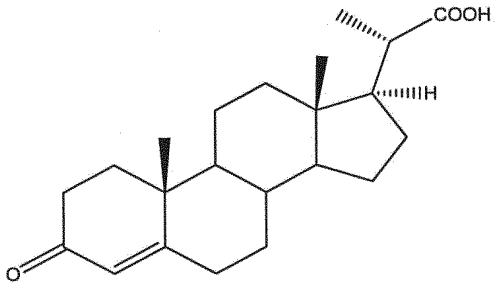
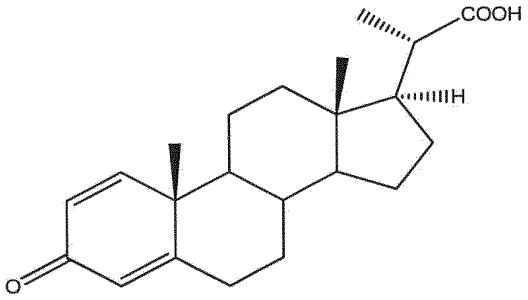


wherein R can be hydroxyl or oxo;

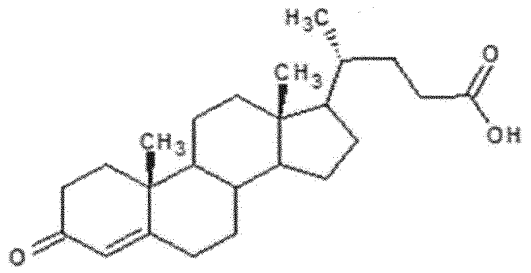


3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC);

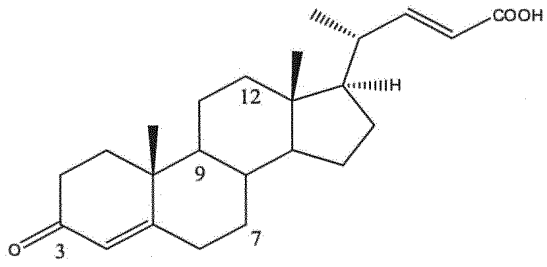


3-oxo-23,24-bisnor-1,4-choladiene-22-oic acid (1,4-BNC); or variants thereof.

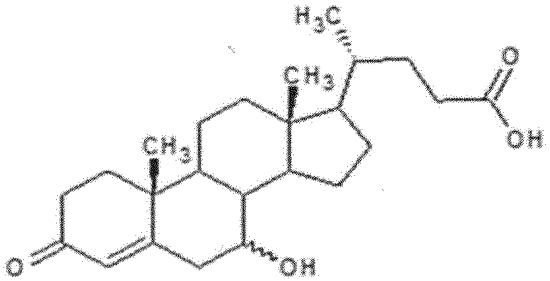
In other preferred embodiments, the steroidal product of interest may be



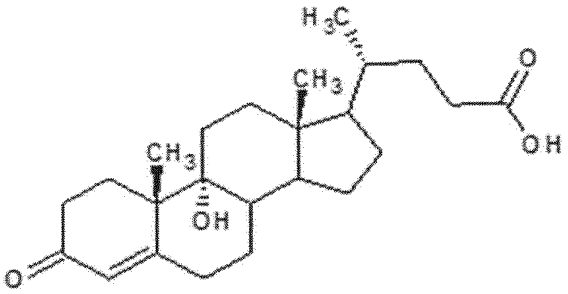
3-oxo-4-cholenic acid;



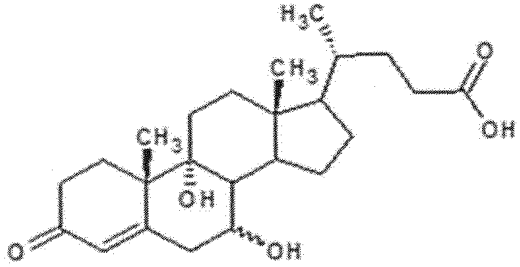
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



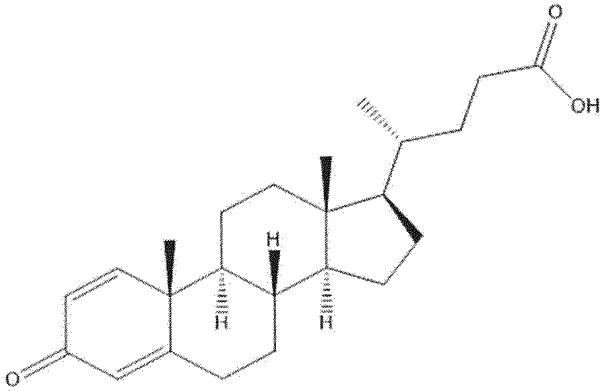
3-oxo-7-hydroxy-4-cholenic acid;



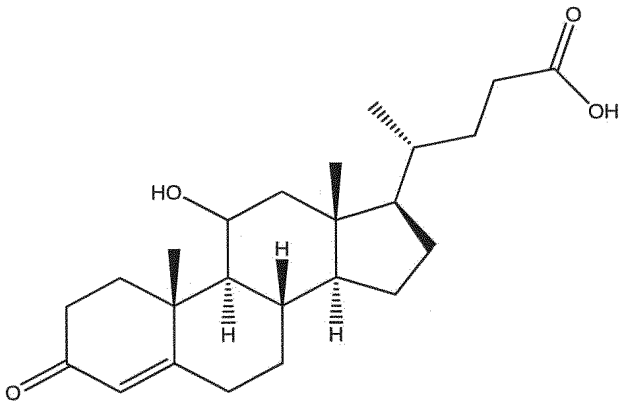
3-oxo-9-hydroxy-4-cholenic acid;



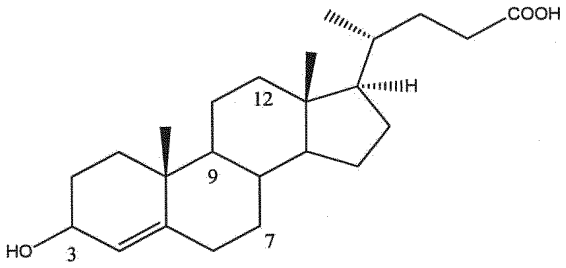
3-oxo-7,9-dihydroxy-4-cholenic acid;



3-oxo-1,4-choladienoic acid;



3-oxo-11-hydroxy-4-cholenic acid;



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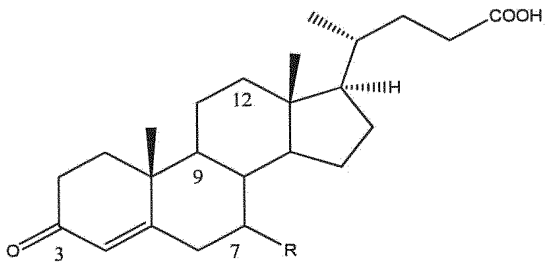
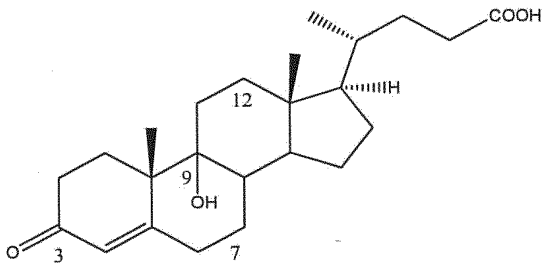


wherein R can be hydroxyl, oxo, or a halogen;



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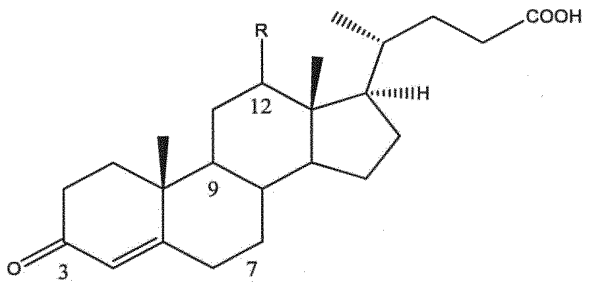


wherein R can be hydroxyl or oxo; or variants thereof.

In some embodiments, the genetically-modified bacterium may be of the *Actinobacteria* class or the *Gammaproteobacteria* class.

In certain embodiments, a genetically modified bacterium of the *Actinobacteria* class may be a *Rhodococcus* species, a *Mycobacterium* species, a *Nocardia* species, a *Corynebacterium* species, or an *Arthrobacter* species.

Where the bacterium is of a *Rhodococcus* species, the *Rhodococcus* species may be *Rhodococcus rhodochrous*, *Rhodococcus erythropolis, Rhodococcus jostii*, *Rhodococcus ruber*, preferably *Rhodococcus rhodochrous.*

Where the bacterium is of a *Mycobacterium* species, the *Mycobacterium* species may be *Mycobacterium neoaurum*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis, or Mycobacterium fortuitum,* preferably *Mycobacterium neoaurum.*

Where the bacterium is of a *Nocardia* species, the *Nocardia* species may be *Nocardia restrictus*, *Nocardia corallina*, or *Nocardia opaca.*

Where the bacterium is of a *Arthrobacter* species, the *Arthrobacter* species may be *Arthrobacter simplex.*

In some embodiments, the genetically-modified bacterium comprises one or more genetic modifications. In certain embodiments, the genetic modification of the genetically-modified bacterium may comprise inactivation of the genes: *kshA1* (SEQ ID NO: 1), *kshA2* (SEQ ID NO: 2), *kshA3* (SEQ ID NO: 3), *kshA4* (SEQ ID NO: 4), *kshA5* (SEQ ID NO: 5), or homologs thereof.

By "genetic modification" we include the meaning of an artificial alteration or addition to the genetic material present in an organism. For example, a genetic modification may be a directed deletion of a gene or genomic region, a directed mutagenesis of a gene or genomic region (e.g. a point mutation), the addition of a gene or genetic material to the genome of the organism (e.g. an integration), or, in the case of bacteria, the transformation of such cells with plasmid.

By "homolog" we include the meaning of a second gene or polypeptide that has a similar biological function to a first gene or polypeptide and may also have a degree of sequence similarity to the first gene or polypeptide. A homologous gene may encode a polypeptide that exhibits a degree of sequence similarity to a polypeptide encoded by the corresponding first gene. For example, a homolog may be a similar gene in a different species derived from a common ancestral gene (ortholog), or a homolog may be a second similar gene within the genome of a single species that is derived from a gene duplication event (paralog). A homologous gene or polypeptide may have a nucleotide or amino acid sequence that varies from the nucleotide or amino acid sequence of the first gene or polypeptide, but still maintains functional characteristics associated with the first gene or polypeptide (e.g. in the case where a homologous polypeptide is an enzyme, the homologous polypeptide catalyses the same reaction as the first polypeptide). The variations that can occur in a nucleotide or amino acid sequence of a homolog may be demonstrated by nucleotide or amino acid differences in the overall sequence or by deletions, substitutions, insertions, inversions or additions of nucleotides or amino acids in said sequence.

In some embodiments, the genetic modification further comprises re-introduction of a wild type copy of the *kshA5* gene comprising SEQ ID NO: 5, or a homolog thereof.

In other embodiments, the genetic modification comprises inactivation of the genes: *kshA1* (SEQ ID NO: 1), *kshA2* (SEQ ID NO: 2), *kshA3* (SEQ ID NO: 3), and *kshA4* (SEQ ID NO: 4), or homologs thereof.

In some embodiments, the genetic modification of the genetically-modified bacterium further comprises inactivation of the genes: *kstD1*(SEQ ID NO: 6), *kstD2* (SEQ ID NO: 7), and *kstD3* (SEQ ID NO: 8), or homologs thereof.

In some embodiments, the genetic modification comprises inactivation of one or more of the genes: *fadE34* (SEQ ID NO: 9; SEQ ID NO: 12), *fadE34#2* (SEQ ID NO: 10), or homologs thereof.

In other preferred embodiments, the genetic modification of the genetically-modified bacterium further comprises inactivation of the gene: *fadE26* (SEQ ID NO: 11), or homologs thereof.

In some embodiments, where the genetic modification comprises a gene inactivation, the gene activation is by gene deletion.

By "gene deletion" we include the meaning of removal of all or substantially all of a gene or genomic region from the genome of an organism, such that the functional polypeptide product(s) encoded by that gene or genomic region is no longer produced by the organism.

In certain embodiments, the homolog has a nucleotide sequence with at least 50% sequence identity with the nucleotide sequence of a first gene. In other embodiments, the homolog has a nucleotide sequence that has a sequence identity of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% with the nucleotide sequence of a first gene.

In some embodiments, the homolog encodes a polypeptide that has an amino acid sequence with at least 50% sequence identity with the amino acid sequence of a first polypeptide. The homolog encodes a polypeptide that has an amino acid sequence identity of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

By "sequence identity" we include the meaning of the extent to which two nucleotide or amino acid sequences are similar, measured in terms of a percentage identity. Optimal alignment is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the nucleotide or amino acid sequence in the comparison window may comprise additions or deletions (e.g. gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide base or amino acid residue occurs in both

sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (USA) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used.

In certain embodiments, the genetically-modified *Rhodococcus rhodochrous* bacterium may be of strain: LM9 (Accession No. NCIMB 43058), LM19 (Accession No. NCIMB 43059), or LM33 (Accession No. NCIMB 43060).

In certain embodiments, the genetically-modified *Mycobacterium neoaurum* bacterium may be of strain: NRRL B-3805 *Mneo-ΔfadE34* (Accession No. NCIMB 43057).

In a second aspect, the invention provides a genetically-modified bacterium according to the first aspect for use in the conversion of a steroidal substrate into a steroidal compound of interest.

In a third aspect, the invention provides a method of converting a steroidal substrate into a steroidal product of interest, comprising the steps of:

(a) inoculating culture medium with genetically-modified bacteria according to the first or second aspect and growing the bacterial culture until a target OD_{600} is reached;

(b) adding a steroidal substrate to the bacterial culture when the target OD_{600} is reached;

(c) culturing the bacterial culture so that the steroidal substrate is converted to the steroidal product of interest; and,

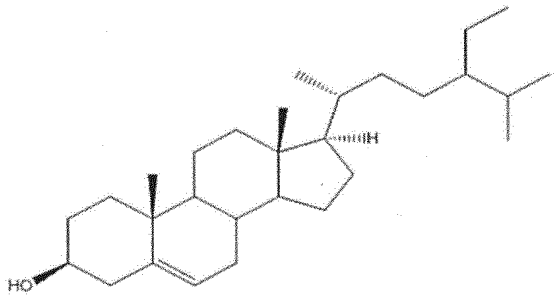
(d) extracting and/or purifying the steroidal product of interest from the bacterial culture.

By "culture medium" we include the meaning of a solid, liquid, or semi-solid medium designed to support the growth of microorganisms or cells.

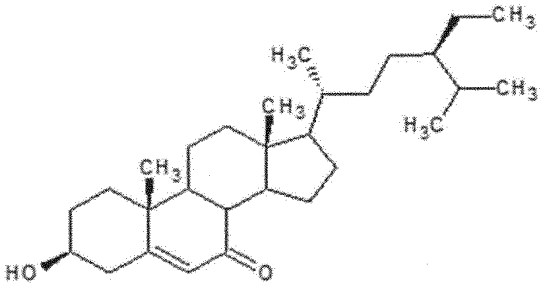
In some embodiments, the culture medium may be Luria-Bertani (LB) medium (10g/L tryptone; 5g/L yeast extract; 10g/L NaCl) or minimal medium (4.65g/L K_2HPO_4; 1.5g/L $NaH_2PO_4.H_2O$; 3g/L NH_4Cl; 1g/L $MgSO_4.7H_2O$; 1ml/L Vishniac trace element solution).

In certain embodiments, in step (a) of the method the bacterial culture may be grown to a target OD_{600} of at least 0.25, at least 0.5, at least 0.75, at least 1.0, at least 1.5, at least 2.0, at least 2.5, at least 3.0, at least 3.5, at least 4.0, at least 4.1, at least 4.2, at least 4.3, at least 4.4, at least 4.5, at least 4.6, at least 4.7, at least 4.8, at least 4.9, or at least 5.0. Preferably, the target OD_{600} may be at least 1.0, more preferably at least 4.0, yet more preferably at least 4.5, most preferably at least 5.0.

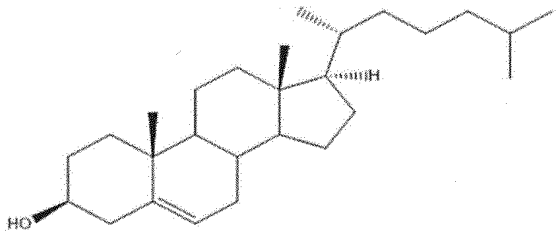
In some embodiments of the method, the steroidal substrate may be a sterol substrate. In certain embodiments, the sterol substrate may comprise:



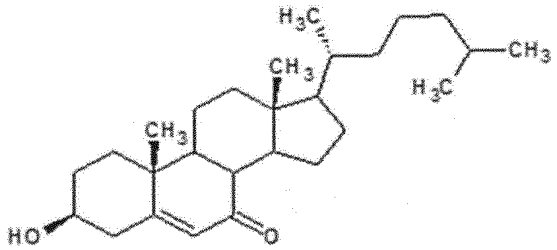
β-sitosterol;



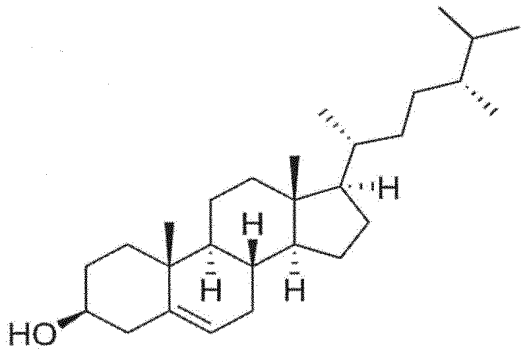
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



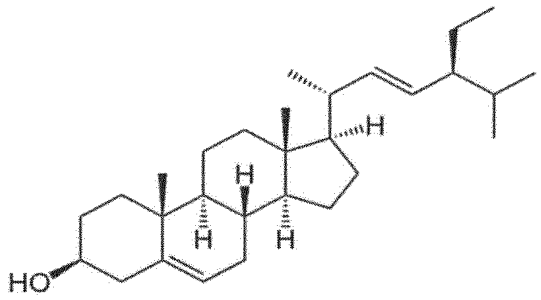
cholesterol;



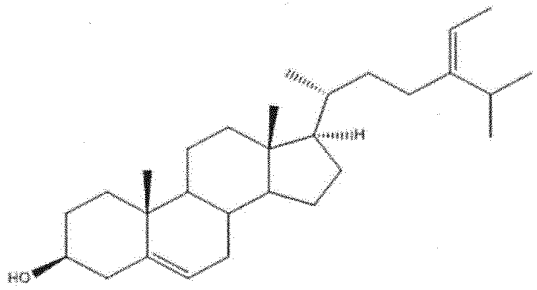
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



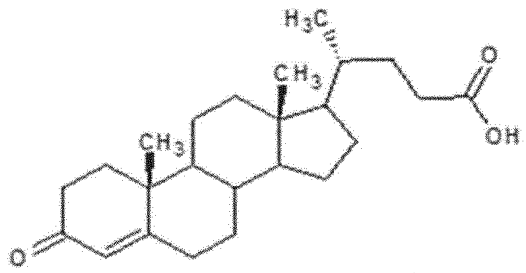
stigmasterol;



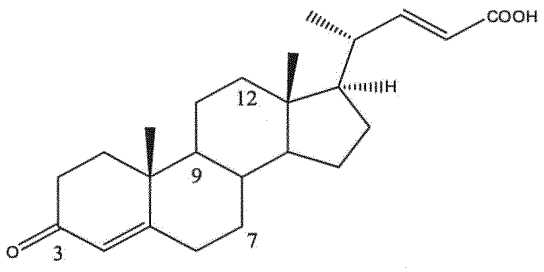
fucosterol;

7-oxo-phytosterol; or a combination thereof.

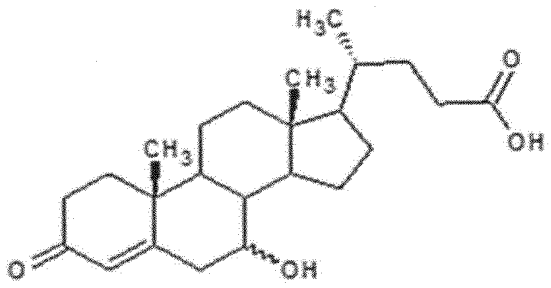
In some embodiments of the method, the steroidal product of interest may comprise an intact polycyclic ring system. In certain embodiments, the steroidal product of interest may be a steroidal compound with a side-chain having a backbone of five carbons.



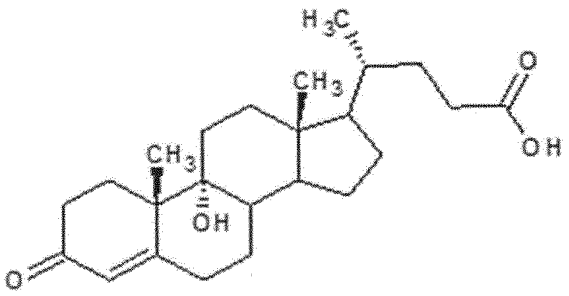
3-oxo-4-cholenic acid;



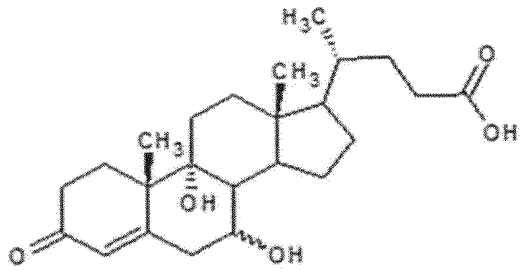
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



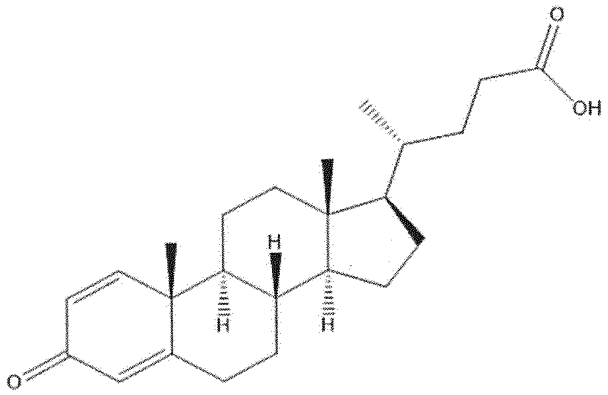
3-oxo-7-hydroxy-4-cholenic acid;



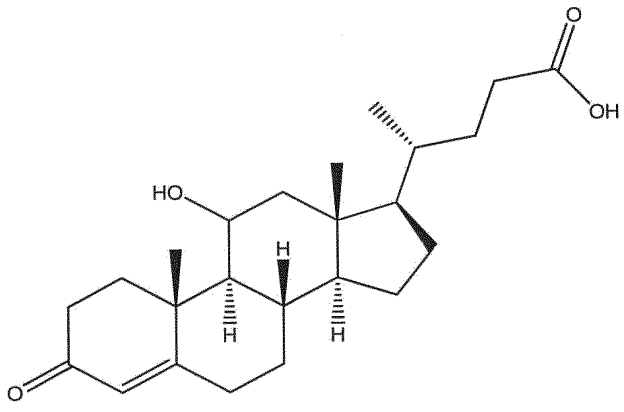
3-oxo-9-hydroxy-4-cholenic acid;



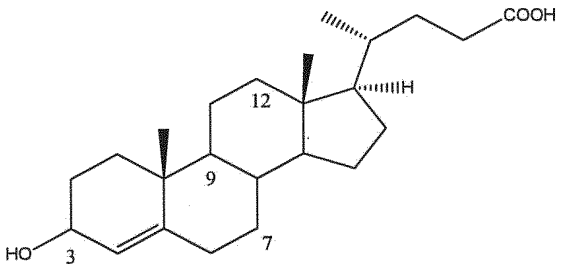
3-oxo-7,9-dihydroxy-4-cholenic acid;



3-oxo-1,4-choladienoic acid;



3-oxo-11-hydroxy-4-cholenic acid;



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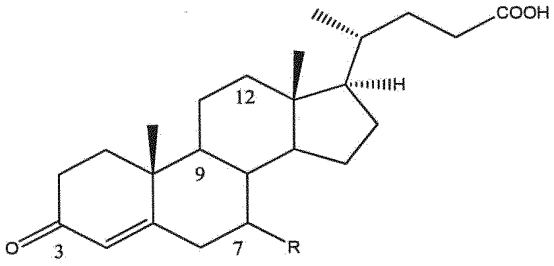
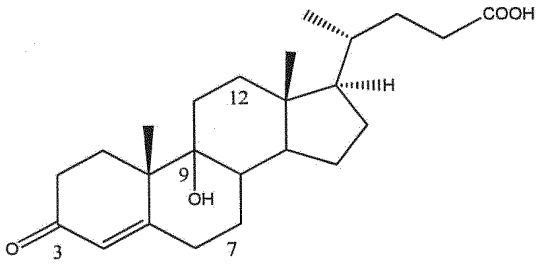


wherein R can be hydroxyl, oxo, or a halogen;



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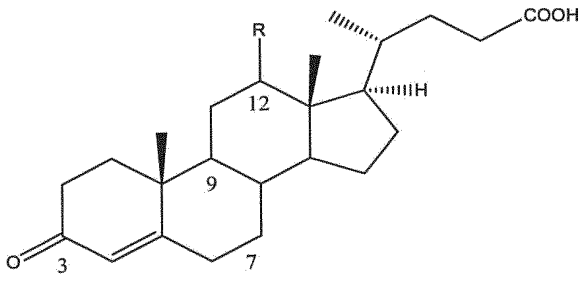
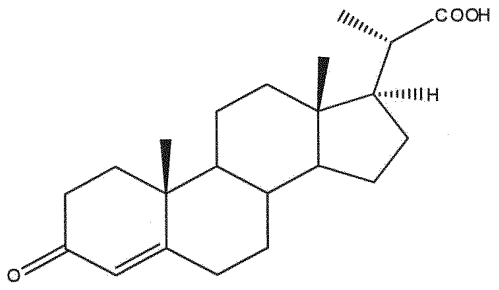
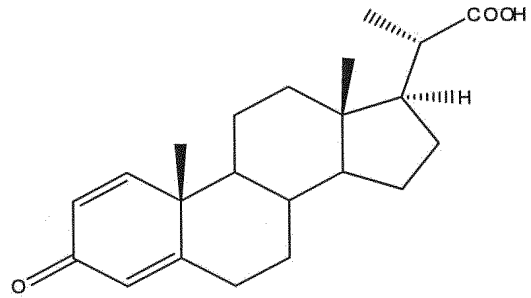


wherein R can be hydroxyl or oxo;



4-BNC

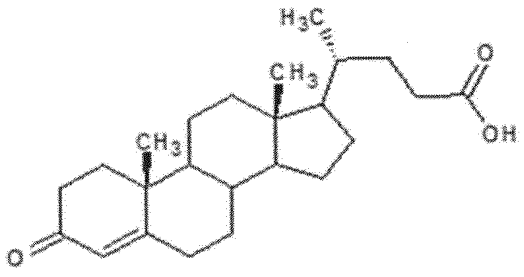
3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC);



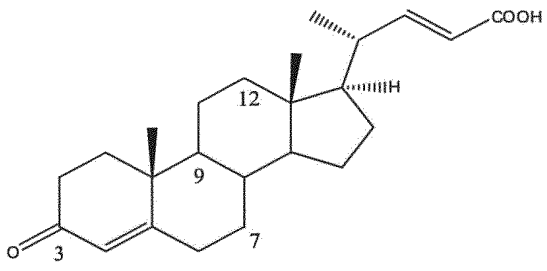
1,4-BNC

3-oxo-23,24-bisnor-1,4-choladiene-22-oic acid (1,4-BNC); or variants thereof.

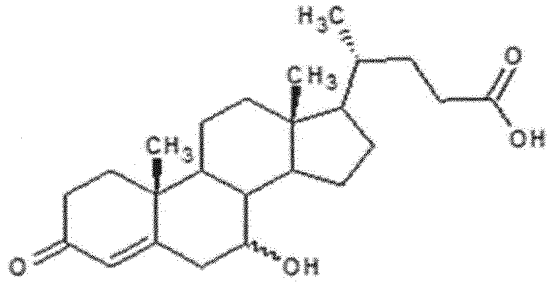
In some preferred embodiments, the steroidal product of interest may be:



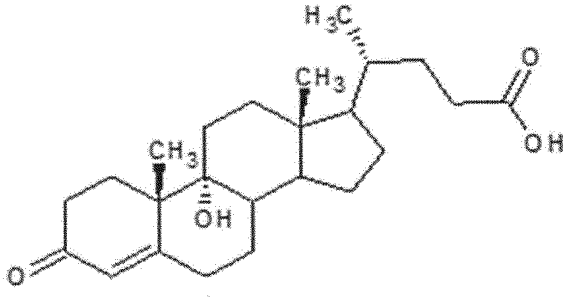
3-oxo-4-cholenic acid;



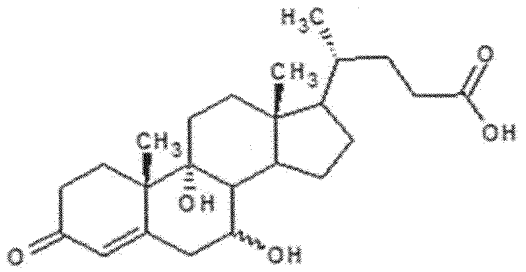
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



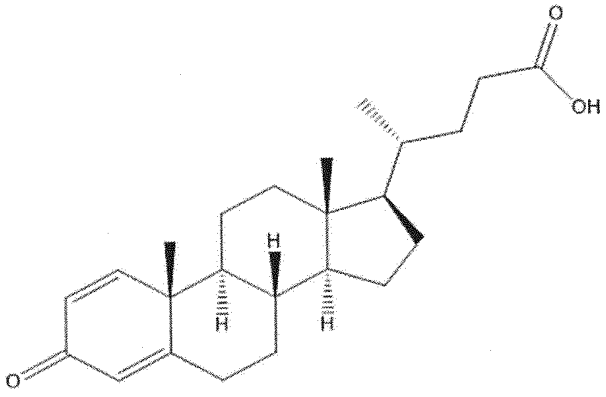
3-oxo-7-hydroxy-4-cholenic acid;



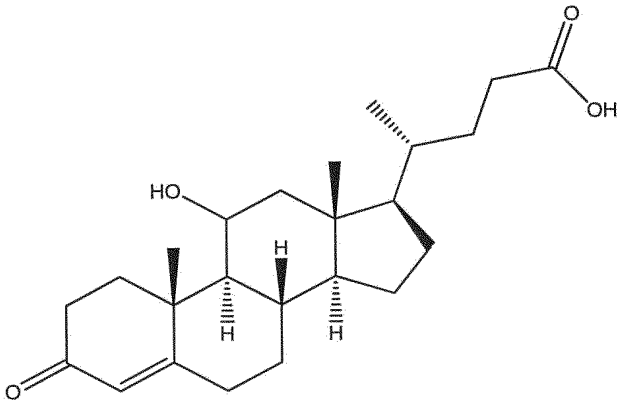
3-oxo-9-hydroxy-4-cholenic acid;



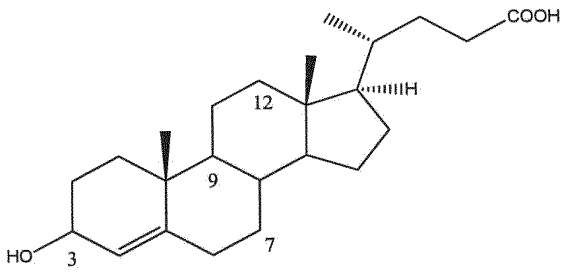
3-oxo-7,9-dihydroxy-4-cholenic acid;



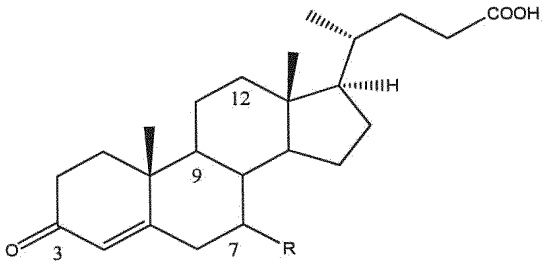
3-oxo-1,4-choladienoic acid;



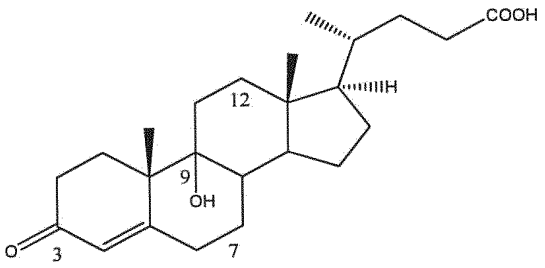
3-oxo-11-hydroxy-4-cholenic acid;



;



wherein R can be hydroxyl, oxo, or a halogen;



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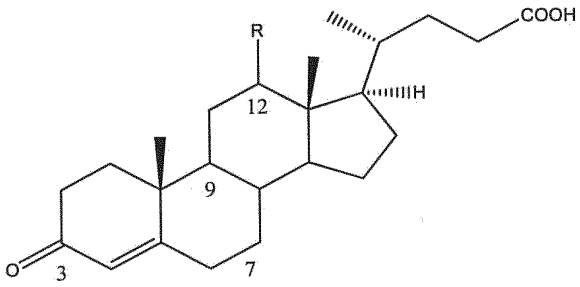


; or variants thereof.

In some embodiments, in step (b) of the method, the steroidal substrate may be added at a concentration of at least 0.1mM, at least 0.2mM, at least 0.3mM, at least 0.4mM, at least 0.5mM, at least 0.6mM, at least 0.7mM, at least 0.8mM, at least 0.9mM, at least 1.0mM, at least 1.1mM, at least 1.2mM, at least 1.3mM, at least 1.4mM, at least 1.5mM, at least 1.6mM, at least 1.7mM, at least 1.8mM, at least 1.9mM, or at least 2.0mM. Preferably,

the steroidal substrate may be added at a concentration of at least 1mM, more preferably at least 1.5mM, most preferably at least 2.0mM.

In some embodiments, in step (b) of the method a cyclodextrin may be added to the culture medium.

By "cyclodextrin" we include the meaning of a compound made up of sugar molecules bound together in a ring, where the ring is composed of 5 or more α-D-glucopyranoside units linked 1→4. Non-limiting examples of cyclodextrins include: α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, methyl-β-cyclodextrin, and 2-OH-propyl-β-cyclodextrin.

In certain embodiments, the cyclodextrin may be a β-cyclodextrin or a γ-cyclodextrin. Where the cyclodextrin is a β-cyclodextrin, it may be a methyl-β-cyclodextrin or a 2-OH-propyl-β-cyclodextrin.

In some embodiments, the cyclodextrin is added at a concentration of 1mM to 50mM, 2mM to 45mM, 3mM to 40mM, 4mM to 35mM, 5mM to 30mM, 6mM to 29mM, 7mM to 28mM, 8mM to 27mM, 9mM to 26mM, 10mM to 25mM, 11mM to 24mM, 12mM to 23mM, 13mM to 22mM, 14mM to 21mM, 15mM to 21mM, 16mM to 20mM, 17mM to 19mM, 1mM to 18mM. Preferably, the cyclodextrin may be added at a concentration of 1mM to 25mM, more preferably 5mM to 25mM.

In other embodiments, the cyclodextrin is added at a concentration of at least 1mM, at least 2mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 11mM, at least 12mM, at least 13mM, at least 14mM, at least 15mM, at least 16mM, at least 17mM, at least 18mM, at least 19mM, at least 20mM, at least 21mM, at least 22mM, at least 23mM, at least 24mM, at least 25mM, at least 30mM, at least 35mM, at least 40mM, at least 45mM, or at least 50mM. Preferably the cyclodextrin is added at a concentration of at least 1mM, preferably at least 5mM, more preferably at least 12.5mM, most preferably at least 25mM.

In some embodiments, in step (b) of the method an organic solvent may be added to the culture medium.

