Biosynthesis of forskolin and related compounds

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(54) **Title**: BIOSYNTHESIS OF FORSKOLIN AND RELATED COMPOUNDS

(57) **Abstract**: The invention described materials and methods for producing oxidised 13R-MO, such as forskolin. In particular, the invention describes P450s involved in oxidation of 13R- MO including CYP76AH8, CYP76AH11, CYP76AH15, CYP76AH17, CYP71D381 and CYP76AH9. Host organisms expressing one or more of these P450s are useful in the production of oxidized 13R-MO.

Biosynthesis of forskolin and related compounds

Field of invention

The present invention relates to the field of biosynthesis of terpenoids. More specifically the invention relates to methods for biosynthesis of forskolin and related compounds, such as to biosynthesis of oxidised 13R-MO.

Background of invention

Forskolin is a complex functionalised derivative of 13R-MO requiring region- and stereospecific oxidation of five carbon positions. Forskolin is a diterpene naturally produced by *Coleus forskohlii*. Both Forskolin and oxidized variants of forskolin have been suggested as useful in treatment in a number of clinical conditions. Forskolin has the ability to decrease the intraocular pressure therefore it is used today as an antiglaucoma agent (Wagh K, Patil P, Surana S, Wagh V. Forskolin: Upcoming antiglaucoma molecule, J Postgrad Med 2012, 58(3):199-202), in the form of eye drops. Moreover a water-soluble analogue of forskolin (NKH477) has been approved for commercial use in Japan for treatment of acute heart failure and heart surgery complications because of its vasodilatory effects when administered intravenously (Kikura M, Morita K, Sato S. Pharmacokinetics and a simulation model of colforsin daropate, new forskolin derivative inotropic vasodilator, in patients undergoing coronary artery bypass grafting. Pharmacol Res 2004, 49: 275-281). Forskolin also acts as bronchodilator so it could be used for asthma treatments (Yousif MH and Thulesius O. Forskolin reverses tachyphylaxis to the bronchodilator effects of salbutamol: an in-vitro study on isolated guinea-pig trachea. J Pharm Pharmacol, 1999. 51:181-186). Forskolin may help additionally to treat obesity by contributing to higher rates of body fat burning and promoting lean body mass formation (Godard MP, Johnson BA, Richmond SR. Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men. Obes Res 2005, 13:1335-1343)

Summary of invention

Hitherto forskolin has been purified from *Coleus forskohlii* or produced chemically. Here novel methods for biosynthesis of forskolin and other oxidised 13R-MOs are presented.

Oxidised 13R-MO may be valuable on its own account or as precursors for production of forskolin.

Thus, it is an aspect of the invention to provide methods of producing an oxidsed 13R-manoyl oxide (13R-MO), said method comprising the steps of:

a) providing a host organism comprising

I. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or catalysing oxidation of the hydroxyl group to form an oxo-group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO;

said host organism optionally comprising one or more of the following:

II. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position;

III. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position

IV. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position

V. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position

VI. A heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO

b) Incubating said host organism in the presence of 13R-MO under conditions allowing growth of said host organism;

c) Optionally isolating oxidised 13R-MO from the host organism and/or from its surroundings.

If the host organism is a microorganism, then the oxidsed 13R-MO may be isolated from the cultivation medium used for cultivation of the host organism.

Incubating the host organism in the presence of 13R-MO may for example be accomplished by the host organism being capable of producing 13R-MO or 13R-MO may be added to the host organism. In preferred embodiments the host organism is capable of producing 13R-MO.

It is also an aspect of the invention to provide host organisms comprising

I. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or catalysing oxidation of the hydroxyl group to form an oxo-group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO;

said host organism optionally comprising one or more of the following:

II. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position;

III. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position;

IV. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position;

V. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position

VI. A heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO.

It is also an aspect of the invention to provide enzymes capable of

I. catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or
II. catalysing oxidation of the hydroxyl group at the 11 position of 11-hydroxyl-13R-manoyl oxide and/or an oxidised 11-hydroxyl-13R-MO.

It is also an aspect of the invention to provide enzymes capable of catalysing hydroxylation of 11-keto-13R-manoyl oxide (13R-MO) and/or an oxidised 11-keto-13R-MO derivative at one or more of the positions 1, 6, 7 and/or 9.

It is also an aspect of the invention to provide enzymes capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO at one or more of the positions 1, 6, 7 and/or 9.

Description of Drawings

Figure 1A shows a scheme of the full oxidation of (1) 13R-MO to (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one), (4a) keto-hydroxy-13R-MO and (4b) keto-hydroxy-13R-MO and (7a) keto-dihydroxy-13R-MO by *Coleus forskohlii* CYP76AH8. * indicates possible position of –OH group(s).

Figure 1B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing (1) 13R-MO and expressing CYP76AH8 of SEQ ID NO:1. GC-MS trace with relative detector response and retention time in minutes. (1) 13R-MO [only traces detected] (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one), (4a) keto-hydroxy-13R-MO and (4b) keto-hydroxy-13R-MO and (5) keto-dihydroxy-13R-MO are detected. IS, internal standard are shown.

Figure 2A shows a scheme of the full oxidation of (1) 13R-MO to (3c) hydroxy-13R-MO, (5b) dihydroxy-13R-MO and (5c) dihydroxy -13R-MO and (8b) trihydroxy-13R-MO by *Coleus forskohlii* CYP76AH11. * indicates possible position of –OH group(s).

Figure 2B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP76AH11 of SEQ IDNO:2. 1) 13R-MO to (3c) hydroxy-13R-MO, (5b) dihydroxy-13R-MO and (5c) dihydroxy -13R-MO and (8b) trihydroxy-13R-MO are detected. A GC-MS trace with relative detector response and retention time in minutes. * indicates possible position of –OH group(s).

Figure 3A shows selected observed oxidation reactions of 13R-MO en route to forskolin. CYP76AH8 and CYP76AH11 effectively catalyse the first four reactions going via compound (2), 4d, 7a and 8a. Also compound 6a is produced* indicates possible position of –OH group(s). With respect to compound 6a * indicates possible position of =O or –OH group.

Figure 3B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP76AH8 of SEQ ID NO:1 and CYP76AH11 of SEQ ID NO:2. (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one), (6a) hydroxy-di-keto-13R-MO and (4d) keto-hydroxy-13R-MO, (4) keto-dihydroxy-13R-MO and (5) keto-trihydroxy-13R-MO are detected. A GC-MS trace with relative detector response and retention time in minutes

Figure 4A shows selected observed oxidation reactions of 13R-MO. CYP71D381 effectively catalyse two hydroxylation reactions going from compound (1) to either (3a) 2-hydroxy-13R-manoyl oxide or (3b) 20-hydroxy-13R-manoyl oxide.

Figure 4B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP71D381. (1) 13R-MO, (3a) 2-hydroxy-13R-MO and (3b)20- hydroxy-13R-MO are detected. A GC-MS trace with relative detector response and retention time in minutes.

Figure 5A shows selected observed oxidation reactions of 13R-MO. CYP76AH9 catalyse two hydroxylation reactions going from compound (1) to either (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one) or (9) hydroxy-13R-MO. * indicates possible position of –OH group(s).

Figure 5B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP76AH9. (1) 13R-MO, (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one) and (9) hydroxy-13R-MO are detected. A GC-MS trace with relative detector response and retention time in minutes

Figure 6 shows 13R-manoyl-oxide and oxidised 13R-manoyl-oxides found in *C. forskohlii* and that have pharmaceutical properties.

Figure 7 shows selected observed oxidation reactions of 13R-MO en route to forskolin and other pharmacological relevant 13R-MO derived compounds. CYP76AH8 and CYP76AH11 effectively catalyse the first four reactions, towards forskolin. CYP71D381, CYP76AH9 and CYP76AH11 catalyse hydroxylation reactions of 13R-MO towards other 13R-MO derived compounds with potential pharmacological relevance. With respect to compound 6a * indicates possible position of =O or –OH group(s). With respect to all other compounds * indicates possible position of –OH group(s).

Figure 8 shows a proposed biosynthetic route to forskolin in *C. forskohlii* proposed by Asada et al., Phytochemistry 79 (2012) 141-146.

Figure 9 shows an overview of the biosynthesis of forskolin. Each column shows the compounds produced by CYP76AH8, CYP76AH11 and by the combined action of CYP76AH8 and CYP76AH11. The right hand column shows the structure of the compounds as well as one route to forskolin. The numbers in the table refers to the compound numbers used in figures 1 to 3. The chemical names and structures of the compounds are provided in figures 1 to 3. More specifically, (2) is (8,13-epoxy-labd-14-ene-11-one), (5c) is 7-11-dihydroxy-13R-manoyl oxide, (7a) is 1-9-deoxydeacethylforskolin and (8a) is 1-deoxydeacethylforskolin.

Figure 10 shows GC-MS analysis of extracts from assays with S. cerevisiae producing 13R-MO and expressing either CYP76AH11 (upper panel) or CYP76AH8 (lower panel). In the upper panel compounds 6a, 6b and 4c are detected and in the lower panel compound 8 is detected. Chemical formula of the compounds are provided in figure 9.

Figure 11A shows a scheme of the full oxidation of (1) 13R-MO to (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one), (3a) keto-hydroxy-13R-MO and (3b) keto-hydroxy-13R-MO and (4) keto-dihydroxy-13R-MO by *Coleus forskohlii* CYP76AH8, CYP76AH15 or CYP76AH17. * indicates possible position of –OH group(s).

Figure 11B shows GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing (1) 13R-MO and expressing CYP76AH8 of SEQ ID NO:1. GC-MS trace with relative detector response and retention time in minutes. (1) 13R-MO [only traces detected] (2) keto-13R-MO (8,13-epoxy-labd-14-ene-11-one), (3a) keto-hydroxy-13R-MO and (3b) keto-hydroxy-13R-MO and (5) keto-dihydroxy-13R-MO are detected. IS, internal standard are shown.

Detailed description of the invention

Methods of preparing oxidised 13R-MO

It is one aspect of the present invention to provide biosynthetic methods for preparing oxidised 13R-MO. The methods of the invention generally comprise the steps of:

1) Providing a host organism comprising one or more of the following:
   I. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or catalysing oxidation of the hydroxyl group to form an oxo-group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO;
   II. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position;
   III. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position
   IV. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position

V. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position

VI. A heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO

2) Incubating said host organism in the presence of 13R-MO under conditions allowing growth of said host organism
3) Optionally isolating oxidised 13R-MO from the host organism.

The structure of 13R-MO is provided herein below in the section "Oxidised 13R-MO.

The oxidised 13R-MO may be any of the oxidised 13R-MO described herein below in the section "Oxidised 13R-MO".

The term "oxidised 11-hydroxyl-13R-MO" as used herein refers to 11-hydroxyl-13R-MO further substituted at one or more of the positions 1, 6, 7 and 9 with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl.

The term "oxidised hydroxylated-13R-MO" as used herein refers to 13R-MO, which is substituted with hydroxyl on at least one of the positions 1, 6, 7 and 9, and which further is substituted at one or more of the others positions 1, 6, 9 and 11 with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl.

The term "oxidised 11-keto-13R-MO" as used herein refers to 13R-MO, which is substituted with oxo at the 11 position and which further is substituted at one or more of the positions 1, 6, 9 and 11 with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl.

The heterologous nucleic acid I. may for example be a heterologous encoding any of the enzymes described in the section "I. Enzyme catalysing hydroxylation of 13R-MO at the 11 position" herein below. The heterologous nucleic acid II. may for example be a heterologous encoding any of the enzymes described in the section "II. Enzyme catalysing hydroxylation of 13R-MO at the 1 position" herein below. The heterologous nucleic acid III. may for example be a heterologous encoding any of the enzymes

described in the section "III. Enzyme catalysing hydroxylation of 13R-MO at the 6 position" herein below. The heterologous nucleic acid IV. may for example be a heterologous encoding any of the enzymes described in the section "IV. Enzyme catalysing hydroxylation of 13R-MO at the 7 position" herein below. The heterologous nucleic acid V. may for example be a heterologous encoding any of the enzymes described in the section "V. Enzyme catalysing hydroxylation of 13R-MO at the 9 position" herein below. The heterologous nucleic acid VI. may for example be a heterologous encoding any of the enzymes described in the section "VI. Enzyme catalysing transfer of an acyl group" herein below.

The host organism may comprise one of more of the heterologous nucleic acids I., II., III., IV., V. and VI, such as at least 2, for example at least 3, such as at least 4, for example at least 5, such as all of heterologous nucleic acids I., II., III., IV., V. and VI.

Incubating said host organism in the presence of 13R-MO may be obtained in several manners. For example, 13R-MO may be added to the host organism. If the host organism is a microorganism, then 13R-MO may be added to the cultivation medium of said microorganism. If the host organism is a plant, then 13R-MO may be added to the growing soil of the plant or it may be introduced into the plant by infiltration. Thus, if the heterologous nucleic(s) are introduced into the plant by infiltration, then 13R-MO may be co-infiltrated together with the heterologous nucleic acid(s).

It is also comprised within the invention that the host organism is capable of producing 13R-MO. In such embodiments incubating said host organism in the presence of 13R-MO simply requires cultivating said host organism.

In one embodiment of the invention the host organism comprises one or more heterologous nucleic acids encoding enzymes involved in the production of 13R-MO. Thus, in preferred embodiments of the invention the host organism comprises in addition to the heterologous nucleic acids I., II., III., IV., V. and/or VI at least one of the following heterologous nucleic acids:

IX. A heterologous nucleic acid encoding TPS2, such as any of the TPS2 described herein below in the section "IX. TPS2"

X. A heterologous nucleic acid encoding TPS3, such as any of the TPS3 described herein below in the section "X. TPS3", and/or

XI. A heterologous nucleic acid encoding TPS4, such as any of the TPS4 described herein below in the section "XI. TPS4"

Such host organisms are in general capable of producing 13R-MO and thus, no 13R-MO needs to be added to such host organisms. In such embodiments it is preferable that the host organism is incubated in the presence of GGPP. Many host organisms are capable of producing GGPP, and thus incubation in the presence of GGPP may be simply require cultivation of the host organism.

The host organism according to the invention may also comprise one or more additional heterologous nucleic acids, in addition to the heterologous nucleic acids described herein.

The methods of the invention may also be performed in vitro. Thus, the method of producing an oxidised 13R-MO may comprise the steps of

i. providing a host organism comprising one or more of the heterologous nucleic acids I., II., III., IV., V. and VI, and preferably comprising at least the heterologous nucleic acid I.,
b) preparing an extract of said host organism;
c) providing 13R-MO
d) incubating said extract with 13R-MO

thereby producing oxidised 13R-MO.

The method of producing an oxidised 13R-MO may also comprise the steps of

a) providing a host organism comprising one or more of the heterologous nucleic acids I., II., III., IV., V., VI, IX, X and XI, preferably comprising at least the heterologous nucleic acid I., IX and X,
b) preparing an extract of said host organism;
d) incubating said extract in the presence of GGPP

thereby producing oxidised 13R-MO.

The host organism may be any of the host organisms described herein below in the section "Host organism".