By "organic solvent" we include the meaning of a carbon-based solvent capable of dissolving other substances. Non-limiting examples of organic solvents include: ethanol,

dimethylformamide (DMF), acetone, methanol, isopropanol, dimethyl sulfoxide (DMSO), and toluene.

In certain embodiments, the organic solvent may be ethanol, dimethylformamide (DMF), or acetone. Preferably, the organic solvent may be ethanol.

In some embodiments, the organic solvent is added the culture medium at a volume/volume (v/v) concentration of 1% to 20%, 2% to 19%, 3%, to 18%, 4% to 17%, 5% to 16%, 6% to 15%, 7% to 14%, 8%, to 13%, 9% to 12%, 10 % to 11%. Preferably, the organic solvent may be added at a volume/volume (v/v) concentration of 5% to 20%, more preferably 5% to 15%.

In some embodiments, the organic solvent is added to the culture medium at a volume/volume (v/v) concentration of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, or at least 20%. Preferably, the organic solvent may be added at a volume/volume (v/v) concentration of at least 1%. More preferably, the organic solvent may be added at a volume/volume (v/v) concentration of at least 5%.

In some embodiments, in step (b) of the method a cyclodextrin and an organic solvent are added to the culture medium.

In certain embodiments, where a cyclodextrin and an organic solvent are added to the culture medium, the cyclodextrin is added at a concentration of 1mM to 25mM, 2mM to 24mM, 3mM to 23mM, 4mM to 22mM, 5mM to 21mM, 6mM to 20mM, 7mM to 19mM, 8mM to 18mM, 9mM to 17mM, 10mM to 16mM, 11mM to 15mM, 12mM to 14mM, 1mM to 13mM, and the organic solvent is added at a volume/volume (v/v) concentration of 1% to 20%, 2% to 19%, 3%, to 18%, 4% to 17%, 5% to 16%, 6% to 15%, 7% to 14%, 8%, to 13%, 9% to 12%, 10 % to 11%. Preferably, the cyclodextrin may be added at concentration of 1mM to 25mM and the organic solvent may be added at a volume/volume (v/v) concentration of 1% to 10%. More preferably, the cyclodextrin may be added at concentration of 1mM to 10mM and the organic solvent may be added at a volume/volume (v/v) concentration of 1% to 10%. Yet more preferably, the cyclodextrin may be added at concentration of 1mM to 5mM and the organic solvent may be added at a volume/volume (v/v) concentration of 1% to 5%. Most preferably, the cyclodextrin may be added at

concentration of 5mM and the organic solvent may be added at a volume/volume (v/v) concentration of 5%.

In other embodiments, where a cyclodextrin and an organic solvent are added to the culture medium, the cyclodextrin is added at a concentration of at least 1mM, at least 2mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 11mM, at least 12mM, at least 13mM, at least 14mM, at least 15mM, at least 16mM, at least 17mM, at least 18mM, at least 19mM, at least 20mM, at least 21mM, at least 22mM, at least 23mM, at least 24mM, at least 25mM, and the organic solvent is added at a volume/volume (v/v) concentration of at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, or at least 20%. Preferably, the cyclodextrin may be added at concentration of at least 1mM and the organic solvent may be added at a volume/volume (v/v) concentration of at least 1%. More preferably, the cyclodextrin may be added at concentration of at least 5mM and the organic solvent may be added at a volume/volume (v/v) concentration of 5%.

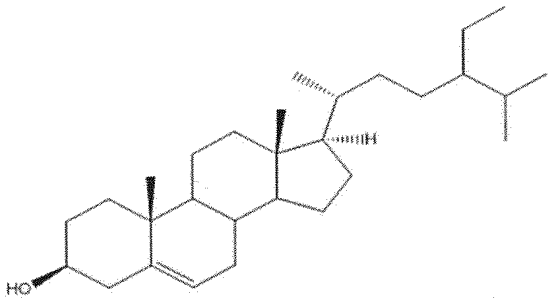
In a fourth aspect, the invention provides a steroidal product of interest produced by the method of the third aspect.

In a fifth aspect, the invention provides a kit for converting a steroidal substrate into a steroidal product of interest, wherein the kit comprises:

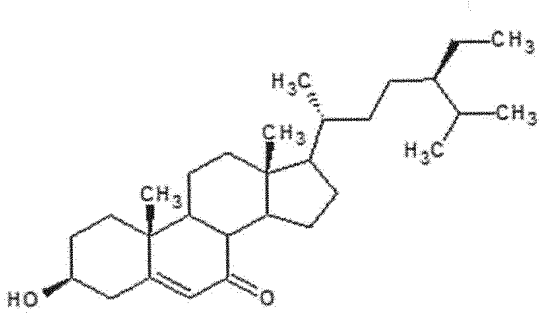
(a) a genetically-modified bacterium according to the first aspect; and,
(b) instructions for using the kit.

The kit may further comprise a steroidal substrate.

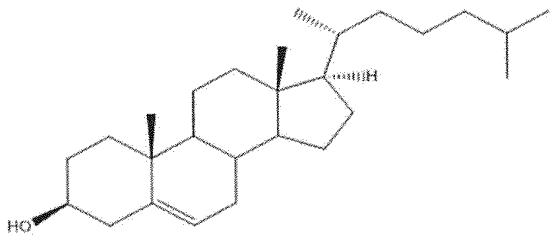
In some embodiments, the steroidal substrate may be a sterol substrate. In certain embodiments, the sterol substrate comprises:



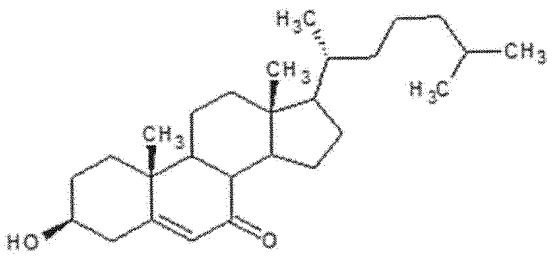
β-sitosterol;



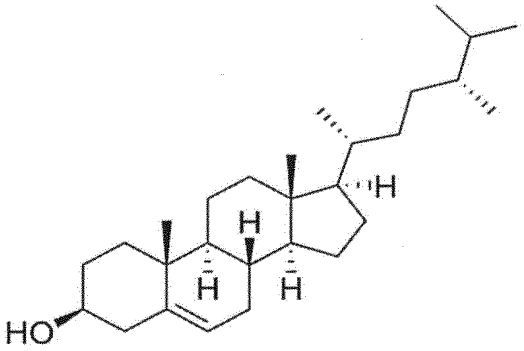
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



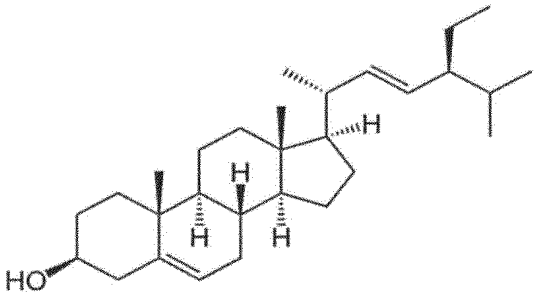
cholesterol;



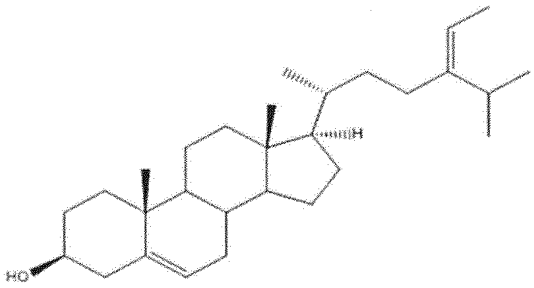
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



stigmasterol;



fucosterol;

7-oxo-phytosterol; or a combination thereof.

In some embodiments, the kit may further comprise a cyclodextrin such as a β-cyclodextrin or a γ-cyclodextrin. Preferably, the cyclodextrin is a β-cyclodextrin, more preferably a methyl-β-cyclodextrin or a 2-OH-propyl-β-cyclodextrin.

In some embodiments, the kit may further comprise an organic solvent. In certain embodiments, the organic solvent is ethanol, dimethylformamide (DMF), or acetone, preferably ethanol.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

The deposits referred to in this disclosure (Accession Nos. NCIMB 43057, NCIMB 43058, NCIMB 43059, and NCIMB 43060) were deposited at the National Collection of Industrial, Food and Marine Bacteria, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, UK by Cambrex Karlskoga AB on 29 May 2018.

The present invention will now be described in more detail with reference to the following non-limiting figures and examples.

DESCRIPTION OF THE FIGURES

FIGURE 1. Overview of cholesterol catabolic pathway.

FIGURE 2. Overview of cholesterol side-chain degradation pathway.

FIGURE 3. Overview of β-sitosterol side-chain degradation pathway.

FIGURE 4. Examples of steroidal compounds

FIGURE 5. Examples of steroidal substrates.

FIGURE 6. Total ion chromatogram obtained by LC-MS for LM3 cultured when cholesterol is the starting substrate. Peaks at 7.67 minutes and 8.25 minutes indicate accumulation of 4-androstene-3,17-dione (AD) and 3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC) respectively. NL: Normalisation Level = base peak intensity.

FIGURE 7. Product ion mass spectra obtained by LC-MS for LM9 when cholesterol is the starting substrate. (A) Peak at Peak at m/z of 345.24 (positive mode) corresponds to 4-BNC being accumulated when cholesterol is the starting substrate. (B) Peak at m/z

of 373.27 (positive mode) corresponds to 3-oxo-4-cholenic acid being accumulated when cholesterol is the starting substrate. NL: Normalisation Level = base peak intensity.

FIGURE 8. Product ion mass spectra obtained by LC-MS for LM9 when cholesterol, β-sitosterol, or 7-oxo-sterol is the starting substrate. (A, top) Peak at m/z of 389.27 (positive mode) corresponds to production of 3-oxo-7-hydroxy-4-cholenic acid when 7-oxo-sterol is the starting substrate. (B, middle) Peak at m/z of 373.27 (positive mode) corresponds to production of 3-oxo-4-cholenic acid when β-sitosterol is the starting substrate. (C, bottom) Peak at m/z of 373.27 (positive mode) corresponds to production of 3-oxo-4-cholenic acid when cholesterol is the starting substrate. NL: Normalisation Level = base peak intensity.

FIGURE 9. Extracted ion chromatograms obtained by LC-MS for LM19 and LM9 when cholesterol or β-sitosterol is the starting substrate. (A) Strain = LM9; Substrate = Cholesterol. Peak at 9.70 minutes corresponds to production of 3-oxo-4-cholenic acid by LM9. (B) Strain = LM19; Substrate = Cholesterol. Peak at 8.07 minutes corresponds to production of 3-oxo-9-OH-4-cholenic acid by LM19. (C) Strain = LM9; Substrate = β-sitosterol. Peak at 9.68 minutes corresponds to production of 3-oxo-4-cholenic acid by LM9. (D) Strain = LM19; Substrate = β-sitosterol. Peak at 8.09 minutes corresponds to production of 3-oxo-9-OH-4-cholenic acid by LM19. NL: Normalisation Level = base peak intensity.

FIGURE 10. Product ion mass spectra obtained by LC-MS confirming identity of peaks produced by LM9 and LM19 when cholesterol or β-sitosterol is the starting substrate. (A) Strain = LM19; Substrate = Cholesterol or β-sitosterol. Peak at m/z of approximately 389.27 (positive mode) corresponds to production of 3-oxo-9-OH-4-cholenic acid by LM19. (B) Strain = LM9; Substrate = Cholesterol or β-sitosterol. Peak at m/z of 373.27 (positive mode) corresponds to production of 3-oxo-4-cholenic acid by LM9. NL: Normalisation Level = base peak intensity.

FIGURE 11. Product ion mass spectra obtained by LC-MS for LM19 when 7-oxo-sterol is the starting substrate. Peak at m/z of 405.26 (positive mode) corresponds to production of 3-oxo-7,9-dihydroxy-4-cholenic acid by LM19. NL: Normalisation Level = base peak intensity.

FIGURE 12. HPLC analysis comparing the steroidal products produced by LM9 and LM33 when β-sitosterol is the starting substrate and the culture medium is

supplemented with methyl-β-cyclodextrins. The upper line on the graph represents the HPLC trace for the steroidal compounds produced by LM9 and the lower line represents the HPLC trace for the steroidal compounds produced by LM33.

FIGURE 13. HPLC analysis comparing the activity of LM9 and LM33 towards 3-oxo-4-cholenic acid as the starting substrate and the culture medium is supplemented with methyl-β-cyclodextrins. The upper line on the graph represents the HPLC trace for the steroidal compounds produced by LM9 (T= 72h) and the lower line represents the HPLC trace for the steroidal compounds produced by LM33 (T =72h).

FIGURE 14. Product ion mass spectrum obtained by LC-MS for LM9 when β-sitosterol is the starting substrate and the culture medium is supplemented with 2-OH-propyl-β-cyclodextrins. Peak at m/z of 373.27 (positive mode) corresponds to production of 3-oxo-4-cholenic acid by LM9. NL: Normalization Level = base peak intensity.

FIGURE 15. HPLC analysis of steroidal compounds produced by LM9 β-sitosterol is the starting substrate and the culture medium is supplemented with 2-OH-propyl-β-cyclodextrins. (A) LM9 products at T = 24h; (B) LM9 products at T = 48h; (C) LM9 products at T = 72h; (D) 3-oxo-4-cholenic acid standard (0.025mg/mL).

FIGURE 16. Extracted ion chromatograms obtained by LC-MS for LM9 when 7-oxosterols is the starting substrate and the culture medium is supplemented with 2-OH-propyl-β-cyclodextrins. (A) LM9 products in the presence of 2-OH-propyl-β-cyclodextrins (T = 48h). Peak at 7.74 minutes corresponds to production of 3-oxo-7-hydroxy-4-cholenic acid. (B) LM9 products in the absence of 2-OH-propyl-β-cyclodextrins (T = 48h). Peak at 7.76 minutes corresponds to production of 3-oxo-7-hydroxy-4-cholenic acid. (C) LM9 products in the presence of 2-OH-propyl-β-cyclodextrins but no substrate (T = 48h). NL: Normalization Level = base peak intensity.

FIGURE 17. HPLC analysis of steroidal compounds produced by *Mycobacterium neoaurum* NRRL B-3805 (parent strain) and *MneoΔfadE34* when cholesterol is the starting substrate. The upper line on the graph represents the HPLC trace for the steroidal compounds produced by the parent strain (T =72h) and the lower line represents the HPLC trace for the steroidal compounds produced by *MneoΔfadE34*.

FIGURE 18. HPLC analysis of steroidal compounds produced by *Mycobacterium neoaurum* NRRL B-3805 (parent strain) and *MneoΔfadE34* when β-sitosterol is the starting substrate. The upper line on the graph represents the HPLC trace for the steroidal compounds produced by *MneoΔfadE34* (T =72h) and the lower line represents the HPLC trace for the steroidal compounds produced by the parent strain

FIGURE 19. HPLC analysis of steroidal compounds produced by *Mycobacterium neoaurum* NRRL B-3805 (parent strain) and *MneoΔfadE34* when 7-oxo-sterols are the starting substrate. The upper line on the graph represents the HPLC trace for the steroidal compounds produced by *MneoΔfadE34* (T =72h) and the lower line represents the HPLC trace for the steroidal compounds produced by the parent strain.

FIGURE 20. HPLC analysis of steroidal compounds produced by *MneoΔfadE34* when phytosterol mix (Aturex 90) is the starting substrate and the culture medium is supplemented with methyl-β-cyclodextrins. From bottom to top the traces shown correspond to the steroidal compounds produced by *MneoΔfadE34* at T = 0h, 24h, 48h, 72h, 96h, and 168h respectively.

FIGURE 21. HPLC analysis of steroidal compounds produced by *MneoΔfadE34* when 3-oxo-4-cholenic acid is the starting substrate and the culture medium is supplemented with methyl-β-cyclodextrins. From bottom to top the traces shown correspond to the steroidal compounds produced by *MneoΔfadE34* at T = 0h, 24h, 48h, 72h, 96h, and 168h respectively.

FIGURE 22. NMR analysis of steroidal compounds produced by *LM33* after fermentation with phytosterol compounds in the presence of hydroxypropyl-β-cyclodextrin. (A) The ^{1}H-spectrum obtained from the product of the fermentation; **(B)** Magnified view of the spectrum of Figure 22A showing peaks in the region 0.65 to 2.55 ppm only; **(C)** The ^{13}C-spectrum obtained from the product of the fermentation; **(D)** Magnified view of the spectrum of Figure 22C showing peaks in the region 11 to 58 ppm only. Both the ^{1}H-spectrum and the ^{13}C-spectrum indicate the presence of 3-oxo-4-cholenic acid in the culture; **(E)** Data parameters used to obtain the ^{1}H-spectrum shown in Figures 22A and 22B; **(F)** Data parameters used to obtain the ^{13}C-spectrum shown in Figures 22C and 22D.

Examples

Example 1 - Construction of strains

Materials and methods

Construction of RG41 strain

RG41 was originally constructed from the parent strain RG32 which was made by unmarked gene deletion of five homologs of 3-ketosteroid-9α-hydroxylase (kshA1-5) as reported by (Wilbrink *et al*, 2011. *Appl Environ Microbiol.*, 77(13): 4455-4464).

RG32 was used as parent strain for the construction of *R. rhodochrous* strain RG41 by deletion of 3 homologs of 3-ketosteroid-Δ1-dehydrogenase (kstDs) as detailed below.

The construction of a mutagenic plasmid for *kstD3* unmarked deletion was performed as follows. A genomic library of *R. rhodochrous* DSM43269 was obtained as explained in (Petrusma *et al*, 2009. *Appl Environ Microbiol.*,75(16): 5300-5307), which was used for isolation of a clone (pKSH800; Wilbrink *et at.*, 2011) carrying *kshA3* and also *kstD3*. A 4 kb *Eco*RI fragment of pKSH800 was ligated into *Eco*RI-digested pZErO-2.1, which was subsequently digested with *Bgl*II/*Eco*RI. Next, a 2.7 kb *Bgl*II/*Eco*RI fragment was ligated into *Bam*HI/*Eco*RI-digested pK18mobsacB, which was then digested with *Eco*RV/*Nru*I and finally self-ligated, rendering the plasmid pKSH841 for *kstD3* gene deletion in *R. rhodochrous* RG32 strain => RG32Δ*kstD3* = strain RG35 (Appendix C).

The construction of a mutagenic plasmid for *kstD1* unmarked deletion was performed as follows. Specific *kstD1* primers (kstD1-F and kstD1-R, Appendix D) were used for the amplification of a 2.4 kb PCR product that was ligated into *Eco*RV-digested pBluescript, which was then digested with *Stu*I/*Sty*I, blunt-ended by Klenow and self-ligated. Then, the construct was digested with *Bam*HI/*Hin*dIII and, finally, a 1.3 kb *Bam*HI/*Hin*dIII fragment was ligated into *Bam*HI/*Hin*dIII-digested pK18mobsacB, rendering the plasmid pKSH852 for *kstD1* gene deletion in RG35 => RG32Δ*kstD1*Δ*kstD3* = strain RG36 (Table Appendix C).

The construction of a mutagenic plasmid for *kstD2* unmarked deletion was performed as follows. Chromosomal DNA of *R. rhodochrous* RG36 was isolated using a genomic DNA isolation kit (Sigma-Aldrich), digested by *Xho*I, and ligated into *Xho*I-digested pZErO-2.1. Transformation of *E. coli* DH5α with the ligation mixture generated a genomic library of approximately 12,000 transformants. A clone carrying the *kstD2* gene (pKSD321) was identified by means of PCR using specific *kstD2* primers (kstD2-F and kstD2-R, Appendix D) and isolated from the genomic library of strain RG36. Then, pKSD321 was digested

with *Xmn*I, self-ligated and subsequently digested with *Sma*I/*Xho*I. Finally, a 2.2 kb *Sma*I/*Xho*I was ligated into *Sma*I/*Sal*-digested pK18mobsacB, rendering the plasmid pKSD326 for the *kstD2* gene deletion in RG36 => RG32Δ*kstD1*Δ*kstD2*Δ*kstD3* = strain RG41 (Appendix C).

Mutagenic plasmids were transferred to *Escherichia coli* S17-1 by transformation and subsequently mobilized to the corresponding *R. rhodochrous* strain by conjugation as described previously (van der Geize *et al*, 2001. *FEMS Microbiol. Lett.*, 205(2): 197-202). All mutants were verified by PCR using specific primers (Appendix D) to confirm deletion of the target gene(s).

Therefore, strain RG41 is a *kshA null* + *ΔkstD1ΔkstD2ΔkstD3* mutant (8-fold mutant), which was then used as parent strain for the construction of deletion mutants in genes involved in side-chain degradation of steroids.

Construction of deletion mutation strains

The single mutant strains LM3 (Δ*fadE34#1)*, LM15 (Δ*fadE34#2)* were constructed by deletion of *fadE34#1* or *fadE34#2* from the parent strain RG41 (*kshA* null + Δ*kstD1* + Δ*kstD2*+ Δ*kstD3*).

Unmarked in frame gene deletion mutants were constructed using the *sacB* counter-selection marker (van der Geize *et al*, 2001). PCR amplification of the upstream and downstream flanking regions of the target genes was performed from wild-type *R. rhodochrous* DSM43269 template using the primers listed in Appendix D. The obtained 1.5 kb PCR products (called UP and DOWN) were cloned together into pK18mobsacB vector, yielding pk18_*fadE34*-UP+DOWN and pk18_*fadE34#2*-UP+DOWN constructs. pDEL-*fadA6*, previously constructed by Wilbrink *et al.*, 2011, was used for the deletion of *fadA6*. Mutagenic plasmids were transferred to *Escherichia coli* S17-1 by transformation and subsequently mobilized to the corresponding *R. rhodochrous* strain by conjugation as described previously (van der Geize *et al*, 2001). All mutants were verified by PCR using specific primers (Appendix D) to confirm deletion of the target gene(s). LM3 and LM15 single mutant strains were constructed by deletion of *fadE34* or *fadE34#2*, respectively, using RG41 as parent strain.

Example 2 - Bioconversions using strains LM3 (Δ*fadE34#1*) and LM15 (Δ*fadE34#2*)

Materials and methods

Mutant strains were inoculated in 100ml Luria-Bertani (LB) medium and incubated at 30°C and 200rpm for 48 hours. When the OD_{600nm}=5, the LB preculture was divided into 10ml cultures and the starting sterol substrate added at 2mM (dissolved in acetone to 4% final concentration).

The time of addition of the starting sterol substrate was treated as T=0 hours. Cultures were incubated at 30°C and 200rpm for several days. 250µl aliquots were taken from the culture at 0 hours, 24 hours, 48 hours, and 72 hours, and frozen at -20°C until needed.

Samples were prepared for HPLC and/or LC-MS analysis by thawing at room temperature and adding 1ml MeOH before briefly vortexing and centrifuging at 4°C and 12,000rpm for 10-15 minutes. The supernatants were then filtered (0.2µm filter size) and analysed by HPLC and/or LC-MS.

HPLC was performed using a Kinetex C18 column (250x4.6mm, particle size 5 µm). A mobile phase of 80% MeOH and 0.1% formic acid was used at a flow rate of 1ml/min and a column temperature of 35°C. 20µl of sample was injected. A 30-minute detection time was used, and steroidal compounds were detected at 254nm. Quantification of the steroidal products produced was achieved by construction of a calibration line of peak areas measured from a known standard. This was used to calculate the amount of product produced in g/l, followed by back calculation of the percentage yield.

LC-MS analysis was carried out using an Accella1250™ HPLC system coupled with the benchtop ESI-MS Orbitrap Exactive™ (Thermo Fisher Scientific, San Jose, CA). A sample of 5 µl was injected into a Reversed Phase C18 column (Shim Pack Shimadzu XR-ODS 3x75 mm) operating at 40°C and flow rate 0.6 ml/min. Analysis was performed using a gradient from 2% to 95% of acetonitrile:water (adding 0.1% formic acid) as follows: 2 min 2% acetronitrile, 8 minutes gradient from 2% to 95% acetonitrile, 4 min 95% acetonitrile. The column fluent was directed to the ESI-MS Orbitrap operating at the scan range (m/z 80 – 1600 Da) switching positive / negative modes. Voltage parameters for positive mode were: 4.2 kV spray, 57.5 V capillary and 95 V tube lens. Voltage parameters for negative mode were: 3kV spray, -25V capillary and -75V tube lens. Capillary temperature 325°C, sheath gas flow 70, auxiliary gas off. Thermo XCalibur™ processing software was used for the data analysis. All the products reported in this work were detected in the positive mode ($M+H^+$).

Results

The total ion chromatogram obtained by LC-MS for the LM3 strain shows an accumulation of AD and 4-BNC from the starting cholesterol substrate (Figure 6), indicating there is no blockage of side-chain degradation in the LM3 single mutant strain. The same result was obtained for the LM15 single mutant strain (data not shown).

Example 3 – Bioconversions using LM9 (Δ*fadE34#1*/Δ*fadE34#2*)

Materials and methods

The same culture conditions, sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

The total ion chromatogram obtained by LC-MS for the LM9 strain (Figure 7, product ion mass spectra shown) using cholesterol as the starting substrate, shows an accumulation of both 4-BNC (peak at m/z of 345.24, positive mode) (Figure 7A, top) and 3-oxo-4-cholenic acid (peak at m/z of 373.27, positive mode) (Figure 7B, bottom). Extracted ion chromatograms, produced by extracting data for the mass to charge ratio (m/z) of the compound of interest, show that 3-oxo-4-cholenic acid is produced by LM9 when cholesterol (Figure 8C, bottom trace, peak at m/z of 373.27) or β-sitosterol (Figure 8B, middle trace, peak at m/z of 373.27) is the starting substrate, and that 3-oxo-7-hydroxy-4-cholenic acid is produced when 7-oxo-sterol is the starting substrate (Figure 8A, top trace, peak at m/z of 389.27). These results indicate that there is some blockage of side-chain degradation in the LM9 strain.

Example 4 – Bioconversions using strain LM19 (Δ*fadE34#1*/Δ*fadE34#2* complemented with *kshA5*)

Materials and methods

Construction of LM19 strain

A wild-type copy of the *kshA5* gene and its flanking regions was amplified by PCR using the primers kshA5-complem-F and kshA5-complem-R (Appendix D). The obtained PCR product of 2.2 kb was cleaned-up, restricted with *BamH*I/*Hin*dIII and subsequently ligated into pk18mobsacB, yielding the construct pk18+*kshA5*-complementation. This construct was transformed into *E. coli* S17-1 and transferred to strain LM9 by conjugation. The resulting complemented mutant LM19, in which the deleted copy of *kshA5* was replaced by the wild-type one, was obtained following the same conjugation protocol used for the construction of the mutant strains, as described in van der Geize *et al*, 2001.

Bioconversions with LM19

As described above, *kshA5* and its flanking regions was reintroduced into strain LM9 to produce strain LM19, in which hydroxylase activity is restored to produce variant compounds with a 9-hydroxyl group. The expected compounds accumulated were 3-oxo-9-OH-4-cholenic acid (from β-sitosterol and cholesterol) and 3-oxo-7,9-dihydroxy-4-cholenic acid (from 7-oxo-sterols).

The same culture conditions, sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

Comparison of the extracted ion chromatograms produced for LM9 and LM19 strains shows that 3-oxo-9-OH-4-cholenic acid (peak at 8.07-8.09 minutes) is produced by LM19 only when the starting sterol is cholesterol or β-sitosterol (Figure 9A and 9C respectively) and 3-oxo-4-cholenic acid (peak at 9.68-9.70 minutes) is produced by LM9 only when the starting sterol is cholesterol or β-sitosterol (Figure 9B and 9D respectively). Those peaks were confirmed as 3-oxo-9-OH-4-cholenic acid (peak at m/z of approximately 389.27, positive mode) is produced by LM19 when the starting sterol is cholesterol or β-sitosterol (Figure 10A) and 3-oxo-4-cholenic acid (peak at m/z of 373.27, positive mode) is produced by LM9 when the starting sterol is cholesterol or β-sitosterol (Figure 10B).

When the starting sterol is 7-oxo-sterol the expected product is 3-oxo-7,9-dihydroxy-4-cholenic acid. The extracted ion chromatogram for LM19 in Figure 11 has a peak corresponding to 3-oxo-7,9-dihydroxy-4-cholenic acid (peak at m/z of 405.26, positive mode). However, this peak is of lower intensity than those produced for LM19 in Figure 10. In overview, these results indicate the successful use of LM19 in the production of variant steroidal compounds with a 9-hydroxy group.

Example 5 – Bioconversions using strain LM33 (Δ*fadE34#1*/Δ*fadE34#2*/Δ*fadE26*)

Materials and methods

An additional mutant strain Δ*fadE34#1*/Δ*fadE34#2*/Δ*fadE26* (LM33) was produced by deletion of *fadE26* from the LM9 strain. *FadE26* is involved in the first cycle of β-oxidation (Figures 2 and 3) and may also use 3-oxo-4-cholenic acid as a substrate (Yang *et al*, 2015. *ACS Infect. Dis.*, 1(2):110-125), thereby limiting its accumulation. Thus, it was thought

that deletion of *fadE26* might lead to a reduction in unwanted oxidation of 3-oxo-4-cholenic acid.

The same culture conditions, sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

A comparison of the bioconversion of β-sitosterol by the LM9 and LM33 strains in the presence of 25mM methyl-β-cyclodextrins (MCDs) (see Example 7 below), shows that the major peak in the HPLC trace for the LM33 sample is 3-oxo-4-cholenic acid and the peaks corresponding to AD and 4-BNC are much smaller, while the converse is observed in the HPLC trace for LM9 (Figure 12). This indicates that the additional deletion of *fadE26* in LM33 enables the further accumulation of 3-oxo-4-cholenic acid, suggesting that unwanted oxidation of 3-oxo-4-cholenic acid is reduced.

Furthermore, a comparison of the activity of the LM9 and LM33 strains towards 3-oxo-4-cholenic acid as the starting substrate in the presence of 25mM methyl-β-cyclodextrins (MCDs) shows that the major peak in the HPLC trace for the LM33 sample remains as 3-oxo-4-cholenic acid and peaks corresponding to AD and 4-BNC are very small. In contrast, in the HPLC trace for LM9 (Figure 13) the peak for 3-oxo-4-cholenic acid is decreased and the peaks for AD and 4-BNC are much more prominent. This indicates that in LM9 the concentration of 3-oxo-4-cholenic acid decreases with time as AD and 4-BNC are formed but in LM33, where *fadE26* is also deleted, the conversion of 3-oxo-4-cholenic acid to AD and 4-BNC is significantly reduced. Those results therefore suggest that unwanted oxidation of 3-oxo-4-cholenic acid is reduced in LM33.

Example 6 – Bioconversions using LM9 in a culture medium supplemented with 2-OH-propyl-β-cyclodextrins

Materials and methods

The addition of 2-OH-propyl-β-cyclodextrins to the culture medium was attempted to improve the solubility of the hydrophobic sterol starting compounds.

The LM9 strain was cultured as described in Example 2 until the OD_{600nm}=5 after approximately 48 hours. The culture was centrifuged at room temperature and 4,500rpm for 15-20 minutes. The cells were resuspended in the same volume of minimal medium (K_2HPO_4 (4.65g/l), $NaH_2PO_4 \cdot H_2O$ (1.5g/l), NH_4Cl (3g/l), $MgSO_4 \cdot 7H_2O$ (1g/l), and Vishniac

trace element solution (1ml/l)). This was divided into 10ml cultures and 25mM 2-OH-propyl-β-cyclodextrins, 25mM $NaHCO_3$ and 2mM sterols were added in powder form.

The same sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

The extracted ion chromatogram obtained by LC-MS of the LM9 strain using β-sitosterol as the starting substrate shows that 3-oxo-4-cholenic acid (peak at m/z of 373.27, positive mode) is produced by LM9 in the presence of 2-OH-propyl-β-cyclodextrins (Figure 14). In order to quantify the amount of 3-oxo-4-cholenic acid produced HPLC analysis was performed (Figure 15), with a yield of 11.64% observed in the sample taken at the 72-hour time point (Table 1 below).

Time point (hours)	Percentage yield (%) of 3-oxo-4-cholenic acid
24	6.53
48	9.78
72	11.64

Table 1. Percentage yields of 3-oxo-4-cholenic acid in LM9 cultures in the presence of 2-OH-propyl-β-cyclodextrins at T = 24h/48h/72h.

Similar experiments were performed using 7-oxo-sterols as the starting substrate, and the extracted ion chromatograms show the production of 3-oxo-7-hydroxy-4-cholenic acid at T = 48h (Figure 16). Comparison of the LC-MS spectra in the presence and absence of 2-OH-propyl-β-cyclodextrins (Figure 16A and 16B) reveals a more intense base peak (evidenced by the NL values on the traces in Figure 16) in the presence of 2-OH-propyl-β-cyclodextrins, indicating a higher yield of 3-oxo-7-hydroxy-4-cholenic acid in those cultures. However, due to the lack of an available standard for HPLC quantification, there is no available data on obtainable percentage yields.

Equivalent experiments were carried out in which the culture was not supplemented with $NaHCO_3$ (data not shown). In those experiments there was no significant difference from the results shown in Figures 14, 15, and 16 and presented in Table 1, thereby indicating

that the presence of $NaHCO_3$ is not required to produce a positive effect on yield in cultures supplemented with 2-OH-propyl-β-cyclodextrins.

Example 7 – Bioconversions using LM9 and LM33 in a culture medium supplemented with methyl-β-cyclodextrins

Materials and methods

The addition of methyl-β-cyclodextrins to the culture medium was attempted to further improve the solubility of the hydrophobic sterol starting compounds.

The LM9 strain was cultured as described in Example 2 until the OD_{600nm}=5 after approximately 48 hours. The culture was centrifuged, and the cells resuspended in the same volume of minimal medium, as described in Example 6. This was divided into 10ml cultures and 25mM methyl-β-cyclodextrins and 2mM sterols were added in powder form.

In an attempt to further maximise the yield of 3-oxo-4-cholenic acid, methyl-β-cyclodextrins were added to the LM33 strain (see Figure 12). The LM33 strain was cultured in LB medium as described in Example 2 until the OD_{600nm}=5 after approximately 48 hours. Then, the preculture was divided into 10ml cultures and 25mM methyl-β-cyclodextrins and 2mM sterols were added in powder form. Alternatively, the culture was centrifuged, and the cells resuspended in the same volume of minimal medium. This was divided into 10ml cultures and 25mM methyl-β-cyclodextrins and 2mM sterols were added in powder form.

The same sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

Table 2 below summarises the maximum percentage yields of 3-oxo-4-cholenic acid obtained by HPLC analysis for LM9 in the presence of methyl-β-cyclodextrins using β-sitosterol as the starting substrate and compares those yields to the yields obtained in the presence of 2-OH-propyl-β-cyclodextrins (see Example 6 above). The overall result indicates that yields are higher in the presence of methyl-β-cyclodextrins.

Culture conditions	Percentage yield of 3-oxo-4-cholenic acid (%)

2-OH-propyl-cyclodextrin (25mM), 72h	11.64
Methyl-β-cyclodextrins (25mM), 72h	23.08

Table 2. Percentage yields of 3-oxo-4-cholenic acid in LM9 cultures supplemented with cyclodextrins at T = 72h.

Quantification of the amount of product produced by LM9 in the presence of methyl-β-cyclodextrins (25mM) was carried out using HPLC analysis. β-sitosterol was the starting substrate and the analysed sample was collected at the 72-hour timepoint. The percentage yields were calculated as outlined in Example 2 above and are presented in Table 3 below.

Steroidal compound	Percentage yield (%) of steroidal compound
3-oxo-4-cholenic acid	23.08
4-BNC	14.80
AD	19.00

Table 3. Percentage yields of steroidal compounds in LM9 cultures supplemented with methyl-β-cyclodextrins (25mM) at T = 72h.