I. Enzyme catalysing hydroxylation of 13R-MO at the 11 position

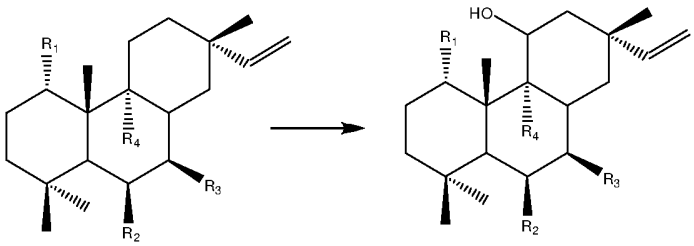
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of

a) catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or

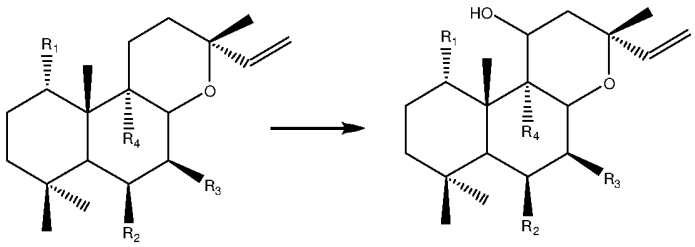
b) catalysing oxidation of the hydroxyl group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO to form an oxo-group.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme I". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at the 11 position with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl, and in particular in embodiments of the invention, wherein the oxidsed 13R-MO to be produced is substituted at the 11 position with oxo (=O).

The enzyme I may be an enzyme having one or two functions. In particular it is preferred that the enzyme I is capable of catalysing the following reaction Ia:



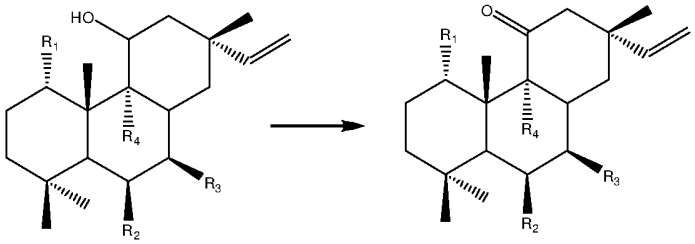
and/or the reaction Id:



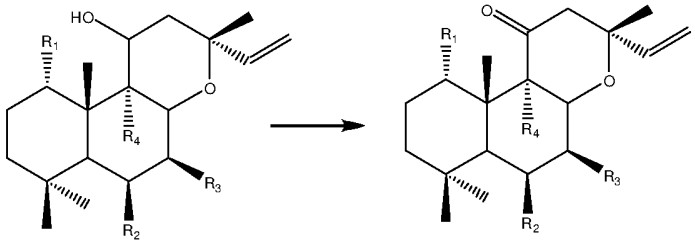
wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2, R_3 and R_4 is –H, for example at least two of R_1, R_2, R_3 and R_4 is –H, for example at least three of R_1, R_2, R_3 and R_4 is –H. In one embodiment all of R_1, R_2, R_3 and R_4 is –H.

It is also preferred that the enzyme I is capable of catalysing the following reaction Ib:



and/or reaction Ie:

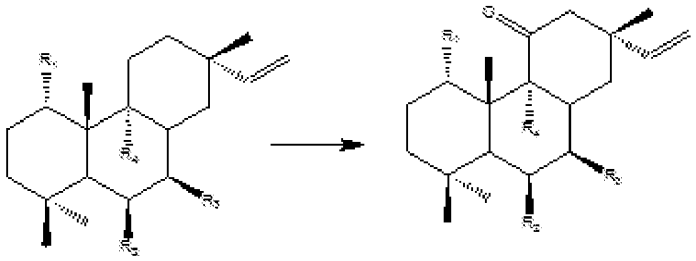


wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

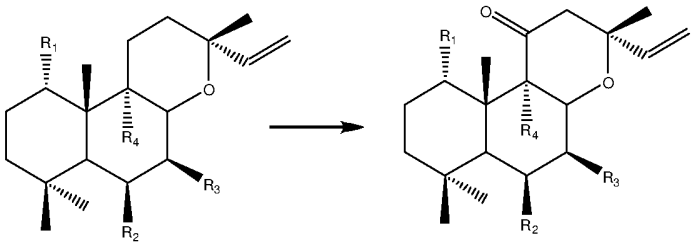
In particular, at least one of R_1, R_2, R_3 and R_4 is –H, for example at least two of R_1, R_2, R_3 and R_4 is –H, for example at least three of R_1, R_2, R_3 and R_4 is –H. In one embodiment all of R_1, R_2, R_3 and R_4 is –H.

It is even more preferred that enzyme I is capable of catalysing both of reactions Ia and Ib outlined above. It is very preferred that enzyme I is capable of catalysing both of reactions Id and Ie outlined above.

It is also possible that enzyme I is capable of catalysing the reaction Ic:



and/or the reaction If:



In particular, at least one of R_1, R_2, R_3 and R_4 is –H, for example at least two of R_1, R_2, R_3 and R_4 is –H, for example at least three of R_1, R_2, R_3 and R_4 is –H. In one embodiment all of R_1, R_2, R_3 and R_4 is –H.

Enzyme I may be any useful enzyme with above mentioned activities, in particular enzyme I may be a CYP450. Enzyme I may be derived from any suitable source, but in a preferred embodiment enzyme I is an enzyme from *Coleus forskohlii.* Thus enzyme I may be a CYP450 from *Coleus forskohlii.*

In a preferred embodiment of the invention, enzyme I is CYP76AH8, preferably enzyme I is CYP76AH8 of SEQ ID NO:1 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as

at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH8is a polypeptide also capable of catalysing reactions Ia, Ib, Id and/or Ie described above.

In another embodiment of the invention, enzyme I is CYP76AH17, preferably enzyme I may be CYP76AH17 of SEQ ID NO:10 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH17 is a polypeptide also capable of catalysing reactions Ia, Ib, Id and/or Ie described above.

In another embodiment of the invention, enzyme I is CYP76AH15, preferably enzyme I may be CYP76AH15 of SEQ ID NO:11 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH15 is a polypeptide also capable of catalysing reactions Ia, Ib, Id and/or Ie described above.

In embodiments of the invention, wherein enzyme I catalyses reaction Ia and/or Id, then enzyme I may be CYP76AH11, preferably enzyme I may be CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The

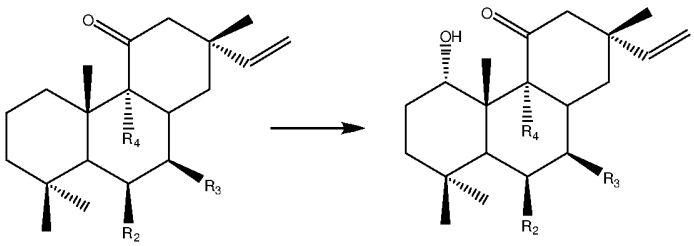
sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH11 may be a polypeptide capable of catalysing reaction Ia and/or Id described above.

II. Enzyme catalysing hydroxylation of 13R-MO at the 1 position

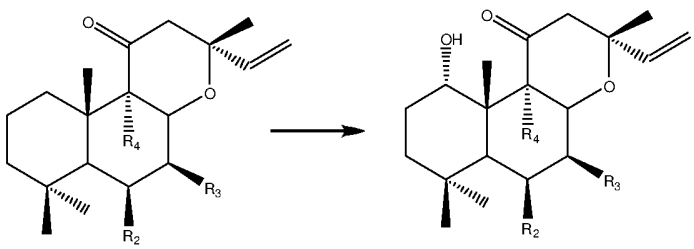
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position. For example, said enzyme may be capable of catalysing hydroxylation of oxidised 11-keto-13R-MO at the 1 position.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme II". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at least at the 1 position with a moiety selected from the group consisting of -OH and OR, wherein R preferably is acyl.

It is preferred that the enzyme II is capable of catalysing the following reaction IIa:



and/or the reaction IIc:

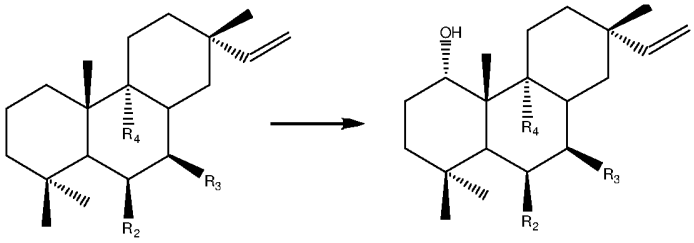


wherein R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

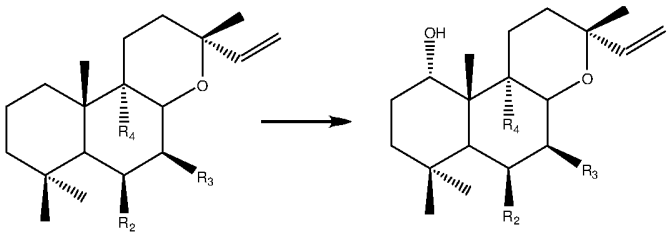
In particular, at least one of R_2, R_3 and R_4 is –H, for example at least two of R_2, R_3 and R_4 is –H, for example all of R_2, R_3 and R_4 is –H.

In one preferred embodiment enzyme II is capable of catalysing reaction IIa, wherein R_2 and R_3 is –OH and R_4 is –H.

It is also preferred that enzyme II is capable of catalysing the following reaction IIb:



and/or the reaction IId:



wherein R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_2, R_3 and R_4 is –H, for example at least two of R_2, R_3 and R_4 is –H, for example all of R_2, R_3 and R_4 is –H.

Thus, enzyme II may be capable of catalysing reaction IIa or reaction IIb or both of reactions IIa and IIb outlined above. Enzyme II may also be capable of catalysing reaction IIc or reaction IId or both of reactions IIc and IId outlined above. It is also

comprised within the invention that said enzyme in addition to being able to catalyse reactions IIa and/or IIb outlined above also may be able to catalyse other reactions, e.g. reactions IIIa, IIIb, IIIc, IIId, IVa, IVb, IVc, IVd, Va, Vb, Vc or Vd outlined below.

Enzyme II may be any useful enzyme with above mentioned activities, in particular enzyme II may be a CYP450. Enzyme II may be derived from any suitable source, but in a preferred embodiment enzyme II is an enzyme from *Coleus forskohlii.* Thus enzyme II may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme II is selected from the group consisting of CYP76AH11, CYP71D381 and CYP76AH9. Thus enzyme II may be selected from the group consisting CYP76AH11 of SEQ ID NO:2, CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 and functional homologues of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In particular, in embodiments of the invention wherein enzyme II is capable of catalysing reaction IIa and/or IIc, then enzyme II may be CYP76AH11, such as CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. Thus, enzyme II may be CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof in embodiments of the invention, wherein enzyme II is capable of catalysing any of reactions IIa, IIb, IIc and/or IId , wherein R_2 and R_3 is –OH and R_4 is –H.

In embodiments of the invention wherein enzyme II is capable of catalysing reaction IIb or IId, then enzyme II may in particular be CYP71D381 or CYP76AH9, such as CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such

as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH11, CYP71D381 and CYP76AH9 may be capable of catalysing reactions IIa, IIb, IIc and/or IId described above.

In another embodiment of the invention, enzyme II is selected from the group consisting of CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH8, CYP76AH15 or CYP76AH17 may be a polypeptide also capable of catalysing reactions IIc and/or IId described above.

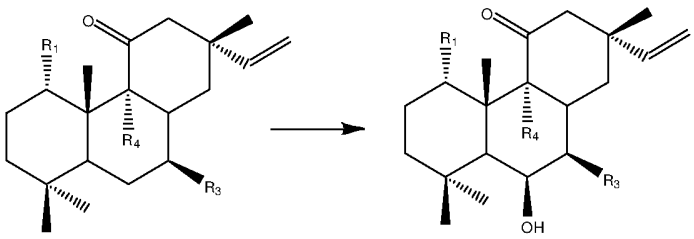
III. Enzyme catalysing hydroxylation of 13R-MO at the 6 position

The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position. For example, said enzyme may be capable of catalysing hydroxylation of oxidised 11-keto-13R-MO at the 6 position.

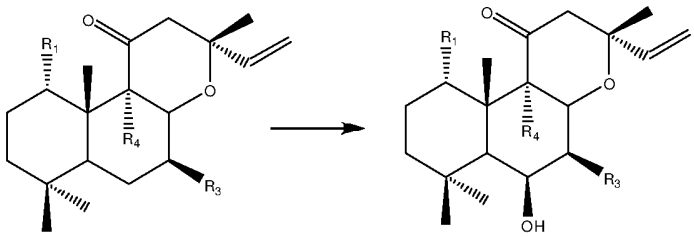
Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme III". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is

substituted at least at the 6 position with a moiety selected from the group consisting of -OH and OR, wherein R preferably is acyl.

It is preferred that the enzyme III is capable of catalysing the following reaction IIIa:



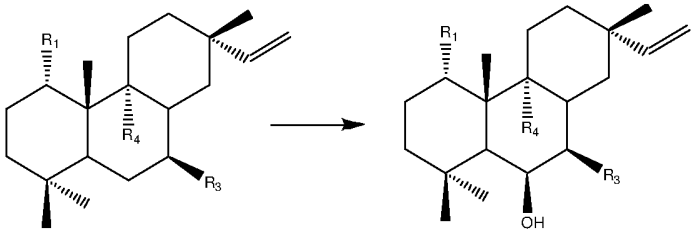
and/or the reaction IIIc:



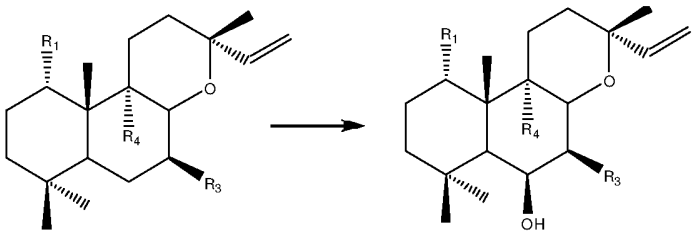
wherein R_1, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_3 and R_4 is –H, for example at least two of R_1, R_3 and R_4 is –H, for example all of R_1, R_3 and R_4 is –H.

It is also preferred that enzyme III is capable of catalysing the following reaction IIIb:



and/or the reaction IIId



wherein R_1, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_3 and R_4 is –H, for example at least two of R_1, R_3 and R_4 is –H, for example all of R_1, R_3 and R_4 is –H.

In one preferred embodiment enzyme III is capable of catalysing reaction IIIa and/or IIIc, wherein all of R_1, R_3 and R_4 are –H.

Thus, enzyme III may be capable of catalysing reaction IIIa or reaction IIIb or both of reactions IIIa and IIIb outlined above. Enzyme III may also be capable of catalysing reactions IIIc or reaction IIId or both of reactions IIIc and IIId outlined above. It is also comprised within the invention that said enzyme in addition to being able to catalyse reactions IIIa, IIIb, IIIc and/or IIId outlined above also may be able to catalyse other reactions, e.g. reactions IIa, IIb, IIc, IId, IVa, IVb, IVc, IVd, Va, Vb, Vc or Vd outlined herein.

Enzyme III may be any useful enzyme with above mentioned activities, in particular enzyme III may be a CYP450. Enzyme III may be derived from any suitable source, but in a preferred embodiment enzyme III is an enzyme from *Coleus forskohlii.* Thus enzyme III may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme III is selected from the group consisting of CYP76AH11, CYP71D381 and CYP76AH9. Thus enzyme III may be selected from the group consisting CYP76AH11 of SEQ ID NO:2, CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 and functional homologues of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at

least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In particular, in embodiments of the invention wherein enzyme III is capable of catalysing reaction IIIa and/or IIIc, then enzyme III may be CYP76AH11, such as CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In embodiments of the invention wherein enzyme III is capable of catalysing reaction IIIb and/or IIId, then enzyme III may in particular be CYP71D381 or CYP76AH9, such as CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section “Sequence identity”. A functional homologue of CYP76AH11, CYP71D381 and CYP76AH9 may be capable of catalysing reactions IIIa, IIIb, IIIc and/or IIId described above.