Similarly, Table 4 below compares bioconversions in LM9 in the presence of methyl-β-cyclodextrins using 7-oxo-sterols as the starting substrate. Due to the lack of available standard for 3-oxo-7-hydroxy-4-cholenic acid, peak areas obtained by HPLC are compared rather than expressed as a percentage yield. However, the results still demonstrate that larger peak areas are achieved in the presence of methyl-β-cyclodextrins compared with 2-OH-propyl-β-cyclodextrins.

Culture conditions	Peak area
2-OH-propyl-cyclodextrin (25mM), 72h	21.21
Methyl-β-cyclodextrins (25mM), 72h	44.22

Table 4. Peak area measurements for 3-oxo-7-hydroxy-4-cholenic acid in LM9 cultures supplemented with cyclodextrins (25mM) at T = 72h.

Table 5 below summarises the percentage yields of 3-oxo-4-cholenic acid obtained by HPLC analysis for LM33 using both cholesterol and β-sitosterol as starting substrates and

culturing in both LB and minimal medium in the presence of methyl-β-cyclodextrins. Comparing the data in Table 3 above and Table 5 below shows that culturing LM33 in the presence of methyl-β-cyclodextrins results in the highest percentage yield of 3-oxo-4-cholenic acid when β-sitosterol is the starting substrate.

Culture conditions	Percentage yield of 3-oxo-4-cholenic acid (%)
B-sitosterol, LB medium, 72h	37.31
B-sitosterol, minimal medium, 72h	39.74
Cholesterol, LB medium, 72h	50.51
Cholesterol, minimal medium, 72h	66.82

Table 5. Percentage yields of 3-oxo-4-cholenic acid in LM33 cultures supplemented with methyl-β-cyclodextrins at T = 72h.

Example 8 – Bioconversions using LM33 in culture medium supplemented with organic solvents and cyclodextrins

Materials and methods

The LM33 strain was cultured as described in Example 1 until the OD_{600nm}=5 after approximately 48 hours. The culture was centrifuged at 4,500rpm at room temperature for 15-20mins. The cells were resuspended in the same volume of minimal medium and the culture divided into 10ml cultures. 2mM β-sitosterol was added dissolved in ethanol (5% or 10% final volume/volume concentration) and different amounts of methyl-β-cyclodextrins (5mM, 12.5mM, or 25mM) were added in powder form.

The same sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

HPLC data for all concentrations of ethanol and methyl-β-cyclodextrins was processed as described in Example 2 to obtain the percentage yields of 3-oxo-4-cholenic acid displayed in Table 6 below. Overall, the use of 5% ethanol and 5mM methyl-β-cyclodextrins in combination results in the highest percentage yield.

Sample conditions	Percentage yield of 3-oxo-4-cholenic acid (%)
0mM MCDs, 5% ethanol, 72h	6.97
5mM MCDs, 5% ethanol, 72h	71.30
12.5mM MCDs, 5% ethanol, 72h	65.11
25mM MCDs, 5% ethanol, 72h	62.16
5mM MCDs, 10% ethanol, 72h	13.05
12.5mM MCDs, 10% ethanol, 72h	34.01
25mM MCDs, 10% ethanol, 72h	32.24

Table 6. Percentage yields of 3-oxo-4-cholenic acid in LM33 cultures supplemented with methyl-β-cyclodextrins and ethanol at T = 72h.

Example 9 – Bioconversions using *Mycobacterium neoaurum* NRRL B-3805 Δ*fadE34* (*MneoΔfadE34)*

Materials and methods

The *Mycobacterium neoaurum* NRRL B-3805 Δ*fadE34* strain was produced by introducing a deletion of *fadE34* into the parent strain NRRL B-3805 (Marsheck *et al*, 1972. *Applied Microbiology*, 3(1):72-77), with the aim of preventing the oxidation of 3-oxo-4-cholenic acid. This followed the same strategy described in Example 1, using the parent strain NRRL B-3805 template and the primers listed in Appendix D, pk18_*fadE34*_Mneo-UP+DOWN plasmid was constructed. This mutagenic plasmid was transferred to NRRL B-3805 strain by electroporation (2.5kV, 25μF, 600Ω). The mutant strain was verified by PCR using specific primers (Appendix D) to confirm deletion of *fadE34*.

MneoΔfadE34 precultures were grown to an OD_{600nm}=5 (~72h at 37°C). The culture was centrifuged, and the cells suspended in the same volume of minimal medium. 2mM of the starting steroid substrate was added in powder form.

The same sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

The HPLC traces of Figure 17, Figure 18 and Figure 19 compare the compounds produced by the *Mneo*-parent strain and *MneoΔfadE34* strain when cholesterol, β-sitosterol and 7-oxosterols are the respective starting substrates. In the case of cholesterol (Figure 17) and β-sitosterol (Figure 18), the *MneoΔfadE34* strain accumulates higher levels of 3-oxo-4-cholenic acid and lower levels of AD and ADD than the *Mneo*-parent strain. These results indicate that the *MneoΔfadE34* strain is blocked in side-chain oxidation at the 3-oxo-4-cholenic acid step. The *Mneo* parent strain NRRL B-3805 was described as lacking KSH and KstD, however, it was observed that there is also a peak that corresponds to production of 3-oxo-1,4-choladienoic acid, indicating that *MneoΔfadE34* (and therefore the parent strain NRRL B-3805) may have residual KstD activity.

When 7-oxosterols are the starting substrate (Figure 19), the traces obtained for the *Mneo* parent strain NRRL B-3805 and *MneoΔfadE34* are very similar, indicating that 7-OH compounds are not able to be accumulated.

Example 10 – Bioconversions using *Mycobacterium neoaurum* NRRL B-3805 *ΔfadE34* (*MneoΔfadE34)* in culture medium supplemented with methyl-β-cyclodextrins

Materials and methods

The same strains and culture conditions were used as outlined in Example 9 above, and 25mM methyl-β-cyclodextrins was added in powder form. 2mM phytosterol mix (Aturex 90) or 3-oxo-4-cholenic acid were added as the starting compounds. The same sample preparation techniques and HPLC/LC-MS protocol were used as outlined in Example 2 above.

Results

Mneo-ΔfadE34 accumulates a possible peak of 3-oxo-1,4-choladienoic acid when those cells are cultured in minimal medium in the presence of methyl-β-cyclodextrins and phytosterol mix is the starting substrate (Figure 20).

When 3-oxo-4-cholenic acid is the starting substrate, there is no accumulation of 3-oxo-1,4-cholenic acid (Figure 21), indicating it is likely that its production is not activated by the presence of 3-oxo-4-cholenic acid.

Example 11 – Bioconversions using LM33 in culture medium supplemented with hydroxy-propyl-ß-Cyclodextrin

Materials and methods

The bioconversion was carried out with growing cells i.e. with bioconversion reagents added to the reactor at the beginning of the fermentation. A pre-culture was prepared as follows:

1) 50 mL LB medium was added to 100 mL conical flask;
2) 200 µL *R. rhodochrous* LM33 was inoculated from glycerol stock;
3) The culture was incubated at 400 RPM on an orbital shaker for 48 hours at 30°C;
4) 1% (5 mL) of OD 5 culture was inoculated into the bioreactor.

The bioreactor was loaded with (final concentrations in brackets): Tryptone (10 g/L); Yeast Extract (10 g/L); NaCL (0.5 g/L); Antifoam DR204 (0.015 g/L); Hydroxy-propyl-ß-Cyclodextrin (23.3 g/L); a premade mixture of Phytosterols AS-7 (70 g/L) and Tween-80 (17.5 g/L); and water. The mixture was autoclaved in the reactor at 121°C for 3 minutes. The bioconversion was run at 30˚C, pH 7.0 with aeration from surface at 200 mL/min and dO_2 set point at 40%.

The initial growth lasted for less than 12 hours as judged from oxygen consumption and a slight CO_2 production. After 48 hours from the start of the experiment there was no CO_2 production and the bioconversion was reinoculated from a fresh pre-culture, at an inoculation rate of 10% (50 mL). After the second inoculation, there was also an initial oxygen consumption phase, which lasted for 6 hours, again followed by a reduction in oxygen consumption. However, after that reduction the culture recovered and started consuming oxygen and producing CO_2 again.

Formation of 3-oxo-4-cholenic acid was detected at the 112th hour. The experiment was concluded after 160 hours, at which point the product concentration had reached 6.09 mM.

The biomass and unreacted phytosterols were separated by first increasing the pH of the culture solution to pH 10 by the addition of 2M NaOH, followed by centrifugation at 4700 g for 10 minutes at 4 °C, affording a clear solution (453 g) containing the product. From this solution, 360g (pH=7.2) was extracted with 4x100 ml MTBE, then adjusted to pH=2.1 with diluted HCl and extracted again with 2x100 ml toluene. The majority of 3-oxo-4-cholenic

acid was detected in MTBE extracts and a minority in toluene extracts. The extracts were evaporated to dryness and pooled by dissolving in MTBE. The solution was washed with diluted HCl and concentrated on rotavap. From the obtained residue, 3-oxo-4-cholenic acid was precipitated by overnight stirring. The identity of 3-oxo-4-cholenic acid was confirmed by NMR (Figure 22).

Results

The spectra of Figure 22 confirm the identity of the isolated product as 3-oxo-4-cholenic acid. Figure 22(A) and (B) depict the ^{1}H-spectrum and Figure 22(C) and (D) depict the ^{13}C-spectrum obtained from the products. The labelling of the peaks corresponds to the functional groups depicted in the formula of 3-oxo-4-cholenic acid shown in Figure 22(A) and (C).

APPENDIX A – Nucleotide sequences

Name and SEQ ID NO.	GENBANK Accession No.	Nucleotide sequence
kshA1 Rhodococcus rhodochrous (SEQ ID NO: 1)	HQ425873.1	GTGAGCCTCGGCACTTCCGAACAATCCGAAATCCGTGA GATCGTCGCCGGGTCGGCTCCCGCCCGCTTCGCCCGCG GCTGGCACTGCCTCGGCCTGGCGAAGGATTTCAAGGAC GGCAAGCCGCATTCCGTGCACGCCTTCGGTACCAAACT CGTGGTGTGGGCCGACAGCAACGACGAGATCAGGATCC TCGACGCGTACTGCCGGCACATGGGCGGCGATCTCAGC CAGGGCACCGTCAAGGGCGACGAGATCGCGTGCCCGTT CCACGACTGGCGCTGGGGCGGCAACGGCCGCTGCAAGA ACATCCCGTACGCACGTCGTGTTCCCCCGATCGCGAAG ACCCGCGCGTGGCACACGCTCGATCAGGACGGGCTGCT GTTCGTCTGGCACGACCCCCAGGGCAATCCGCCGCCGG CCGACGTGACGATCCCGCGCATCGCGGGTGCGACGAGC GACGAGTGGACCGACTGGGTCTGGTACACCACCGAGGT CGACACCAACTGCCGCGAGATCATCGACAACATCGTCG ACATGGCGCACTTCTTCTACGTGCACTACTCCTTCCCG GTGTACTTCAAGAACGTCTTCGAAGGACACGTCGCCAG CCAGTTCATGCGCGGTCAGGCCCGTGAGGACACCCGTC CGCACGCGAACGGTCAACCGAAGATGATCGGAAGCCGA TCCGATGCAAGCTATTTCGGCCCGTCCTTCATGATCGA CGATCTCGTCTACGAGTACGAGGGATACGACGTCGAGT CGGTCCTCATCAACTGCCACTACCCGGTCTCCCAGGAC AAGTTCGTCCTGATGTACGGCATGATCGTCAAGAAGTC CGACCGTCTCGAGGGCGAGAAGGCGTTGCAGACCGCGC AGCAGTTCGGCAACTTCATCGCGAAGGGTTTCGAGCAG GACATCGAGATCTGGCGCAACAAGACCCGCATCGACAA CCCGCTCCTGTGCGAGGAGGACGGCCCCGTCTACCAGC TGCGTCGCTGGTACGAGCAGTTCTACGTCGACGTCGAG GACGTCGCGCCCGAGATGACCGACCGCTTCGAGTTCGA GATGGACACCACCCGTCCCGTCGCGGCGTGGATGAAGG AGGTCGAGGCGAACATCGCCCGCAAGGCCGCCCTCGAC ACGGAAACTCGTTCTGCACCAGAGCAGTCCACCACCGC GGGCTAG
kshA2 Rhodococcus rhodochrous (SEQ ID NO: 2)	HQ425874.1	GTGGGTTCCACAGACACCGAAGATCAGGTCCGCACCAT CGATGTGGGCACGCCGCCGGAGCGCTACGCGCGAGGAT GGCACTGCCTGGGGCTCGTACGCGATTTCGCCGACGGC AAGCCCCACCAGGTCGACGCGTTCGGGACCTCGCTCGT GGTGTTCGCCGGTGAGGACGGAAAGCTCAACGTTCTGG

		ACGCCTACTGCAGGCACATGGGTGGAAATCTGGCCCAG GGATCCGTGAAGGGCAACACCATCGCCTGTCCGTTCCA CGACTGGCGCTGGCGCGGTGACGGGAAGTGTGCCGAGA TTCCCTATGCGCGCCGTGTTCCACCGCTCGCCCGTACC CGGACGTGGCCGGTGGCGGAGGTGAGCGGTCAGCTCTT CGTGTGGCACGACCCGCAGGGCAGCAAGCCGCCGGCGG AGCTCGCCGTTCCGGAGGTTCCCACCTACGGCGATCCC GGGTGGACCGACTGGGTGTGGAACTCGATCGAGGTGAC CGGATCCCACTGTCGCGAGATCGTGGACAACGTCGTCG ACATGGCGCACTTTTTCTACGTCCACTACGGGATGCCG ACCTACTTCCGAAACGTGTTCGAAGGTCATACGGCCAC CCAGGTCATGCGGTCCCTGCCCCGGGCGGACGCCGTAG GCGTCAGCCAGGCCACCAATTACAGTGCCGAGAGCAGA TCCGATGCAACGTATTACGGTCCCTCGTACATGATCGA CAAGCTGTGGAGCGCCGGCCGTGATCCCGAGTCGACGC CGAACATCTATCTGATCAACTGCCACTACCCCATCTCT CCGACCTCCTTCCGCCTGCAGTACGGCGTGATGGTGGA AAGGCCCGAGGGAGTGCCCCCGGAGCAGGCGGAACAGA TCGCCCAGGCCGTCGCCCAGGGCGTCGCGATCGGATTC GAGCAGGACGTCGAGATCTGGAAGAACAAGTCGCGGAT CGACAACCCCCTGCTGTGCGAGGAGGACGGTCCCGTCT ACCAACTGCGGCGGTGGTACGAACAGTTCTACGTCGAC GTCGAAGACATCCGACCCGAGATGGTCAACCGGTTCGA GTACGAGATCGACACCACGCGCGCCCTGACGAGCTGGC AGGCCGAAGTCGACGAGAACGTCGCGGCCGGACGTAGT GCCTTCGCCCCGAACCTCACCCGGGCTCGTGAAGCAGC CTCCGCCGAATCGGGATCCTGA
kshA3 Rhodococcus rhodochrous (SEQ ID NO: 3)	HQ425875.1	ATGGCACAGATTCGCGAGATCGACGTCGGAGAGGTCCG GACGCGTTTCGCGCGAGGCTGGCACTGCCTCGGCCTCA GTCGCACGTTCAAGGACGGCAAGCCCCACGCCGTCGAG GCCTTCGGCACGAAACTCGTGGTGTGGGCCGACAGCAA CGGCGAACCGAAGGTGCTCGACGCGTACTGCCGTCACA TGGGCGGCGACCTGTCACAGGGCGAGATCAAGGGCGAT TCGGTTGCGTGCCCGTTCCACGACTGGCGCTGGGGCGG CAACGGCAAGTGCACGGACATCCCGTATGCCAGGCGCG TTCCCCCGCTGGCCCGCACCCGTTCGTGGATAACGATG GAGAAGCACGGCCAGCTGTTCGTGTGGAACGACCCCGA GGGCAACACCCCGCCCCCGGAGGTCACGATCCCCGAGA TCGAGCAGTACGGCTCGGACGAGTGGACGGACTGGACC TGGAACCAGATCCGGATCGAAGGTTCCAACTGTCGCGA

		GATCATCGACAACGTCGTCGACATGGCGCACTTCTTCT ACATCCACTACGCCTTCCCCACGTTCTTCAAGAACGTC TTCGAAGGGCACATCGCGGAGCAGTACCTCAACACCCG GGGCCGGCCGGACAAGGGCATGGCGACGCAGTACGGCC TGGAGTCGACCCTCGAGTCGTACGCGGCCTACTACGGC CCCTCCTACATGATCAATCCGCTCAAGAACAACTACGG CGGGTACCAGACCGAATCCGTACTGATCAACTGCCATT ACCCGATCACGCACGATTCGTTCATGCTGCAGTACGGC ATCATCGTCAAGAAGCCGCAGGGCATGTCACCCGAGCA GTCCGACGTGCTGGCCGCCAAGCTCACCGAGGGTGTCG GTGAAGGCTTCCTGCAGGACGTCGAGATCTGGAAGAAC AAGACCAAGATCGAGAATCCGCTGCTGTGCGAGGAGGA TGGTCCGGTCTACCAGCTCCGTCGCTGGTACGAGCAGT TCTACGTCGACGTCGCCGACGTGACGGAGAAGATGACG GGCCGCTTCGAGTTCGAGGTCGACACCGCCAAGGCCAA CGAGGCCTGGGAGAAGGAGGTCGCCGAGAATCTCGAGC GCAAGAAGCGCGAGGAAGAACAGGGCAAGCAGGAAGCG GAGGTGTGA
kshA4 Rhodococcus rhodochrous (SEQ ID NO: 4)	HQ425876.1	ATGACCGTCCCTCAGGAGCGGATCGAGATCCGCAACAT CGATCCCGGTACCAATCCCACCCGCTTCGCGCGCGGAT GGCACTGCATCGGCCTCGCCAAGGATTTCCGCGACGGA AAGCCGCACCAGGTCAAGGTGTTCGGCACCGACCTAGT GGTCTTCGCCGACACGGCCGGAAAGTTGCACGTGCTCG ACGCCTTCTGCCGGCACATGGGCGGCAACCTCGCTCGC GGCGAGATCAAGGGCGACACCATCGCGTGCCCGTTCCA CGACTGGCGCTGGAACGGCCAGGGCCGTTGCGAAGCGG TGCCGTACGCGCGCCGCACGCCGAAGCTCGGCCGTACC AAGGCGTGGACGACGATGGAGCGCAACGGCGTTCTGTT CGTCTGGCACTGCCCGCAGGGTAGTGAGCCCACTCCCG AGCTCGCGATCCCCGAGATCGAGGGCTACGAGGACGGG CAGTGGAGCGACTGGACGTGGACGACTATCCACGTCGA AGGATCGCACTGCCGCGAGATCGTCGACAACGTCGTCG ACATGGCGCACTTCTTCTACGTGCACTTCCAGATGCCC GAGTACTTCAAGAACGTCTTCGACGGGCACATCGCCGG CCAGCACATGCGCTCCTACGGGCGCGACGACATCAAGA CCGGTGTGCAGATGGACCTTCCGGAGGCGCAGACCATC TCGGATGCCTTCTACTACGGTCCGTCCTTCATGCTCGA CACCATCTACACGGTCTCCGAAGGCACGACCATCGAGT CGAAGCTGATCAACTGCCACTACCCGGTCACGAACAAC TCGTTCGTGCTGCAGTTCGGCACCATCGTCAAGAAGAT

		CGAGGGCATGTCCGAGGAGCAGGCCGCGGAGATGGCGA CGATGTTCACCGACGGTCTCGAGGAGCAGTTCGCCCAG GACATCGAGATCTGGAAGCACAAGTCCCGCATCGAGAA TCCGCTCCTCACCGAGGAGGACGGCCCGGTCTACCAGC TGCGTCGCTGGTACAACCAGTTCTACGTCGACCTCGAG GACGTCACACCGGACATGACCCAGCGTTTCGAGTTCGA GGTGGACACCTCCCGTGCGCTCGAGTCGTGGCACAAGG AGGTCGAGGAAAACCTCGCCGGTACGGCGGAGTGA
kshA5 Rhodococcus rhodochrous (SEQ ID NO: 5)	HQ425877.1	ATGTCCATCGACACCGCACGGTCCGGTTCGGACGACGA CGTCGAGATCCGCGAGATCCAGGCTGCGGCCGCTCCCA CCCGCTTCGCACGGGGCTGGCACTGCCTCGGCCTGCTC CGAGACTTCCAGGACGGCAAGCCGCACTCCATCGAGGC CTTCGGAACCAAGCTGGTCGTGTTCGCCGACAGCAAGG GGCAGCTCAACGTCCTCGATGCCTACTGCCGGCACATG GGTGGCGACCTGAGCCGCGGCGAGGTCAAGGGCGACTC GATCGCGTGCCCGTTCCACGACTGGCGCTGGAACGGCA AGGGCAAGTGCACCGACATCCCCTACGCCCGGCGCGTC CCGCCGATCGCGAAGACCCGCGCCTGGACGACCCTCGA ACGCAACGGCCAGCTGTACGTCTGGAACGACCCGCAGG GCAATCCGCCGCCGGAGGATGTCACCATCCCGGAGATC GCCGGTTACGGCACCGACGAGTGGACGGACTGGAGCTG GAAGAGCCTGCGCATCAAGGGCTCCCACTGCCGTGAGA TCGTCGACAACGTCGTCGACATGGCGCACTTCTTCTAC ATCCACTACTCGTTCCCGCGCTACTTCAAGAACGTCTT CGAGGGCCACACCGCCACGCAGTACATGCACTCGACCG GTCGTGAGGACGTCATCTCCGGCACCAACTACGACGAC CCCAACGCCGAACTGCGTTCCGAGGCAACCTATTTCGG TCCGTCGTACATGATCGACTGGCTCGAATCCGATGCCA ACGGCCAGACCATCGAGACCATCCTCATCAACTGCCAC TACCCGGTGAGCAACAACGAGTTCGTGCTGCAGTACGG CGCGATCGTCAAGAAGCTCCCGGGGGTGTCGGACGAGA TCGCCGCCGGGATGGCCGAGCAGTTCGCCGAGGGCGTG CAGCTCGGTTTCGAGCAGGACGTCGAGATCTGGAAGAA CAAGGCACCCATCGACAATCCGCTGCTGTCCGAGGAGG ACGGCCCGGTCTACCAGCTGCGTCGCTGGTACCAGCAG TTCTACGTCGATGTCGAGGACATCACCGAGGACATGAC CAAGCGCTTCGAGTTCGAGATCGACACCACCCGGGCGG TCGCGAGCTGGCAGAAGGAGGTCGCGGAGAACCTCGCG AAGCAGGCCGAAGGCTCCACCGCGACCCCCTAG

kstD1 Rhodococcus rhodochrous (SEQ ID NO: 6)	N/A	ATGGCGGAGTGGGCGGAAGAATGTGACGTCCTCGTGGT GGGGTCGGGAGCCGGAGGGTGCTGCGGTGCGTACACCG CTGCGCGCGAAGGGCTGTCGGTGATCCTCGTCGAGGCG TCCGAGTACTTCGGCGGCACCACGGCGTACTCCGGGGG CGGCGGCGTCTGGTTCCCCACCAACGCGGTCCTGCAGC GCGCCGGTGACGATGACACCATCGAGGATGCGCTGACC TACTACCACGCGGTCGTCGGCGACCGCACCCCGCACGA GCTGCAGGAGGCCTACGTTCGCGGCGGCGCCCCGCTGA TCGACTACCTCGAGTCCGACGACGACCTCGAATTCATG GTGTACCCGTGGCCCGACTACTTCGGCAAGGCGCCCAA GGCCCGTGCCCAGGGACGGCACATCGTCCCGTCGCCGC TGCCCATCGCCGGCGATCCCGAGCTCAACGAGTCGATC CGCGGCCCGCTCGGCCGTGAACGCATCGGCGAACCCCT GCCCGACATGCTCATCGGCGGTCGTGCGCTCGTCGGAC GATTCCTCATCGCCCTGCGCAAGTACCCGAACGTGGAC CTGTACCGGAACACCCCGCTCGAGGAACTGATCGTCGA GGACGGCGTGGTCGTGGGCGCGGTCGTCGGGAACGACG GTGAGCGACGTGCGATCCGCGCGCGCAAGGGCGTCGTC CTGGCCGCCGGCGGTTTCGATCAGAACGACGAGATGCG CGGCAAGTACGGGGTACCGGGTGCCGCGCGGGACTCGA TGGGACCGTGGTCGAACCTCGGCAAGGCCCACGAGGCG GGCATCGCCGTCGGCGCCGACGTGGATCTGATGGATCA GGCCTGGTGGTCACCGGGACTGACCCATCCGGACGGAC GCTCGGCGTTCGCGCTGTGCTTCACGGGCGGCATCTTC GTCGACCAGGACGGTGCGCGGTTCACCAACGAGTACGC ACCCTACGACCGTCTGGGCCGCGACGTCATCGCCCGCA TGGAGCGCGGCGAGATGACGTTGCCGTTCTGGATGATC TACGACGACCGGAACGGTGAGGCCCCGCCGGTCGGGGC GACGAACGTGCCGCTCGTCGAGACCGAGAAGTACGTCG ACGCGGGACTGTGGAAGACCGCCGACACCCTCGAGGAG CTCGCCGGGCAGATCGGTGTGCCCGCCGAATCCCTGAA GGCGACCGTCGCGCGGTGGAACGAGCTGGCCGCGAAGG GAGTCGACGAAGACTTCGGTCGCGGGGACGAACCCTAC GATCTCGCCTTCACCGGCGGTGGGTCCGCGCTGGTCCC GATCGAGCAGGGCCCCTTCCACGCGGCGCAGTTCGGCA TCTCCGATCTCGGCACCAAGGGCGGTCTGCGGACCGAC ACCGTCGGGCGCGTGCTCGACAGCGAGGGTGCTCCGAT CCCCGGTCTGTACGCGGCGGGCAACACGATGGCAGCAC CGAGCGGCACCGTCTACCCCGGCGGTGGCAACCCGATC

		GGCGCGAGCGCGCTGTTCGCGCACCTGTCCGTGATGGA CGCTGCGGGACGCTGA
kstD2 Rhodococcus rhodochrous (SEQ ID NO: 7)	N/A	ATGGCCAAGACCCCTGTACCGGCCGTGACCACAGCCCG CGATACGACCGTGGACCTGCTCGTGATCGGGTCCGGTA CCGGCATGGCCGCTGCGCTCACCGCGCACGAGGCGGGC CTGTCCGCTCTCATCGTGGAGAAGTCGGCCTACGTCGG CGGATCGACCGCCCGTTCCGGCGGTGCATTCTGGGTGC CGGCCAATCCGGTACTCACCGCGGCGGGAAGCGGCGAC ACCATCGAGCGCGGCCACACCTACGTGCGGACGGTCGT CGACGGCACGGCGCCGGTCGAGCGGGGCGAGGCCTTCG TCGACAACGGTGTCGCCACCATCGAGATGCTCCAGCGC ACCACCCCCATGAAGCTGTTCTGGGCCGAGGGCTACTC CGACTATCACCCCGAACTGGCGGGTGGTTCGGCGGTCG GCCGCAGCTGCGAGTGCCTGCCCCTCGACCTGTCGGTC CTCGGTGAGGAGCGCGGTCGACTGCGTCCGGGCCTCAT GGAGGCGAGCCTGCCGATGCCCACCACCGGTGCCGACT ACAAGTGGATGAACCTCATGCTGCGCGTGCCGCACAAG GGTTTTCCGCGCATCTTCAAGCGGCTCGCCCAGGGTGT CGCCGGTCTCGCCGTCAAGCGTGAATATGTCGCGGGTG GACAGGCGATCGCCGCCGGTCTGTTCGCGGGTGTGCTG AAGGCCGGTGTCCCGGTGTGGACCGAGACGTCGCTGGT GCGTCTGCTCACCGACGGGGACCGTGTCACCGGTGCCG TCGTCGAGCAGAACGGACGTGAGGTGACGGTGACCGCG CGTCGCGGGGTGGTGCTCGCCGCCGGCGGTTTCGACCA CGACATGGAGATGCGGCGCAAGTTCCAGTCCGAGCGTC TGCTCGACCACGAGAGCCTGGGAGCGGAGACCAACACC GGCGACGCGATCAAGGCGGCCCAGGAGGTCGGTGCAGA TCTCGCCCTCATGGACCAGGCCTGGTGGTTCCCTGCCG TCGCGCCGACCCGCACGGGAAAGCCGCCGATGGTCATG CTCGCCGAGCGGTCGCTGCCGGGTTCGTTCATCGTCGA CCAGACGGGCCGCCGGTTCACCAACGAGTCGTCGGACT ACATGTCGTTCGGACAGTTGGTGCTCGAACGTGAGCGT GCCGGCGATCCGATCGAGTCGATGTGGATCGTCTTCGA CCAGAAGTACCGCAACAGCTACGTCTTCGCGGCCGGGG TGTTCCCGCGTCAACCGCTCCCGGAAGCCTGGTACGAG GCGGGCATCGCCCACCGTGGCACCACCGCTGCGGAACT CGCGGCGTCGATGGGCGTGCCGGTGGACACCTTCGCCG CGACGTTCGACAGGTTCAACGAGGACGCGGCGGCGGGA ACGGATTCCGAGTTCGGACGCGGCGGCAGTGCCTACGA CCGCTACTACGGTGATCCGACCGTCCAGCCGAACCCGA

		ACCTGCGGCCCCTCACGCACGGCCCGCTCTACGCGGTG AAGATGACGCTGAGCGATCTCGGCACGTGCGGTGGCGT GCGCGCCGACGAGCGGGCGCGGGTCCTCCGCGAGGACG GCAGCCCCATCGCCGGTCTCTACGCTATCGGCAACACC GCGGCCAACGCGTTCGGCCACCGCTATCCCGGTGCCGG CGCCACGATCGGCCAGGGCCTGGTCTTCGGGTACATCG CGGCACGCGACGCAGCATCGTCGGACGCACCGGTCGCC TGA
kstD3 Rhodococcus rhodochrous (SEQ ID NO: 8)	HQ425875.1	ATGACGAAGCAGGAGTACGACATCGTTGTCGTCGGCAG CGGTGCCGGCGGAATGACCGCCGCCATCACCGCAGCCC GCAAGGGCGCCGACGTGGTCCTGATCGAGAAGGCGCCA CGCTACGGCGGGTCGAGCGCCCGATCGGGCGGCGGTGT GTGGATCCCCAACAACGAGGCCCTGAAGGCCGCCGGGG TGGACGACACACCCGAGGAGGCCCGGAAATACCTCCAC AGCATCATCGGCGACGACGTACCCGCCGAGAAGATCGA CACCTACATCGATCGCGGACCGGAGATGCTCTCCTTCG TCCTGAAGAACAGCGCACTCGAACTGCAGTGGGTGCCG GGCTATTCCGACTACTACCCCGAGGCGCCGGGCGGACG TCCCGGTGGCCGTTCGGTGGAACCGACACCCTTCGACG GTCGCCGTCTCGGCGAGGATCTCGCTCTCCTCGAACCC GACTACGCCCGCGCTCCCAAGAACTTCGTCATCACCCA GGCCGACTACAAGTGGCTGAACCTGCTCATGCGGAACC CGCGCGGACCGATTCGCGCCATGCGGGTCGGCGCCCGG TTCGTCTGGGCGAACATCACCAAGAAGCACCTGCTCGT CCGAGGCCAGGCACTCATGGCCGGTCTGCGGATCGGTC TGCGTGACGCCGGTGTGCCCCTGCTGCTGGAGACGGCG CTCACCGACCTCGTCGTCGAGGGCGGCGCCGTGCGCGG CGTCAAGGTGGTCGCGAACGGCGAGACGCGCGTCATCC GTGCCCGCAAGGGCGTGATCATCGCGAGCGGCGGTTTC GAGCACAACGCCGAGATGCGGGCGCAATACCAGCGTCA GCCGATCGGCACCGAGTGGACCGTGGGGGCGAAGGCGA ACACCGGCGACGGAATCCGCGCCGGACAGAAGCTGGGC GCCGCAGTCGATTTCATGGACGACGCCTGGTGGGGACC GTCCTTCACCCTCACCGGCGGCCCGTGGTTCGCACTGT CGGAACGCAGCCTCCCCGGGTGCCTCATGGTCAACGCC GCGGGCAAGCGTTTCGTCAACGAGTCGGCGCCCTACGT CGAAGCGACGCATGCGATGTACGGCGGCAAGCACGGAC GCGGCGAGGGACCGGGCGAGAACATCCCCAGCTGGCTG ATCCTCGATCAGCGCTACCGCGACCGCTACACCTTCGC CGGCATCACCCCCCGCACTCCCTTCCCCCGCCGGTGGC

		TCGAGGCCGGGGTGCTCGTCAAGGCCGGTTCCGTCGCC GAACTCGCCGAGAAGATCGGGGTACCGGCCGACGCCCT CACCGAGACGGTGCAGCGGTTCAACGGCTTCGCCCGGG CCGGCAAGGACGAGGACTTCGGCCGCGGCGAATCCCAC TATGACCACTACTACGGGGATCCGCGCAACAAGCCGAA TCCGAGCCTCGGCGTGGTCGATAAGGCCCCGTTCTACG CGTTCAAGGTGGTCCCCGGCGATCTCGGCACCAAGGGC GGGCTCGTCACCGACGTCCACGGCCGGGTGGTGCGCGA GGACGGCAGCGTGATCGACGGCCTGTACGCGACCGGTA ACGCCAGCTCCCCGGTCATGGGTCACACCTACGCCGGG CCCGGTGCCACCATCGGACCGGCGATGACCTTCGGCTA TCTCGCGGCCCTCGACATCCTGGATCGCACGGGTGACG AACGCACCGAGGAACTGCGAGAATCCGCCGACACCGTG TGA
fadE34 Rhodococcus rhodochrous (SEQ ID NO: 9)	N/A	GTGAGTATCGCCACGACCGAGGAGCAGCGGGCCGTCCA GGCGTCTGTCCAGGCCTGGTCACGTGCCGTAGACCCCA TGTCGACGATACGTCGCGCAGGTGATGCGACGTGGCGC GACGGCTGGTCCTCCCTCGCAGAACTCGGAATCTTCGG TGTTGCCGTCCCGGAGGAGGCGGGCGGCCTCGGCGCGA CCGCCGTGGATCTGGCCGTCATGCTCGAGCAGGCCGCC CACGAACTCGCGCCGGGTCCGGTCCTGACCACCGCCGT GGCGGCCCTCGTGTTCGGCCGTGCCGGTGAGACCGTCG CCAAGACGGCGGAGCGACTCGCCGAGGGTGAGGTCCCC ACCGCACTCGCTCTCGACTCCGGCGTGACCGTGGAGCC GGCGGGTGACGGAGTCCTGCTGCGCGGTGAGGCCGGGC CGGCCGTGGGTGCCGAAGCCGGGGTCGCCGTGCTCGTC CGTGTCGCGGGGGAAGGTGATCCGGCCGTCGAGAGCTG GGCGCTCGTCGAGGCGGACGATCCGGGTCTGCACATCG AACCGCTCGAGACCATCGACGCCTCCCGCGCGGTGGCC CGCGTCCGCCTCGACGGCGCGACGGTCCCGGCCGACCG GGTCGCGACCGTCCCGGCCGGCTTCGTGCGCGACCTCA CCGCCGGTCTCGCCGCCGCGGAGCTGGCCGGTCTCGCC GGTTGGGCGCTGACCACCGCCGTCGAGTACGCGAAGAT CCGCGAGCAGTTCGGAAAACCGATCGGTTCGTTCCAGG CCGTCAAGCACATCTGTGCCGAAATGCTCTGCCGCACC GAGAAGATCCGGGCCATGGCCTGGGATGCTGCGGTCAC CGTCGACGCGCAGCCCGACGAACTGCCGATCGCCGCGG CTGCCGCCGTGGCGGTCGCACTCGATGCCGCGGTGCAG ACCGCCAAGGATGCGATCCAGGTGCTCGGCGGCATCGG GTTCACGTGGGAACACGACGCGCACTTCTATCTTCGCC