In another embodiment of the invention, enzyme III is selected from the group consisting of CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section “Sequence identity”. A functional homologue of

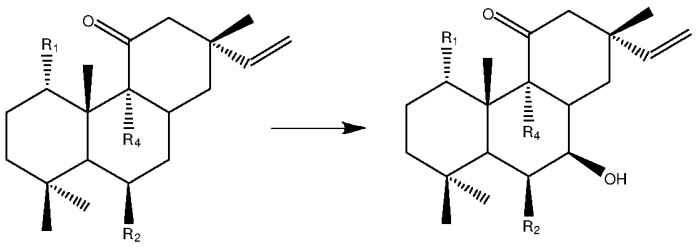
CYP76AH8, CYP76AH15 or CYP76AH17 may be a polypeptide also capable of catalysing reactions IIIc and/or IIId described above.

IV. Enzyme catalysing hydroxylation of 13R-MO at the 7 position

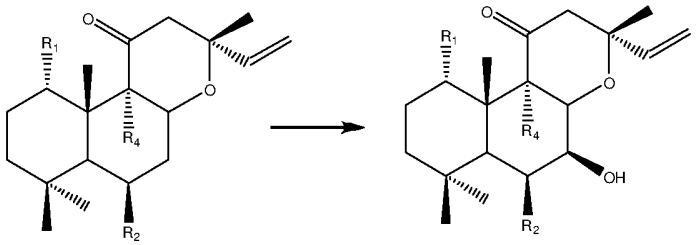
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position. For example, said enzyme may be capable of catalysing hydroxylation of oxidised 11-keto-13R-MO at the 7 position.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme IV". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at least at the 7 position with a moiety selected from the group consisting of -OH and OR, wherein R preferably is acyl.

It is preferred that the enzyme IV is capable of catalysing the following reaction IVa:



and/or reaction IVc:

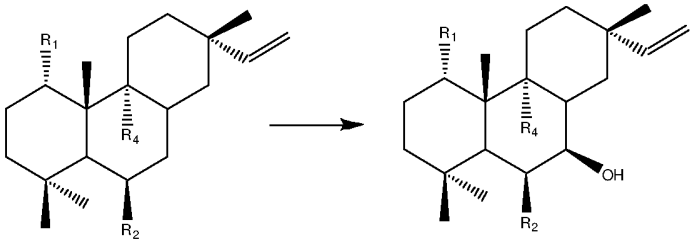


wherein R_1, R_2 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

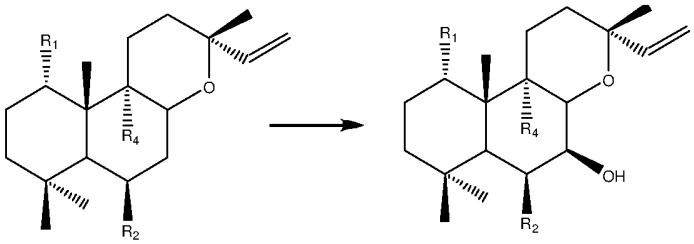
In particular, at least one of R_1, R_2 and R_4 is –H, for example at least two of R_1, R_2 and R_4 is –H, for example all of R_1, R_2 and R_4 is –H.

In one preferred embodiment enzyme IV is capable of catalysing reaction Iva and/or IVc, wherein R_1 and R_4 are –H and R_2 is –OH.

It is also preferred that enzyme IV is capable of catalysing the following reaction IVb:



and/or reaction IVd:



wherein R_1, R_2 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2 and R_4 is –H, for example at least two of R_1, R_2 and R_4 is –H, for example all of R_1, R_2 and R_4 is –H.

Thus, enzyme IV may be capable of catalysing reaction IVa or reaction IVb or both of reactions IVa and IVb outlined above. Enzyme IV may also be capable of catalysing reaction IVc or reaction IVd or both of reactions IVc and IVd outlined above. It is also comprised within the invention that said enzyme in addition to being able to catalyse reactions Iva, IVb, IVc and/or IVd outlined above also may be able to catalyse other reactions, e.g. reactions Ia, Ib, Id, Ie, IIa, IIb, IIc, IId, Va, Vb, Vc or Vd outlined herein.

Enzyme IV may be any useful enzyme with above mentioned activities, in particular enzyme IV may be a CYP450. Enzyme IV may be derived from any suitable source, but in a preferred embodiment enzyme III is an enzyme from *Coleus forskohlii.* Thus enzyme IV may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme IV is selected from the group consisting of CYP76AH11, CYP71D381 and CYP76AH9. Thus enzyme IV may be selected from the group consisting CYP76AH11 of SEQ ID NO:2, CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 and functional homologues of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In particular, in embodiments of the invention wherein enzyme IV is capable of catalysing reaction IVa and/or IVc, then enzyme IV may be CYP76AH11, such as CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. Thus, enzyme IV may be CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof in embodiments of the invention, wherein enzyme IV is capable of catalysing reaction Iva and/or IVc, wherein R_1 and R_4 are –H and R_2 is –OH.

In embodiments of the invention wherein enzyme IV is capable of catalysing reaction IVb and/or IVd, then enzyme IV may in particular be CYP71D381 or CYP76AH9, such as CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at

least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH11, CYP71D381 and CYP76AH9 may be capable of catalysing reactions Iva, IVb, IVc and/or IVd described above.

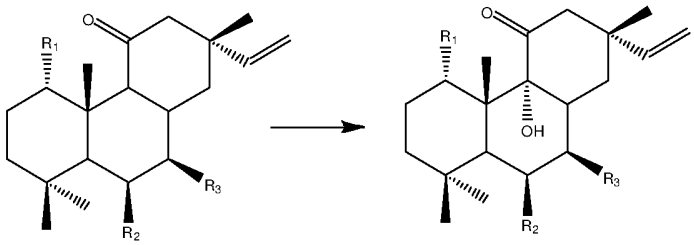
In another embodiment of the invention, enzyme IV is selected from the group consisting of CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH8, CYP76AH15 or CYP76AH17 may be a polypeptide also capable of catalysing reactions IVc and/or IVd described above.

V. Enzyme catalysing hydroxylation of 13R-MO at the 9 position

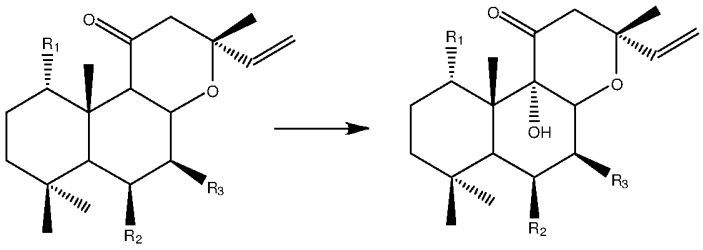
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position. For example, said enzyme may be capable of catalysing hydroxylation of oxidised 11-keto-13R-MO at the 9 position.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme V". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at least at the 9 position with a moiety selected from the group consisting of -OH and OR, wherein R preferably is acyl.

It is preferred that the enzyme V is capable of catalysing the following reaction Va:



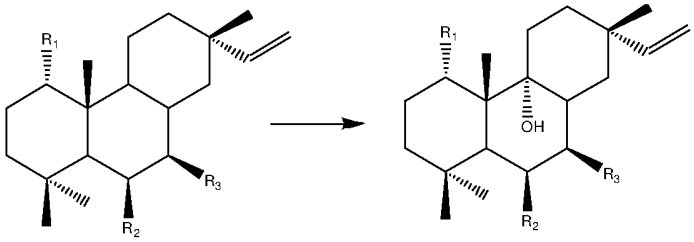
and/or the reaction Vc:



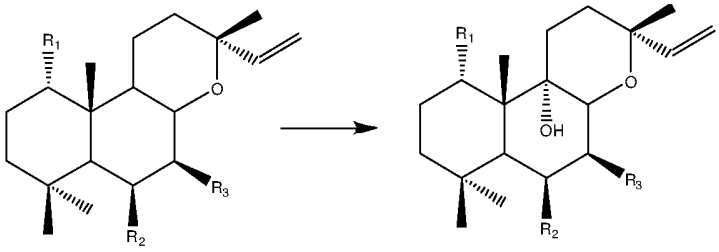
wherein R_1, R_2 and R_3 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2 and R_3 is –H, for example at least two of R_1, R_2 and R_3 is –H, for example all of R_1, R_2 and R_3 is –H.

It is also preferred that enzyme V is capable of catalysing the following reaction Vb:



and/or the reaction Vd



wherein R_1, R_2 and R_3 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2 and R_3 is –H, for example at least two of R_1, R_2 and R_3 is –H, for example all of R_1, R_2 and R_3 is –H.

Thus, enzyme V may be capable of catalysing reaction Va or reaction Vb or both of reactions Va and Vb outlined above. Enzyme V may also be capable of catalysing reaction Vc or reaction Vd or both of reactions Vc and Vd outlined above It is also comprised within the invention that said enzyme in addition to being able to catalyse reactions Va, Vb, Vc and/or Vd outlined above also may be able to catalyse other reactions, e.g. reactions Ia, Ib, Id, Ie, IIa, IIb, IIc, IId, IVa, IVb, IVc or IVd outlined herein.

Enzyme V may be any useful enzyme with above mentioned activities, in particular enzyme V may be a CYP450. Enzyme V may be derived from any suitable source, but in a preferred embodiment enzyme III is an enzyme from *Coleus forskohlii.* Thus enzyme V may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme V is selected from the group consisting of CYP76AH11, CYP71D381 and CYP76AH9. Thus enzyme V may be selected from the group consisting CYP76AH11 of SEQ ID NO:2, CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 and functional homologues of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In particular, in embodiments of the invention wherein enzyme V is capable of catalysing reactions Va and/or Vc, then enzyme V may be CYP76AH11, such as CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

In embodiments of the invention wherein enzyme V is capable of catalysing reaction Vb and/or Vd, then enzyme V may in particular be CYP71D381 or CYP76AH9, such as CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH11, CYP71D381 and CYP76AH9 may be capable of catalysing reactions Vc and/or Vd described above.

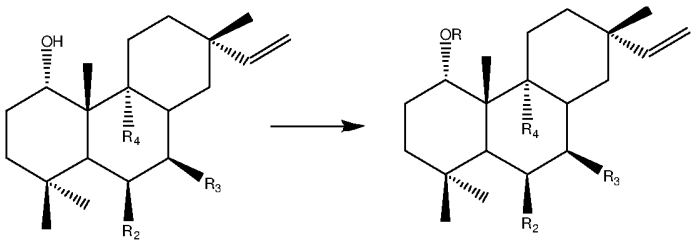
In another embodiment of the invention, enzyme V is selected from the group consisting of CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith. The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP76AH8, CYP76AH15 or CYP76AH17 may be a polypeptide also capable of catalysing reactions Vc and/or Vd described above.

VI. Enzyme catalysing transfer of an acyl group

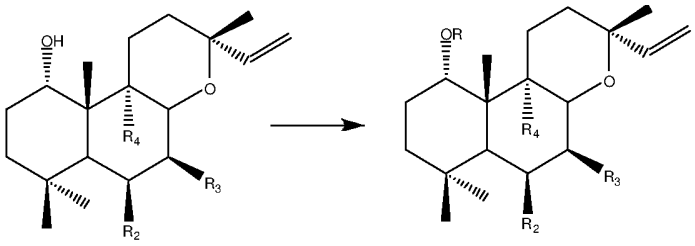
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme VI". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at one of the positions 1, 6, 7, 9 or 11 with -OR, wherein R preferably is acyl.

The enzyme VI may for example be capable of catalysing the following reaction VIa:



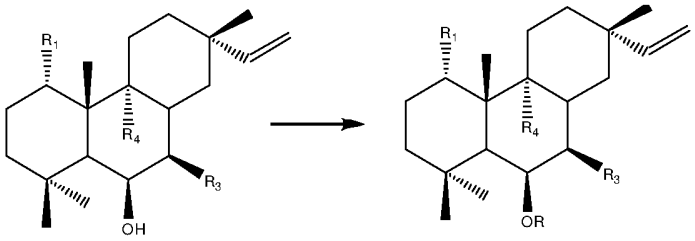
and/or the reaction VId:



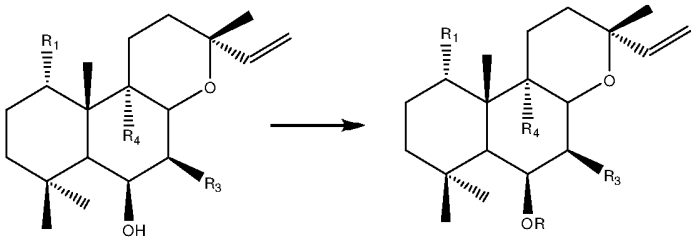
wherein R is acyl, more preferably R is acetyl and
R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OX, wherein X preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_2, R_3 and R_4 is –H or -OH, for example at least two of R_2, R_3 and R_4 is –H or -OH, for example all of R_2, R_3 and R_4 is –H or -OH.

The enzyme VI may for example be capable of catalysing the following reaction VIb:



and/or the reaction VIe:

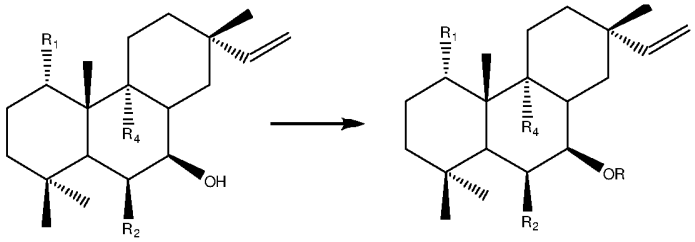


wherein R is acyl, more preferably R is acetyl and

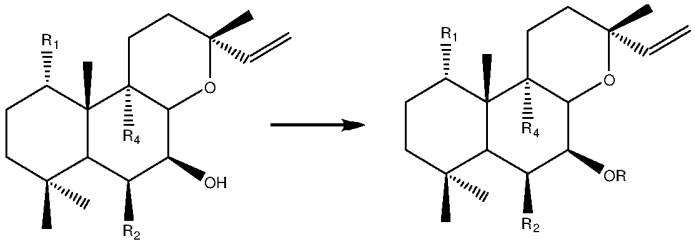
R_1, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OX, wherein X preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_3 and R_4 is –H or -OH, for example at least two of R_1, R_3 and R_4 is –H or -OH, for example all of R_1, R_3 and R_4 is –H or -OH.

The enzyme VI may for example be capable of catalysing the following reaction VIc:



and/or the reaction VIf:



wherein R is acyl, more preferably R is acetyl and

R_1, R_2 and R_4 individually are selected from the group consisting of –H, -OH and –OX, wherein X preferably is acyl. acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2 and R_4 is –H or -OH, for example at least two of R_1, R_2 and R_4 is –H or -OH, for example all of R_1, R_2 and R_4 is –H or -OH.

The enzyme VI may be capable of catalysing one or more of the reactions VIa, VIb, VIc, VId, VIe and VIf outlined above.