		GTGCGGTCGCCACCCGCCAGGTGCTCGGTGGTTCGACC GTGTGGCGTTCGCGGCTGACGACCCTGGTCCGCGCAGG CGCACGTCGTCACCTCGGTATCGACCTGTCCGATCACG AGGAGGAGCGCGCACGGATCCGTGCGGAAGTCGAGAAG ATCGCCGCCGCACCGGAATCCGAGCGCCGCGTCGCCCT CGCCGAGTCGGGTCTGCTCGCGCCGCACTGGCCGCAGC CGTACGGTCGCGGAGCCGGTGCCGCCGAACAGCTCGTC GTCCAGGAGGAGCTCGCCGCCGCCGGTATCGAACGTCC CGATCTCGTGATCGGCTGGTGGGCGGTTCCGACTATCC TCGAACACGGAACACCCGAGCAGATCGAGCGTTTCGTG ATGCCCACCCTGCGCGGCGATGTGGTGTGGTGCCAGCT CTTCTCCGAGCCCGGCGCCGGCTCGGACCTCGCGGCGC TGCGCACGAGCGCGGAGAAGGCCGACGGCGGATGGGTG CTGCGCGGGCAGAAGGTGTGGACCTCCCTCGCGCAGCA GGCGGACTGGGCGATCTGCCTCGCCCGCACCGACCGCG ACGTCCCCAAGCACAAGGGCATCACCTATTTCCTCGTC GACATGAAGTCGGCGGGCATCACGATCTCGCCGCTGCG CGAGATCACCGGCGACGCGTTGTTCAACGAGGTCTTCC TCGATTCGGTCTTCGTGCCGGACGACTGCGTGGTCGGC AATCTCGGTGACGGCTGGAAGCTGGCCCGCACGACTCT CGCCAACGAGCGTGTCGCGATGGGCGGCAAGTCGTCGC TGGGGCAGAGCATCGAGGAACTGCTCGAACTGTCGACC CCCGGTGATCCCGTCGCAGAGGACCGCATCGCGACGCA GATCGGCGAGGCGACCGTCGGTTCGCTCCTGGATCTGC GGGCGACCCTCGCGCAGCTCGAAGGTCAGGATCCGGGC GCCGCGTCCAGCGTCCGCAAGCTCATCGGTGTGCGGCA GCGGCAGGACACCGCCGAGCTCGCCATGGATCTCGCGG GCGAGGCCGGCTGGGTGGAAGGTCCGCTCACCCGGGAG TTCCTCAACACCCGGTGCCTGACGATCGCCGGCGGGAC CGAGCAGATCCTGCTCACCGTGGCGGCCGAGCGGCTGC TGGGCCTGCCGCGGGGTTGA
fadE34#2 Rhodococcus rhodochrous (SEQ ID NO: 10)	N/A	ATGACTCTGGGATTGAGCGACGAGGACCGCGAACTCCG CGACTCCGTGCGCGGCTGGGCGGCACGACACGCCACAC CCGACGTGATCCGCACGGCCGTCGAAGCGAAGACGGAA GCCCGCCCGACGTACTGGAGCTCGTTCGCCGAACTCGG CATGCTGGGATTGCACCTGCCCGAAGAGGTCGGAGGCG CCGGTTTCGGTCTGCTCGAAACGGCGATCGTCGCAGAG GAACTCGGACGGGCCATGGTGCCCGGCCCGTTCCTTCC GACCGTGATCGTGTCCGCGGTCCTCGACGAGGCCGGCC GTCGCAGCGAACTCGACGGGCTCGCGGACGGTTCGCTG

		TTCGGTGCGGTCGCCCTGCAGCCGGGGGACCTGCGCGT
		GGAGCGCGACGGCGATTCCGTCACGCTCTCGGGAACCT
		CCGGTGTCGCTCTCGGCGGCCAGGTCGCGGATGTCTTC
		CTGCTCGCGGCCGACGACGGTGGTGAGCGGGTATTCGT
		CGTCGTGACCCGTGACCGGGTCGAGGTCACGAACCTGC
		CCAGCTACGACGTGATCCGCCGCAACGCCGAGATCACC
		GTGAGTGCCGTGCCGCTGTCCGACGGGGACGTGCTGGA
		GTCGGATCCGCATCGGATCGTCGATATCGCCGCGACCT
		TGTTCGCCGCCGAAGCCGCCGGTCTCGCGGACTGGGCC
		ACCACCACCGCCGCGGACTATGCGCGGGTCCGCAAGCA
		GTTCGGCCGCGTCATCGGACAGTTCCAGGGTGTCAAGC
		ACACCGTCGCCCGGATGCTCTGCCTCACCGAACAGGCG
		CGGGTCGTGGCCTGGGACGCCGCGCGAGCGCGGCGCGA
		GGACGTGCCGGACGACGAGGCGTCGCTGGCCGTGGCGG
		TCGCCGCGTCCATCGCCCCCGAGGCCGCCTTCCAGGTC
		ACCAAGAACTGCATCCAGGTGCTCGGCGGTATCGGCTA
		CACCTGGGAGCACGACGCCCACCTGTACATGCGCCGCG
		CCCAGTCGCTCCGAATCCTGCTCGGCTCCACGGCGTCC
		TGGCGGCGCCGGGTCGCCCACCTCACGCTCGGCGGTGC
		CCGCCGCGTGCTGAGCGTCGATCTGCCGCCCGAGGCGG
		AACGGATCCGCGCCGACGTCCGTGCCGAACTCGAGCCG
		GCGAAGTCGCTGGAGAACGCAGCGCGGAAGGCGTATCT
		GGCGGAGAAGGGTTACACCGCTCCCCATCTGCCCGAAC
		CGTGGGGCAAGGCCGCCGACGCCGTCACGCAACTCGTC
		GTCGCCGAGGAACTGCGCGCCGCCGAACTCGAACCGCA
		CGACATGATCATCGGCAACTGGGTGGTGCCGACCCTCA
		TCGCGCACGGCAGTACCGAGCAGATCGAGCGATTCGTC
		CCGCAGTCGCTGCGCGGGGATCTCGTGTGGTGTCAGCT
		CTTCTCCGAACCCGGCGCCGGATCCGACCTCGCGGGCC
		TGTCCACCAAGGCCGTCAAGGTGGACGGCGGATGGAGG
		CTCGACGGCCAGAAGGTGTGGACGTCGATGGCACGGGT
		CGCGGATTGGGGCATCTGCCTCGCCCGCACCGACGCGG
		AAGCGCCCAAACACAAAGGCCTGTCCTACTTCCTGATC
		GACATCAGGAACACCGAGGGTCTCGACATCCGGCCGCT
		GCGAGAGATCACCGGCGAAGCCCTGTTCAACGAGGTGT
		TCCTCGACGGCGTGTTCGTGCCCGACGAGTGCCTCGTC
		GGCGAGCCCGGGGACGGATGGAAGCTCGCCCGTACCAC
		CCTCGCGAACGAACGCGTCTCCCTCTCGCACGATTCGA
		CTTTCGGTGCCGGCTGCGAGACTCTCATAGCGCTCGCG
		AACGGTATGCCCGGTGGACCGGACGACGAACAACTCAC

		CGTCCTCGGCAAGGTTCTCGGCGATGCCGCGTCCGGTG GCCTCATGGGTCTGCGTACCGCTCTACGGTCCCTGGCC GGCGCACAGCCGGGTGCCGAGTCCTCCGTCGCCAAGCT CCTCGGCGTCGAGCACCTCCAGCAGGTCTGGGAGACCG CGATGGACTGGGCCGGTACTGCGTCGTTGCTCGACGAC CAGGACCGAACTTCGGCGACCCACATGTTCCTCAACGT GCAGTGCATGTCCATCGCCGGTGGGACGACCAACGTCC AGCTGAACATCATCGGTGAGCGGCTTCTCGGCCTGCCC CGCGATCCCGAACCCGGAAAGTGA
fadE26 Rhodococcus rhodochrous (SEQ ID NO: 11)	HM588720.1	GTGGACATCTCCTACACCCCCGGGCAACAAGCCCTCCG CGAGGAATTGCGGGCCTATTTCGCACAGATCATGACCC CCGAGCGCCGCGAGGCGCTCGCGGCCACGACCGGGGAG TACGGCTCCGGCAACGTGTACCGCGAGGTCGTGCAGCA GATGGGCAAGGACGGCTGGCTCACCCTCGGGTGGCCCG AGGAATACGGCGGCCAGAACCGTTCCGCGATGGACCAA TTGATCTTCACCGACGAGGCGGCCATCGCCGGCGCGCC CGTCCCGTTCCTCACCATCGACTCGGTCGCGCCGACGA TCATGCACTACGGCACGGACGAGCAGAAGGAGTTCTTC CTCCCCCGCATCTCCGCGGGAGAACTGCACTTCTCGAT CGGCTATTCCGAACCCGGCGCCGGCACCGACCTCGCCT CGCTGCGCACCACCGCCGTGCGCGACGGCGACGAGTGG GTCATCAACGGGCAGAAGATGTGGACGAGCCTGATCGC CTACGCCGACTACGTCTGGCTCGCCGCGCGCACCAACC CGGATGTCAAGAAGCACAAGGGGATCAGCGTCTTCATC GTGCCGACCGACGCTCCCGGCTTCTCGTACACCCCCGT GCACACCATGGCCGGCCCCGACACGAGCGCCACCTACT ACCAGGACGTGCGCGTCCCGGCGTCCGCGCTCGTCGGT GAGGTCGACGGCGGCTGGGCGCTCATCACCAACCAGCT CAATCACGAGCGGGTCGCACTCACCTCCGCCGGTCCCG TGCGCACCGCGCTGACCGAGGTCCGGCGCTGGGCGCAG GAGACGCACCTGCCCGACGGACGACGGGTGATCGACCA GGAATGGGTGCAGATCAACCTGGCACGCGTCCATGCCA AGGCCGAATACCTGCAGCTGATGAACTGGGACATCGCC TCGAGCGCCGGCACGACCCCGCTCGGTCCGGAGGCCGC CTCGGCCAACAAGGTGTTCGGCACCGAATTCGCGACCG AGGCCTACCGGTTGCTCATGGAGGTCCTCGGACCCGCG GCGACGGTACGGCAGAACTCGGCCGGCGCACTGCTCCG CGGCCGGATCGAACGCATGCACCGCAGTTCCCTCATCC TCACCTTCGGTGGCGGCACCAACGAGGTCCAGCGCGAC

		ATCATCGCGATGACCGCTCTCGGCCAGCCGCCCGCCAA GCGTTAG
fadE34 Mycobacterium neoaurum (SEQ ID NO: 12)	N/A – full *Mycobacterium neoaurum* genome (CP011022.1)	GTGTCTGTGCTGTCCGTCCCGACCGATACATCGGATGA GGCCGCGGCCCGTGAACTGGTCAGAGACTGGGTTCCGA GCTCTGGGTCGATCACCGCGATCCGCAACGTCGAACTC GGCGATCCGCAGGCCTGGCGCACGCCGTTTGCCGGCTT CGCCGAACTAGGGGTATTCGGCGTCGCGGTGCCCGAGG AGTACGGCGGGGCCGGCAGCACGGTGGCGGATCTGCTC GCGATGATCGACGAGGCGGCCGCCGGCCTGATCCCGGG ACCCGTCGCGGGGACCGCACTTGCCACCCTCGTCGCCG ATGATCCGGCCGTCCTGGAGGCGTTGGCCACCGGGGAG CGCAGCGCCGGGATCGCCATGACGTCCGACATCACGGT CGATTCCGGTACCGCCACCGGCACCGCGCCCCACGTGC TGGGTGCCGATCCCGGCGGGGTCCTCATCCTGCCTGCC GGGCAGCATTGGATCCTGGTGGACGCGAGTTCCGACGG GGTGACCATCGACCCGCTGGAGGCCACCGACTTCTCCC GACCGCTGGCCCGGGTGACGCTGACATCGGCACCGGCG CAGCAGCTGAATGCCTCGGCGCAGCGGGTCACCGACCT GATGGCGACTGTGCTGGCGGCCGAGCTGGCCGGGTTGT CGCGCTGGCTGCTCAACACCGCCAACGAGTACGCCAAG GTGCGCGAACAGTTCGGCAAGCCGATCGGCAGCTTCCA GGCCGTCAAACACATGTGCGCGGAGATGCTGCTGCGTA GCCAGCAGGTCACCGTCGCCGCCGCCGACGCGATCGCG GCCGCTGCCGGTGACGACGCCGACCAGCTGTCCGTCGC CGCGGCGGTGGCGGCGGCCATCGGTATCGACGCCGCGA AGCTGAACGCGCGCGACTGCATCCAGGTGCTCGGCGGG ATCGGCATCACCTGGGAGCACGATGCGCACCTGTACCT GCGTCGGGCATATGCGAACGCGCAGTTCCTCGGTGGCC GGTCGCGTTGGTTGCGTCGCGTCGTCGAACTGACCCGT GCCGGCGTGCGCCGCGAACTGCACGTCGACACCGCTGA TGCCGATGCCATCCGTCCCGAGATCGCCGCGGCCGCCG CCCGCATCGCCGCGCTGCCCGAGGACCAACGAGGGCGG GCACTCGCCGAATCCGGGCTGCTGGCCCCGCATTGGCC GACGCCGTACGGGCGGGACGCGACCCCGGCCGAACAGT TGGTGATCGACGAGGAACTGGCGGCTGCCGAGGTGGCG CGCCCCGATATCTCGATCGGCTGGTGGGCCGCTCCGAC GATCCTTGCCGCCGGTACGCCCGAACAGATCGATCGGT TCATCCCCGGCACCCTCAACGGCGACATCTTCTGGTGC CAGCTGTTCTCCGAGCCCGGCGCGGGGTCGGATCTGGC GGCGTTGCGCACCAAGGCCGTTCGTGTGGAGAAGGATG

		GCCGCACTGGCTGGTCTCTGACCGGACAGAAGGTGTGG ACCTCCAACGCGCACCGCGCCAACTGGGGCATCTGCCT GGCCCGGACCAACCCGGACGCTCCGAAACACAAGGGCA TCTCCTATTTCCTGGTCGATATGAGCTCACCGGGTATC GATATCCGGCCGCTGCGCGAGATCACCGGTGAGGCCCT GTTCAACGAGGTCTTCTTCGATGACCTGTTCGTTCCCG ACGACTGCGTGGTCGGTGAGGTGGACGGTGGCTGGCCG CTGGCCCGTACCACGCTGGCCAACGAGCGCGTCGCCAT CGCCACCGGCGGGGCACTGGACAAGGGCATGGAGCATC TGCTTGCCGTGATCGGTGACCGGGAGCTCGACGGCGCC GAGGCCGATCGGCTCGGTGCCCTGATCACCCTGGCCCA GGTCGGTTCGCTGCTGGATCAGCTCATCGCGCGGATGG CGTTGGGCGGCAATGATCCTGGTGCTCCGTCGAGCGTG CGCAAGCTGATCGGCGTGCGTTATCGACAGGGGTTGGC CGAGGCGGCGATGGAGTTCCAGGACGGTGGCGGCATCG TCGACTCGCCCGATGTCCGGTACTTCCTCAACACCCGC TGCTTGAGCATCGCCGGGGGCACCGAGCAGATCCTGCT CACCCTCGCCGGTGAGCGGCTGCTGGGGTTGCCGCGCT AG

APPENDIX B – Amino acid sequences

Name and SEQ ID NO.	GENBANK Accession No.	Amino acid sequence
kshA1 Rhodococcus rhodochrous (SEQ ID NO: 13)	ADY18310.1	VSLGTSEQSEIREIVAGSAPARFARGWHCLGLAKDFKD GKPHSVHAFGTKLVVWADSNDEIRILDAYCRHMGGDLS QGTVKGDEIACPFHDWRWGGNGRCKNIPYARRVPPIAK TRAWHTLDQDGLLFVWHDPQGNPPPADVTIPRIAGATS DEWTDWVWYTTEVDTNCREIIDNIVDMAHFFYVHYSFP VYFKNVFEGHVASQFMRGQAREDTRPHANGQPKMIGSR SDASYFGPSFMIDDLVYEYEGYDVESVLINCHYPVSQD KFVLMYGMIVKKSDRLEGEKALQTAQQFGNFIAKGFEQ DIEIWRNKTRIDNPLLCEEDGPVYQLRRWYEQFYVDVE DVAPEMTDRFEFEMDTTRPVAAWMKEVEANIARKAALD TETRSAPEQSTTAG
kshA2 Rhodococcus rhodochrous (SEQ ID NO: 14)	ADY18316.1	VGSTDTEDQVRTIDVGTPPERYARGWHCLGLVRDFADG KPHQVDAFGTSLVVFAGEDGKLNVLDAYCRHMGGNLAQ GSVKGNTIACPFHDWRWRGDGKCAEIPYARRVPPLART RTWPVAEVSGQLFVWHDPQGSKPPAELAVPEVPTYGDP GWTDWVWNSIEVTGSHCREIVDNVVDMAHFFYVHYGMP TYFRNVFEGHTATQVMRSLPRADAVGVSQATNYSAESR SDATYYGPSYMIDKLWSAGRDPESTPNIYLINCHYPIS PTSFRLQYGVMVERPEGVPPEQAEQIAQAVAQGVAIGF EQDVEIWKNKSRIDNPLLCEEDGPVYQLRRWYEQFYVD VEDIRPEMVNRFEYEIDTTRALTSWQAEVDENVAAGRS AFAPNLTRAREAASAESGS
kshA3 Rhodococcus rhodochrous (SEQ ID NO: 15)	ADY18318.1	MAQIREIDVGEVRTRFARGWHCLGLSRTFKDGKPHAVE AFGTKLVVWADSNGEPKVLDAYCRHMGGDLSQGEIKGD SVACPFHDWRWGGNGKCTDIPYARRVPPLARTRSWITM EKHGQLFVWNDPEGNTPPPEVTIPEIEQYGSDEWTDWT WNQIRIEGSNCREIIDNVVDMAHFFYIHYAFPTFFKNV FEGHIAEQYLNTRGRPDKGMATQYGLESTLESYAAYYG PSYMINPLKNNYGGYQTESVLINCHYPITHDSFMLQYG IIVKKPQGMSPEQSDVLAAKLTEGVGEGFLQDVEIWKN KTKIENPLLCEEDGPVYQLRRWYEQFYVDVADVTEKMT GRFEFEVDTAKANEAWEKEVAENLERKKREEEQGKQEA EV
kshA4 Rhodococcus rhodochrous (SEQ ID NO: 16)	ADY18323.1	MTVPQERIEIRNIDPGTNPTRFARGWHCIGLAKDFRDG KPHQVKVFGTDLVVFADTAGKLHVLDAFCRHMGGNLAR GEIKGDTIACPFHDWRWNGQGRCEAVPYARRTPKLGRT KAWTTMERNGVLFVWHCPQGSEPTPELAIPEIEGYEDG

		QWSDWTWTTIHVEGSHCREIVDNVVDMAHFFYVHFQMP EYFKNVFDGHIAGQHMRSYGRDDIKTGVQMDLPEAQTI SDAFYYGPSFMLDTIYTVSEGTTIESKLINCHYPVTNN SFVLQFGTIVKKIEGMSEEQAAEMATMFTDGLEEQFAQ DIEIWKHKSRIENPLLTEEDGPVYQLRRWYNQFYVDLE DVTPDMTQRFEFEVDTSRALESWHKEVEENLAGTAE
kshA5 Rhodococcus rhodochrous (SEQ ID NO: 17)	ADY18328.1	MSIDTARSGSDDDVEIREIQAAAAPTRFARGWHCLGLL RDFQDGKPHSIEAFGTKLVVFADSKGQLNVLDAYCRHM GGDLSRGEVKGDSIACPFHDWRWNGKGKCTDIPYARRV PPIAKTRAWTTLERNGQLYVWNDPQGNPPPEDVTIPEI AGYGTDEWTDWSWKSLRIKGSHCREIVDNVVDMAHFFY IHYSFPRYFKNVFEGHTATQYMHSTGREDVISGTNYDD PNAELRSEATYFGPSYMIDWLESDANGQTIETILINCH YPVSNNEFVLQYGAIVKKLPGVSDEIAAGMAEQFAEGV QLGFEQDVEIWKNKAPIDNPLLSEEDGPVYQLRRWYQQ FYVDVEDITEDMTKRFEFEIDTTRAVASWQKEVAENLA KQAEGSTATP
kstD1 Rhodococcus rhodochrous (SEQ ID NO: 18)	N/A	MAEWAEECDVLVVGSGAGGCCGAYTAAREGLSVILVEA SEYFGGTTAYSGGGGVWFPTNAVLQRAGDDDTIEDALT YYHAVVGDRTPHELQEAYVRGGAPLIDYLESDDDLEFM VYPWPDYFGKAPKARAQGRHIVPSPLPIAGDPELNESI RGPLGRERIGEPLPDMLIGGRALVGRFLIALRKYPNVD LYRNTPLEELIVEDGVVVGAVVGNDGERRAIRARKGVV LAAGGFDQNDEMRGKYGVPGAARDSMGPWSNLGKAHEA GIAVGADVDLMDQAWWSPGLTHPDGRSAFALCFTGGIF VDQDGARFTNEYAPYDRLGRDVIARMERGEMTLPFWMI YDDRNGEAPPVGATNVPLVETEKYVDAGLWKTADTLEE LAGQIGVPAESLKATVARWNELAAKGVDEDFGRGDEPY DLAFTGGGSALVPIEQGPFHAAQFGISDLGTKGGLRTD TVGRVLDSEGAPIPGLYAAGNTMAAPSGTVYPGGGNPI GASALFAHLSVMDAAGR
kstD2 Rhodococcus rhodochrous (SEQ ID NO: 19)	N/A	MAKTPVPAVTTARDTTVDLLVIGSGTGMAAALTAHEAG LSALIVEKSAYVGGSTARSGGAFWVPANPVLTAAGSGD TIERGHTYVRTVVDGTAPVERGEAFVDNGVATIEMLQR TTPMKLFWAEGYSDYHPELAGGSAVGRSCECLPLDLSV LGEERGRLRPGLMEASLPMPTTGADYKWMNLMLRVPHK GFPRIFKRLAQGVAGLAVKREYVAGGQAIAAGLFAGVL KAGVPVWTETSLVRLLTDGDRVTGAVVEQNGREVTVTA RRGVVLAAGGFDHDMEMRRKFQSERLLDHESLGAETNT GDAIKAAQEVGADLALMDQAWWFPAVAPTRTGKPPMVM

		LAERSLPGSFIVDQTGRRFTNESSDYMSFGQLVLERER AGDPIESMWIVFDQKYRNSYVFAAGVFPRQPLPEAWYE AGIAHRGTTAAELAASMGVPVDTFAATFDRFNEDAAAG TDSEFGRGGSAYDRYYGDPTVQPNPNLRPLTHGPLYAV KMTLSDLGTCGGVRADERARVLREDGSPIAGLYAIGNT AANAFGHRYPGAGATIGQGLVFGYIAARDAASSDAPVA
kstD3 Rhodococcus rhodochrous (SEQ ID NO: 20)	ADY18320.1	MTKQEYDIVVVGSGAGGMTAAITAARKGADVVLIEKAP RYGGSSARSGGGVWIPNNEALKAAGVDDTPEEARKYLH SIIGDDVPAEKIDTYIDRGPEMLSFVLKNSALELQWVP GYSDYYPEAPGGRPGGRSVEPTPFDGRRLGEDLALLEP DYARAPKNFVITQADYKWLNLLMRNPRGPIRAMRVGAR FVWANITKKHLLVRGQALMAGLRIGLRDAGVPLLLETA LTDLVVEGGAVRGVKVVANGETRVIRARKGVIIASGGF EHNAEMRAQYQRQPIGTEWTVGAKANTGDGIRAGQKLG AAVDFMDDAWWGPSFTLTGGPWFALSERSLPGCLMVNA AGKRFVNESAPYVEATHAMYGGKHGRGEGPGENIPSWL ILDQRYRDRYTFAGITPRTPFPRRWLEAGVLVKAGSVA ELAEKIGVPADALTETVQRFNGFARAGKDEDFGRGESH YDHYYGDPRNKPNPSLGVVDKAPFYAFKVVPGDLGTKG GLVTDVHGRVVREDGSVIDGLYATGNASSPVMGHTYAG PGATIGPAMTFGYLAALDILDRTGDERTEELRESADTV
fadE34 Rhodococcus rhodochrous (SEQ ID NO: 21)	N/A	VSIATTEEQRAVQASVQAWSRAVDPMSTIRRAGDATWR DGWSSLAELGIFGVAVPEEAGGLGATAVDLAVMLEQAA HELAPGPVLTTAVAALVFGRAGETVAKTAERLAEGEVP TALALDSGVTVEPAGDGVLLRGEAGPAVGAEAGVAVLV RVAGEGDPAVESWALVEADDPGLHIEPLETIDASRAVA RVRLDGATVPADRVATVPAGFVRDLTAGLAAAELAGLA GWALTTAVEYAKIREQFGKPIGSFQAVKHICAEMLCRT EKIRAMAWDAAVTVDAQPDELPIAAAAAVAVALDAAVQ TAKDAIQVLGGIGFTWEHDAHFYLRRAVATRQVLGGST VWRSRLTTLVRAGARRHLGIDLSDHEEERARIRAEVEK IAAAPESERRVALAESGLLAPHWPQPYGRGAGAAEQLV VQEELAAAGIERPDLVIGWWAVPTILEHGTPEQIERFV MPTLRGDVVWCQLFSEPGAGSDLAALRTSAEKADGGWV LRGQKVWTSLAQQADWAICLARTDRDVPKHKGITYFLV DMKSAGITISPLREITGDALFNEVFLDSVFVPDDCVVG NLGDGWKLARTTLANERVAMGGKSSLGQSIEELLELST PGDPVAEDRIATQIGEATVGSLLDLRATLAQLEGQDPG AASSVRKLIGVRQRQDTAELAMDLAGEAGWVEGPLTRE FLNTRCLTIAGGTEQILLTVAAERLLGLPRG

fadE34#2 Rhodococcus rhodochrous (SEQ ID NO: 22)	N/A	MTLGLSDEDRELRDSVRGWAARHATPDVIRTAVEAKTE ARPTYWSSFAELGMLGLHLPEEVGGAGFGLLETAIVAE ELGRAMVPGPFLPTVIVSAVLDEAGRRSELDGLADGSL FGAVALQPGDLRVERDGDSVTLSGTSGVALGGQVADVF LLAADDGGERVFVVVTRDRVEVTNLPSYDVIRRNAEIT VSAVPLSDGDVLESDPHRIVDIAATLFAAEAAGLADWA TTTAADYARVRKQFGRVIGQFQGVKHTVARMLCLTEQA RVVAWDAARARREDVPDDEASLAVAVAASIAPEAAFQV TKNCIQVLGGIGYTWEHDAHLYMRRAQSLRILLGSTAS WRRRVAHLTLGGARRVLSVDLPPEAERIRADVRAELEP AKSLENAARKAYLAEKGYTAPHLPEPWGKAADAVTQLV VAEELRAAELEPHDMIIGNWVVPTLIAHGSTEQIERFV PQSLRGDLVWCQLFSEPGAGSDLAGLSTKAVKVDGGWR LDGQKVWTSMARVADWGICLARTDAEAPKHKGLSYFLI DIRNTEGLDIRPLREITGEALFNEVFLDGVFVPDECLV GEPGDGWKLARTTLANERVSLSHDSTFGAGCETLIALA NGMPGGPDDEQLTVLGKVLGDAASGGLMGLRTALRSLA GAQPGAESSVAKLLGVEHLQQVWETAMDWAGTASLLDD QDRTSATHMFLNVQCMSIAGGTTNVQLNIIGERLLGLP RDPEPGK
fadE26 Rhodococcus rhodochrous (SEQ ID NO: 23)	ADP09632.1	MDISYTPGQQALREELRAYFAQIMTPERREALAATTGE YGSGNVYREVVQQMGKDGWLTLGWPEEYGGQNRSAMDQ LIFTDEAAIAGAPVPFLTIDSVAPTIMHYGTDEQKEFF LPRISAGELHFSIGYSEPGAGTDLASLRTTAVRDGDEW VINGQKMWTSLIAYADYVWLAARTNPDVKKHKGISVFI VPTDAPGFSYTPVHTMAGPDTSATYYQDVRVPASALVG EVDGGWALITNQLNHERVALTSAGPVRTALTEVRRWAQ ETHLPDGRRVIDQEWVQINLARVHAKAEYLQLMNWDIA SSAGTTPLGPEAASANKVFGTEFATEAYRLLMEVLGPA ATVRQNSAGALLRGRIERMHRSSLILTFGGGTNEVQRD IIAMTALGQPPAKR
fadE34 Mycobacterium neoaurum (SEQ ID NO: 24)	N/A	VSVLSVPTDTSDEAAARELVRDWVPSSGSITAIRNVEL GDPQAWRTPFAGFAELGVFGVAVPEEYGGAGSTVADLL AMIDEAAAGLIPGPVAGTALATLVADDPAVLEALATGE RSAGIAMTSDITVDSGTATGTAPHVLGADPGGVLILPA GQHWILVDASSDGVTIDPLEATDFSRPLARVTLTSAPA QQLNASAQRVTDLMATVLAAELAGLSRWLLNTANEYAK VREQFGKPIGSFQAVKHMCAEMLLRSQQVTVAAADAIA AAAGDDADQLSVAAAVAAAIGIDAAKLNARDCIQVLGG IGITWEHDAHLYLRRAYANAQFLGGRSRWLRRVVELTR

		AGVRRELHVDTADADAIRPEIAAAAARIAALPEDQRGR ALAESGLLAPHWPTPYGRDATPAEQLVIDEELAAAEVA RPDISIGWWAAPTILAAGTPEQIDRFIPGTLNGDIFWC QLFSEPGAGSDLAALRTKAVRVEKDGRTGWSLTGQKVW TSNAHRANWGICLARTNPDAPKHKGISYFLVDMSSPGI DIRPLREITGEALFNEVFFDDLFVPDDCVVGEVDGGWP LARTTLANERVAIATGGALDKGMEHLLAVIGDRELDGA EADRLGALITLAQVGSLLDQLIARMALGGNDPGAPSSV RKLIGVRYRQGLAEAAMEFQDGGGIVDSPDVRYFLNTR CLSIAGGTEQILLTLAGERLLGLPR

APPENDIX C – Strains and plasmids referred to in the Examples

Strain code	Full name	Strain description	Reference
DH5α	*E. coli* DH5α	General host for cloning	Bethesda Research Laboratories
S17-1	*E. coli* S17-1	Host strain for conjugal mobilization of pK18mobsacB-derived mutagenic plasmids to *Rhodococcus* strains	DSMZ collection
WT	*Rhodococcus rhodochrous* DSM43269	Wild-type strain	DSMZ collection
RG32	WTΔ*kshA1*Δ*kshA2*Δ*kshA3*Δ*kshA4*Δ*kshA5*	5-fold *kshA* null mutant in WT	Wilbrink *et al* 2011
RG35	RG32Δ*kstD3*	Deletion of *kstD3* in RG32	This work
RG36	RG32Δ*kstD1*Δ*kstD3*	Deletion of *kstD1* in RG35	This work
RG41	RG32Δ*kstD1*Δ*kstD2*Δ*kstD3*	Deletion of *kstD2* in RG36. *kshA* null + *kstD1, 2* and *3* mutant	This work
LM3	RG41Δ*fadE34*	Deletion of *fadE34* in RG41	This work
LM15	RG41Δ*fadE34#2*	Deletion of *fadE34#2* in RG41	This work
LM9	RG41Δ*fadE34*Δ*fadE34#2*	Deletion of *fadE34#2* in LM3	This work
LM33	RG41Δ*fadE34*Δ*fadE34#2*Δ*fadE26*	Deletion of *fadE26* in double mutant LM9	This work
LM19	RG41Δ*fadE34*Δ*fadE34#2* + *kshA5-complem*	Complementation with *kshA5* in LM9	This work
Mneo	*Mycobacterium neoaurum* NRRL B-3805	Parent strain	Marsheck *et al*, 1972
Mneo-Δ*fadE34*	*M. neoaurum* NRRL B-3805-Δ*fadE34*	Deletion of *fadE34* in Mneo	This work
	Plasmid	**Description**	**Reference**
	pBluescript(II)KS	General cloning vector	Stratagene
	pZErO-2.1	General cloning vector	Invitrogene
	pk18mobsacB	Conjugative plasmid for gene mutagenesis in *Rhodococcus;* aphII sacB oriT (RP4) lacZ	Gene (1994) 145: 69
	pKSH800	Clone isolated from genomic library of WT strain carrying *kshA3* and *kstD3*	Wilbrink *et al*, 2011
	pKSH841	pK18mobsacB-derived mutagenic plasmid for deletion of *kstD3* in RG32	This work
	pKSH852	pK18mobsacB-derived mutagenic plasmid for deletion of *kstD1* in RG35	This work
	pKSD321	clone isolated from genomic library of RG36 strain carrying *kstD2*	This work

	pKSD326	pK18mobsacB-derived mutagenic plasmid for deletion of *kstD2* in RG36	This work
	pK18+*fadE34*-UP+DOWN	pK18mobsacB-derived mutagenic plasmid for deletion of *fadE34* in RG41	This work
	pK18+*fadE34#2*-UP+DOWN	pK18mobsacB-derived mutagenic plasmid for deletion of *fadE34#2* in RG41 and LM3	This work
	pDEL-*fadE26*	pK18mobsacB-derived mutagenic plasmid for deletion of *fadE26* in LM9	Wilbrink *et al*, 2011
	pK18+*kshA5*-complementation	pK18mobsacB-derived mutagenic plasmid for complementation with *kshA5* in LM9	This work
	pK18+*fadE34*_Mneo -UP+DOWN	pK18mobsacB-derived mutagenic plasmid for deletion of *fadE34* in Mneo	This work

APPENDIX D – Primers referred to in the Examples

Target Gene	PCR amplicon	Size	Primer name	Primer sequence (5'-3')
kstD1	Construction and checking deletion *kstD1*	WT: 2.4kb / Δ*kstD1*: 1.3kb	kstD1-F	TGGCAGCAGAACTCGCCGGG (SEQ ID NO: 25)
			kstD1-R	CCGGAACGACACCGATGCGCCG (SEQ ID NO:26)
kstD2	Construction and checking deletion *kstD2*	WT: 0.8kb / Δ*kstD2*: no amplif	kstD2-F	CTACAGCGACTACCACCCCGATTT (SEQ ID NO:27)
			kstD2-R	CTGTTGCGGTACTTCTGGTCGAA (SEQ ID NO:28)
kstD3	Checking deletion *kstD3*	WT: 2.9kb / Δ*kstD3*: 2kb	kstD3-F	CGACCTGTCACAGGGCGAGAT (SEQ ID NO:29)
			kstD3-R	GGACCACCTTGAACGCGTAGAAC (SEQ ID NO:30)
fadE34	Upstream region for deletion *fadE34*	1.5kb	FadE34-UP_F	GCGATAAGATCTTGGTGGCGGATG ACGTCGAG (SEQ ID NO:31)
			FadE34-UP_R	GCGATATCTAGAGGCCCGCTGCTC CTCGGTC (SEQ ID NO:32)
	Downstream region for deletion *fadE34*	1.5kb	FadE34-DOWN_F	GCGATATCTAGAATCGCCGGCGGG ACCGAG (SEQ ID NO:33)
			FadE34-DOWN_R	GCGATAAAGCTTGCAGGAACTTCC GCTTCT (SEQ ID NO:34)
fadE34 #2	Upstream region for deletion *fadE34#2*	1.5kb	FadE34#2-UP_F	GCGATAAGATCTCCTTCTGCTGGT CGATCTG (SEQ ID NO:35)
			FadE34#2-UP_R	CGCTATTCTAGAGAGTTCGGCGAA CGAGCTCC (SEQ ID NO:36)
	Downstream for deletion region *fadE34#2*	1.5kb	FadE34#2-DOWN_F	GCGATATCTAGATTGCTCGACGAC CAGGACCGAACTTC (SEQ ID NO:37)
			FadE34#2-DOWN_R	CGCTATAAGCTTAGCTGTGCGGTG GCGCCGCTG (SEQ ID NO:38)
fadE34	Checking deletion *fadE34*	WT: 5.4kb / Δ*fadE34*: 3.4kb	Flanking_fadE34-F	GAACGCGAGCGCGGCGATGACCTC T (SEQ ID NO:39)
			Flanking_fadE34-R	GGTCCAGCTGAAGCCGGGATCCTT G (SEQ ID NO:40)
fadE34 #2	Checking deletion *fadE34#2*	WT: 5.7kb / Δ*fadE34#2*: 3.8kb	Flanking_fadE34#2_F	GAGGTCGCCGAACTCGCCGGTGTC GCCATC (SEQ ID NO:41)
			Flanking_fadE34#2_R	GCGTGCACCTGTTCGCGGTCGGTG ACATCC (SEQ ID NO:42)

kshA5	Construction and checking complementation *kshA5*	Δ*kshA5:* 1.2kb / complemented: 2.2kb	kshA5-complem-F	GCGATAGGATCCGGCCCGGATTGTCGCTGATG (SEQ ID NO:43)
			kshA5-complem-R	CGCTATAAGCTTGATCACGTGCAGCATGC (SEQ ID NO:44)
*fadE34*_Mneo	Upstream region for deletion *fadE34*_Mneo	1.5kb	FadE34_Mneo-UP-F	GCGATAGGATCCGACACCGACTTCCTGCTGTTG (SEQ ID NO:45)
			FadE34_Mneo-UP-R	CGCTATTCTAGACCGATGTCCGGTACTTCCTC (SEQ ID NO:46)
	Downstream region for deletion *fadE34*_Mneo	1.5kb	FadE34_Mneo-DOWN-F	GCGATATCTAGAGATCGCCGAGTTCGACGTTG (SEQ ID NO:47)
			FadE34_Mneo-DOWN-R	CGCTATAAGCTTGTGACGATCACCGCGAACTC (SEQ ID NO:48)
*fadE34*_Mneo	Checking deletion *fadE34*_Mneo	parent: 2.5kb / Δ*fadE34*: 0.5kb	FadE34_Mneo-F	AGATTCGGTGCAGACCGATTG (SEQ ID NO:49)
			FadE34_Mneo-R	AAGCTGCATGCGGATCCAC (SEQ ID NO:50)

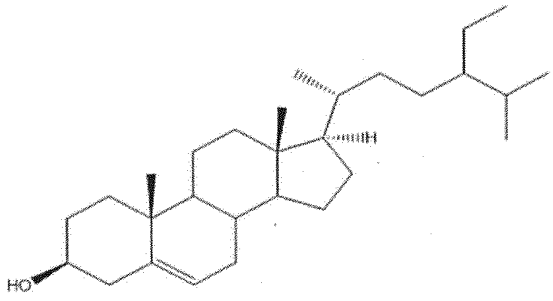
CLAIMS

1. A genetically-modified bacterium blocked in the steroid metabolism pathway prior to degradation of the polycyclic steroid ring system, wherein the bacterium is disrupted in the steroid side-chain degradation pathway, and wherein the bacterium converts a steroidal substrate into a steroidal product of interest.

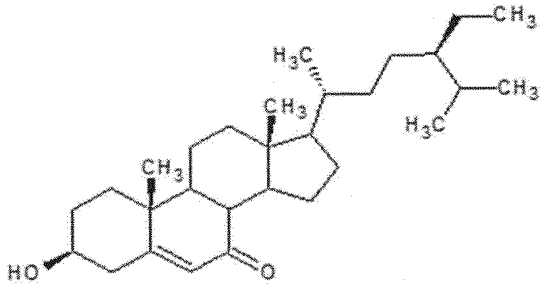
2. The genetically-modified bacterium of Claim 1, wherein the disruption in the steroid side-chain degradation pathway occurs after the first cycle of β-oxidation.

3. The genetically-modified bacterium of Claim 1 or Claim 2, wherein the steroidal substrate is a sterol substrate.

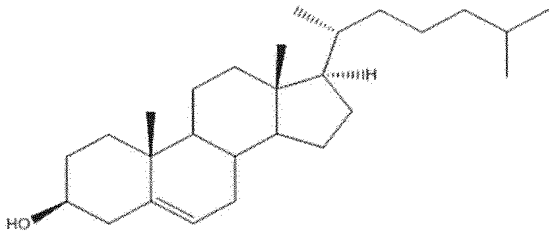
4. The genetically-modified bacterium of Claim 3, wherein the sterol substrate comprises:



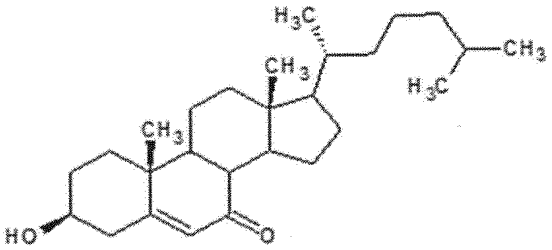
β-sitosterol;



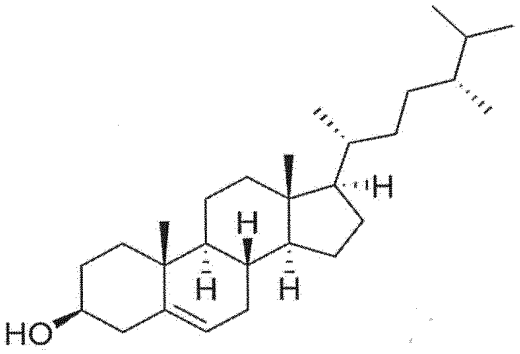
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



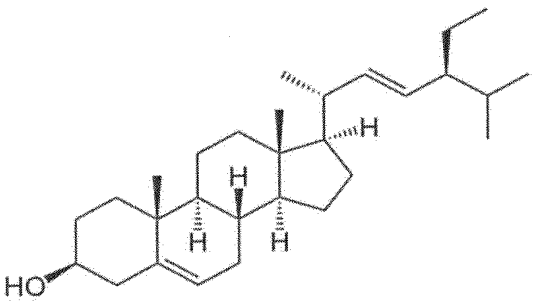
cholesterol;



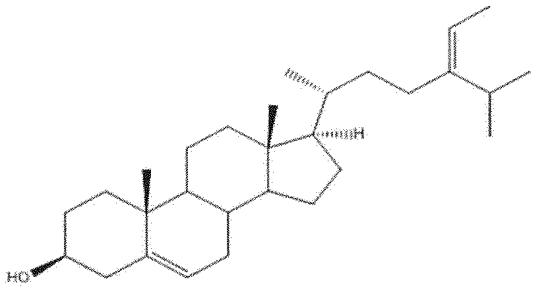
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



stigmasterol;



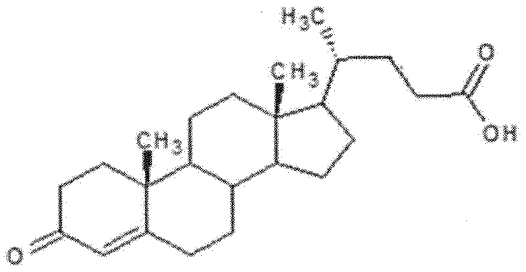
fucosterol;

7-oxo-phytosterol; or a combination thereof.

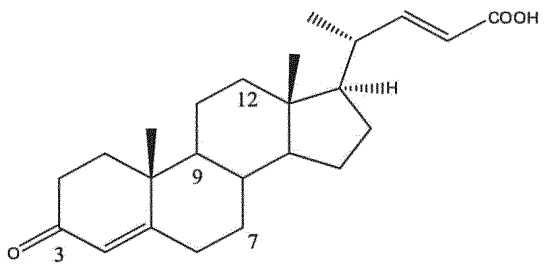
5. The genetically-modified bacterium of any of Claims 1 to 4, wherein the steroidal product of interest comprises an intact polycyclic ring system.

6. The genetically-modified bacterium of any of Claims 1 to 5, wherein the steroidal product of interest is a steroidal compound with a side-chain having a backbone of five carbons.

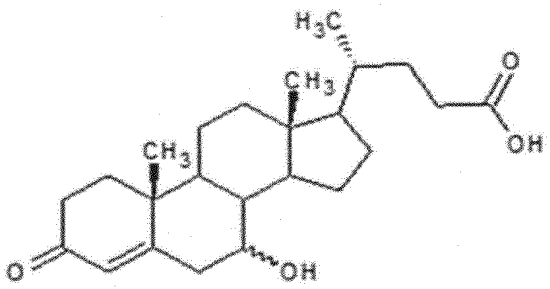
7. The genetically-modified bacterium of Claim 5, wherein the steroidal product of interest is:



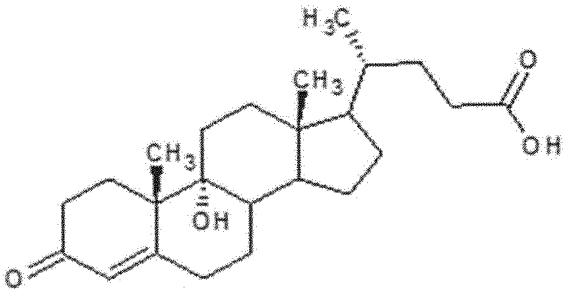
3-oxo-4-cholenic acid;



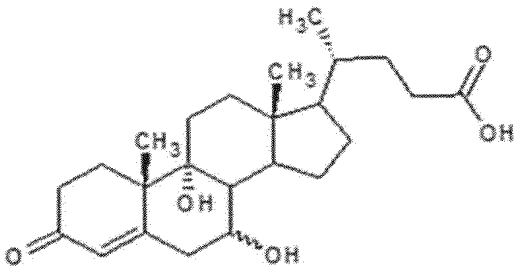
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



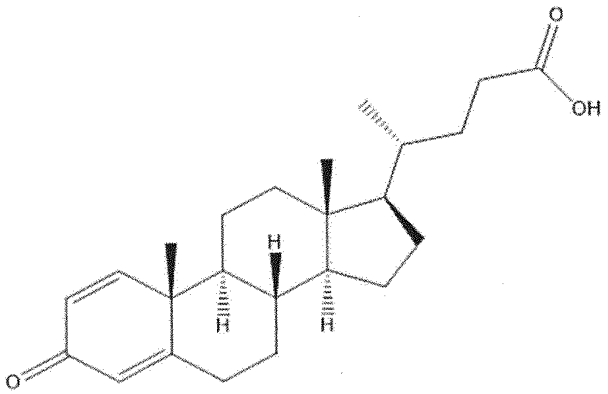
3-oxo-7-hydroxy-4-cholenic acid;



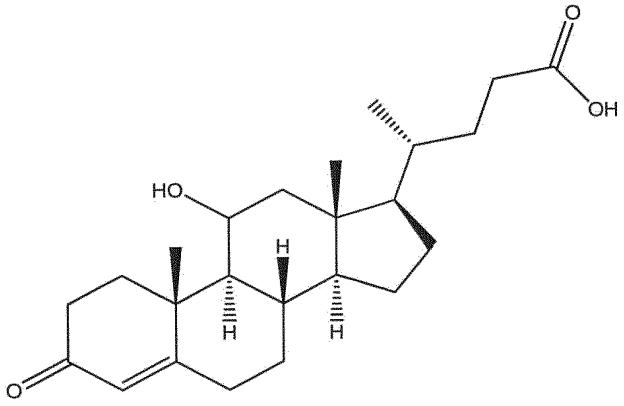
3-oxo-9-hydroxy-4-cholenic acid;



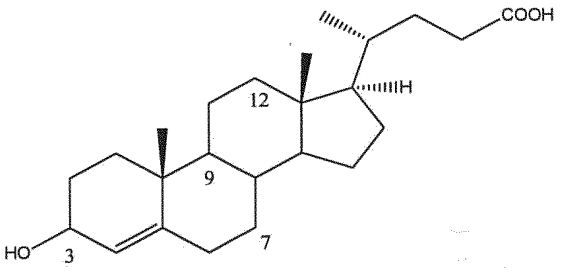
3-oxo-7,9-dihydroxy-4-cholenic acid;



3-oxo-1,4-choladienoic acid;



3-oxo-11-hydroxy-4-cholenic acid;



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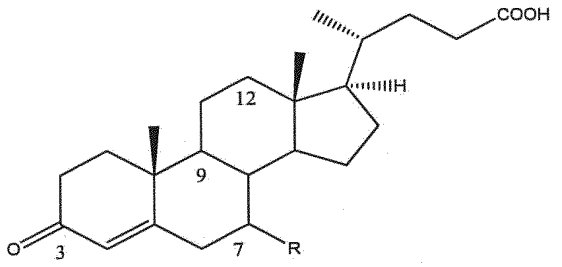
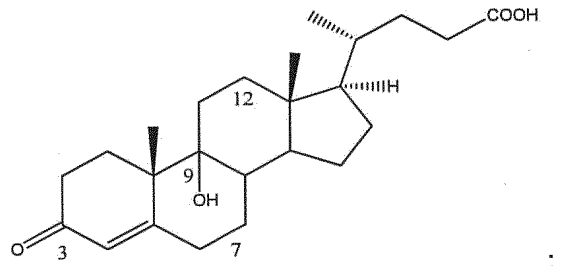


wherein R can be hydroxyl, oxo, or a halogen;



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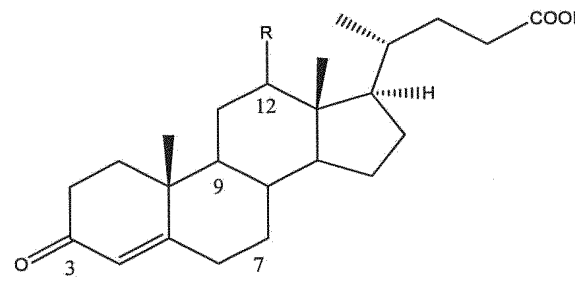
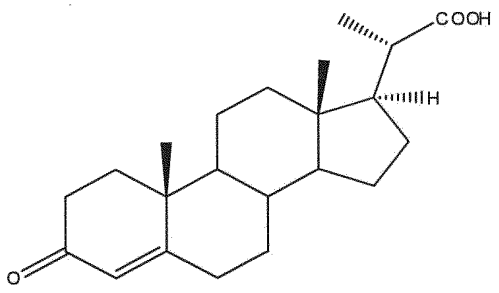
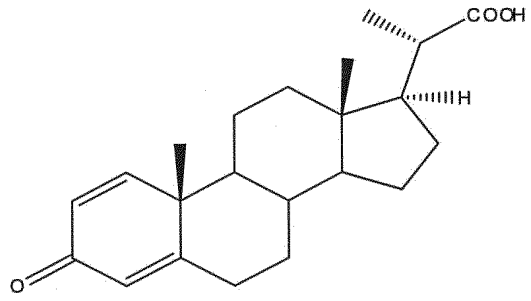


wherein R can be hydroxyl or oxo;



4-BNC

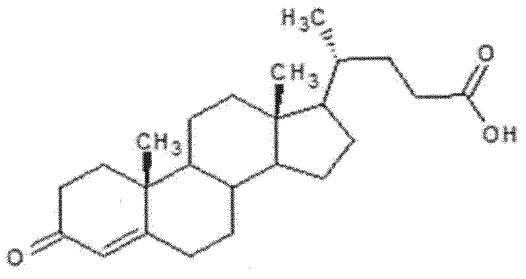
3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC);



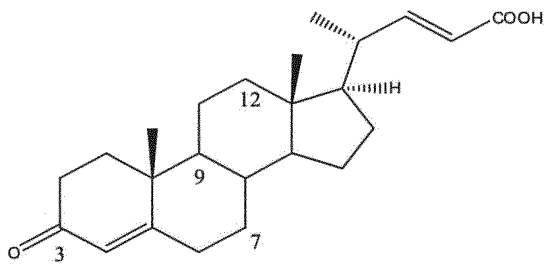
1,4-BNC

3-oxo-23,24-bisnor-1,4-choladiene-22-oic acid (1,4-BNC); or variants thereof.

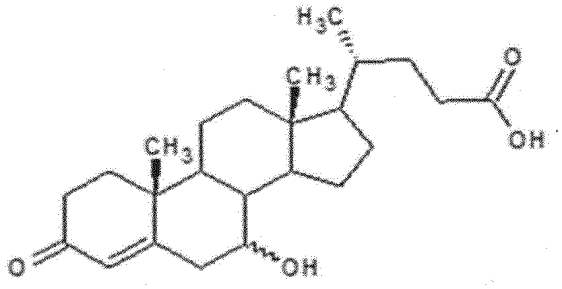
8. The genetically-modified bacterium of Claim 5 or Claim 6, wherein the steroidal product of interest is:



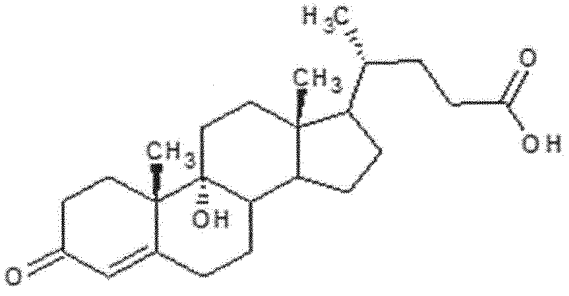
3-oxo-4-cholenic acid;



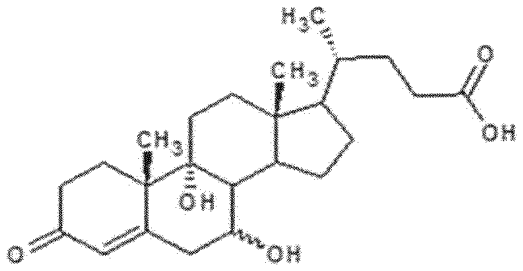
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



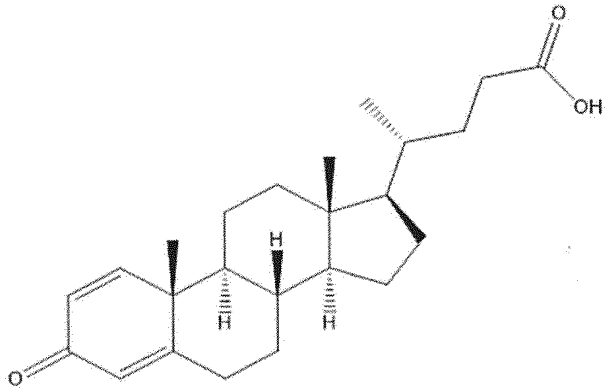
3-oxo-7-hydroxy-4-cholenic acid;



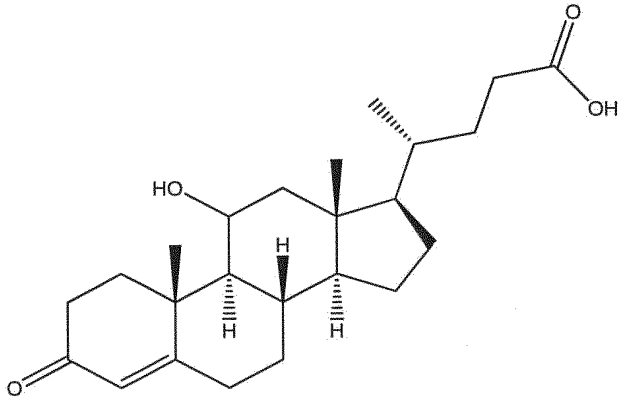
3-oxo-9-hydroxy-4-cholenic acid;



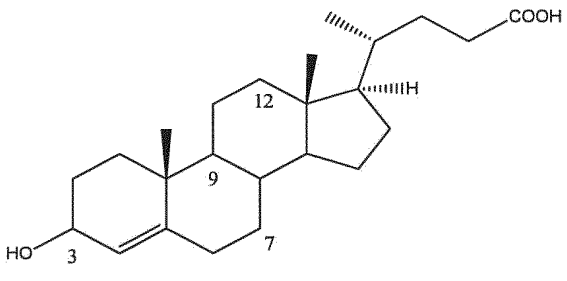
3-oxo-7,9-dihydroxy-4-cholenic acid;



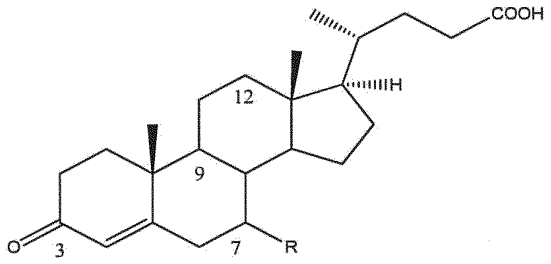
3-oxo-1,4-choladienoic acid;



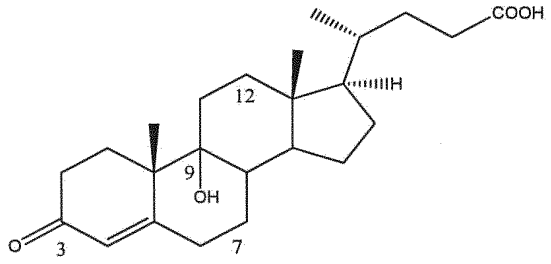
3-oxo-11-hydroxy-4-cholenic acid;



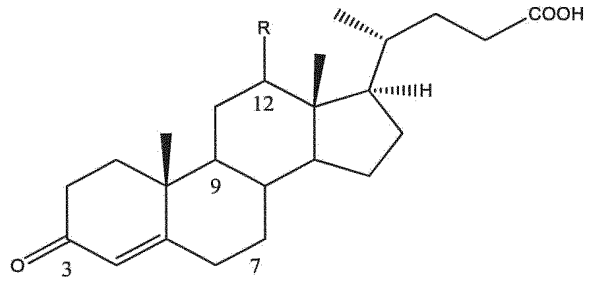
;



wherein R can be hydroxyl, oxo, or a halogen;



;



wherein R can be hydroxyl or oxo; or variants thereof.

9. The genetically-modified bacterium of any of Claims 1 to 8, wherein the bacterium is of the *Actinobacteria* class or the *Gammaproteobacteria* class.

10. The genetically-modified bacterium of Claim 9, wherein the bacterium of the *Actinobacteria* class is a *Rhodococcus* species, a *Mycobacterium* species, a *Nocardia* species, a *Corynebacterium* species, or an *Arthrobacter* species.

11. The genetically-modified bacterium of Claim 10, wherein the *Rhodococcus* species is *Rhodococcus rhodochrous*, *Rhodococcus erythropolis*, *Rhodococcus jostii*, *Rhodococcus ruber*, preferably *Rhodococcus rhodochrous*.

12. The genetically-modified bacterium of Claim 10, wherein the *Mycobacterium* species is *Mycobacterium neoaurum*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis, or Mycobacterium fortuitum,* preferably *Mycobacterium neoaurum*.

13. The genetically-modified bacterium of Claim 10, wherein the *Nocardia* species is *Nocardia restrictus*, *Nocardia corallina*, or *Nocardia opaca*.

14. The genetically-modified bacterium of Claim 10, wherein the *Arthrobacter* species is *Arthrobacter simplex*.

15. The genetically-modified bacterium of any of Claims 1 to 14, wherein the genetic modification comprises inactivation of the genes: *kshA1* (SEQ ID NO: 1), *kshA2* (SEQ ID NO: 2), *kshA3* (SEQ ID NO: 3), *kshA4* (SEQ ID NO: 4), and *kshA5* (SEQ ID NO: 5), or homologs thereof.

16. The genetically-modified bacterium of any of Claim 15, wherein the genetic modification further comprises re-introduction of a wild type copy of the *kshA5* gene comprising SEQ ID NO: 5, or a homolog thereof.

17. The genetically-modified bacterium of any of Claims 1 to 14, wherein the genetic modifications comprise inactivation of the genes: *kshA1* (SEQ ID NO: 1), *kshA2* (SEQ ID NO: 2), *kshA3* (SEQ ID NO: 3), and *kshA4* (SEQ ID NO: 4), or homologs thereof.

18. The genetically-modified bacterium of any of Claims 15 to 17, wherein the genetic modification further comprises inactivation of the genes: *kstD1* (SEQ ID NO: 6), *kstD2* (SEQ ID NO: 7), and *kstD3* (SEQ ID NO: 8), or homologs thereof.

19. The genetically-modified bacterium of any of Claims 1 to 18, wherein the genetic modification comprises inactivation of one or more of the genes: *fadE34* (SEQ ID NO: 9; SEQ ID NO: 12), *fadE34#2* (SEQ ID NO: 10), or homologs thereof.

20. The genetically-modified bacterium of Claim 19, wherein the genetic modification further comprises inactivation of the gene: *fadE26* (SEQ ID NO: 11), or homologs thereof.

21. The genetically-modified bacterium of any of Claims 15 to 20, wherein the gene inactivation is by gene deletion.

22. The genetically-modified bacterium of any of Claims 15 to 21, wherein the homolog has a nucleotide sequence with at least 50% sequence identity with the nucleotide sequence of a gene defined in those claims.

23. The genetically-modified bacterium of Claim 22, wherein the homolog has a nucleotide sequence that has a sequence identity of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

24. The genetically-modified bacterium of any of Claims 15 to 21, wherein the homolog encodes a polypeptide that has an amino acid sequence with at least 50% sequence identity with the amino acid sequence of a polypeptide encoded by a gene defined in those claims.

25. The genetically-modified bacterium of Claim 24, wherein the homolog encodes a polypeptide that has an amino acid sequence identity of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

26. A genetically-modified *Rhodococcus rhodochrous* bacterium of strain: LM9 (Accession No. NCIMB 43058), LM19 (Accession No. NCIMB 43059), or LM33 (Accession No. NCIMB 43060)

27. A genetically-modified *Mycobacterium neoaurum* bacterium of strain: NRRL B-3805 *Mneo*-Δ*fadE34* (Accession No. NCIMB 43057).

28. A genetically-modified bacterium according to any of Claims 1 to 27 for use in the conversion of a steroidal substrate into a steroidal compound of interest.

29. A method of converting a steroidal substrate into a steroidal product of interest, comprising the steps of:

(a) inoculating culture medium with genetically-modified bacteria according to any of Claims 1 to 27 and growing the bacterial culture until a target OD_{600} is reached;

(b) adding a steroidal substrate to the bacterial culture when the target OD_{600} is reached;

(c) culturing the bacterial culture so that the steroidal substrate is converted to the steroidal product of interest; and,

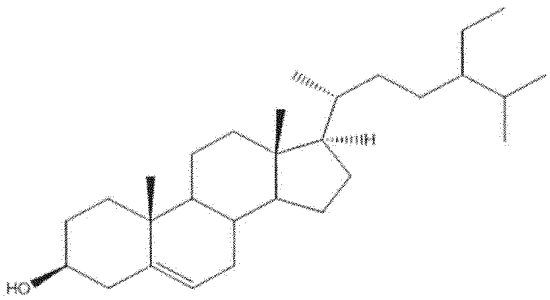
(d) extracting and/or purifying the steroidal product of interest from the bacterial culture.

30. The method according to Claim 29, wherein the culture medium is LB medium or minimal medium.

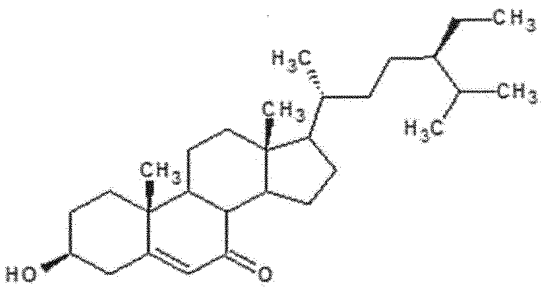
31. The method according to Claim 29 or Claim 30, wherein in step (a) the bacterial culture is grown to a target OD_{600} of at least 1.0, preferably at least 4.0, more preferably at least 4.5, most preferably at least 5.0.

32. The method according to any of Claims 29 to 31, wherein the steroidal substrate is a sterol substrate.

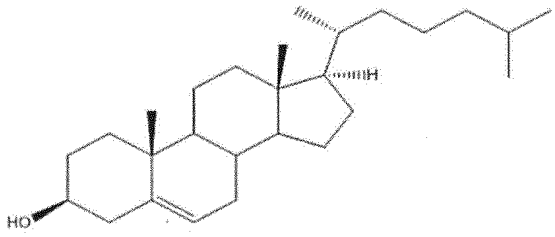
33. The method according to Claim 32, wherein the sterol substrate comprises:



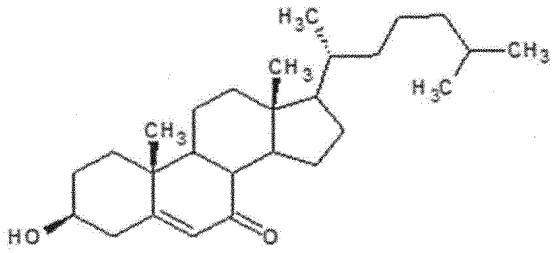
β-sitosterol;



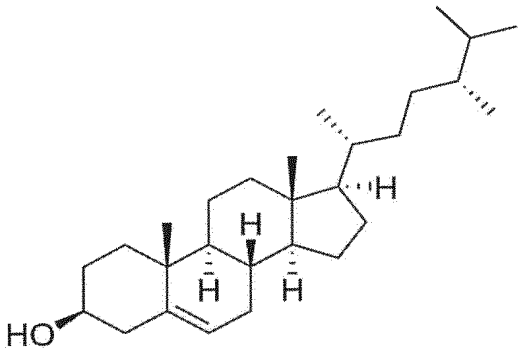
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



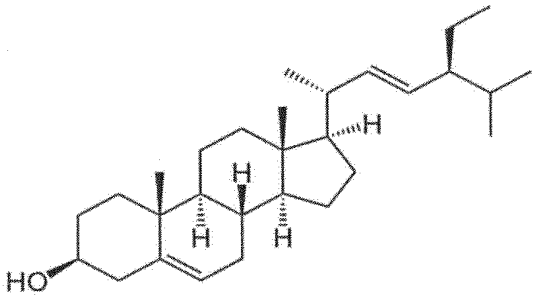
cholesterol;



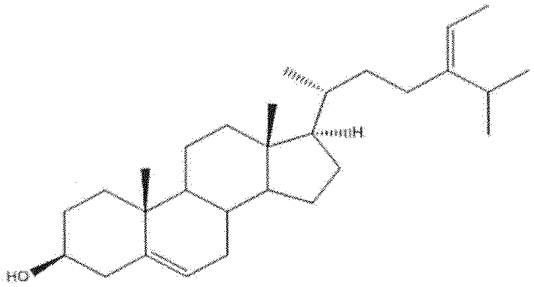
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



stigmasterol;



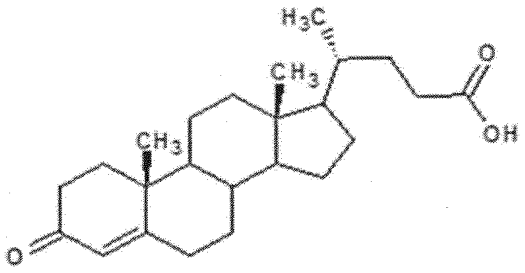
fucosterol;

7-oxo-phytosterol; or a combination thereof.

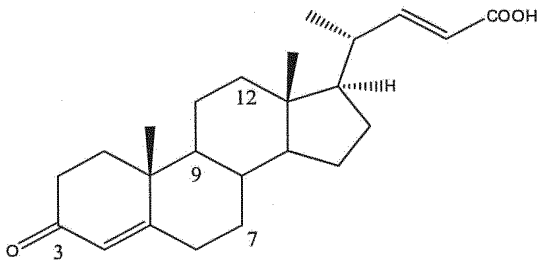
34. The method according to of any of Claims 29 to 33, wherein the steroidal product of interest comprises an intact polycyclic ring system.

35. The method according to of any of Claims 29 to 34, wherein the steroidal product of interest is a steroidal compound with a side-chain having a backbone of five carbons.

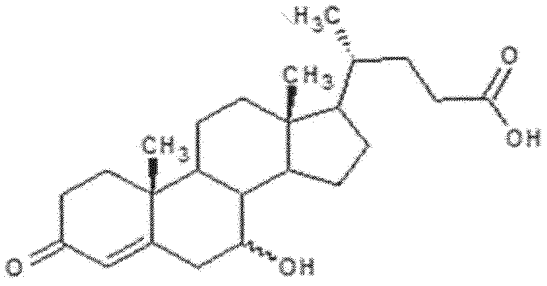
36. The method according to Claim 34, wherein the steroidal product of interest is:



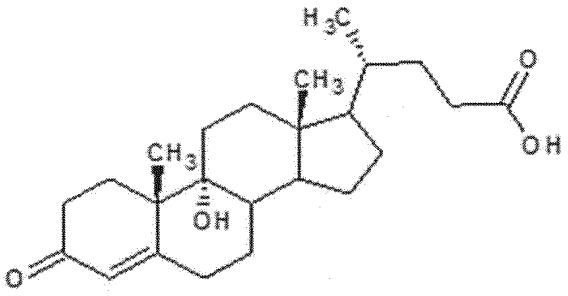
3-oxo-4-cholenic acid;



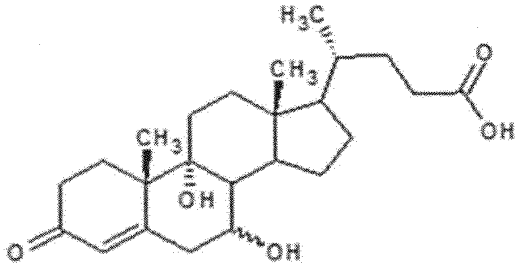
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



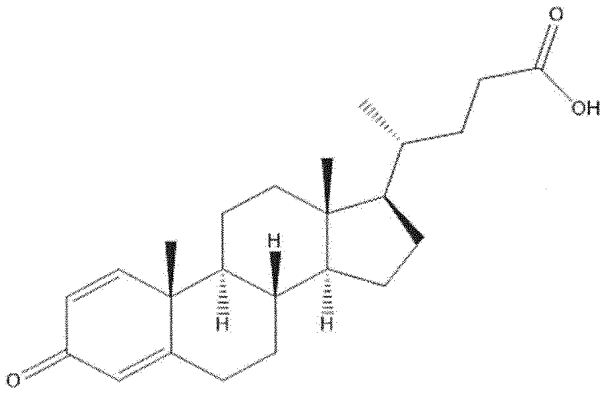
3-oxo-7-hydroxy-4-cholenic acid;



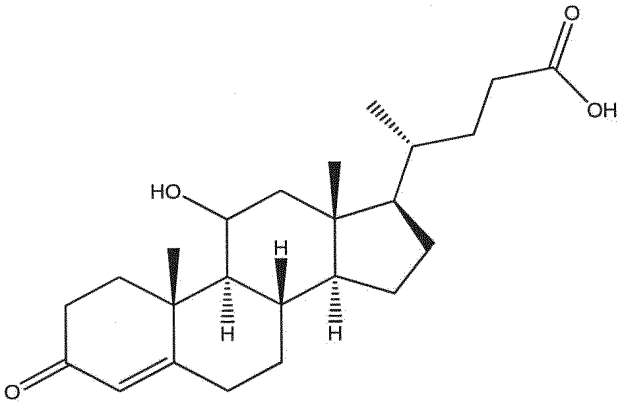
3-oxo-9-hydroxy-4-cholenic acid;



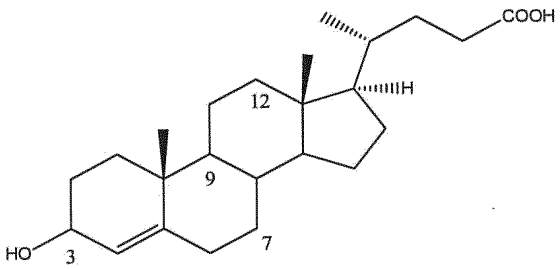
3-oxo-7,9-dihydroxy-4-cholenic acid;



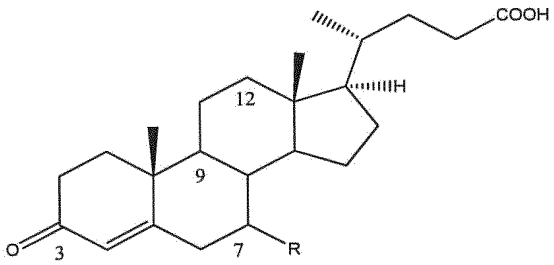
3-oxo-1,4-choladienoic acid;



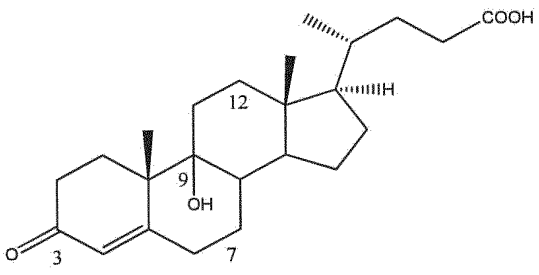
3-oxo-11-hydroxy-4-cholenic acid;



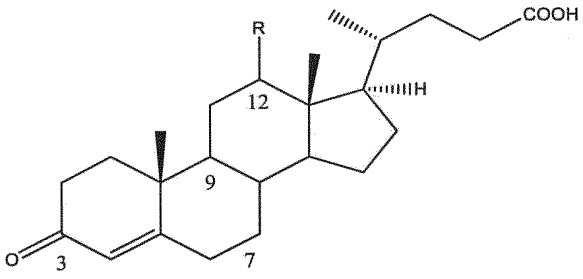
;



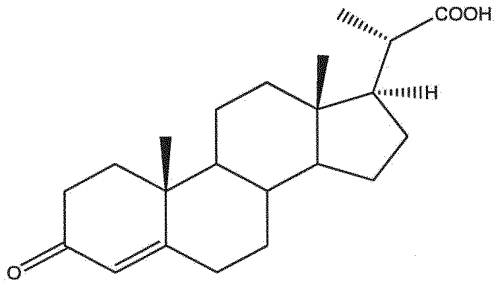
wherein R can be hydroxyl, oxo, or a halogen;



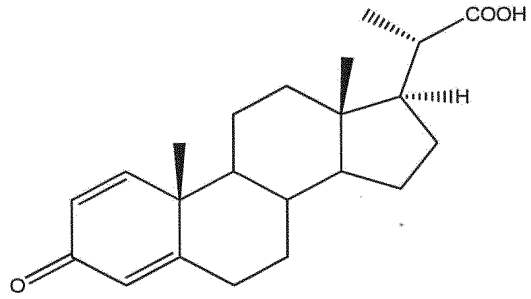
;



wherein R can be hydroxyl or oxo;

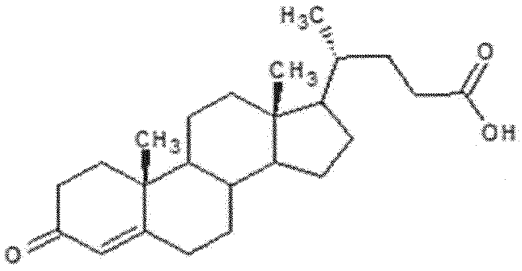


3-oxo-23,24-bisnor-4-cholene-22-oic acid (4-BNC);

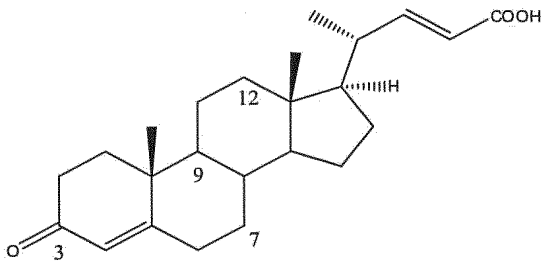


3-oxo-23,24-bisnor-1,4-choladiene-22-oic acid (1,4-BNC); or variants thereof.