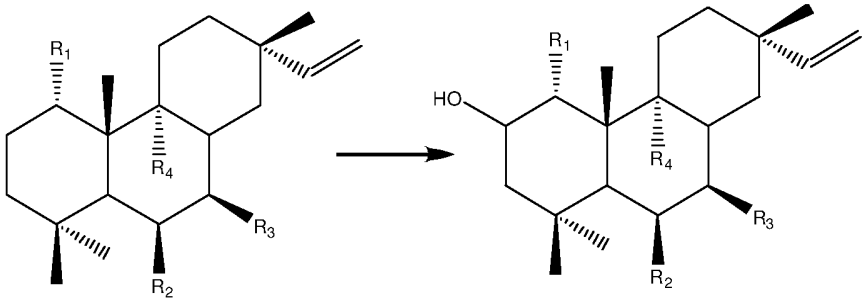
Enzyme VI may be any useful enzyme with above mentioned activities, in particular enzyme I may be an acyl transferase. Enzyme VI may be derived from any suitable source, but in a preferred embodiment enzyme VI is an enzyme from *Coleus forskohlii.* Thus enzyme VI may be a acyl transferase from *Coleus forskohlii.*

VII. Enzyme catalysing hydroxylation of 13R-MO at the 2 position

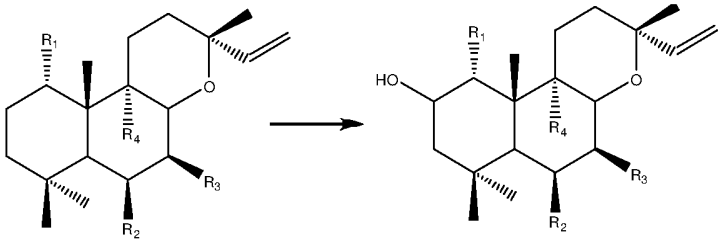
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 2 position, wherein said oxidised 13R-MO carries a –H at the 2-position.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme VII". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted at least at the 2 position with -OH.

It is preferred that the enzyme VII is capable of catalysing the following reaction VII:



and/or reaction VIIb



wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2, R_3 and R_4 is –H, for example at least two of R_1, R_2, R_3 and R_4 are –H, for example all of R_1, R_2, R_3 and R_4 are –H.

Enzyme VII may be any useful enzyme with above mentioned activities, in particular enzyme VII may be a CYP450. Enzyme VII may be derived from any suitable source, but in a preferred embodiment enzyme VII is an enzyme from *Coleus forskohlii.* Thus enzyme VII may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme VII may be CYP71D381, such as CYP71D381 of SEQ ID NO:3 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

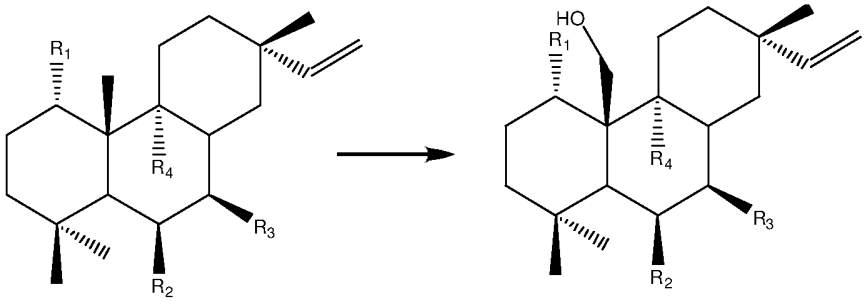
The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP71D381 may be capable of catalysing reaction VII and/or reaction VIIb described above.

VIII. Enzyme catalysing hydroxylation of 13R-MO at the methyl on the 10 position

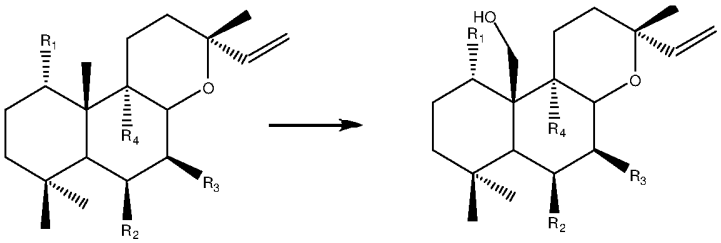
The host organisms to be used with the present invention comprise one or more heterologous nucleic acids. Thus, the host organism may comprise a heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO on the methyl group at the 10 position, wherein said oxidised 13R-MO carries a $-CH_3$ at the 10-position.

Said enzyme may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme VIII". It is in particular preferred that the host organism comprises a heterologous nucleic acid encoding said enzyme, in embodiments of the invention, wherein the oxidised 13R-MO to be produced is substituted with -OH at least on the methyl at the 10 position.

It is preferred that the enzyme VIII is capable of catalysing the following reaction VIII:



and/or reaction VIIIb



wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl. Acyl is as defined in the section "Oxidised 13R-MO" herein below.

In particular, at least one of R_1, R_2, R_3 and R_4 is –H, for example at least two of R_1, R_2, R_3 and R_4 are –H, for example all of R_1, R_2, R_3 and R_4 are –H.

Enzyme VIII may be any useful enzyme with above mentioned activities, in particular enzyme VIII may be a CYP450. Enzyme VIII may be derived from any suitable source, but in a preferred embodiment enzyme VIII is an enzyme from *Coleus forskohlii.* Thus enzyme VIII may be a CYP450 from *Coleus forskohlii.*

In one embodiment of the invention, enzyme VIII may be CYP71D381, such as CYP71D381 of SEQ ID NO:3 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

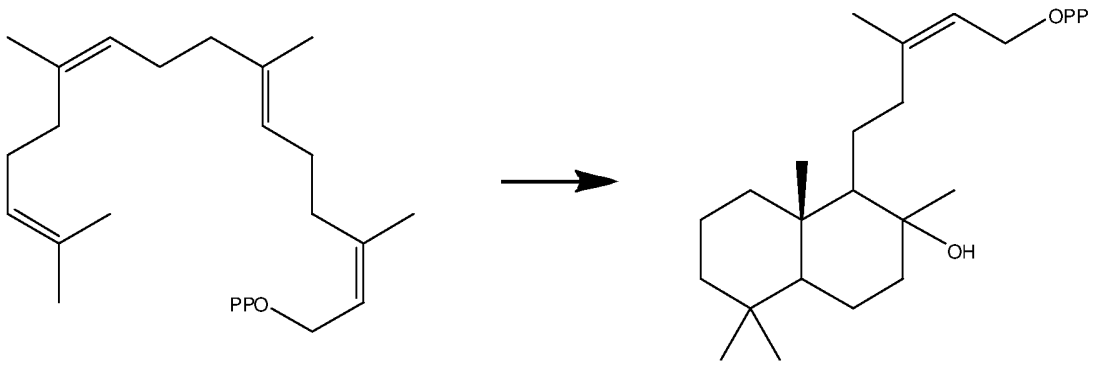
The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of CYP71D381 may be capable of catalysing reaction VIII described above.

IX. TPS2

In addition to the heterologous nucleic acids I, II, III, IV, V and/or VI, the host organism may comprise a heterologous nucleic acid encoding TPS2. It is preferred that in embodiments of the invention where the host organism comprises a nucleic acid encoding TPS2, then the host organism also comprises a heterologous nucleic acid encoding either TPS3 or TPS4.

Said TPS2 may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme IX".

Preferably said TPS2 is an enzyme capable of catalysing the reaction IX:



wherein -OPP refers to diphosphate.

In particular, it is preferred that said TPS2 is TPS2 of *Coleus forskohlii*. In particular, said enzyme IX may be a polypeptide of SEQ ID NO:7 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

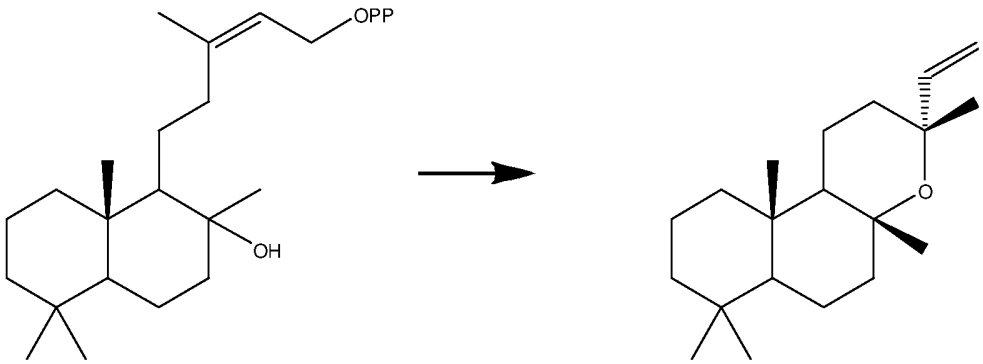
The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of a TPS2 is a polypeptide, which is also capable of catalysing reaction IX described above.

X. TPS3

In addition to the heterologous nucleic acids I, II, III, IV, V and/or VI, the host organism may comprise a heterologous nucleic acid encoding TPS3. It is preferred that in embodiments of the invention where the host organism comprises a nucleic acid encoding TPS3, then the host organism also comprises a heterologous nucleic acid encoding TPS2.

Said TPS3 may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme X".

Preferably said TPS3 is an enzyme capable of catalysing the reaction X:



In particular, it is preferred that said TPS3 is TPS3 of *Coleus forskohlii*. In particular, said enzyme X may be a polypeptide of SEQ ID NO:8 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

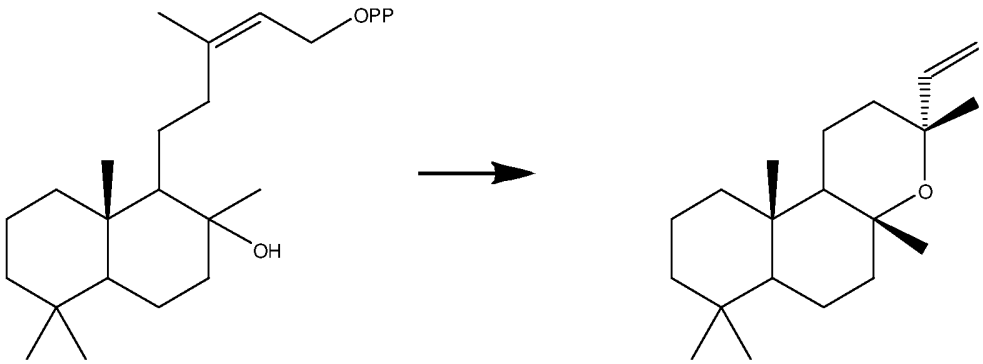
The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of a TPS3 is a polypeptide, which is also capable of catalysing reaction X described above.

XI. TPS4

In addition to the heterologous nucleic acids I, II, III, IV, V and/or VI, the host organism may comprise a heterologous nucleic acid encoding TPS4. It is preferred that in embodiments of the invention where the host organism comprises a nucleic acid encoding TPS4, then the host organism also comprises a heterologous nucleic acid encoding TPS2.

Said TPS4 may for example be any of the enzymes described herein in this section and may also be referred to herein as "enzyme XI".

Preferably said TPS4 is an enzyme capable of catalysing the reaction XI:



In particular, it is preferred that said TPS4 is TPS4 of *Coleus forskohlii*. In particular, said enzyme XI may be a polypeptide of SEQ ID NO:9 or a functional homologue thereof sharing at least 70%, such as at least 80%, for example at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section "Sequence identity". A functional homologue of a TPS4 is a polypeptide, which is also capable of catalysing reaction XI described above.

Sequence identity

A high level of sequence identity indicates likelihood that the first sequence is derived from the second sequence. Amino acid sequence identity requires identical amino acid sequences between two aligned sequences. Thus, a candidate sequence sharing 80% amino acid identity with a reference sequence, requires that, following alignment, 80% of the amino acids in the candidate sequence are identical to the corresponding amino acids in the reference sequence. Identity according to the present invention is determined by aid of computer analysis, such as, without limitations, the ClustalW computer alignment program (Higgins D., Thompson J., Gibson T., Thompson J.D., Higgins D.G., Gibson T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680), and the default parameters suggested therein. The ClustalW software is available from as a ClustalW

WWW Service at the European Bioinformatics Institute http://www.ebi.ac.uk/clustalw. Using this program with its default settings, the mature (bioactive) part of a query and a reference polypeptide are aligned. The number of fully conserved residues are counted and divided by the length of the reference polypeptide.

The ClustalW algorithm may similary be used to align nucleotide sequences. Sequence identities may be calculated in a similar way as indicated for amino acid sequences.
In one important embodiment, the cell of the present invention comprises a nucleic acid sequence coding, as define herein.

Heterologous nucleic acid

The term "heterologous nucleic acid" as used herein refers to a nucleic acid sequence, which has been introduced into the host organism, wherein said host does not endogenously comprise said nucleic acid. For example, said heterologous nucleic acid may be introduced into the host organism by recombinant methods. Thus, the genome of the host organism has been augmented by at least one incorporated heterologous nucleic acid sequence. It will be appreciated that typically the genome of a recombinant host described herein is augmented through the stable introduction of one or more heterologous nucleic acids encoding one or more enzymes.

Suitable host organisms include microorganisms, plant cells, and plants, and may for example be any of the host organisms described herein below in the section "Host organism".

In general the heterologous nucleic acid encoding a polypeptide (also referred to as "coding sequence" in the following) is operably linked in sense orientation to one or more regulatory regions suitable for expressing the polypeptide. Because many microorganisms are capable of expressing multiple gene products from a polycistronic mRNA, multiple polypeptides can be expressed under the control of a single regulatory region for those microorganisms, if desired. A coding sequence and a regulatory region are considered to be operably linked when the regulatory region and coding sequence are positioned so that the regulatory region is effective for regulating transcription or translation of the sequence. Typically, the translation initiation site of the translational

reading frame of the coding sequence is positioned between one and about fifty nucleotides downstream of the regulatory region for a monocistronic gene.

"Regulatory region" refers to a nucleic acid having nucleotide sequences that influence transcription or translation initiation and rate, and stability and/or mobility of a transcription or translation product. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, protein binding sequences, 5´ and 3´ untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, introns, and combinations thereof. A regulatory region typically comprises at least a core (basal) promoter. A regulatory region also may include at least one control element, such as an enhancer sequence, an upstream element or an upstream activation region (UAR). A regulatory region is operably linked to a coding sequence by positioning the regulatory region and the coding sequence so that the regulatory region is effective for regulating transcription or translation of the sequence. For example, to operably link a coding sequence and a promoter sequence, the translation initiation site of the translational reading frame of the coding sequence is typically positioned between one and about fifty nucleotides downstream of the promoter. A regulatory region can, however, be positioned at further distance, for example as much as about 5,000 nucleotides upstream of the translation initiation site, or about 2,000 nucleotides upstream of the transcription start site.

The choice of regulatory regions to be included depends upon several factors, including the type of host organism. It is a routine matter for one of skill in the art to modulate the expression of a coding sequence by appropriately selecting and positioning regulatory regions relative to the coding sequence. It will be understood that more than one regulatory region may be present, *e.g.*, introns, enhancers, upstream activation regions, transcription terminators, and inducible elements.

It will be appreciated that because of the degeneracy of the genetic code, a number of nucleic acids can encode a particular polypeptide; *i.e.*, for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. Thus, codons in the coding sequence for a given polypeptide can be modified such that optimal expression in a particular host organisms obtained, using appropriate codon bias tables for that host (e.g., microorganism). Nucleic acids may also be optimized to

a GC-content preferable to a particular host, and/or to reduce the number of repeat sequences. As isolated nucleic acids, these modified sequences can exist as purified molecules and can be incorporated into a vector or a virus for use in constructing modules for recombinant nucleic acid constructs.

A non-limiting example of a heterologous nucleic acid encoding CYP76AH8 is provided herein as SEQ ID NO:5. Thus, the heterologous nucleic acid encoding enzyme I may comprise or consist of SEQ ID NO:5 or a sequence sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith. Said heterologous nucleic acid is particularly useful in embodiments of the invention where the host is a yeast cell, such as S. cerevisiae.
A non-limiting example of a heterologous nucleic acid encoding CYP76AH11 is provided herein as SEQ ID NO:6. Thus, the heterologous nucleic acid encoding enzyme II, enzyme III or enzyme may comprise or consist of SEQ ID NO:6 or a sequence sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith. Said heterologous nucleic acid is particularly useful in embodiments of the invention where the host is a yeast cell, such as S. cerevisiae.