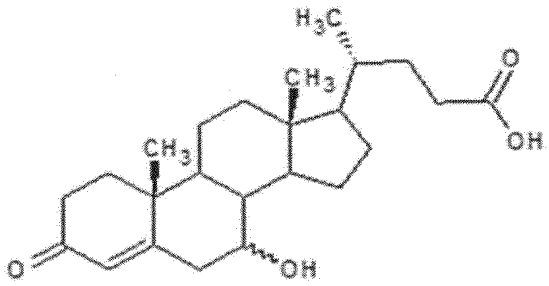
37. The method according to Claim 34 or Claim 35, wherein the steroidal product of interest is:



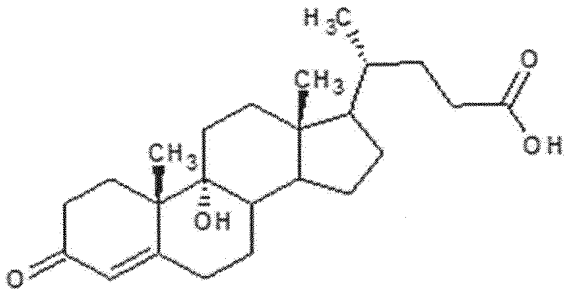
3-oxo-4-cholenic acid;



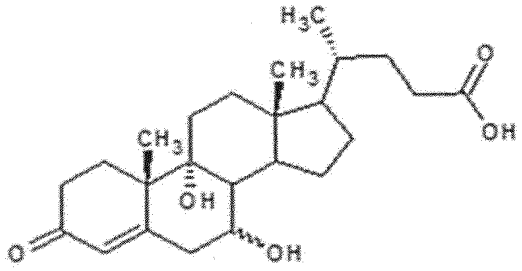
Chola 4,22-dien-24-oic acid, 3-oxo (CAS 59648-73-6, or CAS 82637-22-7 for pure E isomer);



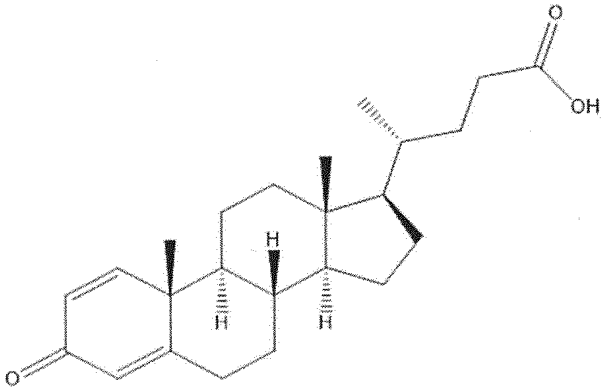
3-oxo-7-hydroxy-4-cholenic acid;



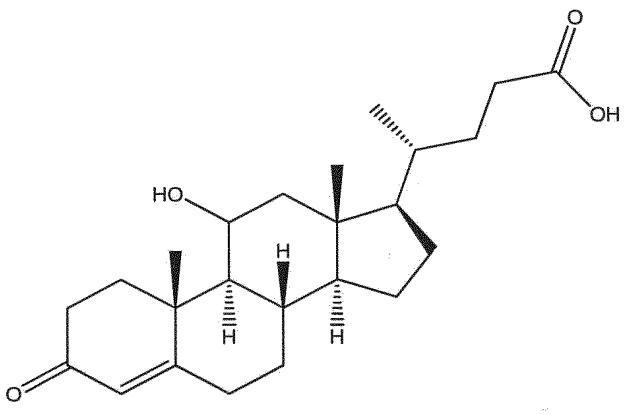
3-oxo-9-hydroxy-4-cholenic acid;



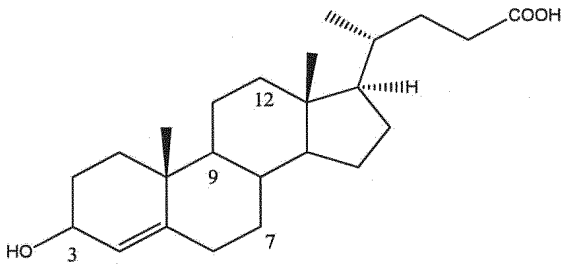
3-oxo-7,9-dihydroxy-4-cholenic acid;



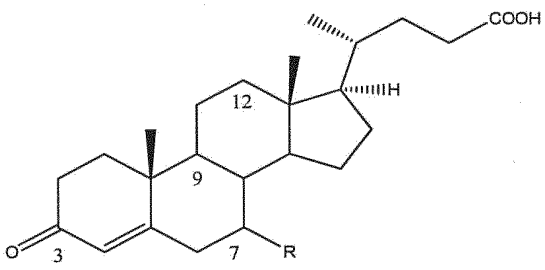
3-oxo-1,4-choladienoic acid;



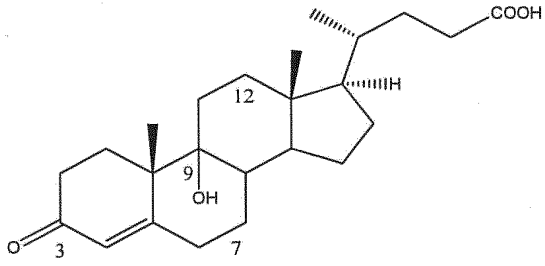
3-oxo-11-hydroxy-4-cholenic acid;



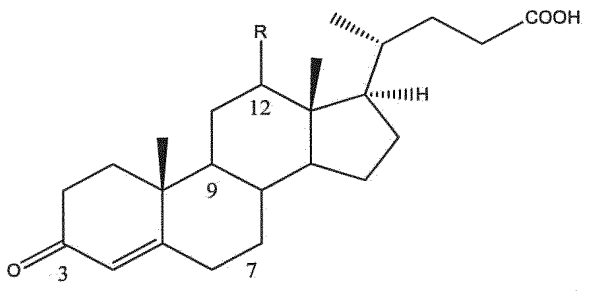
;



wherein R can be hydroxyl, oxo, or a halogen;



;



;

wherein R can be hydroxyl or oxo; or variants thereof.

38. The method of any of Claims 29 to 37, wherein in step (b) the steroidal substrate is added at a concentration of at least 0.1mM, at least 0.2mM, at least 0.3mM, at least 0.4mM, at least 0.5mM, at least 0.6mM, at least 0.7mM, at least 0.8mM, at least 0.9mM, at least 1.0mM, at least 1.1mM, at least 1.2mM, at least 1.3mM, at least 1.4mM, at least 1.5mM, at least 1.6mM, at least 1.7mM, at least 1.8mM, at least 1.9mM, or at least 2.0mM.

39. The method of Claim 38, wherein the steroidal substrate is added at a concentration of at least 1mM, preferably at least 1.5mM, more preferably at least 2.0mM.

40. The method according to any of Claims 29 to 39, wherein in step (b) a cyclodextrin is added to the culture medium, preferably a β-cyclodextrin or a γ-cyclodextrin.

41. The method according to Claim 40, wherein the cyclodextrin is a β-cyclodextrin, preferably a methyl-β-cyclodextrin or a 2-OH-propyl-β-cyclodextrin.

42. The method according to Claim 40 or Claim 41, wherein the cyclodextrin is added at a concentration of 1mM to 25mM, preferably 5mM to 25mM.

43. The method according to Claim 40 or 41, wherein the cyclodextrin is added at a concentration of at least 1mM, preferably at least 5mM, more preferably at least 12.5mM, most preferably at least 25mM.

44. The method according to any of Claims 29 to 43, wherein in step (b) an organic solvent is added to the culture medium.

45. The method according to Claim 44, wherein the organic solvent is ethanol, dimethylformamide (DMF), or acetone, preferably ethanol.

46. The method according to Claim 44 or Claim 45, wherein the organic solvent is added at a volume/volume (v/v) concentration of 1% to 20%, 2% to 19%, 3%, to 18%, 4% to 17%, 5% to 16%, 6% to 15%, 7% to 14%, 8%, to 13%, 9% to 12%, 10 % to 11%, preferably 5% to 20%, more preferably 5% to 15%.

47. The method according to any of Claims 29 to 46, wherein in step (b) a cyclodextrin and an organic solvent are added to the culture medium.

48. The method according to Claim 47, wherein the cyclodextrin is added at concentration of 1mM to 25mM and the organic solvent is added at a volume/volume (v/v) concentration of 1% to 10%, more preferably the cyclodextrin is added at concentration of 1mM to 10mM and the organic solvent is added at a volume/volume (v/v) concentration of 1% to 10%, yet more preferably the cyclodextrin is added at concentration of 1mM to 5mM and the organic solvent is added at a volume/volume (v/v) concentration of 1% to 5%, most preferably the cyclodextrin is added at concentration of 5mM and the organic solvent is added at a volume/volume (v/v) concentration of 5%.

49. The method according to Claim 47, wherein the cyclodextrin is added at a concentration of at least 1mM and the organic solvent may be added at a volume/volume (v/v) concentration of at least 1%, preferably the cyclodextrin is added at concentration of at least 5mM and the organic solvent may be added at a volume/volume (v/v) concentration of 5%.

50. A steroidal product of interest produced by the method of any of Claims 29 to 49.

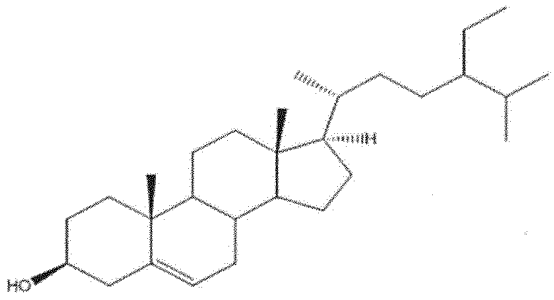
51. A kit for converting a steroidal substrate into a steroidal product of interest, wherein the kit comprises:

(a) a genetically-modified bacterium according to any of Claims 1 to 27; and,
(b) instructions for using the kit.

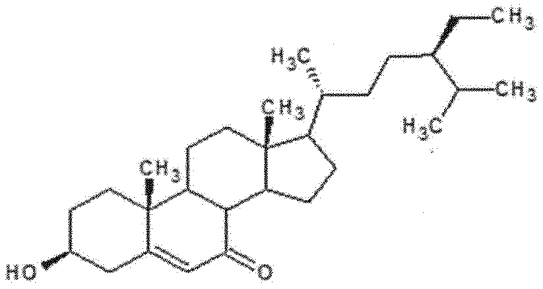
52. The kit according to Claim 51, wherein the kit further comprises a steroidal substrate.

53. The kit according to Claim 52, wherein the steroidal substrate is a sterol substrate.

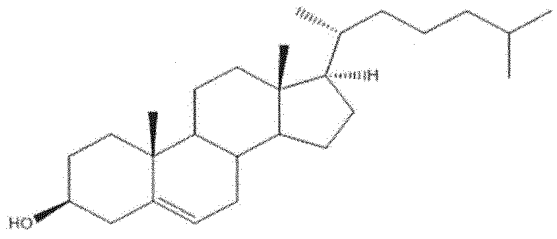
54. The kit according to Claim 53, wherein the sterol substrate comprises:



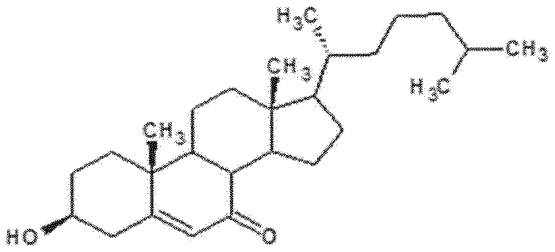
β-sitosterol;



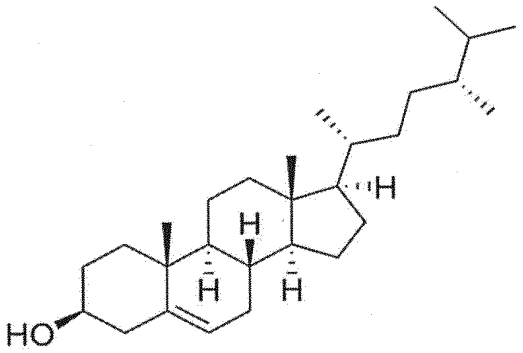
7-oxo-β-sitosterol or 7-hydroxy-β-sitosterol;



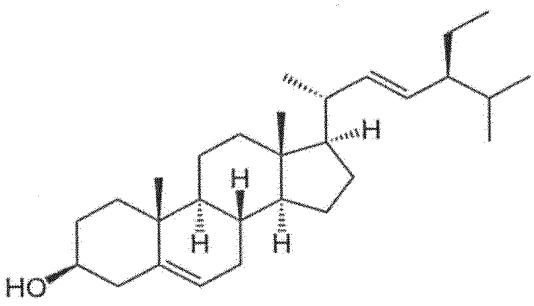
cholesterol;



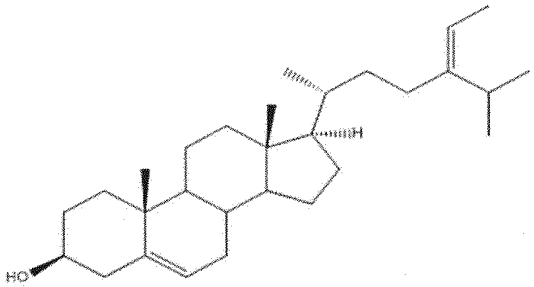
7-oxo-cholesterol or 7-hydroxy-β-cholesterol;



campesterol;



stigmasterol;



fucosterol;

7-oxo-phytosterol; or a combination thereof.

55. The kit according to any of Claims 51 to 54, wherein the kit further comprises a cyclodextrin, preferably a β-cyclodextrin or a γ-cyclodextrin.

56. The kit according to Claim 55, wherein the cyclodextrin is a β-cyclodextrin, preferably a methyl-β-cyclodextrin or a 2-OH-propyl-β-cyclodextrin.

57. The kit according to any of Claims 51 to 56, wherein the kit further comprises an organic solvent.

58. The kit according to Claim 57, wherein the organic solvent is ethanol, dimethylformamide (DMF), or acetone, preferably ethanol.

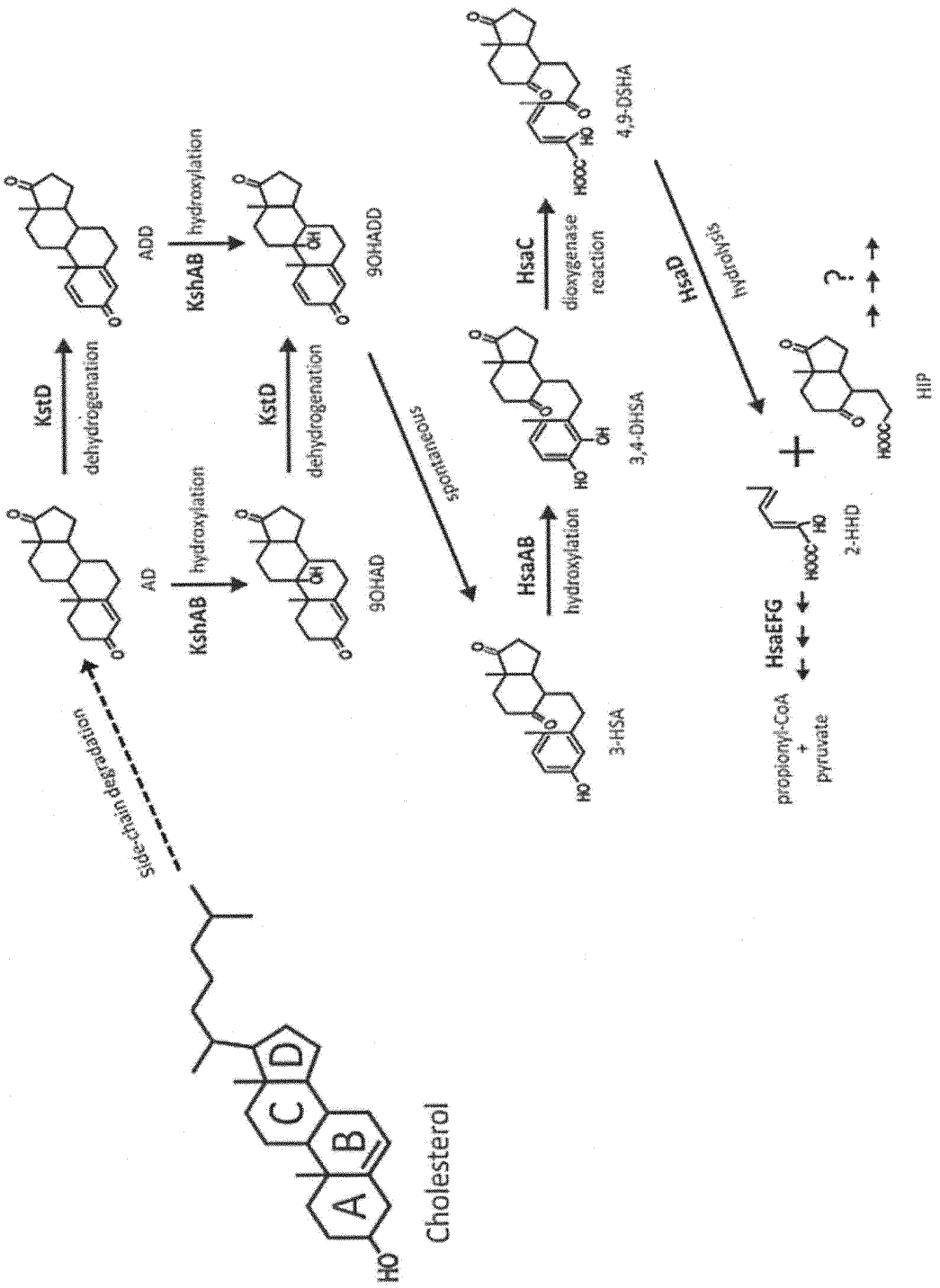

Cholesterol
Side-chain degradation
AD
ADD
9OHAD
9OHADD
KstD
dehydrogenation
KshAB
hydroxylation
spontaneous
HsaAB
3-HSA
3,4-DHSA
HsaC
dioxygenase reaction
4,9-DSHA
HsaD
hydrolysis
HsaEFG
propionyl-CoA
+
pyruvate
2-HHD
HIP
?

Figure 1

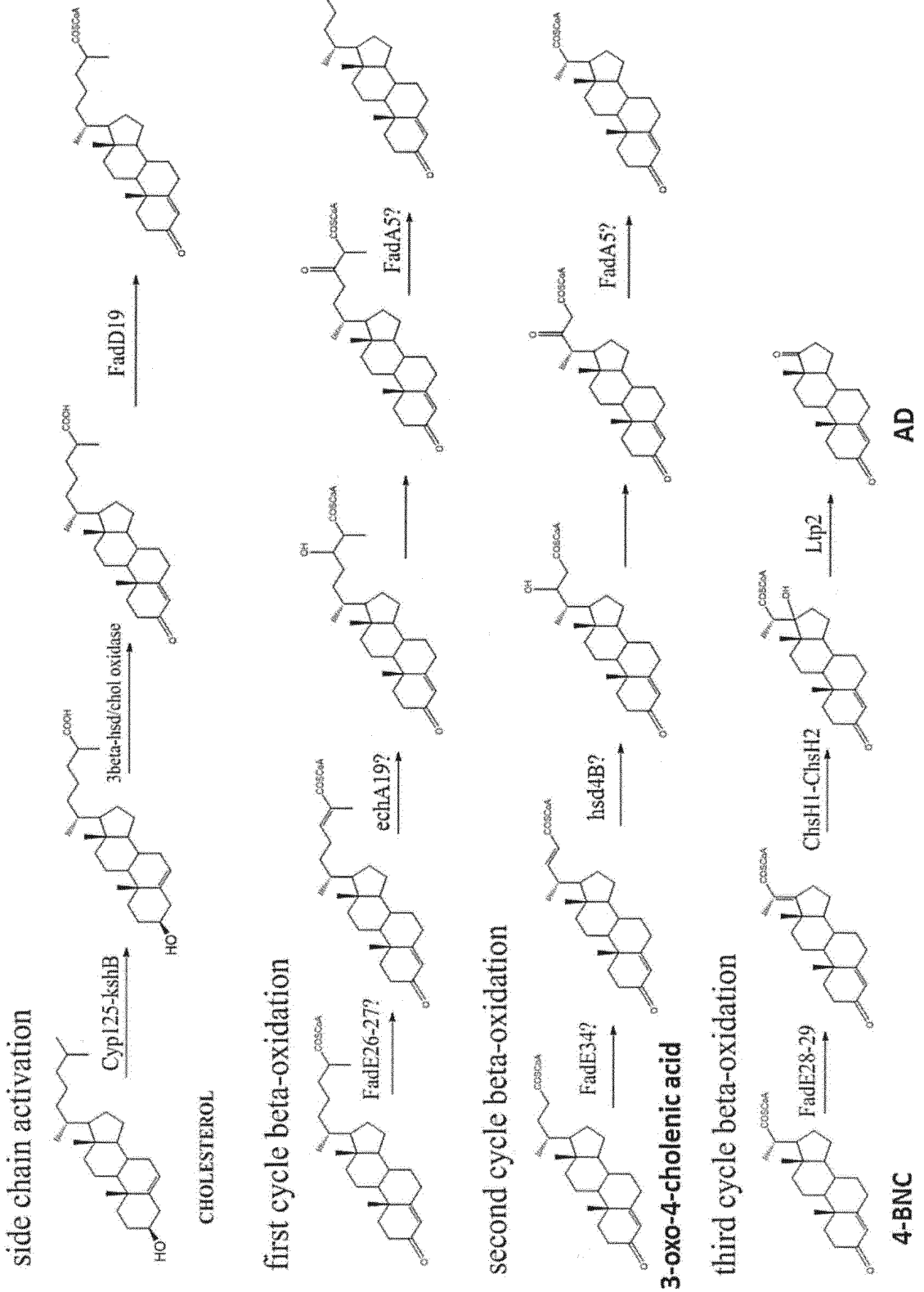

side chain activation
Cyp125-kshB
3beta-hsd/chol oxidase
FadD19
CHOLESTEROL
first cycle beta-oxidation
FadE26-27?
echA19?
FadA5?
second cycle beta-oxidation
FadE34?
hsd4B?
FadA5?
3-oxo-4-cholenic acid
third cycle beta-oxidation
FadE28-29
ChsH1-ChsH2
Ltp2
4-BNC
AD

Figure 2

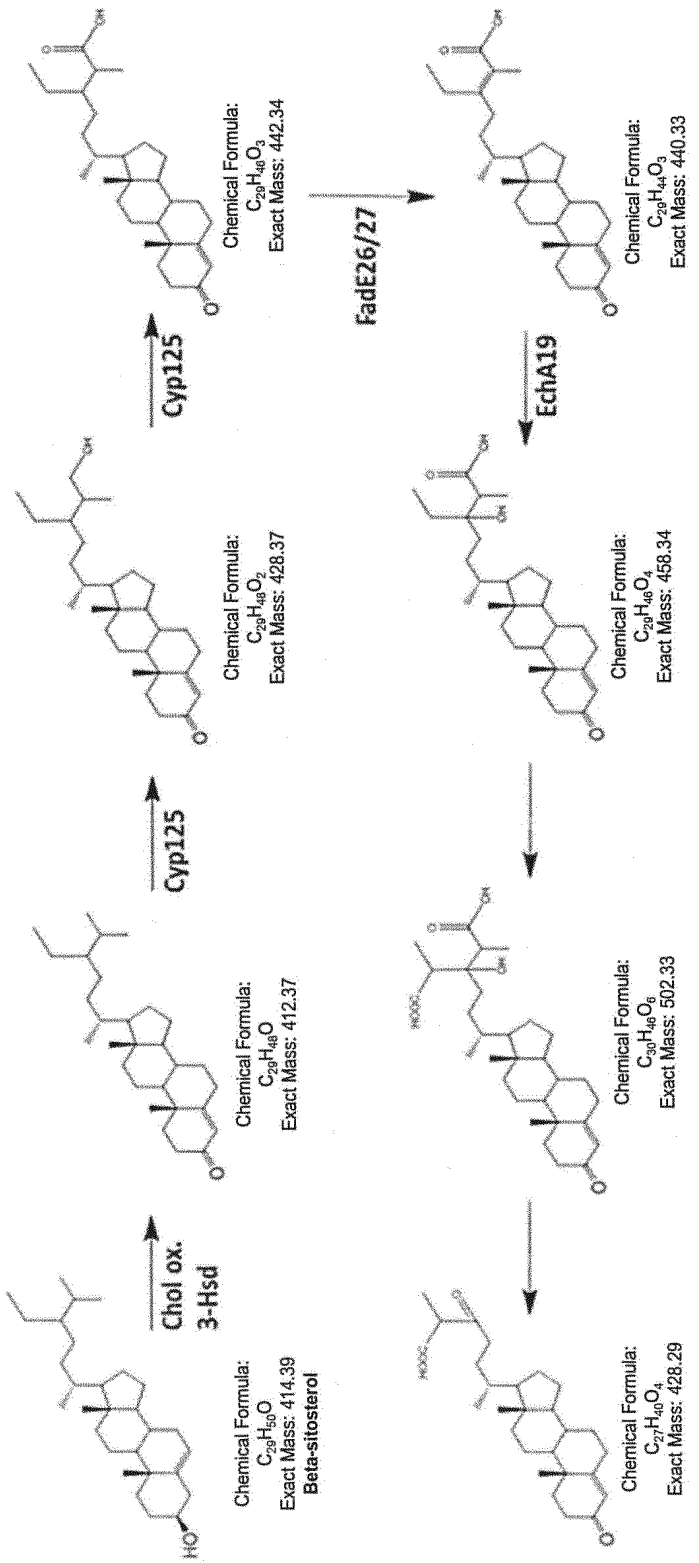
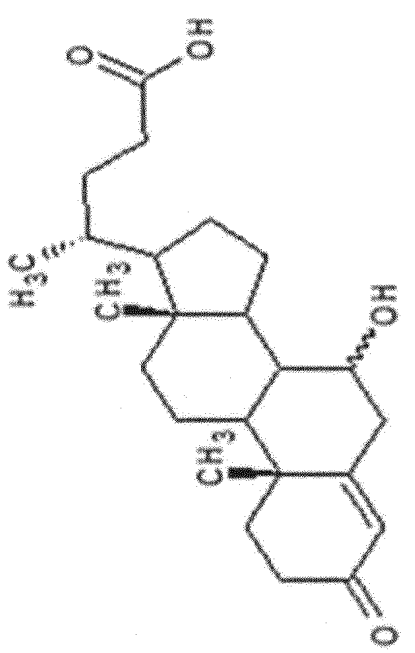
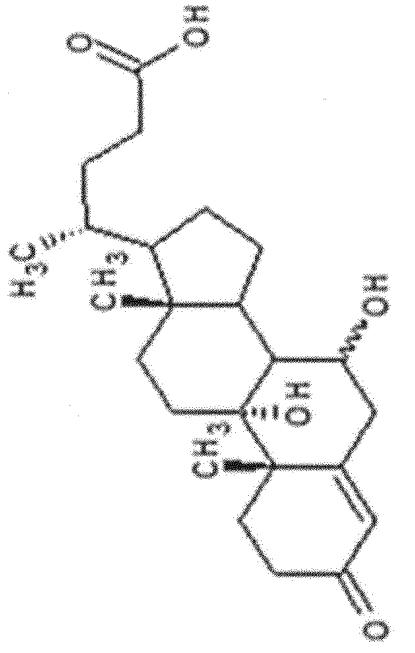

Chol ox.
3-Hsd
Cyp125
Cyp125
Cyp125
FadE26/27
EchA19
FadE34
FadE28/29
Chemical Formula: C29H50O
Exact Mass: 414.39
Beta-sitosterol
Chemical Formula: C28H48O
Exact Mass: 412.37
Chemical Formula: C28H48O2
Exact Mass: 428.37
Chemical Formula: C29H48O3
Exact Mass: 442.34
Chemical Formula: C27H40O4
Exact Mass: 428.29
Chemical Formula: C30H46O6
Exact Mass: 502.33
Chemical Formula: C29H46O4
Exact Mass: 458.34
Chemical Formula: C29H44O3
Exact Mass: 440.33
Chemical Formula: C24H36O3
Exact Mass: 372.27
3-oxo-4-cholenic acid
Chemical Formula: C22H32O3
Exact Mass: 344.24
4-BNC
Chemical Formula: C19H26O2
Exact Mass: 286.19
AD

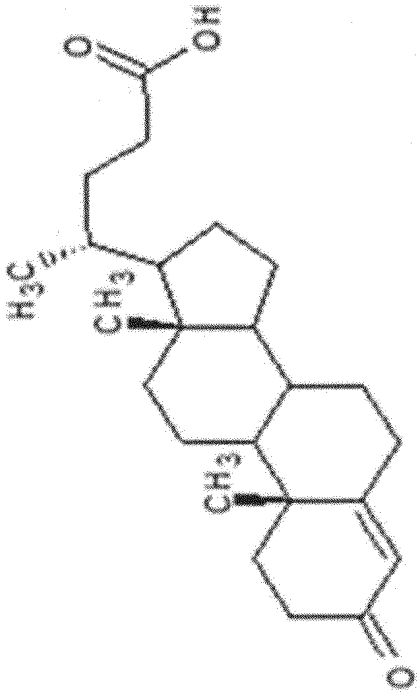
Figure 3



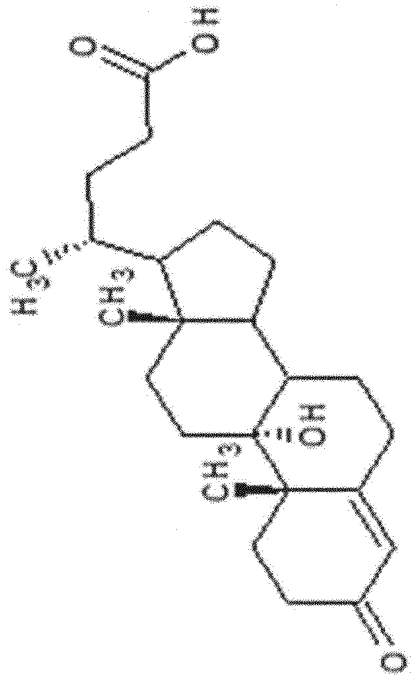
3-oxo-7-hydroxy-4-cholenic acid



3-oxo-7,9-dihydroxy-4-cholenic acid

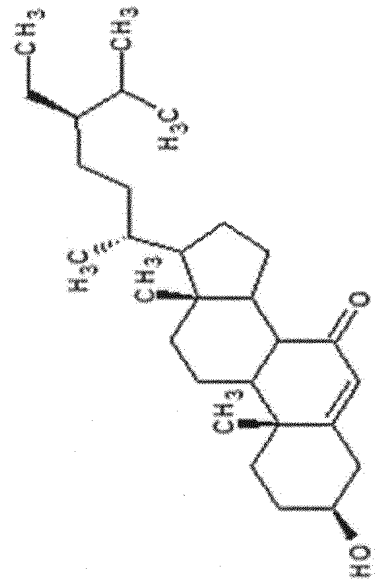


3-oxo-4-cholenic acid



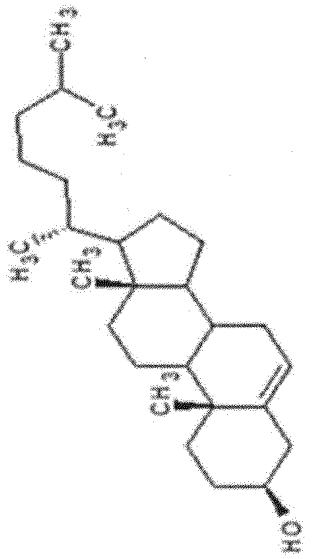
3-oxo-9-hydroxy-4-cholenic acid

Figure 4

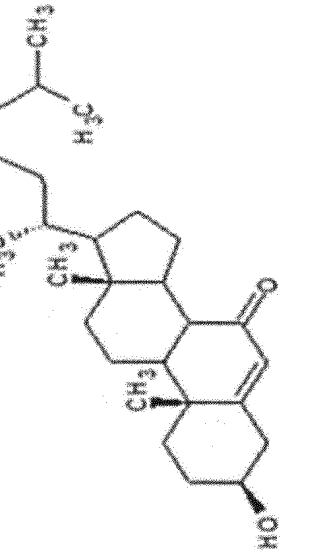


β-Sitosterol (Pure & Mixed)

7–Oxo–β-Sitosterol (Pure & Mixed)
or 7-Hydroxy-β-sitosterol



Cholesterol (Pure & Mixed)