Oxidised 13R-MO

The present invention relates to methods for producing forskolin and related compounds. In particular, the invention relates to methods for producing oxidised 13R-MO.

The term “oxidised 13R-MO” as used herein refers to 13R-manoyl-oxide (13R-MO) substituted at one or more positions with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl.

The term “substituted with a moiety” as used herein in relation to chemical compounds refers to hydrogen group(s) being substituted with said moiety.

The term “acyl” as used herein denoted a substituent of the formula –(C=O)-alkyl. “Alkyl” as used herein refers to a saturated, straight or branched hydrocarbon chain. The hydrocarbon chain preferably contains of from one to eighteen carbon atoms (C_{1-}

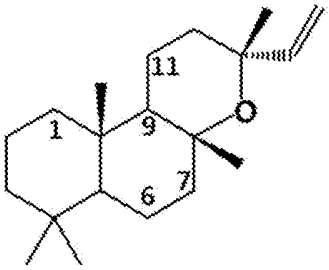
$_{18}$-alkyl), more preferred of from one to six carbon atoms (C_{1-6}-alkyl), including methyl, ethyl, propyl, isopropyl, butyl, isobutyl, secondary butyl, tertiary butyl, pentyl, isopentyl, neopentyl, tertiary pentyl, hexyl and isohexyl. In a preferred embodiment alkyl represents a C_{1-3}-alkyl group, which may in particular include methyl, ethyl, propyl or isopropyl. In another preferred embodiment of this invention alkyl represents methyl.

The term "oxo" as used herein refers to a "=O" substituent.

The term "keto-" as used herein is used as a prefix to indicate possession of a carbonyl (C=O) group.

The term "hydroxyl" as used herein refers to a "-OH" substituent.

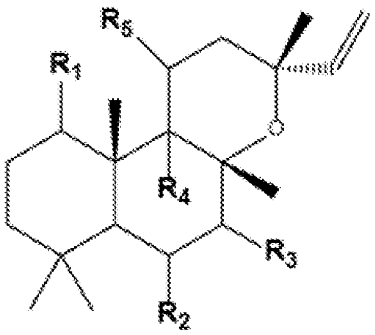
The structure of 13R-manoyl-oxide (13R-MO) is provided below. The structure also provides the numbering of the carbon atoms of the ring structure used herein.



Preferably, the oxidised 13R-MO according to the present invention, is 13R-MO substituted at one or more of the positions 1, 6, 7, 9 and/or 11 with a moiety selected from the group consisting of =O, -OH and OR, wherein R preferably is acyl.

In another embodiment of the invention the oxidised 13R-MO is 13R-MO substituted at the 2 position with –OH. In yet another embodiment of the invention the oxidised 13R-MO is 13R-MO substituted on the methyl at the 10 position with –OH. Thus, the substituent on the 1 position may be $–CH_2$-OH.

Thus, for example the oxidised 13R-MO may be a compound of formula III:



wherein R_1, R_2, R_3, R_4 and R_5 individually are selected from the group consisting of, -H =O, -OH and -OR, wherein R preferably is acyl, more preferably R is –(C=O)-CH_3.

For example, R_1 may be selected from the group consisting of –OH, -H and =O.

For example R_2 may be selected from the group consisting of –OH and –O-acyl, for example R_2 may be selected from the group consisting of –OH and –O-(C=O)-CH_3.

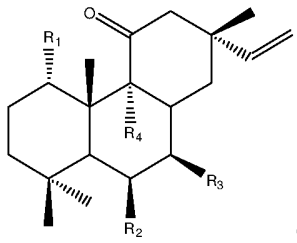
For example R_3 may be selected from the group consisting of –OH and –O-acyl, for example R_3 may be selected from the group consisting of –OH and –O-(C=O)-CH_3.

For example R_4 may be selected from the group consisting of -H and –OH.

For example R_5 may be selected from the group consisting of =O and –O-acyl, for example R_5 may be selected from the group consisting of =O and –O-(C=O)-CH_3.

In particular, the oxidised 13R-MO may be 13R-MO, which is substituted at the 11 position with =O. In addition, to said substitution, the oxidised 13R-MO may be substituted at one or more of the positions 1, 6, 7 and 9 with a moiety selected from the group consisting of =O, -OH and OR, preferably with a moiety selected from the group consisting of =O, -OH and -OR. R may be acyl, wherein acyl is as defined above.

Thus, in one embodiment of the invention said oxidised 13R-MO is a compound of the formula I



(I)

wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl, wherein acyl is as defined above. It is preferred that at least one of R_1, R_2, R_3 and R_4 is –OH or –OR, more preferably at least two of R_1, R_2, R_3 and R_4 is –OH or –OR, for example at least three of least one of R_1, R_2, R_3 and R_4 is –OH or –OR, for example all of R_1, R_2, R_3 and R_4 is –OH or –OR.

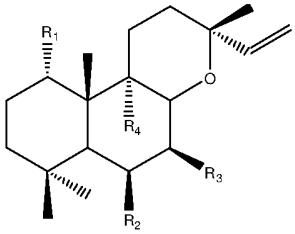
R_1 may be selected from the group consisting of –H, -OH and –OR, wherein R is as defined above. Preferably, R_1 is selected from the group consisting of –H and –OH, in particular R_1 may be –OH.

R_2 may be selected from the group consisting of –H, -OH and –OR, wherein R is as defined above. Preferably, R_2 is selected from the group consisting of –OR and –OH, wherein R is as defined above. For example R_2 may be selected from the group consisting of –O-(C=O)-CH_3 (acetyl), –O-(C=O)-CH_2-CH_3, –O-(C=O)-CH_2-CH_2-CH_3 and –OH. In particular, R_2 may be –OH.

R_3 may be selected from the group consisting of –H, -OH and –OR, wherein R is as defined above. Preferably, R_3 is selected from the group consisting of –OR and –OH, wherein R is as defined above. For example R_3 may be selected from the group consisting of –O-(C=O)-CH_3 (acetyl), –O-(C=O)-CH_2-CH_3, –O-(C=O)-CH_2-CH_2-CH_3 and –OH. In particular, R_3 may be acetyl.

R_4 may be selected from the group consisting of –H, -OH and –OR, wherein R is as defined above. Preferably, R_4 is selected from the group consisting of –H and –OH, in particular R_4 may be –OH.

It is also comprised within the invention that the oxidised 13R-MO is not substituted at the 11 position. Thus, the oxidised 13R-MO may be a compound of the formula (II)



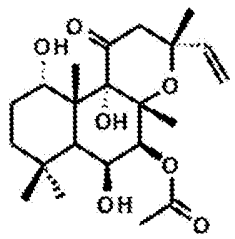
wherein each of R_1, R_2, R_3 and R_4 may be as indicated herein above in relation to compounds of formula I.

For example, the oxidised 13R-MO may be selected from the group consisting of compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14b and 15 shown in figure 8.

The oxidised 13R-MO may also be any of the oxidised 13R-MO shown in figure 7, such as compounds 2, 3a, 3b, 4, 5, 6, 7a, 7b, 8, 9, 10 or 11 of figure 7.

In particular, the oxidised 13R-MO may be selected from the group consisting of forskolin, iso-forskolin, forskolin B, forskolin D, 9-deoxyforskolin, 1,9-dideoxyforskolin and coleoforskolin. The structures of these compounds are provided in figure 6.

In a preferred embodiment of the invention, the invention relates to methods for producing forskolin. The term “forskolin” as used herein refers to a compound of the formula



forskolin

Host organism

The host organism to be used with the methods of the invention, may be any suitable host organism containing one or more of the heterologous nucleic acids I., II., III., IV., V. and/or VI. described herein above.

Suitable host organisms include microorganisms, plant cells, and plants.

The microorganism can be any microorganism suitable for expression of heterologous nucleic acids. In one embodiment the host organism of the invention is a eukaryotic cell. In another embodiment the host organism is a prokaryotic cell.
In a preferred embodiment, the host organism is a fungal cell such as a yeast or filamentous fungus. In particular the host organism may be a yeast cell.

In a further embodiment the yeast cell is selected from the group consisting of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Yarrowia lipolytica, Candida glabrata, Ashbya gossypii, Cyberlindnera jadinii, and Candida albicans.

In general, yeasts and fungi are excellent microorganism to be used with the present invention. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media. For instance yeasts grow on a wide range of carbon sources and are not restricted to glucose. Thus, the microorganism to be used with the present invention may be selected from the group of yeasts described below:

Arxula adeninivorans (Blastobotrys adeninivorans) is a dimorphic yeast (it grows as a budding yeast like the baker's yeast up to a temperature of 42 °C, above this threshold it grows in a filamentous form) with unusual biochemical characteristics. It can grow on a wide range of substrates and can assimilate nitrate. It has successfully been applied to the generation of strains that can produce natural plastics or the development of a biosensor for estrogens in environmental samples.

Candida boidinii is a methylotrophic yeast (it can grow on methanol). Like other methylotrophic species such as Hansenula polymorpha and Pichia pastoris, it provides an excellent platform for the production of heterologous proteins. Yields in a multigram range of a secreted foreign protein have been reported. A computational method, IPRO, recently predicted mutations that experimentally switched the cofactor specificity of Candida boidinii xylose reductase from NADPH to NADH. Details on how to download the software implemented in Python and experimental testing of predictions are outlined in the following paper.

Hansenula polymorpha (Pichia angusta) is another methylotrophic yeast (see Candida boidinii). It can furthermore grow on a wide range of other substrates; it is thermo-

tolerant and can assimilate nitrate (see also Kluyveromyces lactis). It has been applied to the production of hepatitis B vaccines, insulin and interferon alpha-2a for the treatment of hepatitis C, furthermore to a range of technical enzymes.

Kluyveromyces lactis is a yeast regularly applied to the production of kefir. It can grow on several sugars, most importantly on lactose which is present in milk and whey. It has successfully been applied among others to the production of chymosin (an enzyme that is usually present in the stomach of calves) for the production of cheese. Production takes place in fermenters on a 40,000 L scale.

Pichia pastoris is a methylotrophic yeast (see Candida boidinii and Hansenula polymorpha). It provides an efficient platform for the production of foreign proteins. Platform elements are available as a kit and it is worldwide used in academia for the production of proteins. Strains have been engineered that can produce complex human N-glycan (yeast glycans are similar but not identical to those found in humans).

Saccharomyces cerevisiae is the traditional baker's yeast known for its use in brewing and baking and for the production of alcohol. As protein factory it has successfully been applied to the production of technical enzymes and of pharmaceuticals like insulin and hepatitis B vaccines. Also it has been useful for production of terpenoids.

Yarrowia lipolytica is a dimorphic yeast (see Arxula adeninivorans) that can grow on a wide range of substrates. It has a high potential for industrial applications but there are no recombinant products commercially available yet.

In another embodiment the host organism is a microalgae such as Chlorella and Prototheca.

In another embodiment of the invention the host organism is a filamentous fungus, for example Aspergillus.

In further yet another embodiment the host organism is a plant cell. The host organism may be a cell of a higher plant, but the host organism may also be cells from organisms not belonging to higher plants for example cells from the moss Physcomitrella patens.

In another embodiment the host organism is a mammalian cell, such as a human, feline, porcine, simian, canine, murine, rat, mouse or rabbit cell.

As mentioned, the host organism can also be a prokaryotic cell such as a bacterial cell. If the host organism is a prokaryotic cell the cell may be selected from, but not limited to E. coli, Corynebacterium, Bacillus, Pseudomonas and Streptomyces cells.

The host organism may also be a plant.

A plant or plant cell can be transformed by having a heterologous nucleic acid integrated into its genome, *i.e.*, it can be stably transformed. Stably transformed cells typically retain the introduced nucleic acid with each cell division. A plant or plant cell can also be transiently transformed such that the recombinant gene is not integrated into its genome. Transiently transformed cells typically lose all or some portion of the introduced nucleic acid with each cell division such that the introduced nucleic acid cannot be detected in daughter cells after a certain number of cell divisions. Both transiently transformed and stably transformed transgenic plants and plant cells can be useful in the methods described herein.

Plant cells comprising a heterologous nucleic acid used in methods described herein can constitute part or all of a whole plant. Such plants can be grown in a manner suitable for the species under consideration, either in a growth chamber, a greenhouse, or in a field. Plants may also be progeny of an initial plant comprising a heterologous nucleic acid provided the progeny inherits the heterologous nucleic acid. Seeds produced by a transgenic plant can be grown and then selfed (or outcrossed and selfed) to obtain seeds homozygous for the nucleic acid construct.

The plants to be used with the invention can be grown in suspension culture, or tissue or organ culture. For the purposes of this invention, solid and/or liquid tissue culture techniques can be used. When using solid medium, plant cells can be placed directly onto the medium or can be placed onto a filter that is then placed in contact with the medium. When using liquid medium, transgenic plant cells can be placed onto a flotation device, *e.g.*, a porous membrane that contacts the liquid medium.

When transiently transformed plant cells are used, a reporter sequence encoding a reporter polypeptide having a reporter activity can be included in the transformation procedure and an assay for reporter activity or expression can be performed at a suitable time after transformation. A suitable time for conducting the assay typically is about 1-21 days after transformation, *e.g.*, about 1-14 days, about 1-7 days, or about 1-3 days. The use of transient assays is particularly convenient for rapid analysis in different species, or to confirm expression of a heterologous polypeptide whose expression has not previously been confirmed in particular recipient cells.

Techniques for introducing nucleic acids into monocotyledonous and dicotyledonous plants are known in the art, and include, without limitation, *Agrobacterium*-mediated transformation, viral vector-mediated transformation, electroporation and particle gun transformation, U.S. Patent Nos 5,538,880; 5,204,253; 6,329,571; and 6,013,863. If a cell or cultured tissue is used as the recipient tissue for transformation, plants can be regenerated from transformed cultures if desired, by techniques known to those skilled in the art.

The plant comprising a heterologous nucleic acid to be used with the present invention may for example be selected from: corn (Zea. mays), canola (Brassica napus, Brassica rapa ssp.), alfalfa (Medicago sativa), rice (Oryza sativa), rye (Secale cerale), sorghum (Sorghum bicolor, Sorghum vulgare), sunflower (Helianthus annuas), wheat (Tritium aestivum and other species), Triticale, Rye (Secale) soybean (Glycine max), tobacco (Nicotiana tabacum), potato (Solanum tuberosum), peanuts (Arachis hypogaea), cotton (Gossypium hirsutum), sweet potato (Impomoea batatus), cassava (Manihot esculenta), coffee (Cofea spp.), coconut (Cocos nucifera), pineapple (Anana comosus), citrus (Citrus spp.) cocoa (Theobroma cacao), tea (Camellia senensis), banana (Musa spp.), avacado (Persea americana), fig (Ficus casica), guava (Psidium guajava), mango (Mangifer indica), olive (Olea europaea), papaya (Carica papaya), cashew (Anacardium occidentale), macadamia (Macadamia intergrifolia), almond (Primus amygdalus), apple (Malus spp), Pear (Pyrus spp), plum and cherry tree (Prunus spp), Ribes (currant etc.), Vitis, Jerusalem artichoke (Helianthemum spp), non-cereal grasses (Grass family), sugar and fodder beets (Beta vulgaris), chicory, oats, barley, vegetables, and ornamentals.

For example, plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea, sugar beets, sugar cane, soybean, oilseed rape, sunflower and other root, tuber or seed crops. Other important plants maybe fruit trees, crop trees, forest trees or plants grown for their use as spices or pharmaceutical products (Mentha spp, clove, Artemesia spp, Thymus spp, Lavendula spp, Allium spp., Hypericum, Catharanthus spp, Vinca spp, Papaver spp., Digitalis spp, Rawolfia spp., Vanilla spp., Petrusilium spp., Eucalyptus, tea tree, Picea spp, Pinus spp, Abies spp, Juniperus spp,. Horticultural plants which may be used with the present invention may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower, carrots, and carnations and geraniums.