7-oxo-Cholesterol (Pure & Mixed)
or 7-Hydroxy-β-cholesterol

Figure 5

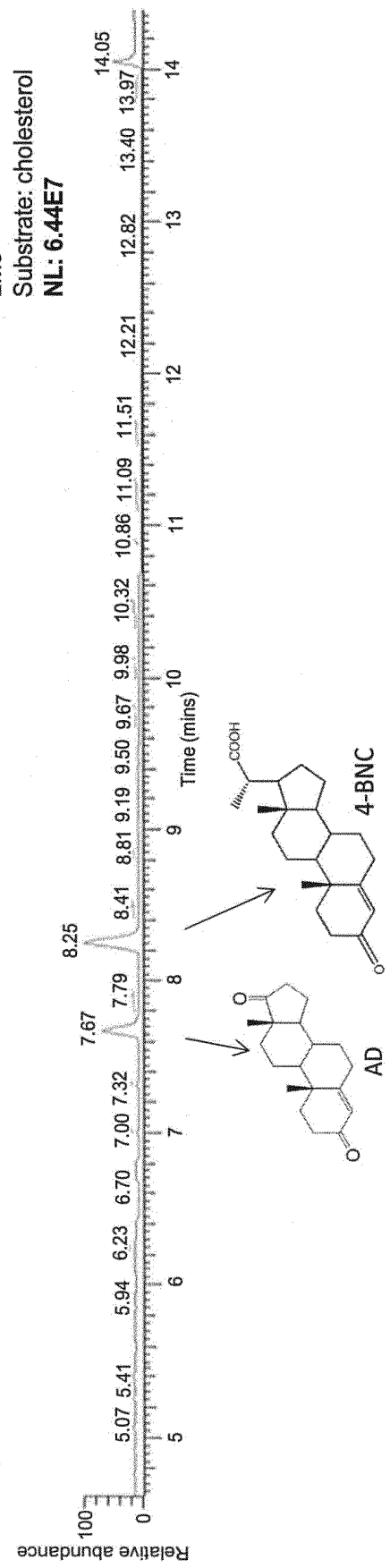

LM9
Substrate: cholesterol
NL: 6.44E7
Relative abundance
Time (mins)
5.07
5.41
5.94
6.23
6.70
7.00
7.32
7.67
7.79
8.25
8.41
8.81
9.19
9.50
9.67
9.98
10.32
10.86
11.09
11.51
12.21
12.82
13.40
13.97
14.05
AD
4-BNC
COOH

Figure 6

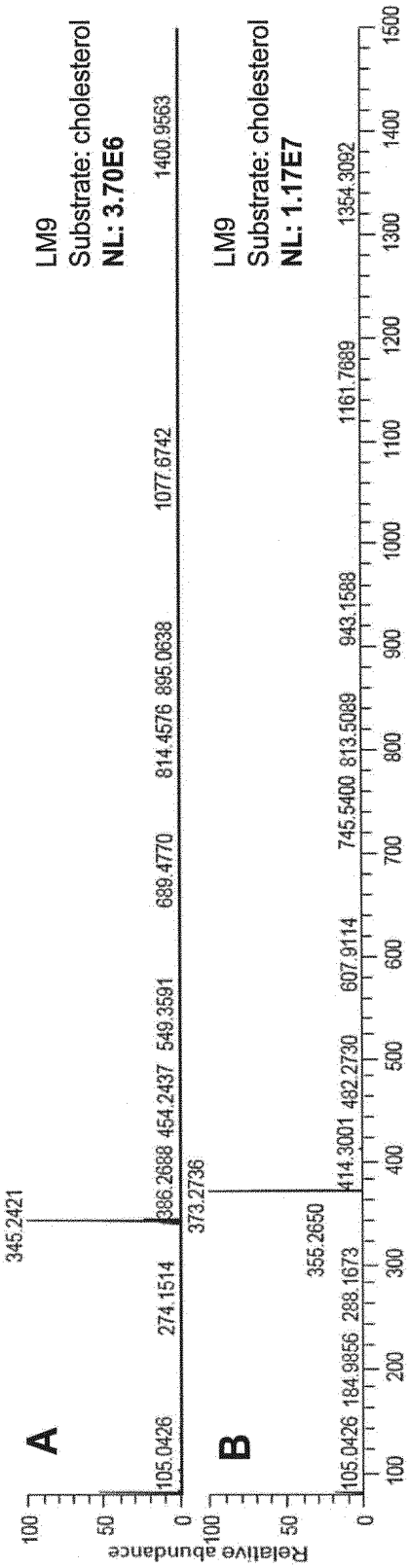

A
LM9
Substrate: cholesterol
NL: 3.70E6
105.0426
274.1514
345.2421
386.2688
454.2437
549.3591
689.4770
814.4576
895.0638
1077.6742
1400.9563
B
LM9
Substrate: cholesterol
NL: 1.17E7
105.0426
184.9856
288.1673
355.2650
373.2736
414.3001
482.2730
607.9114
745.5400
813.5089
943.1588
1161.7689
1354.3092
Relative abundance
m/z

Figure 7

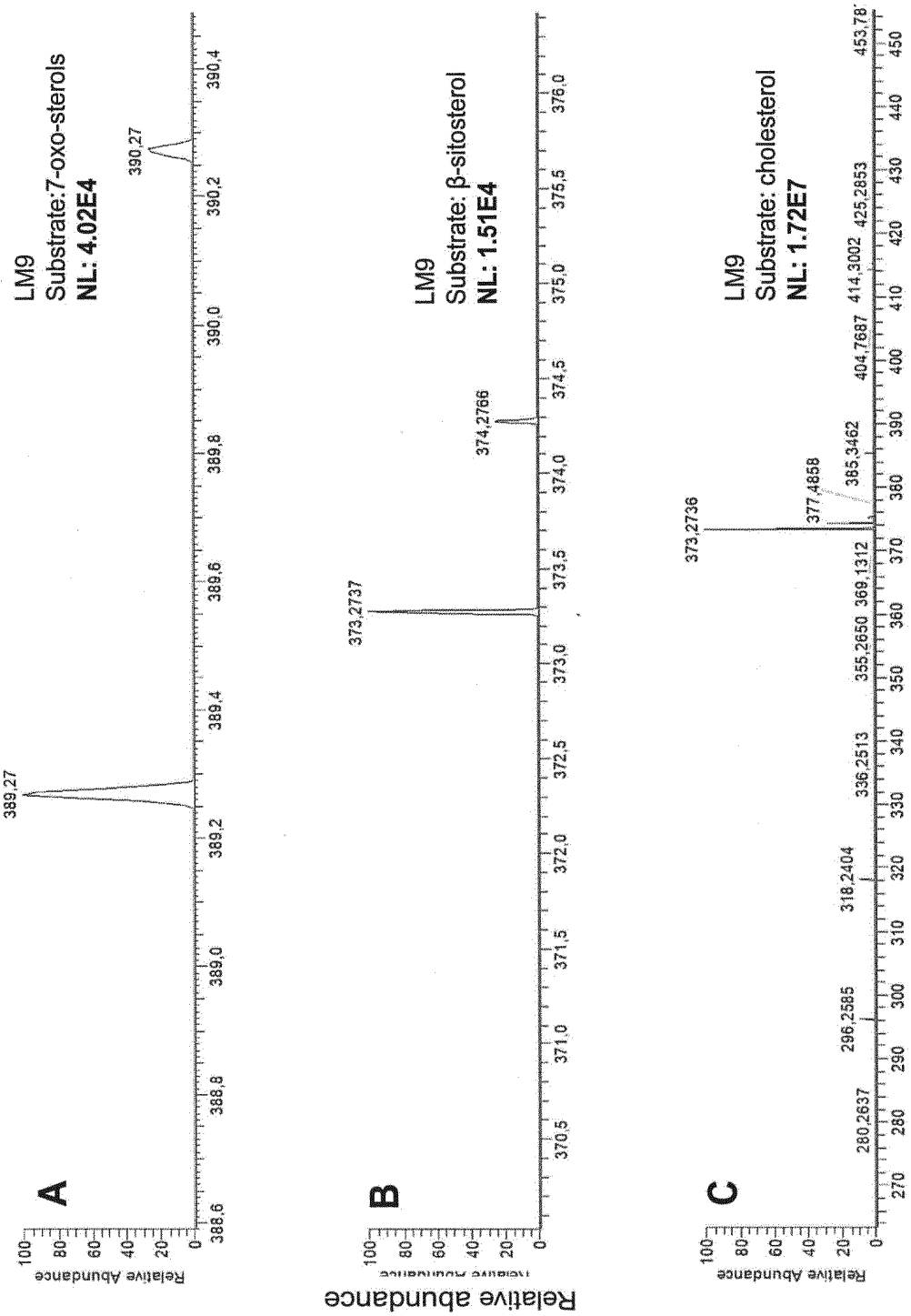

A
LM9
Substrate:7-oxo-sterols
NL: 4.02E4
389,27
390,27
Relative Abundance
B
LM9
Substrate: β-sitosterol
NL: 1.51E4
373,2737
374,2766
Relative abundance
C
LM9
Substrate: cholesterol
NL: 1.72E7
373,2736
377,4858
385,3462
404,7687
414,3002
425,2853
453,78
280,2637
296,2585
318,2404
336,2513
355,2650
369,1312
m/z

Figure 8

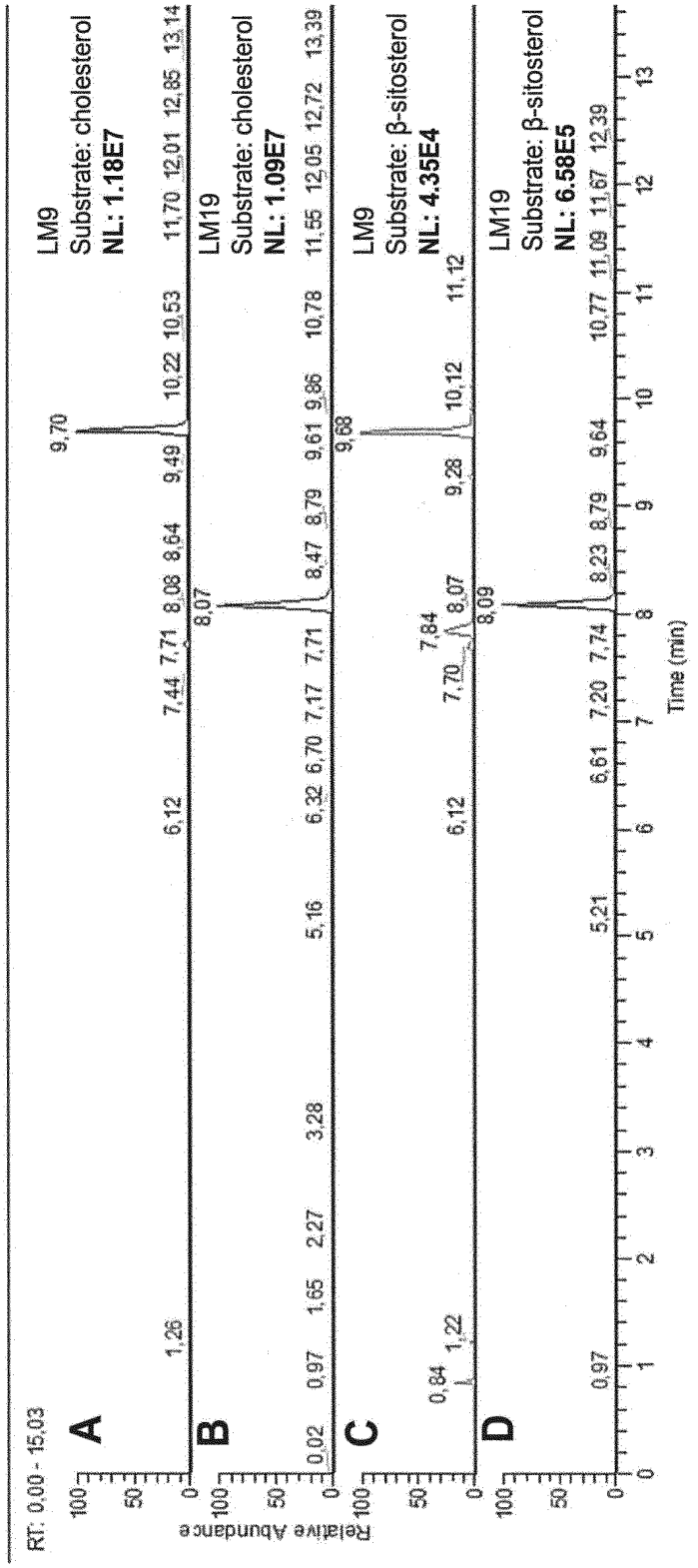

RT: 0,00 - 15,03
A
LM9
Substrate: cholesterol
NL: 1.18E7
B
LM19
Substrate: cholesterol
NL: 1.09E7
C
LM9
Substrate: β-sitosterol
NL: 4.35E4
D
LM19
Substrate: β-sitosterol
NL: 6.58E5
Relative Abundance
Time (min)

Figure 9

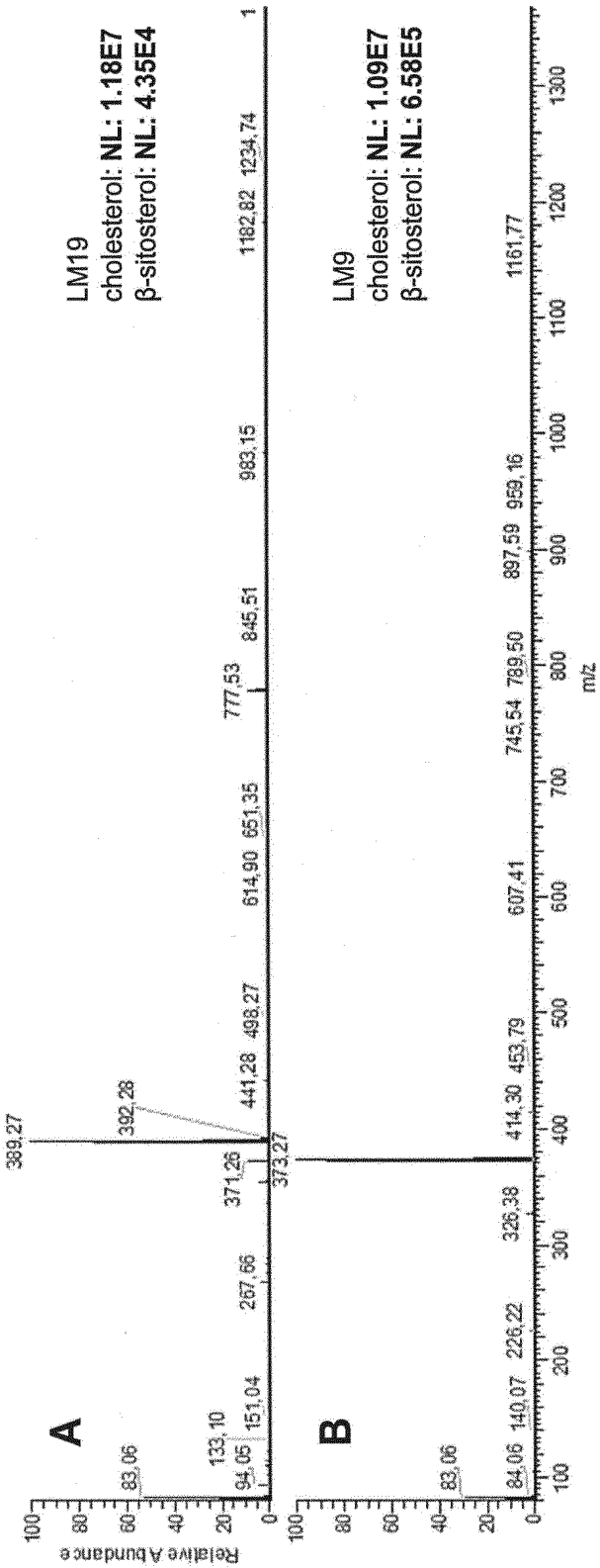

A
LM19
cholesterol: NL: 1.18E7
β-sitosterol: NL: 4.35E4
B
LM9
cholesterol: NL: 1.09E7
β-sitosterol: NL: 6.58E5
Relative Abundance
m/z

Figure 10

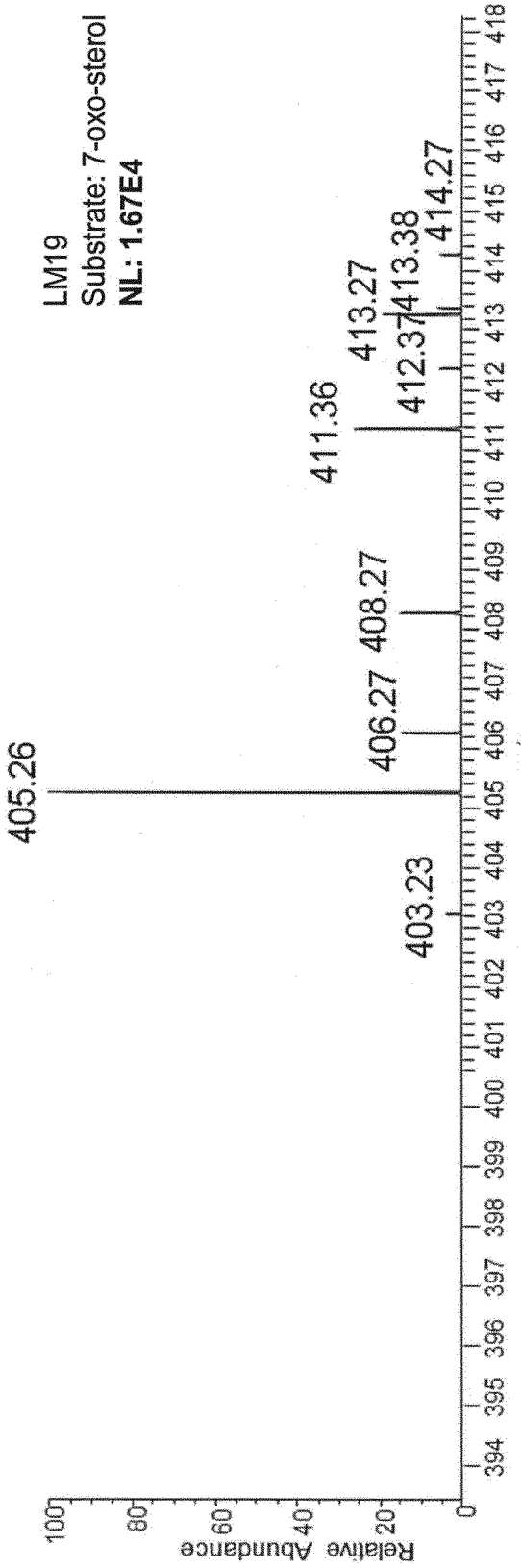

LM19
Substrate: 7-oxo-sterol
NL: 1.67E4
405.26
406.27
408.27
411.36
412.37
413.27
413.38
414.27
403.23
m/z
Relative Abundance

Figure 11

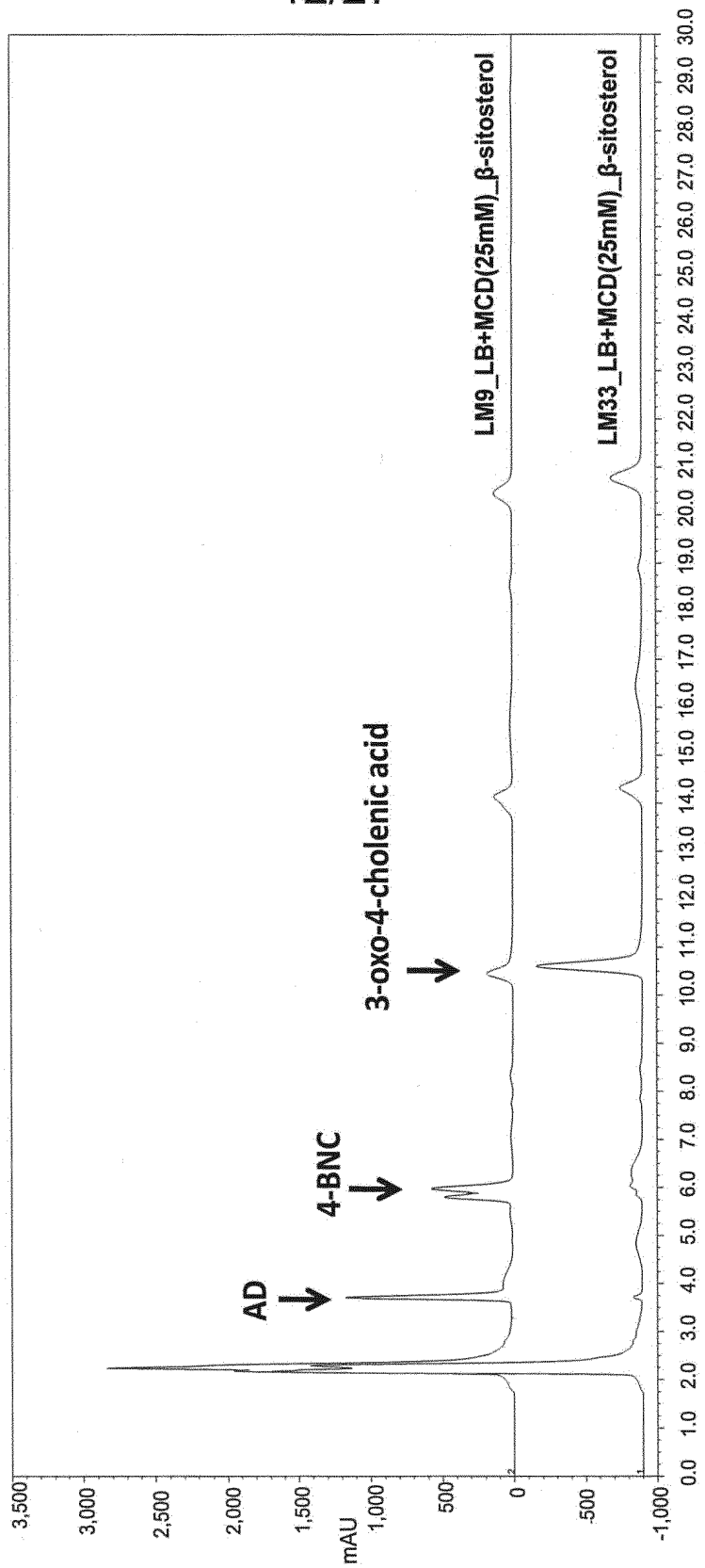

AD
4-BNC
3-oxo-4-cholenic acid
LM9_LB+MCD(25mM)_β-sitosterol
LM33_LB+MCD(25mM)_β-sitosterol
mAU
Time (mins)

Figure 12

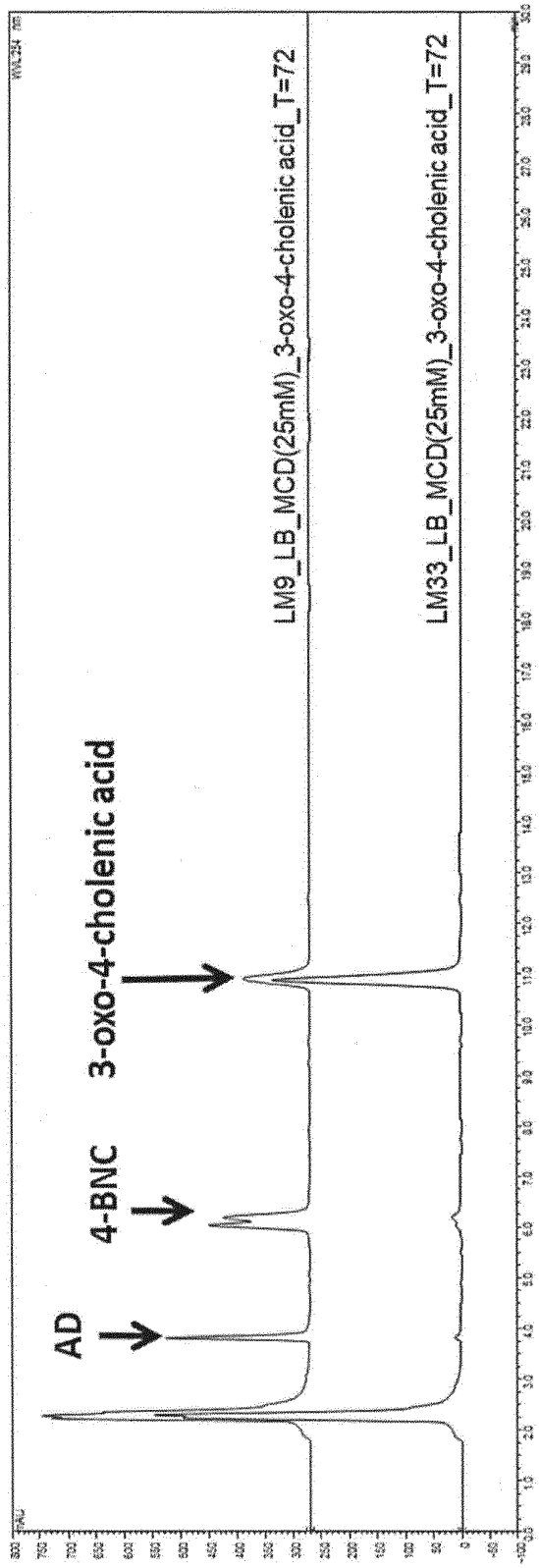

AD
4-BNC
3-oxo-4-cholenic acid
LM9_LB_MCD(25mM)_3-oxo-4-cholenic acid_T=72
LM33_LB_MCD(25mM)_3-oxo-4-cholenic acid_T=72

Figure 13

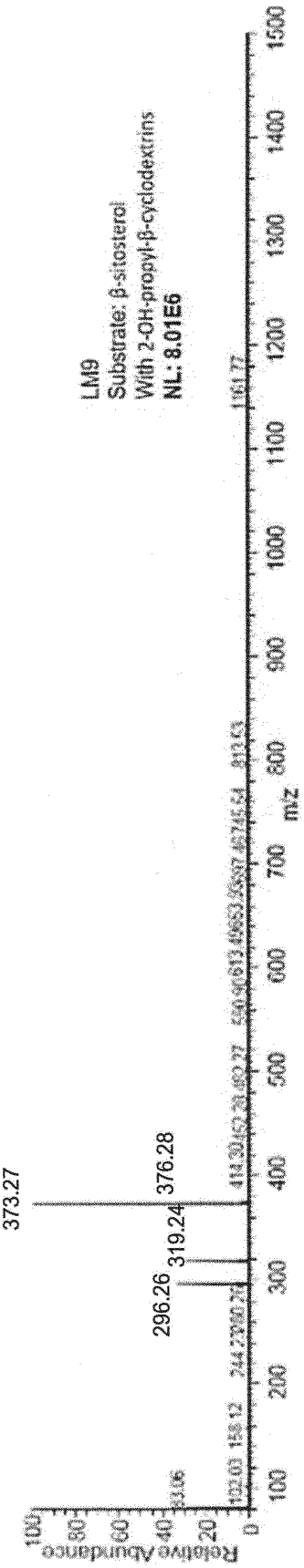

LM9
Substrate: β-sitosterol
With 2-OH-propyl-β-cyclodextrins
NL: 8.01E6
Relative Abundance
m/z
373.27
376.28
319.24
296.26

Figure 14

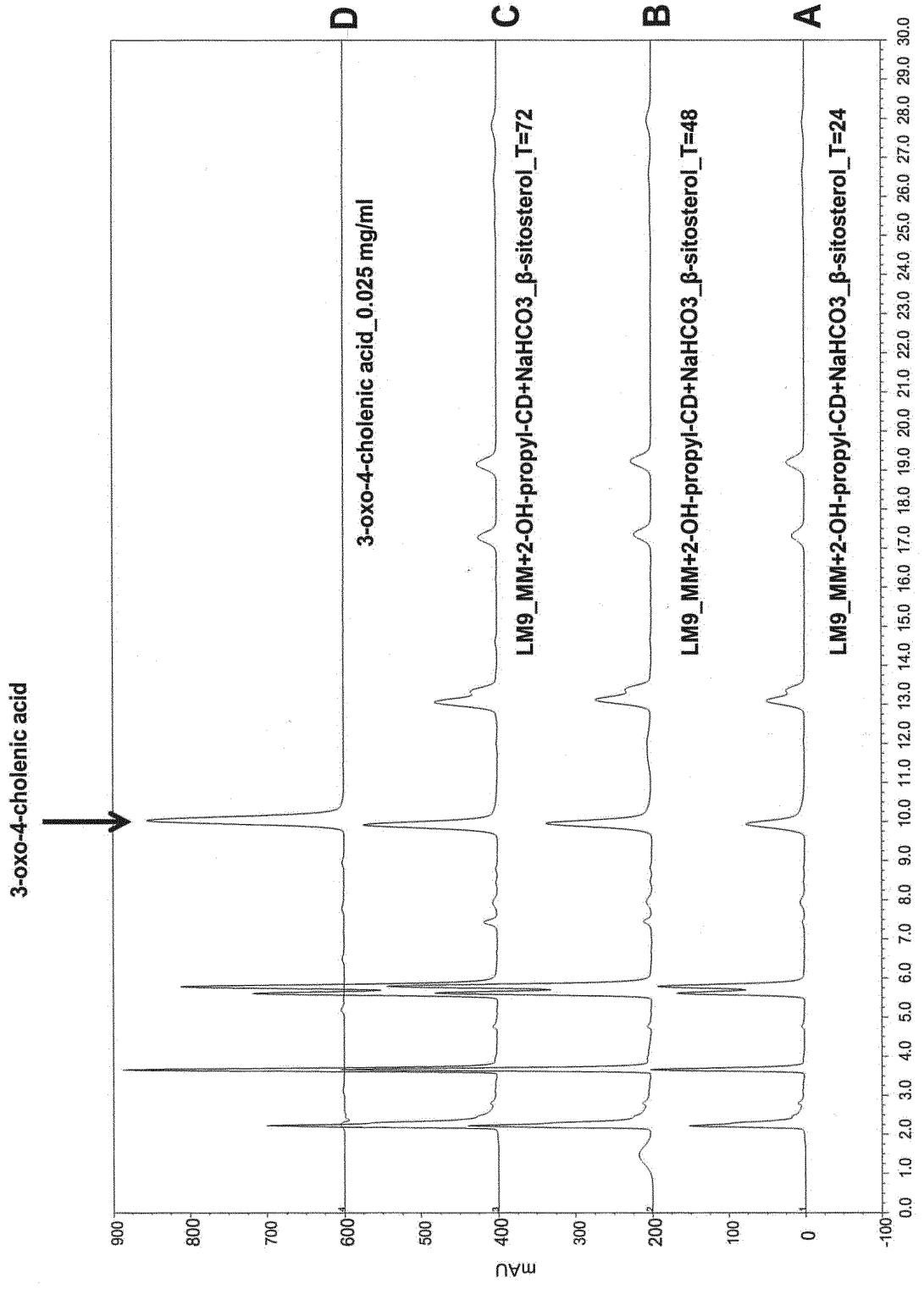

3-oxo-4-cholenic acid
D
3-oxo-4-cholenic acid_0.025 mg/ml
C
LM9_MM+2-OH-propyl-CD+NaHCO3_β-sitosterol_T=72
B
LM9_MM+2-OH-propyl-CD+NaHCO3_β-sitosterol_T=48
A
LM9_MM+2-OH-propyl-CD+NaHCO3_β-sitosterol_T=24
mAU
Time (mins)

Figure 15

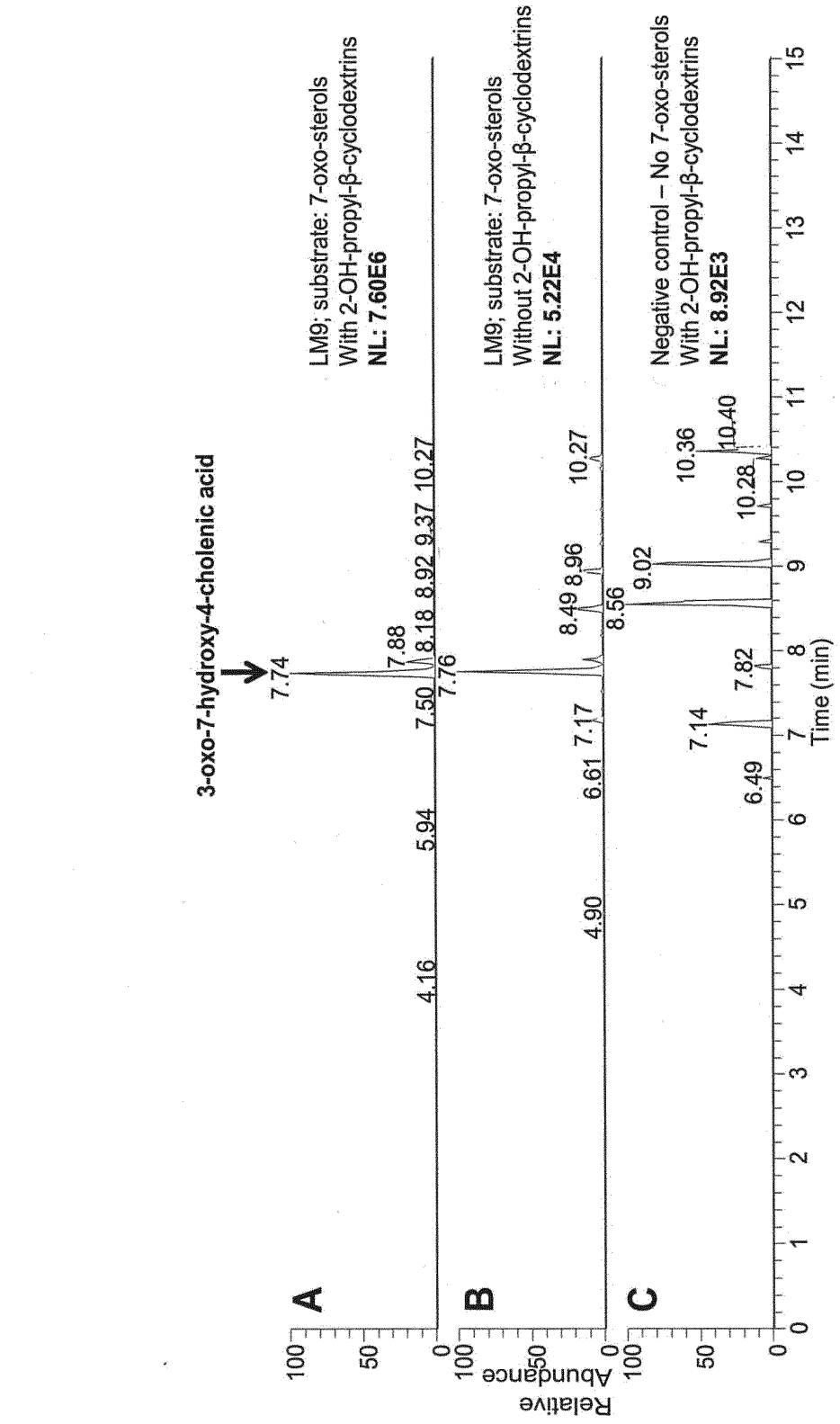

3-oxo-7-hydroxy-4-cholenic acid
LM9; substrate: 7-oxo-sterols
With 2-OH-propyl-β-cyclodextrins
NL: 7.60E6
LM9; substrate: 7-oxo-sterols
Without 2-OH-propyl-β-cyclodextrins
NL: 5.22E4
Negative control – No 7-oxo-sterols
With 2-OH-propyl-β-cyclodextrins
NL: 8.92E3
A
B
C
Relative Abundance
Time (min)