The plant may also be selected from the group consisting of tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper and Chrysanthemum.

The plant may also be a grain plants for example oil-seed plants or leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, sorghum, rye, etc. Oil-seed plants include cotton soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mung bean, lima bean, fava bean, lentils, chickpea.

In a further embodiment of the invention said plant is selected from the following group: maize, rice, wheat, sugar beet, sugar cane, tobacco, oil seed rape, potato and soybean. Thus, the plant may for example be rice.

In one embodiment the plant is tobacco.

The whole genome of *Arabidopsis thaliana* plant has been sequenced (The Arabidopsis Genome Initiative (2000). "Analysis of the genome sequence of the flowering plant Arabidopsis thaliana". *Nature* **408** (6814): 796–815. doi:10.1038/35048692. PMID 11130711). Consequently, very detailed knowledge is available for this plant and it may therefore be a useful plant to work with.

Accordingly, one plant, which may be used with the present invention is an *Arabidopsis* and in particular an *Arabidopsis thaliana*.

It may be preferred that the plant is not *Coleus forskohlii*.

In one embodiment of the invention, the host organism may comprise at least the following heterologous nucleic acids:

a) a heterologous nucleic acid encoding CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith.

In one embodiment of the invention, the host organism may comprise at least the following heterologous nucleic acids:

a) a heterologous nucleic acid encoding CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith.

In one embodiment of the invention, the host organism may comprise at least the following heterologous nucleic acids:

a) a heterologous nucleic acid encoding CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith; and

b) a heterologous nucleic acid encoding CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith.

In one embodiment of the invention, the host organism may comprise at least the following heterologous nucleic acids:

a) a heterologous nucleic acid encoding CYP76AH8 of SEQ ID NO:1, CYP76AH15 of SEQ ID NO:11, CYP76AH17 of SEQ ID NO:10 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith; and

b) a heterologous nucleic acid encoding CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith; and

c) a heterologous nucleic acid encoding TPS2 of SEQ ID NO:7 or a functional homologue thereof sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith; and

d) a heterologous nucleic acid encoding TPS3 of SEQ ID NO:8, TPS4 of SEQ ID NO:9 or a functional homologue of any of the aforementioned sharing at least 70%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 98%, such as at least 99% sequence identity therewith.

Sequence listing

SEQ ID NO:1 – amino acid sequence of CfCYP76AH8
METITLLLALFFIALTYFISSRRRRNLPPGPFPLPIIGNMLQLGSKPHQSFAQLSKKYGPL
MSIHLGSLYTVIVSSPEMAKEILQKHGQVFSGRTIAQAVHACDHDKISMGFLPVANTW
RDMRKICKEQMFSHHSLEASEELRHQKLQQLLDYAQKCCEAGRAVDIREASFITTLNL
MSATMFSTQATEFDSEATKEFKEIIEGVATIVGVANFADYFPILKPFDLQGIKRRADGYF
GRLLKLIEGYLNERLESRRLNPDAPRKKDFLETLVDIIEANEYKLTTEHLTHLMLDLFVG
GSETNTTSLEWIMSELVINPDKMAKVKEELKSVVGDEKLVNESDMPRLPYLQAVIKEV
LRIHPPGPLLLPRKAESDQVVNGYLIPKGTQILFNAWAMGRDPTIWKDPESFEPERFL
NQSIDFKGQDFELIPFGSGRRICPGMPLANRILHMTTATLVHNFDWKLEEGTADADHK
GELFGLAVRRATPLRIIPLKP

SEQ ID NO:2 – amino acid sequence of CfCYP76AH11
MELVQVIAVVAVVVVLWSQLKRKGRKLPPGPSPLPIVGNIFQLSGKNINESFAKLSKIY
GPVMSLRLGSLLTVIISSPEMAKEVLTSKDFANRPLTEAAHAHGHSKFSVGFVPVSDP
KWKQMRRVCQEEMFASRILENSQQRRHQKLQELIDHVQESRDAGRAVTIRDPVFATT
LNIMSLTLFSADATEFSSSATAELRDIMAGVVSVLGAANLADFFPILKYFDPQGMRRKA
DLHYGRLIDHIKSRMDKRSELKKANPNHPKHDDFLEKIIDITIQRNYDLTINEITHLLVDL
YLAGSESTVMTIEWTMAELMLRPESLAKLKAELRSVMGERKMIQESDDISRLPYLNGA
IKEALRLHPPGPLLFARKSEIDVELSGYFIPKGTQILVNEWGMGRDPSVWPNPECFQP
ERFLDKNIDYKGQDPQLIPFGAGRRICPGIPIAHRVVHSVVAALVHNFDWEFAPGGSQ
CNNEFFTGAALVREVPLKLIPLNPPSI

SEQ ID NO:3 – amino acid sequence of CfCYP71D381

MEFDFPSALIFPAVSLLLLLWLTKTRKPKSDLDRIPGPRRLPLIGNLHHLISLTPPPRLFR
EMAAKYGPLMRLQLGGVPFLIVSSVDVAKHVVKTNDVPFANRPPMHAARAITYNYTDI
GFAPYGEYWRNLRKICTLELLSARRVRSFRHIREEENAGVAKWIASKEGSPANLSERV
YLSSFDITSRASIGKATEEKQTLTSSIKDAMKLGGFNVADLYPSSKLLLLITGLNFRIQRV
FRKTDRILDDLLSQHRSTSATTERPEDLVDVLLKYQKEETEVHLNNDKIKAVIMDMFLA
GGETSATAVDWAMAEMIRNPTTLKKAQEEVRRVFDGKGYVDEEEFHELKYLKLVIKE
MLRMHPPLPFLVPRMNSERCEINGYEIPANTRLLINAWAIGRPKYWNDAEKFIPERFE
NSSIDFKGNNLEYIPFGAGRRMCPGMTFGLASVEFTLAMLLYHFDWKMPQGIKLDMT
ESFGASLKRKHDLLMIPTLKRPLRLAP

SEQ ID NO:4 – amino acid sequence of CfCYP76AH9

MDFFTLLAALFLITLTFFLFFKSESKRRGGANLPPGPYPLPIVGNIFQLGKKPHQSLAQL
AKIHGPLMSLHFGSVYTVIVTSPEMAKEIFVKNDQAFLNRTVVEAVHAHDHDKISMAF
MDVGTEWRTLRRICKEQMFSTQSLETSQGLRQEKLQQLHDFVQRCCDSGRVVDIRE
ASFVTTLNLMSATLFSIQATEFDSNATEEFREIMEGVASIVGDPNFADYFPILKRFDPQ
GVKRKAELYFGKMLVLVEDLLQKRQEERRRSPSYAKKDDLLERLVDVLNEKNEYKLT
TKHITHLLLDLFVGGSETTTTSVEWIMSELLINPEKLAKLKEELKTVVGEKKQVQESDIP
QLPYFEAVLKEVFRLHPPGPLLLPRKAECDVQVGSYTIPKETQILVNAWAIGRDPAIWP
NPEAFEPERFLSQKMDYKGQDFELIPFGSGRRICPGLSFANRMLPMTVATLIHNFDWK
LEVEANAEDVHKGEMFGIAVRRAVPLRAYPIQP

SEQ ID NO:5 – DNA sequence encoding CfCYP76AH8

ATGGAAACCATCACCTTGTTGTTGGCCTTGTTTTTCATTGCTTTGACCTACTTCATC
TCCTCCAGAAGAAGAAGAAATTTGCCACCAGGTCCATTTCCATTGCCAATTATTGG
TAACATGTTGCAATTGGGTTCCAAGCCACATCAATCTTTTGCTCAATTGTCCAAAAA
GTACGGTCCATTGATGTCCATTCATTTGGGTTCCTTGTACACCGTTATAGTCTCTT
CACCAGAAATGGCCAAAGAAATCTTGCAAAAACACGGTCAAGTTTTCTCCGGTAG
AACTATTGCTCAAGCTGTTCATGCTTGTGATCACGATAAGATTTCTATGGGTTTTTT
GCCAGTTGCCAACACTTGGAGAGATATGAGAAAGATCTGCAAAGAACAAATGTTC
TCCCACCATTCTTTGGAAGCTAGTGAAGAATTGAGACACCAAAAGTTGCAACAATT
ATTAGACTACGCTCAAAAGTGTTGCGAAGCTGGTAGAGCTGTTGATATTAGAGAA
GCCTCTTTCATTACCACCTTGAACTTGATGTCTGCTACTATGTTTTCTACCCAAGCT
ACCGAATTTGATTCCGAAGCTACAAAAGAATTCAAAGAAATTATCGAAGGTGTCGC
CACTATAGTTGGTGTTGCTAATTTTGCTGATTACTTCCCAATCTTGAAGCCATTTGA
CTTGCAAGGTATTAAGAGAAGAGCTGATGGTTACTTCGGTAGATTATTGAAGTTGA
TCGAAGGTTACTTGAACGAAAGATTGGAATCTAGAAGATTGAACCCAGATGCTCCA
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GTTGACTACTGAACATTTGACCCACTTGATGTTGGATTTGTTTGTTGGTGGTTCTG
AAACTAACACCACATCCTTGGAATGGATCATGTCTGAATTGGTTATCAACCCAGAT
AAGATGGCCAAGGTCAAAGAAGAATTGAAGTCTGTTGTTGGTGACGAAAAGTTGG
TTAACGAATCTGATATGCCAAGATTGCCATACTTGCAAGCCGTTATCAAAGAAGTT
TTGAGAATTCATCCACCTGGTCCTTTGTTGTTGCCAAGAAAAGCTGAATCTGATCA
AGTTGTTAACGGTTATTTGATCCCAAAGGGTACTCAAATTTTGTTCAATGCTTGGG
CTATGGGTAGAGATCCAACTATTTGGAAAGATCCAGAATCCTTCGAACCAGAAAGA
TTCTTGAATCAATCCATCGACTTCAAGGGTCAAGACTTCGAATTGATTCCATTTGG
TTCTGGTAGAAGAATCTGTCCAGGTATGCCATTGGCTAATAGAATCTTGCATATGA
CTACCGCCACTTTGGTTCATAATTTCGATTGGAAATTGGAAGAAGGTACTGCTGAC
GCTGATCATAAGGGTGAATTATTTGGTTTGGCTGTTAGAAGAGCTACCCCATTGAG
AATCATTCCATTGAAACCATAA

SEQ ID NO:6 – DNA encoding CYP76AH11

ATGGAATTGGTCCAAGTTATCGCTGTTGTTGCAGTTGTTGTTGTTTTGTGGTCCCA
ATTGAAAAGAAAGGGTAGAAAATTGCCACCAGGTCCATCTCCATTGCCAATAGTTG
GTAATATCTTCCAATTGTCCGGTAAGAACATCAACGAATCTTTCGCTAAGTTGTCC
AAAATCTACGGTCCAGTTATGTCTTTGAGATTGGGTTCTTTGTTGACCGTCATTATC
TCTTCACCAGAAATGGCCAAAGAAGTCTTGACTTCTAAGGATTTTGCTAACAGACC
ATTGACTGAAGCTGCTCATGCTCATGGTCATTCTAAATTTTCTGTTGGTTTCGTTCC
AGTCTCTGATCCAAAATGGAAACAAATGAGAAGAGTCTGCCAAGAAGAAATGTTC
GCCTCTAGAATTTTGGAAAACTCCCAACAAAGAAGACACCAAAAGTTGCAAGAATT
GATCGACCACGTTCAAGAATCTAGAGATGCTGGTAGAGCTGTTACTATTAGAGATC
CAGTTTTCGCTACCACCTTGAACATTATGTCCTTGACTTTGTTTTCTGCCGATGCTA
CTGAATTCTCTTCTTCTGCTACTGCTGAATTGAGAGATATTATGGCTGGTGTTGTTT
CTGTTTTGGGTGCTGCTAATTTGGCTGATTTCTTCCCAATCTTGAAATACTTCGATC
CACAAGGTATGAGAAGAAAGGCTGACTTGCATTACGGTAGATTGATTGACCATATC
AAGTCCAGAATGGACAAGAGATCTGAATTGAAGAAGGCTAATCCAAACCATCCAA
AGCACGATGATTTCTTGGAAAAGATCATCGACATCACCATTCAAAGAAACTACGAC
TTGACCATTAACGAAATCACCCATTTGTTGGTCGACTTGTATTTGGCTGGTTCTGA
ATCTACTGTTATGACCATTGAATGGACCATGGCCGAATTGATGTTAAGACCAGAAT
CATTGGCTAAATTGAAGGCAGAATTGAGATCCGTTATGGGTGAAAGAAAGATGAT
CCAAGAATCCGACGACATTTCTAGATTGCCATACTTAAACGGTGCTATCAAAGAAG
CCTTAAGATTGCATCCACCTGGTCCTTTGTTGTTTGCTAGAAAGTCTGAAATCGAT
GTTGAATTGTCTGGTTACTTCATCCCAAAGGGTACTCAAATCTTGGTTAATGAATG
GGGTATGGGTAGAGATCCTTCTGTTTGGCCTAATCCAGAATGTTTTCAACCAGAAA
GATTTTTGGATAAGAACATTGACTACAAGGGTCAAGACCCACAATTGATTCCATTT
GGTGCAGGTAGAAGAATTTGTCCAGGTATTCCAATTGCCCATAGAGTTGTTCATTC
AGTTGTTGCTGCTTTGGTTCATAACTTCGATTGGGAATTTGCTCCTGGTGGTTCTC
AATGTAACAACGAATTTTTCACTGGTGCTGCCTTGGTTAGAGAAGTTCCATTGAAG
TTGATTCCTTTGAACCCACCATCCATCTGA

SEQ ID NO:7 is the protein sequence of TPS2 from *Coleus forskohlii*, which is described in Pateraki et al., 2014, Plant Physiology, March 2014, Vol. 164, pp. 1222–1236. The sequence has the GenBank accession number KF444507.

SEQ ID NO:8 is the protein sequence of TPS3 from *Coleus forskohlii*, which is described in Pateraki et al., 2014, Plant Physiology, March 2014, Vol. 164, pp. 1222–1236. The sequence has the GenBank accession number KF444508.

SEQ ID NO:9 is the protein sequence of TPS4 from *Coleus forskohlii*, which is described in Pateraki et al., 2014, Plant Physiology, March 2014, Vol. 164, pp. 1222–1236. The sequence has the GenBank accession number KF444509.

SEQ ID NO:10 AH17 (aa):
MESMNALVVGLLLIALTILFSLRRRRNLAPGPYPFPIIGNMLQLGTKPHQSFAQLSKKY
GPLMSIHLGSLYTVIVSSPEMAKEILQKHGQVFSGRTIAQAVHACDHDKISMGFLPVSN
TWRDMRKICKEQMFSHHSLEGSQGLRQQKLLQLLDYAQKCCETGRAVDIREASFITT
LNLMSATMFSTQATEFESKSTQEFKEIIEGVATIVGVANFGDYFPILKPFDLQGIKRKAD
GYFGRLLKLIEGYLNERLESRKSNPNAPRKNDFLETVVDILEANEYKLSVDHLTHLMLD
LFVGGSETNTTSLEWTMSELVNNPDKMAKLKQELKSVVGERKLVDESEMPRLPYLQA
VIKESLRIHPPGPLLLPRKAETDQEVNGYLIPKGTQILFNVWAMGRDPSIWKDPESFEP
ERFLNQNIDFKGQDFELIPFGSGRRICPGMPLANRILHMATATMVHNFDWKLEQGTDE
ADAKGELFGLAVRRAVPLRIIPLQP

SEQ ID NO:11 amino acid sequence of CYP76AH15:
METMTLLLPLFFIALTYFLSWRRRRNLPPGPFPLPIIGNLLQIGSKPHQSFAQLSKKYGP

LMSVQLGSVYTVIASSPEMAKEILQKHGQVFSGRTIAQAAQACGHDQISIGFLPVATT
WRDMRKICKEQMFSHHSLESSKELRHEKLQKLLDYAQKCCEAGRAVDIREAAFITTLN
LMSATLFSTQATEFDSEATKEFKEVIEGVAVIVGEPNFADYFPILKPFDLQGIKRRANSY
FGRLLKLMERYLNERLESRRLNPDAPKKNDFLETLVDIIQADEYKLTTDHVTHLMLDLF
VGGSETSATSLEWIMSELVSNPSKLAKVKAELKSVVGEKKVVSESEMARLPYLQAVIK
EVLRLHPPGPLLLPRKAGSDQVVNGYLIPKGTQLLFNVWAMGRDPSIWKNPESFEPE
RFLNQNIDYKGQDFELIPFGSGRRICPGMPLADRIMHMTTATLVHNFDWKLEDGAGD
ADHKGDDPFGLAIRRATPLRIIPLKP

SEQ ID NO: 12 cDNA sequence encoding CfCYP76AH15
ATGGAAACCATGACTCTTCTCCTCCCTCTTTTCTTCATCGCTCTGACATATTTCCTC
TCCTGGAGGCGCCGGAGAAACCTTCCTCCGGGGCCTTTTCCTCTTCCAATCATCG
GAAACTTGCTGCAAATCGGCTCCAAACCCCACCAGTCATTCGCCCAACTCTCAAA
GAAATATGGGCCTCTCATGTCCGTCCAACTCGGGAGTGTATACACCGTGATAGCC
TCCTCCCCGGAAATGGCGAAAGAGATACTGCAAAAACACGGCCAAGTGTTTTCCG
GGAGAACCATCGCACAGGCGGCGCAAGCGTGCGGCCACGACCAGATCTCCATC
GGGTTTCTGCCGGTGGCAACCACGTGGCGTGATATGCGTAAAATATGCAAAGAAC
AGATGTTCTCGCATCACAGCCTGGAATCCAGCAAGGAGCTGAGGCACGAGAAGC
TGCAGAAGCTGCTGGACTACGCCCAGAAATGCTGCGAAGCCGGCCGTGCCGTTG
ATATTCGTGAGGCCGCCTTCATTACAACGCTCAACCTCATGTCTGCCACGTTGTTC
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CTTGAAGCCTTTCGATCTTCAGGGGATCAAGCGTAGAGCTAATAGCTACTTTGGAA
GACTGCTCAAGTTAATGGAGAGATATCTGAATGAGAGGCTGGAATCAAGAAGGTT
GAACCCAGATGCCCCCAAGAAGAATGACTTTTTGGAAACCCTGGTGGATATCATC
CAGGCTGATGAATACAAGCTCACGACCGACCACGTCACGCACCTCATGCTTGACT
TATTTGTTGGAGGATCGGAAACAAGCGCGACCTCACTGGAATGGATAATGTCGGA
GTTAGTGAGCAATCCGAGTAAATTGGCGAAGGTGAAAGCGGAGCTCAAGAGCGTT
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CAAGCAGTGATCAAAGAAGTGCTCCGACTTCACCCTCCCGGCCCTCTTCTGCTTC
CTCGCAAGGCAGGGAGTGATCAAGTTGTGAATGGATACCTGATCCCAAAGGGAAC
TCAATTACTCTTCAATGTATGGGCAATGGGCAGAGACCCCAGTATCTGGAAGAAT
CCTGAATCTTTCGAGCCCGAGCGCTTCCTCAATCAAAACATAGACTACAAAGGCC
AAGATTTCGAGCTCATTCCATTCGGGTCCGGGAGAAGAATTTGCCCCGGTATGCC
GCTGGCGGATCGGATTATGCACATGACGACGGCCACTCTGGTTCACAACTTCGAT
TGGAAACTGGAAGACGGAGCAGGTGATGCGGATCACAAGGGAGACGACCCCTTC
GGCTTGGCCATCCGCCGTGCAACTCCTCTCAGGATCATTCCACTTAAGCCATGA

SEQ ID NO:13 – cDNA encoding CfCYP76AH17:
ATGGAAAGCATGAATGCTCTTGTCGTCGGTCTCTTGTTGATCGCTTTGACAATTTT
GTTTTCGTTGAGGCGGCGGAGAAACCTTGCTCCGGGGCCTTATCCTTTTCCGATC
ATCGGAAACATGCTTCAACTGGGCACGAAACCACACCAATCATTCGCCCAGCTGT
CGAAGAAATATGGGCCGCTCATGTCCATCCACCTGGGAAGTTTATACACAGTGAT
CGTTTCGTCGCCGGAAATGGCGAAAGAGATCCTGCAAAAGCACGGCCAAGTGTTT
TCAGGGAGAACCATCGCTCAGGCGGTGCATGCATGCGACCACGACAAGATCTCC
ATGGGGTTTCTGCCGGTGTCGAACACGTGGCGCGATATGCGTAAAATATGCAAAG
AGCAGATGTTCTCGCATCACAGCTTGGAAGGCAGCCAGGGTCTCCGCCAGCAGA
AGCTGCTGCAGCTGCTCGACTACGCCCAGAAGTGCTGCGAAACCGGCCGCGCCG
TTGACATTCGTGAGGCTTCCTTCATCACAACTCTCAACCTCATGTCGGCCACCATG
TTTTCGACTCAAGCTACCGAGTTTGAATCGAAATCTACTCAGGAGTTCAAGGAGAT
CATTGAAGGCGTGGCCACGATTGTGGGCGTGGCTAATTTCGGAGACTACTTCCCA
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GCAGATTGCTGAAATTAATCGAGGGCTATCTCAATGAAAGATTGGAATCCAGAAAA
TCGAACCCAAATGCCCCCAGAAAGAATGACTTTTTGGAAACAGTGGTCGATATCCT
CGAGGCAAATGAGTACAAGTTGTCAGTCGACCACCTCACGCATCTCATGCTGGAT
TTGTTTGTTGGAGGATCGGAAACAAACACGACCTCACTGGAGTGGACAATGTCGG
AGTTAGTGAACAACCCCGACAAAATGGCCAAGCTGAAACAGGAGCTGAAGAGCGT
TGTAGGAGAGAGGAAACTGGTGGATGAGTCGGAGATGCCGAGGCTGCCATATCT
GCAAGCTGTCATCAAAGAATCGCTCCGAATTCACCCACCGGGCCCTCTTCTTCTC
CCTCGCAAAGCAGAGACCGATCAAGAGGTGAATGGATATCTCATCCCAAAAGGGA
CTCAGATTCTCTTCAATGTGTGGGCAATGGGCAGGGATCCTAGCATCTGGAAGGA
TCCTGAATCTTTTGAGCCCGAGCGCTTCCTCAATCAAAACATAGACTTCAAAGGCC
AAGATTTCGAGCTCATTCCATTCGGGTCGGGCCGAAGAATCTGCCCCGGCATGCC
GCTGGCCAATCGGATTCTCCACATGGCCACCGCGACTATGGTTCATAACTTCGAT
TGGAAACTGGAACAAGGAACAGATGAAGCTGATGCCAAAGGAGAGTTGTTTGGAT
TGGCCGTGCGCAGGGCAGTTCCCCTCAGGATCATTCCACTTCAGCCTTAA

SEQ ID NO:1	Amino acid sequence of CYP76AH8 from *Coleus forskohlii*
SEQ ID NO:2	Amino acid sequence of CYP76AH11 from *Coleus forskohlii*
SEQ ID NO:3	Amino acid sequence of CYP71D381 from *Coleus forskohlii* –
SEQ ID NO:4	Amino acid sequence of CYP76AH9 from *Coleus forskohlii* CYP76AH9 may also be referred to as CYP76AH2.
SEQ ID NO:5	DNA sequence encoding CYP76AH8 from *Coleus forskohlii.* DNA sequence codon optimised for expression in yeast
SEQ ID NO:6	DNA sequence encoding CYP76AH11 from *Coleus forskohlii.* DNA sequence codon optimised for expression in yeast
SEQ ID NO:7	Amino acid sequence of TPS2 from *Coleus forskohlii* –
SEQ ID NO:8	Amino acid sequence of TPS3 from *Coleus forskohlii*
SEQ ID NO:9	Amino acid sequence of TPS4 from *Coleus forskohlii.*
SEQ ID NO:10	Amino acid sequence of CYP76AH17 from *Coleus forskohlii*
SEQ ID NO:11	Amino acid sequence of CYP76AH15 from *Coleus forskohlii*
SEQ ID NO:12	cDNA sequence encoding CYP76AH15 from *Coleus forskohlii*
SEQ ID NO:13	cDNA sequence encoding CYP76AH17 from *Coleus forskohlii*

Examples

The invention is further illustrated by the following examples, which however are not intended as being limiting for the invention.

Example 1

The entire pathway to the high-value drug forskolin is active in a single cell type in the root of *Coleus forskohlii*. To identify the genes encoding the enzymes involved in decorating 13R-manoyl oxide (13R-MO) through oxidative functionalization of the backbone, a transcriptome prepared from aforementioned cell type was used. Candidates of the large family of cytochrome P450 enzymes (P450) were identified and build into an exhaustive inventory of cloned full-length sequences (Cf450s). CfP450s were tested in a transient Agrobacterium/*Nicotiana benthamiana* heterologous expression system, which produced 13R-MO. In particular, some Nicotiana benthamiana plants expressed TPS2 of SEQ ID NO:7 and TPS3 of SEQ ID NO:8 and thus were able to produce 13R-MO in addition to one or more CfP450s to be tested. All CfP450s were tested and in gas-chromatography mass-spectrometry analyses of extracts from plants expressing CfCYP76AH9 (SEQ ID NO:4), CYP71D381 of SEQ ID NO:3 and/or CYP76AH8 of SEQ ID NO:1, conversion of 13R-MO was observed, next to accumulation of novel, oxidised products. Specifically with CYP76AH8, conversion of the substrate to keto-dihydroxy 13R-MO was detected, while one ketonated and two further hydroxylated products were found, consistent with the most simple 13R-MO ketone intermediate also detected in the native C. forskohlii. This keto-group is also found in all forskolin derivatives detected in the plant extract, indicating that this represents the first biosynthetic intermediate and the first required step towards forskolin. GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP76AH8shows production of (2) keto-13R-MO by *Coleus forskohlii* CYP76AH8, as well as production of (4a) keto-hydroxy-13R-MO and (4b) keto-hydroxy-13R-MO and (7a) keto-dihydroxy-13R-MO(see fig. 1). The identity of the compounds was confirmed by mass spectrometry. Only traces of 13R-MO are detected. A potential intermediate, hydroxyl-13R-MO, IS, internal standard and minor hydroxylation side products are also detected (see fig. 1).

A similar result was observed in Nicotiana benthamiana plants expressing either CYP76AH15 of SEQ ID NO:11 or CYP76AH17 of SEQ ID NO:10 together with TPS2 of

SEQ ID NO:7 and TPS3 of SEQ ID NO:8. Thus, such plants also produced (2) keto-13R-MO, (3a) keto-hydroxy-13R-MO and (3b) keto-hydroxy-13R-MO and (4) keto-dihydroxy-13R-MO as determined by GC-MS (see fig. 11). Thus, it appears that CfCYP76AH8, CfCYP76AH15 and CfCYP76AH17 have similar activity.

A *N. benthamiana* system producing 13R-MO and expressing CYP76AH11 of SEQ ID NO:2 was also generated. With this efficient and specific oxidation of the 13R-MOinto further hydroxylated derivative was achieved. Similar to CYP76AH8, this conversion is near quantitative. The GC-MS analysis showed production of 3c) hydroxy-13R-MO, (5b) dihydroxy-13R-MO and (5c) dihydroxy-13R-MO and (8b) trihydroxy-13R-MO (see fig. 2). The identity of the compounds was confirmed by mass spectrometry.

A *N. benthamiana* system producing 13R-MO and expressing both CYP76AH8 of SEQ ID NO:1 and CYP76AH11 of SEQ ID NO:2 was also generated. With this efficient and specific oxidation of the 13R-MO ketone intermediate into a single further hydroxylated derivative was achieved. The characteristic mass and the retention time support four different positions with a novel hydroxyl group and the required keto-group in correct regiospecificity. GC-MS analysis of extracts from assays with *Nicotiana benthamiana* plants producing 13R-MO and expressing CYP76AH8of SEQ ID NO:1and CYP76AH11 of SEQ ID NO:2 indicates oxidation of (1) 13R-MO via (2) keto-13R-MO to (4d) hydroxyl-keto-13R-MO,(7a) keto-dihydroxy-13R-MO and (8a) keto-trihydroxy-13R-MO by the pair of *Coleus forskohlii* CYP76AH8and CYP76AH11 (see fig. 3). (6a) hydroxyl-di-keto-13R-MO is also produced. The identity of the compounds was confirmed by mass spectrometry.
The specific identity of the compounds (2), (5c), (7a) and (8A) was further determined by NMR as described below in Example 2.

Compound (2) was identified as 8,13-epoxy-labd-14-ene-11-one.
Compound (5c) was identified as 7-11-dihydroxy-13R-manoyl oxide
Compound (7a) was identified as 1-9-deoxydeacethylforskolin
Compound (8a) was identified as 1-deoxydeacethylforskolin

A *N. benthamiana* system producing 13R-MO and expressing CYP71D3811of SEQ ID NO:3 was also generated. GC-MS analysis of extracts from said *Nicotiana benthamiana* plant indicates production of (1) 13R-MO, (3a) hydroxy-13R-MO and (3b)

hydroxy-13R-MO (see fig. 4). The identity of the compounds was confirmed by mass spectrometry. The identity of the compounds as (3a) 2-hydroxy-13R-manoyl oxide and (3b) 20-hydroxy-13R-manoyl oxide.was further determined by NMR as described in Example 2 below.

A *N. benthamiana* system producing 13R-MO and expressing CYP76AH9 of SEQ ID NO:4 was also generated. GC-MS analysis of extracts from said *Nicotiana benthamiana* plant indicates production of (1) 13R-MO, (2) keto-13R-MO and (9) hydroxy-13R-MO (see fig. 5). The identity of the compounds was confirmed by mass spectrometry.

Forskolin is complex functionalised derivative of 13R-MO requiring region- and stereospecific oxidation of five carbon positions: one double-oxidation leading to a ketone and four single oxidation reactions yielding hydroxyl groups. The results presented herein shows identification of cytochrome P450 mono-oxygenases, which efficiently catalyse independent regiospecific hydroxylations (CYP71D381), one hydroxylation and formation of the ketone (CYP76AH8), and the subsequent regiospecific oxidation of the ketone to a hydroxyl-ketone (CYP76AH8 / CYP76AH11). Figure 9 shows one biosynthetic pathway to forskolin involving use of CYP76AH8 and CYP76AH11. In addition a fourth P450 was found to catalyse specific hydroxylations on the 13R-MO backbone (CYP76AH9) at an efficiency indicating that the enzyme might be more active in catalysing hydroxylation of partly oxidised 13R-MO . This establishes the correct sequence of several the reactions from 13R-MO to forskolin with the respective P450s and also further hydroxylation reactions with an order in the route to be determined.

A summary of the observed oxidation reactions of 13R-MO en route to forskolin are shown in figure 7. CYP76AH8 and CYP76AH11 effectively catalyse the first three reactions, CYP71D381 and CYP76AH9 catalyse hydroxylation reactions.