Figure 16

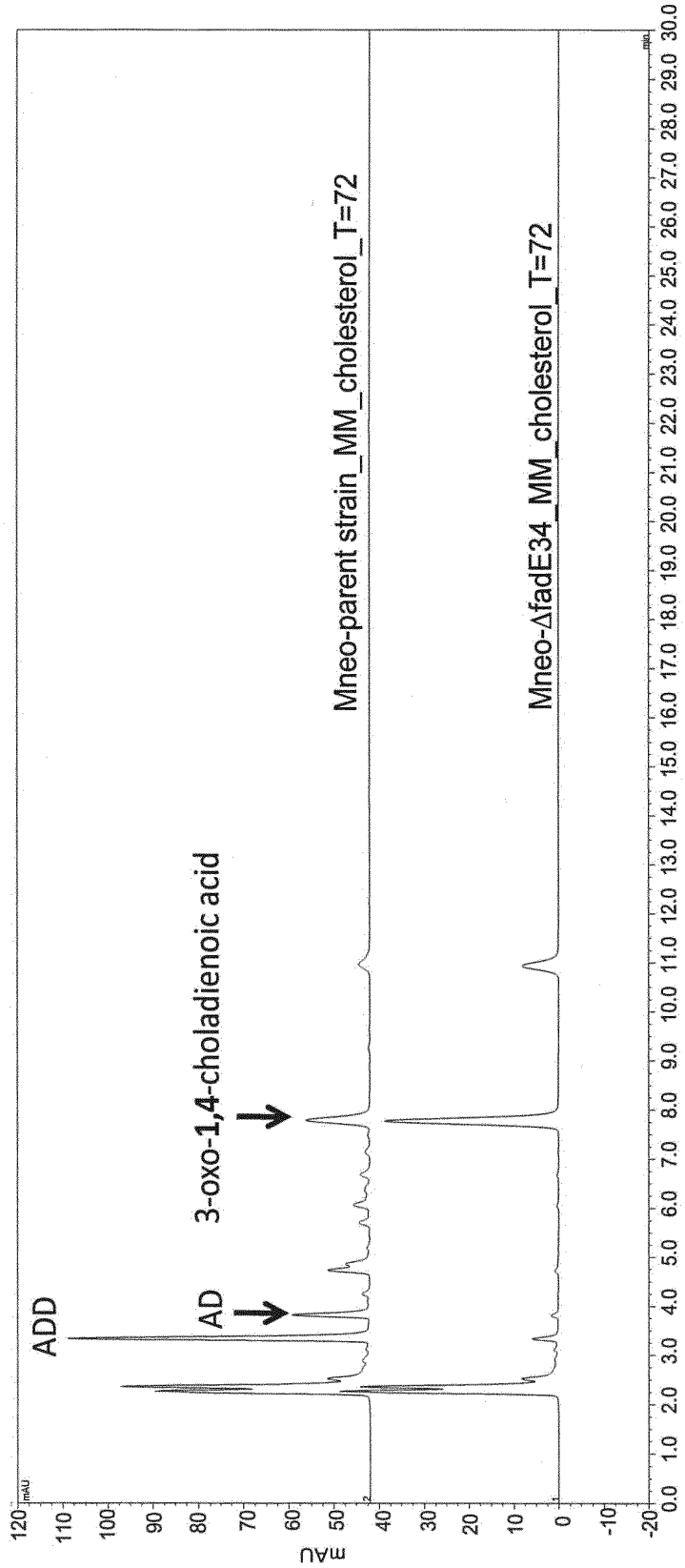

ADD
AD
3-oxo-1,4-choladienoic acid
Mneo-parent strain_MM_cholesterol_T=72
Mneo-ΔfadE34_MM_cholesterol_T=72
mAU
Time (mins)

Figure 17

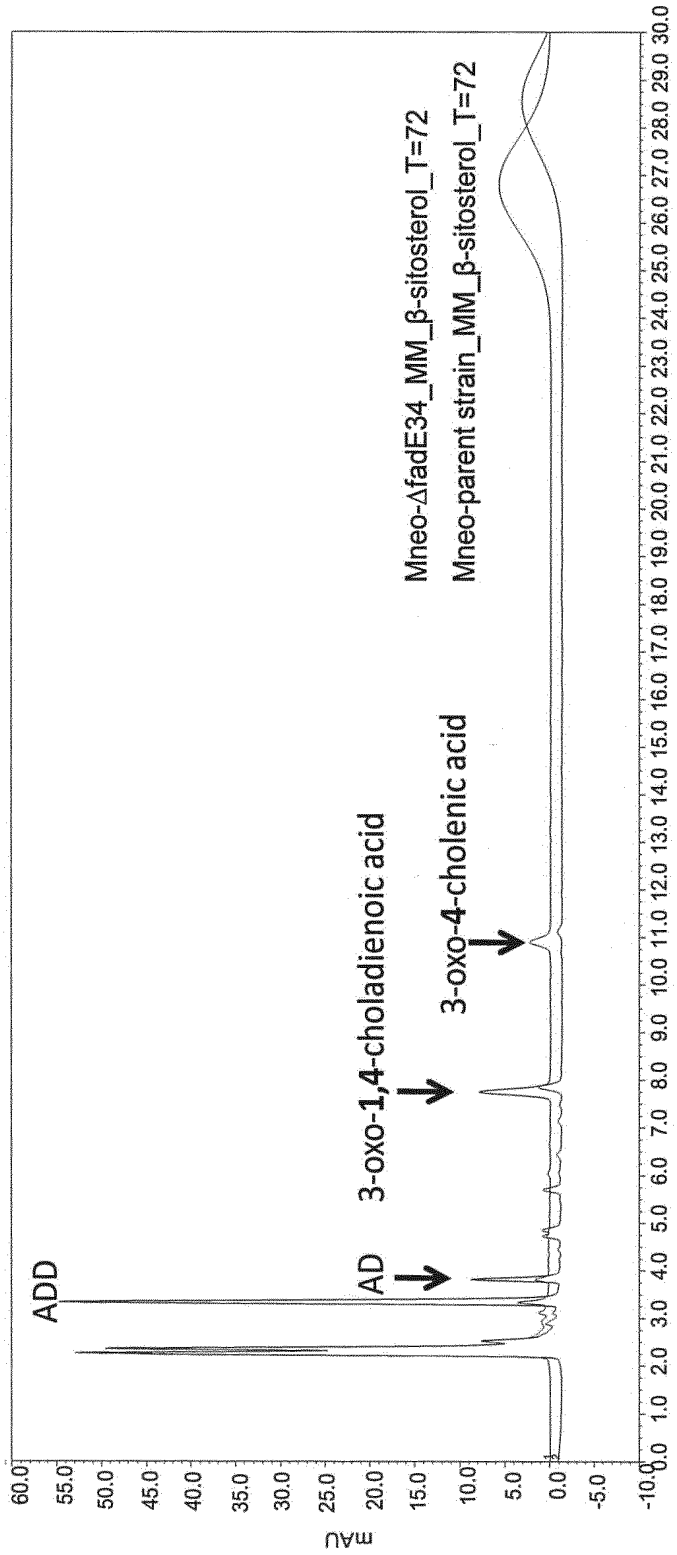

ADD
AD
3-oxo-1,4-choladienoic acid
3-oxo-4-cholenic acid
Mneo-ΔfadE34_MM_β-sitosterol_T=72
Mneo-parent strain_MM_β-sitosterol_T=72
mAU
Time (mins)

Figure 18

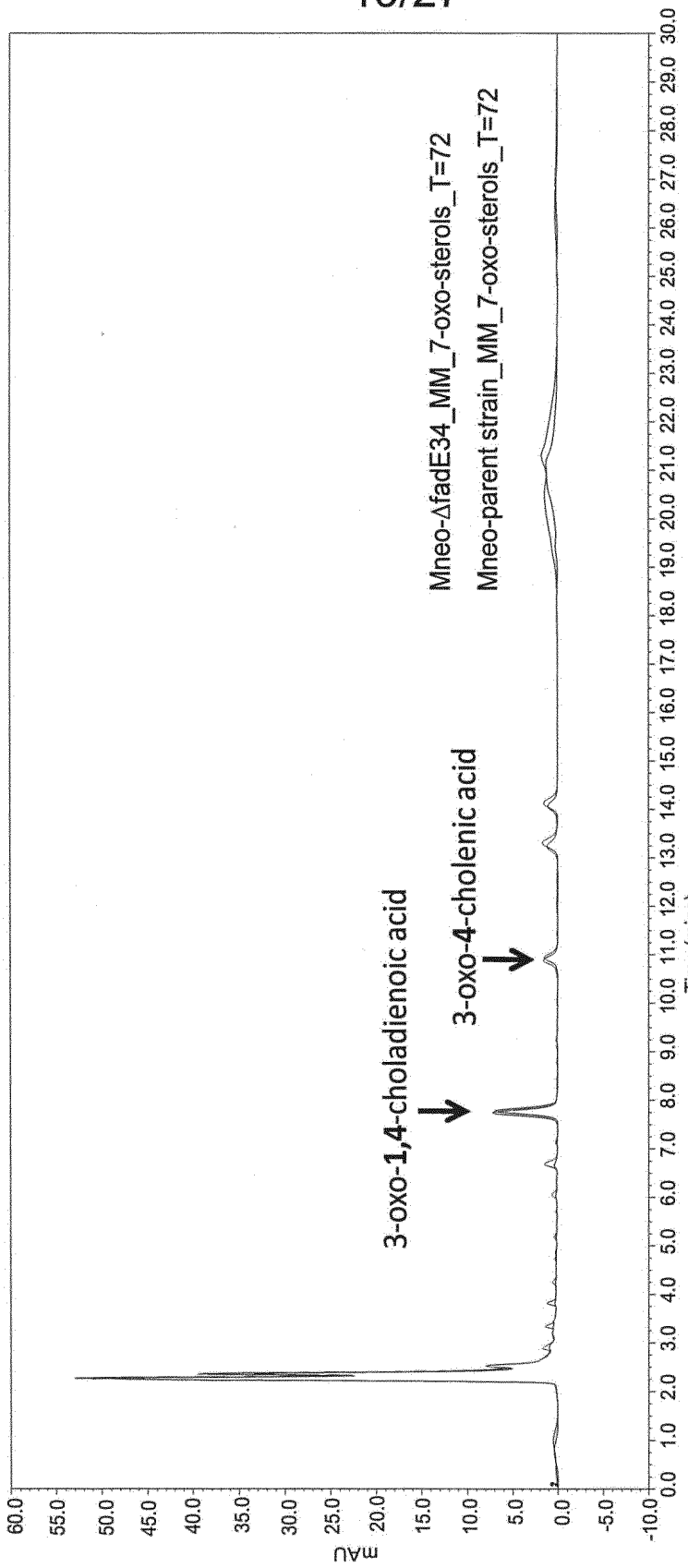

Mneo-ΔfadE34_MM_7-oxo-sterols_T=72
Mneo-parent strain_MM_7-oxo-sterols_T=72
3-oxo-1,4-choladienoic acid
3-oxo-4-cholenic acid
mAU
Time (mins)

Figure 19

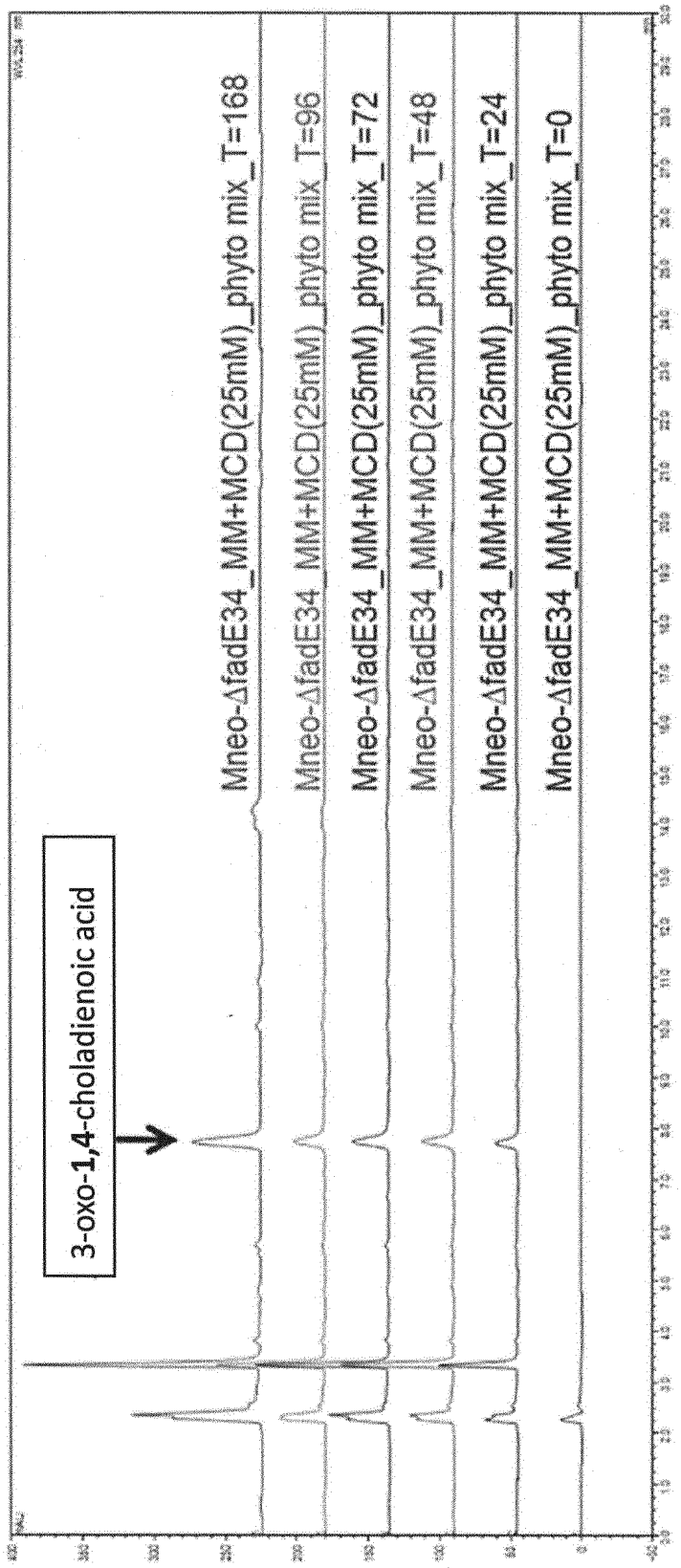

3-oxo-1,4-choladienoic acid
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=168
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=96
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=72
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=48
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=24
Mneo-ΔfadE34_MM+MCD(25mM)_phyto mix_T=0

Figure 20

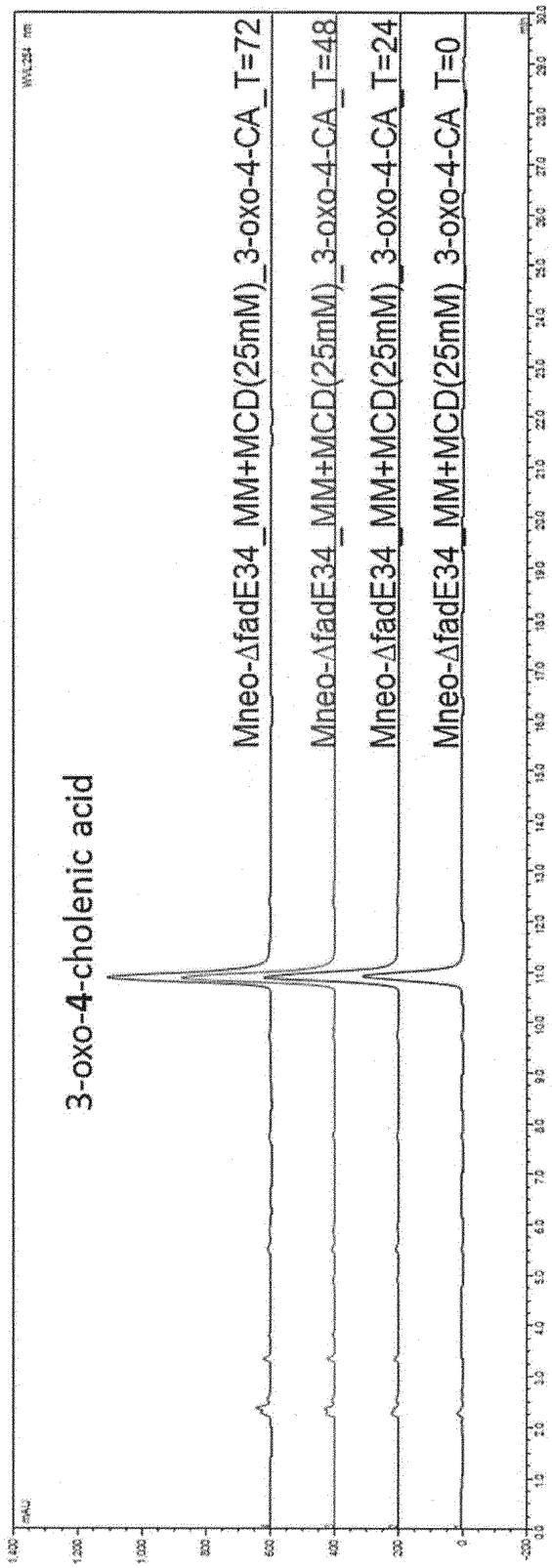

3-oxo-4-cholenic acid
Mneo-ΔfadE34_MM+MCD(25mM)_3-oxo-4-CA_T=72
Mneo-ΔfadE34_MM+MCD(25mM)_3-oxo-4-CA_T=48
Mneo-ΔfadE34_MM+MCD(25mM)_3-oxo-4-CA_T=24
Mneo-ΔfadE34_MM+MCD(25mM)_3-oxo-4-CA_T=0

Figure 21

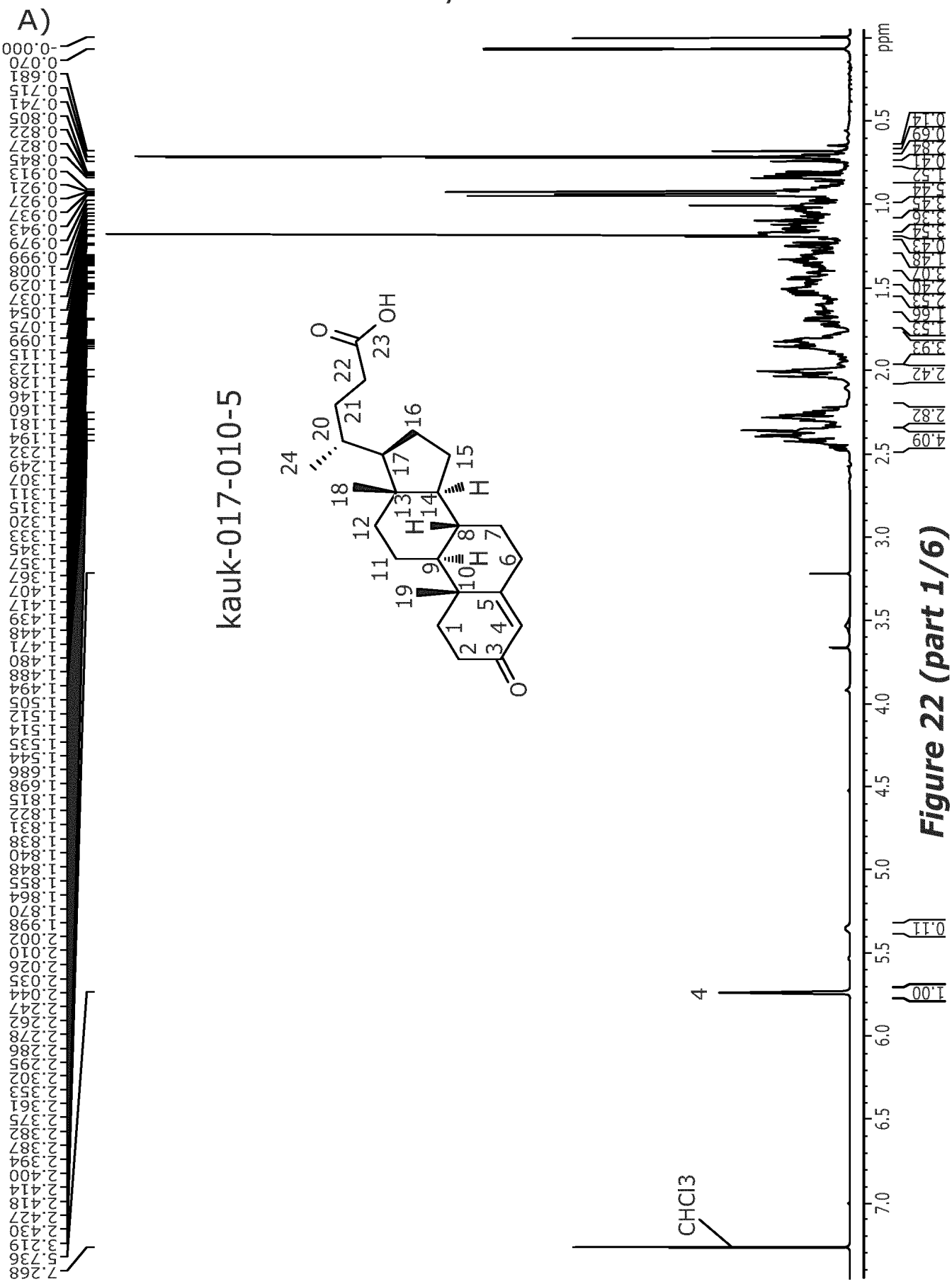


Figure 22 (part 1/6)

B)

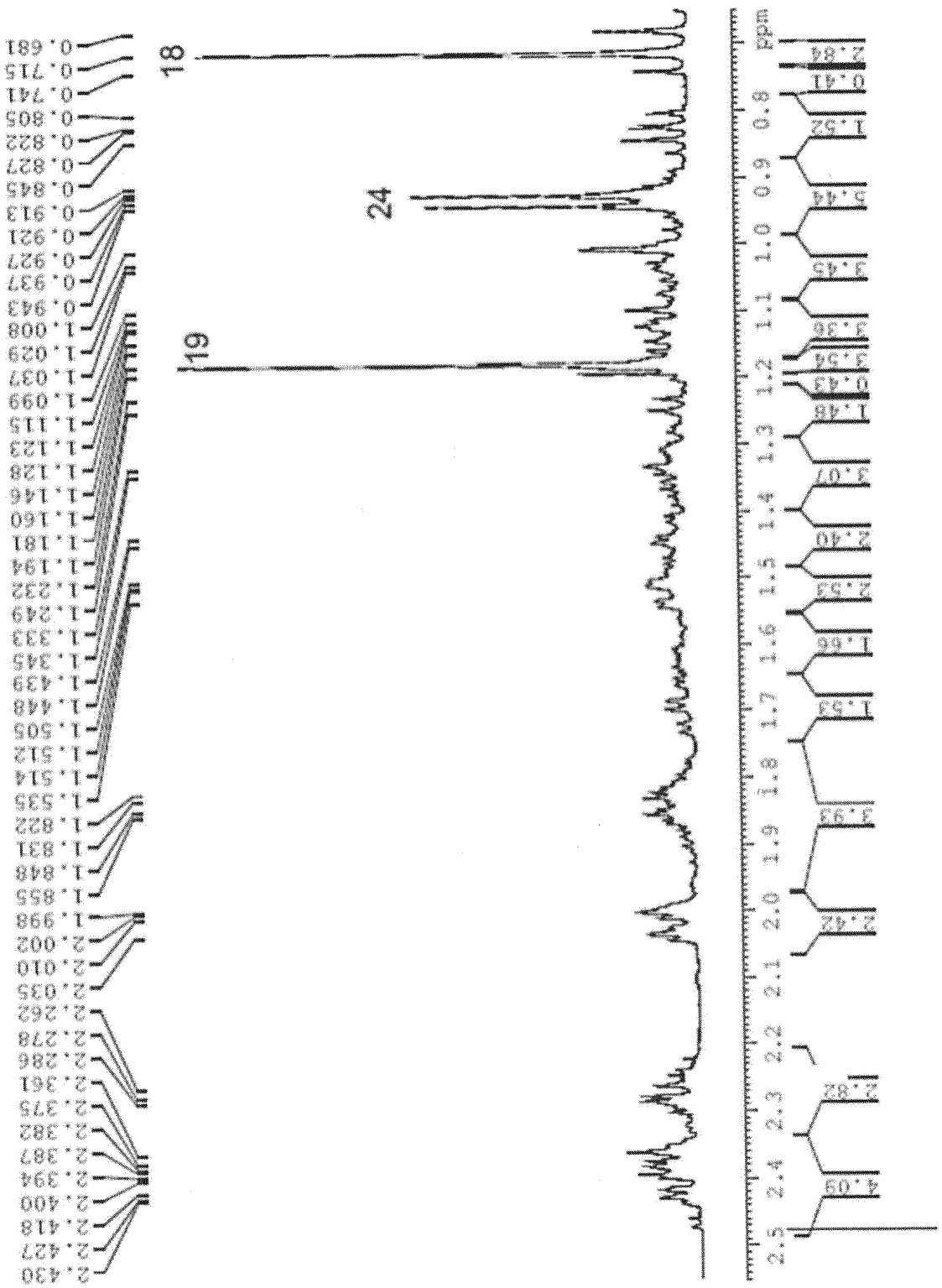


Figure 22 (part 2/6)

C)

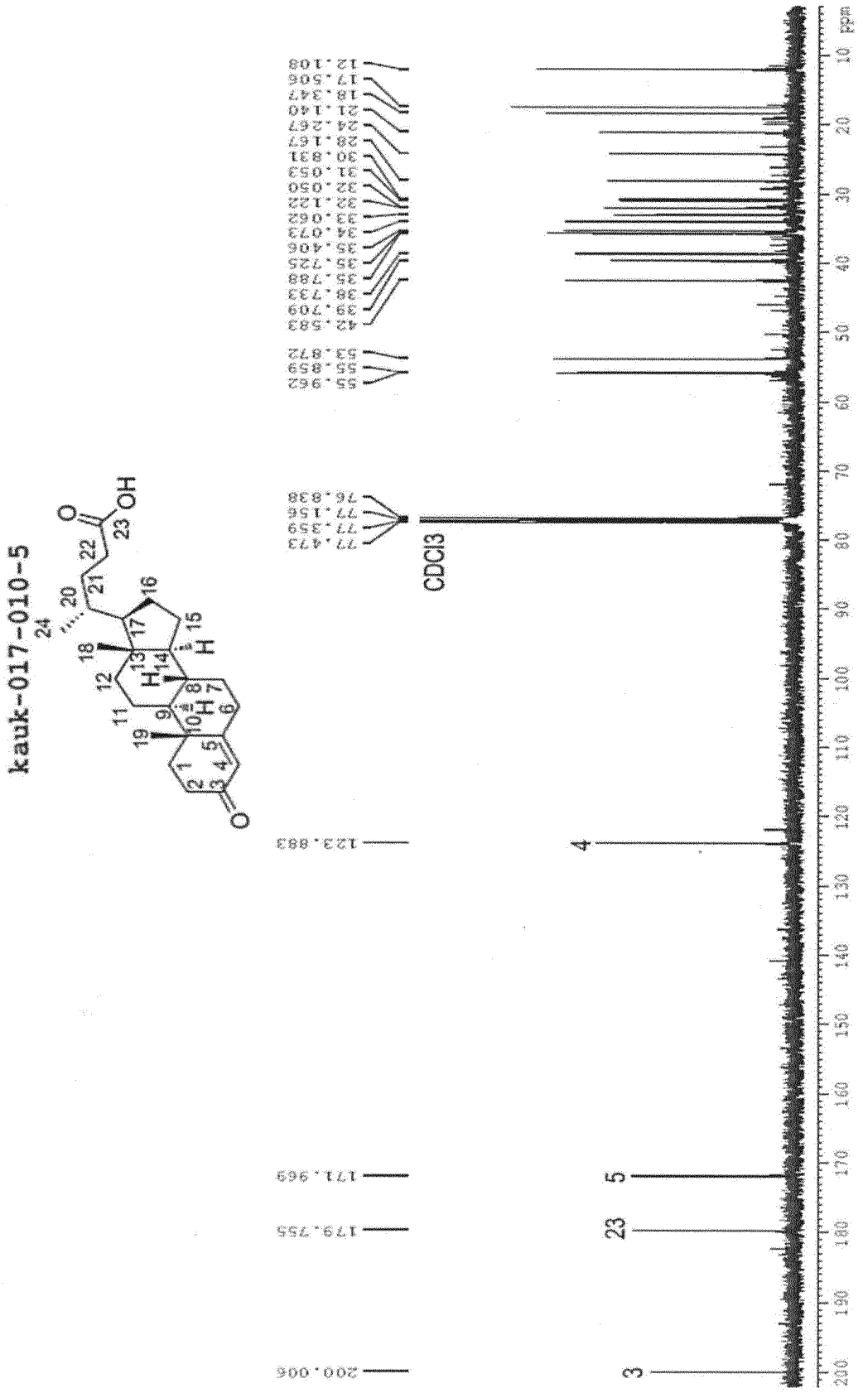


Figure 22 (part 3/6)

D)

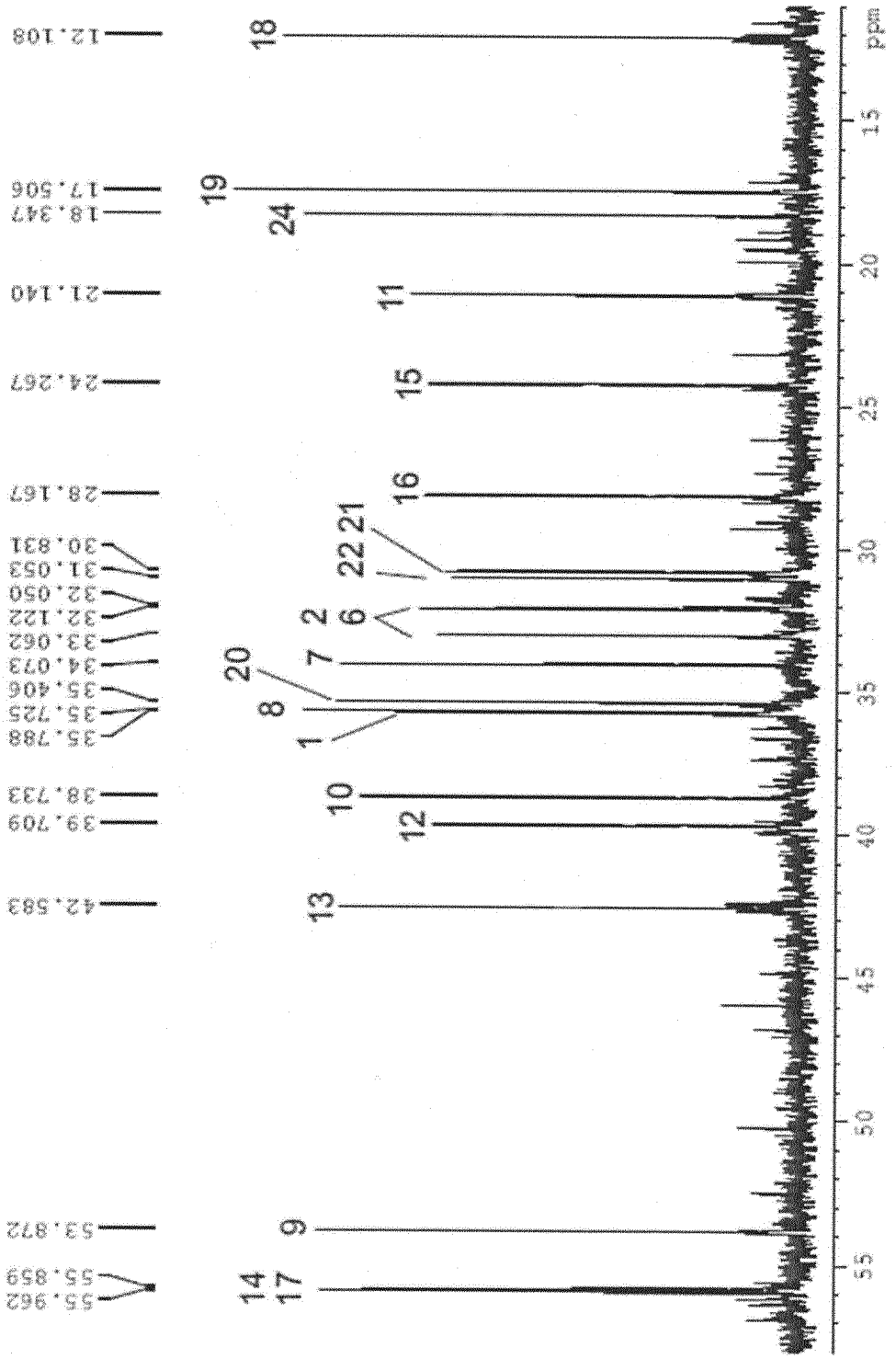


Figure 22 (part 4/6)

E)

```
Current Data Parameters
NAME          pros1809219
EXPNO                  10
PROCNO                  1

F2 - Acquisition Parameters
Date_            20180921
Time                17.47
INSTRUM             spect
PROBHD    5 mm BBO BB-1H
PULPROG              zg30
TD                  65536
SOLVENT             CDCl3
NS                     16
DS                      2
SWH              8223.685 Hz
FIDRES           0.125483 Hz
AQ               3.9845889 sec
RG                     161
DW                60.800 usec
DE                 6.00 usec
TE                296.6 K
D1             1.00000000 sec
TD0                     1

======== CHANNEL f1 ========
NUC1                   1H
P1                 15.30 usec
PL1       0 dB
SFO1         400.0624705 MHz

F2 - Processing parameters
SI                  131072
SF           400.0600097 MHz
WDW                    EM
SSB       0
LB        0 Hz
GB        0
PC                   1.00
```

Figure 22 (part 5/6)

F)

```
Current Data Parameters
NAME         pros1809219
EXPNO                 14
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180922
Time                4.58
INSTRUM            spect
PROBHD   5 mm BBO BB-1H
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                 1024
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ            1.3631488 sec
RG                  203
DW                20.800 usec
DE                 6.00 usec
TE                296.6 K
D1           1.00000000 sec
d11          0.03000000 sec
DELTA        0.89999998 sec
TD0                   1

======== CHANNEL f1 ========
NUC1                 13C
P1                  9.15 usec
PL1                -2.00 dB
SFO1        100.6052265 MHz

======== CHANNEL f2 ========
CPDPRG[2         waltz16
NUC2                 1H
PCPD2              70.00 usec
PL12               13.21 dB
PL13               13.56 dB
PL2       0 dB
SFO2         400.0616002 MHz

F2 - Processing parameters
SI                 262144
SF           100.5951548 MHz
WDW                  EM
SSB       0
LB                  1.00 Hz
GB        0
PC                  1.40
```

Figure 22 (part 6/6)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/071468

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20 C12P33/00 C12N9/02 C12R1/01 C12R1/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GEIZE VAN DER R ET AL: "Molecular and functional characterization of kshA and kshB, encoding two components of 3-ketosteroid 9alpha-hydroxylase, a class IA monooxygenase, in Rhodococcus erythropolis strain SQ1", MOLECULAR MICROBIOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 45, no. 4, August 2002 (2002-08), pages 1007-1018, XP002249467, ISSN: 0950-382X, DOI: 10.1046/J.1365-2958.2002.03069.X	1-6, 9-11, 13-15, 17,18, 21-25, 28-35, 38,39, 50-54
A	the whole document	16,26,27

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
1 November 2019	14/11/2019

Name and mailing address of the ISA/	Authorized officer
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2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	the whole document	44-49, 57,58
X	WO 03/070925 A2 (AKZO NOBEL NV [NL]; VAN DER GEIZE ROBERT [NL] ET AL.) 28 August 2003 (2003-08-28) the whole document	1-3,5,6, 9-11,15, 17,18, 21-25, 28-32, 34,35, 38,39, 50-53
X	CHIN-HSING YEH ET AL: "Deletion of the gene encoding the reductase component of 3-ketosteroid 9alpha-hydroxylase in Rhodococcus equi USA-18 disrupts sterol catabolism, leading to the accumulation of 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid and 1,4-androstadiene-3,17-dione", MICROBIAL CELL FACTORIES,, vol. 13, no. 1, 9 September 2014 (2014-09-09), page 130, XP021198516, ISSN: 1475-2859, DOI: 10.1186/S12934-014-0130-3 the whole document	1-10, 28-39, 50-54

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU ET AL: "Unraveling and engineering the production of 23,24-bisnorcholenic steroids in sterol metabolism", SCIENTIFIC REPORTS,, vol. 6, 22 February 2016 (2016-02-22), pages 1-13, XP002781896, DOI: 10.1038/SREP21928	1-6,9, 10,12, 15,17, 19,20, 28-35, 38-43, 50-56
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/071468

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 03070925 A2	28-08-2003