Example 2

Biosynthesis and isolation of MO derivatives for structural determination by NMR

NMR structural characterization of CfCYP product was achieved by extraction from up to 40 *N. benthamiana* plants. The *N. benthamiana* were modified so that it

produced 13R-MO and furthermore cDNAs encoding the relevant CfCYP(s) catalyzing oxygenations on the MO backbone were introduced. The relevant CYPs and combinations thereof are described above in Example 1. For example, in one experiment cDNAs encoding CYP76AH8 of SEQ ID NO:1and CYP76AH11 of SEQ ID NO:2 were introduced and in another experiment cDNA encoding CYP71D3811of SEQ ID NO:3 was introduced. For infiltration, 0.5 L of agrobacteria cultures for each individual biosynthetic gene was grown overnight using 10 mL starter cultures. The agrobacteria were harvested by centrifugation at 4000xg for 20 min and resuspended in 100mL water. The OD_{600} of the independent samples were normalized and adjusted to a final concentration of OD_{600} of 0.5 before combining for vacuum infiltration of whole *N. benthamiana* plants at -80 mmHg for 30 seconds. Post infiltration growth and extraction was performed similar to small scale extraction using 500 mL n-hexane as extraction solvent. After removal of the solvent by rotor evaporation (Buchi, Schwitzerland) set to 35°C and 220 mbar, the residue was subjected twice to solid phase extraction on a Dual Layer Florisil/Na_2SO_4 6mL PP SPE TUBE (Superleco Analytical), eluting the compounds with 1% ethyl-acetate in n-hexane for removal of fatty acids and other polar components co-extracted with the oxygenated MO. The samples were concentrated by evaporation of the solvent under a stream of nitrogen before chromatography. Final purification was performed using a preparative gas chromatography coupled to a mass spectrometer (7890B GC, 5977A MSD, Agilent technologies) in line with a preparative fraction collector (PFC, Gerstel Inc.) using a preparative scale HP5 column (30m x 250µm x 0.25 µm, Restek Corporation), running with a linear flow of 45 cm/sec using hydrogen as carrier gas and a 1:99 split between the detector and the fraction collection. The oven program was set to 60°C for 1 min, ramp with 20°C/min to 320 °C which was held for 3 min. The ion source and quadropole temperature were set to 230°C and 150°C, respectively. Scan mode from m/z 35 to m/z 500 was used for detection. Peaks of interest were identified by their characteristic mass fragmentation pattern and selected for collection of the compounds in 1µL sample traps (Gerstel) at room temperature, while the temperature for the PFC transfer line and PFC switching device was set to 280°C. Quantities suitable for NMR structural identification were typically collected from 100 injections with a volume of 5 µL. Purified compounds were

directly recovered from the traps with deuterated chloroform ($CDCl_2$), concentrated in an argon stream before transferring into 1.7mm NMR tubes.

NMR analysis for structural identification of hydroxylated MO derivatives

NMR-spectra were acquired using a 600 MHz Bruker Avance III HD equipped with a cryogenically cooled 1.7-mm cryogenically cooled $_1H/_{13}C/_{15}N$ TCI probe head (Bruker). Samples dissolved in $CDCl_2$ (Sigma-Aldrich, 99.8 atom % D) were analyzed at 300 K. Proton spectra, at 600.03 MHz, were acquired using 30°-pulses, a spectral width of 12 kHz, collecting 16 scans with a length of 65536 data points with a relaxation delay of 1.0 sec. FID's were zero-filled to twice the size and exponentially multiplied with a line broadening factor of 0.3 Hz before Fourier transform. HSQC, COSY, HMBC and NOESY spectra were acquired in a similar manner.

The NMR analysis confirmed the specific identity of compounds:

(2) 8,13-epoxy-labd-14-ene-11-one

(3a) 2-hydroxy-13R-manoyl oxide

(3b) 19-hydroxy-13R-manoyl oxide

(5c) 7-11-dihydroxy-13R-manoyl oxide

(7a) 1-9-deoxydeacethylforskolin

(8a) 1-deoxydeacethylforskolin

The 13c NMR spectrometric reference for compound 2 is provided below:

#C	Compound (2)	Gabetta et al. 1989
20	15,63	15,5
2	18,49	18,4
6	19,80	19,7
19	21,76	21,6
18	28,10	27,9
17	31,41	31,2
4	33,40	33,2
16	33,65	33,5
10	37,29	37,1
7	39,59	39,4
1	42,05	41,9
3	43,42	43,3
12	50,38	50,2

5	55,97	55,8
9	66,90	66,7
13	75,11	74,4
8	77,49	77,2
15	112,30	111,9
14	146,87	146
11	207,74	207,1

Gabetta et al., 1989, PhytochemistryV,o l. 28, No. 3, pp. 859-86.

Example 3 Biosynthesis of oxidized manoyl oxide derivatives using CYP76AH8 and CYP76AH11.

C. forskohlii cDNAs/genes were introduced into yeast cells (S. cerevisiae) using standard yeast transformation methods followed by genomic integration.

Heterologous genes were controlled by endogenous constitutively active regulatory elements (promoters).

DNA sequences codon optimized for expression in S. cerevisiae encoding either CYP76AH8 (SEQ ID NO:5) or CYP76AH11 (SEQ ID NO:6) were introduced to yeast cells engineered to produce 13R-MO.

Selection of transformed yeast cells was performed through the selection marker introduced with the transgenes and by genotyping (using PCR techniques).

The selected yeast strains expressing the C. forskohlii genes were cultivated in Synthetic Complete URA dropout medium (SC-URA), at 28°C for 72 hours.

Extraction of diterpenoids from the yeast culture was performed with ethanol: one volume of yeast culture (cells together with medium) was mixed with one volume of ethanol and heated at 80°C for 15 min. Diterpenoids products from CYP76AH8 and CYP76AH11 were extracted from the ethanol-yeast culture mixture by one volume of hexane. Diterpenoids metabolites were analyzed by GC-MS.

Settings for GC-MS:
The oven program was set to 120℃ for 2 min, ramp with 30℃/min to 180 ℃, ramp with 10℃/min to 300 ℃, ramp with 30℃/min to 320 ℃,which was held for 2 min. The

ion source and quadropole temperature were set to 300°C and 150°C, respectively. MS detection was set in scan mode from m/z 50 to m/z 400 was used for detection.

The results of the GC-MS analysis are shown in figure 10. As apparent from figure 10, then yeast cells capable of producing 13R-MO and further expressing CYP76AH11 are capable of producing compounds 4c, 6a and 6b. The structure and chemical formula of compound 4c and the chemical formula of compounds 6a and 6b are shown in figure 9. Yeast cells capable of producing 13R-MO and further expressing CYP76AH8 are capable of producing compound 8. The structure and chemical formula of compound 8 are shown in figure 9.

Claims

1. A method of producing an oxidsed 13R-manoyl oxide (13R-MO), said method comprising the steps of:

a) providing a host organism comprising

I. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or catalysing oxidation of the hydroxyl group to form an oxo-group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO;

said host organism optionally comprising one or more of the following:

II. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position;

III. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position

IV. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position

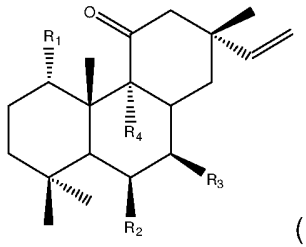
V. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position

VI. A heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO

b) Incubating said host organism in the presence of 13R-MO under conditions allowing growth of said host organism;

c) Optionally isolating oxidised 13R-MO from the host organism.

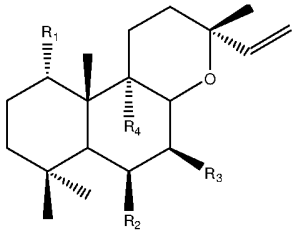
2. The method according to claim 1, wherein the oxidised 13-R-MO is a compound of formula I



(I)

wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl.

3. The method according to claim 1, wherein the oxidised 13R-MO is a compound of the formula (II)



wherein R_1, R_2, R_3 and R_4 individually are selected from the group consisting of –H, -OH and –OR, wherein R preferably is acyl.

4. The method according to any one of claims 2 to 3, wherein R_1 is selected from the group consisting of –H and –OH, preferably R_1 is –OH.

5. The method according to any one of claims 2 to 4, wherein R_2 is selected from the group consisting of –OR and –OH, wherein R is acyl, for example R_2 is selected from the group consisting of –O-(C=O)-CH_3 (acetyl), –O-(C=O)-CH_2-CH_3, –O-(C=O)-CH_2-CH_2-CH_3 and –OH, for example R_2 is –OH.

6. The method according to any one of claims 2 to 5, wherein R_3 is selected from the group consisting of –OR and –OH, wherein R is acyl, for example R_3 is selected from

the group consisting of –O-(C=O)-CH_3 (acetyl), –O-(C=O)-CH_2-CH_3, –O-(C=O)-CH_2-CH_2-CH_3 and –OH, for example R_3 is acetyl.

7. The method according to any one of claims 2 to 6, wherein R_4 is selected from the group consisting of –H and –OH, for example R_4 is –OH.

8. The method according to claim 1, wherein the oxidised 13R-MO is forskolin.

9. A host organism comprising

I. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO derivative at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or catalysing oxidation of the hydroxyl group to form an oxo-group at the 11 position of 11-hydroxyl-13R-MO and/or an oxidised 11-hydroxyl-13R-MO;

said host organism optionally comprising one or more of the following:

II. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 1 position, wherein said oxidised 13R-MO carries a –H at the 1-position;

III. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 6 position, wherein said oxidised 13R-MO carries a –H at the 6-position;

IV. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 7 position, wherein said oxidised 13R-MO carries a –H at the 7-position;

V. A heterologous nucleic acid encoding an enzyme capable of catalysing hydroxylation of 13R-MO and/or oxidised 13R-MO at the 9 position, wherein said oxidised 13R-MO carries a –H at the 9-position

VI. A heterologous nucleic acid encoding an enzyme capable of catalysing transfer of an acyl group to an –OH of a hydroxylated 13R-MO and/or an oxidised hydroxylated-13R-MO.

10. A polypeptide, which is an enzyme capable of

III. catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO at the 11 position, wherein said oxidised 13R-MO carries a –H at the 11-position; and/or

IV. catalysing oxidation of the hydroxyl group at the 11 position of 11-hydroxyl-13R-manoyl oxide and/or an oxidised 11-hydroxyl-13R-MO.

11. The polypeptide according to claim 10, wherein said polypeptide is capable of catalysing formation of a ketone group at the 11 position of 13R-MO or of an oxidised 13R-MO carrying a –H at the 11-position.

12. The polypeptide according to any one of claims 10 to 11, wherein the polypeptide furthermore is capable of catalysing hydroxylation of 13R-MO and/or of oxidised 13R-MO at position 1 and/or at position 6 and/or at position 7 and/or at position 9.

13. The polypeptide according to any one of claims 10 to 11, wherein the polypeptide furthermore is capable of catalysing hydroxylation of 13R-MO and/or of oxidised 13R-MO at position 6 and/or at position 7.

14. The polypeptide according to any one of claims 10 to 13, wherein the polypeptide is an enzyme from *Coleus forskohlii.*

15. The polypeptide according to any one of claims 10 to 14, wherein the polypeptide is a CYP76AH8.

16. The polypeptide according to any one of claims 10 to 15, wherein the polypeptide is a CYP76AH8 of SEQ ID NO:1 or a functional homologue thereof sharing at least 70% sequence identity therewith.

17. The polypeptide according to any one of claims 10 to 14, wherein the polypeptide is a CYP76AH15.

18. The polypeptide according to claim 17, wherein the polypeptide is a CYP76AH15 of SEQ ID NO:11 or a functional homologue thereof sharing at least 70% sequence identity therewith.

19. The polypeptide according to any one of claims 10 to 14, wherein the polypeptide is a CYP76AH17.

20. The polypeptide according to claim 19, wherein the polypeptide is a CYP76AH17 of SEQ ID NO:10 or a functional homologue thereof sharing at least 70% sequence identity therewith.

21. The method according to any one of claims 1 to 8 or the host organism according to claim 9, wherein the heterologous nucleic acid of I. is encoding a polypeptide according to any one of claims 10 to 20.

22. A polypeptide, which is an enzyme capable of catalysing hydroxylation of 11-keto-13R-manoyl oxide (13R-MO) and/or an oxidised 11-keto-13R-MO derivative at one or more of the positions 1, 6, 7 and/or 9.

23. The polypeptide according to claim 22, wherein the polypeptide is an enzyme capable of catalysing hydroxylation of 11-keto-13R-manoyl oxide (13R-MO) and/or an oxidised 11-keto-13R-MO derivative at positions 1, 6 and 7.

24. The polypeptide according to any one of claims 22 to 23, wherein the polypeptide is an enzyme from *Coleus forskohlii.*

25. The polypeptide according to any one of claims 22 to 24, wherein the polypeptide is a CYP76AH11.

26. The polypeptide according to any one of claims 22 to 25, wherein the polypeptide is a CYP76AH11 of SEQ ID NO:2 or a functional homologue thereof sharing at least 70% sequence identity therewith.

27. The method according to any one of claims 1 to 8 and 21, or the host organism according to any one of claims 9 and 21, wherein at least one of the heterologous nucleic acids of II., III., IV. and V. encodes a polypeptide according to any one of claims 22 to 26.

28. A polypeptide, which is an enzyme capable of catalysing hydroxylation of 13R-manoyl oxide (13R-MO) and/or an oxidised 13R-MO at one or more of the positions 1, 6, 7 and/or 9.

29. The polypeptide according to claim 28, wherein the polypeptide is an enzyme from *Coleus forskohlii.*

30. The polypeptide according to claim 28, wherein the polypeptide is selected from the group consisting of CYP71D381 and CYP76AH9.

31. The polypeptide according to claim 28, wherein the polypeptide is a CYP71D381 of SEQ ID NO:3, CYP76AH9 of SEQ ID NO:4, or a functional homologue of any of the aforementioned sharing at least 70% sequence identity therewith.

32. The method according to any one of claims 1 to 8, 21 and 27 or the host organism according to any one of claims 9, 21 and 27, wherein at least one of the heterologous nucleic acids of II., III., IV. and V. encodes a polypeptide according to any one of claims 28 to 31.

33. The method according to any one of claims 1 to 8, 21, 27 and 32, or the host organism according to any one of claims 9, 21, 27 and 32, wherein the host organism is a microorganism.

34. The method or the host organism according to claim 33, wherein the microorganism is yeast.

35. The method according to any one of claims 1 to 8, 21, 27 and 32, or the host organism according to any one of claims 9, 21, 27 and 32, wherein the host organism is a plant.

36. The method according to any one of claims 1 to 8, 21, 27 and 32 to 35, or the host organism according to any one of claims 9, 21, 27 and 32 to 35, wherein the host organism furthermore comprises a heterologous nucleic acid encoding TPS2 of SEQ ID NO:7 or a functional homologue thereof sharing at least 70% sequence identity therewith.

37. The method according to any one of claims 1 to 8, 21, 27 and 32 to 36, or the host organism according to any one of claims 9, 21, 27 and 32 to 36, wherein the host

organism furthermore comprises a heterologous nucleic acid encoding TPS3 of SEQ ID NO:8, TPS4 of SEQ ID NO:9 or a functional homologue of any of the aforementioned sharing at least 70% sequence identity therewith.

38. A method of producing an oxidised 13R-MO, said method comprising the steps of

a) providing a host organism according to any one of claims 9, 21, 27 and 32 to 37;

b) preparing an extract of said host organism;

c) providing 13R-MO

d) incubating said extract with 13R-MO

thereby producing oxidised 13R-MO.

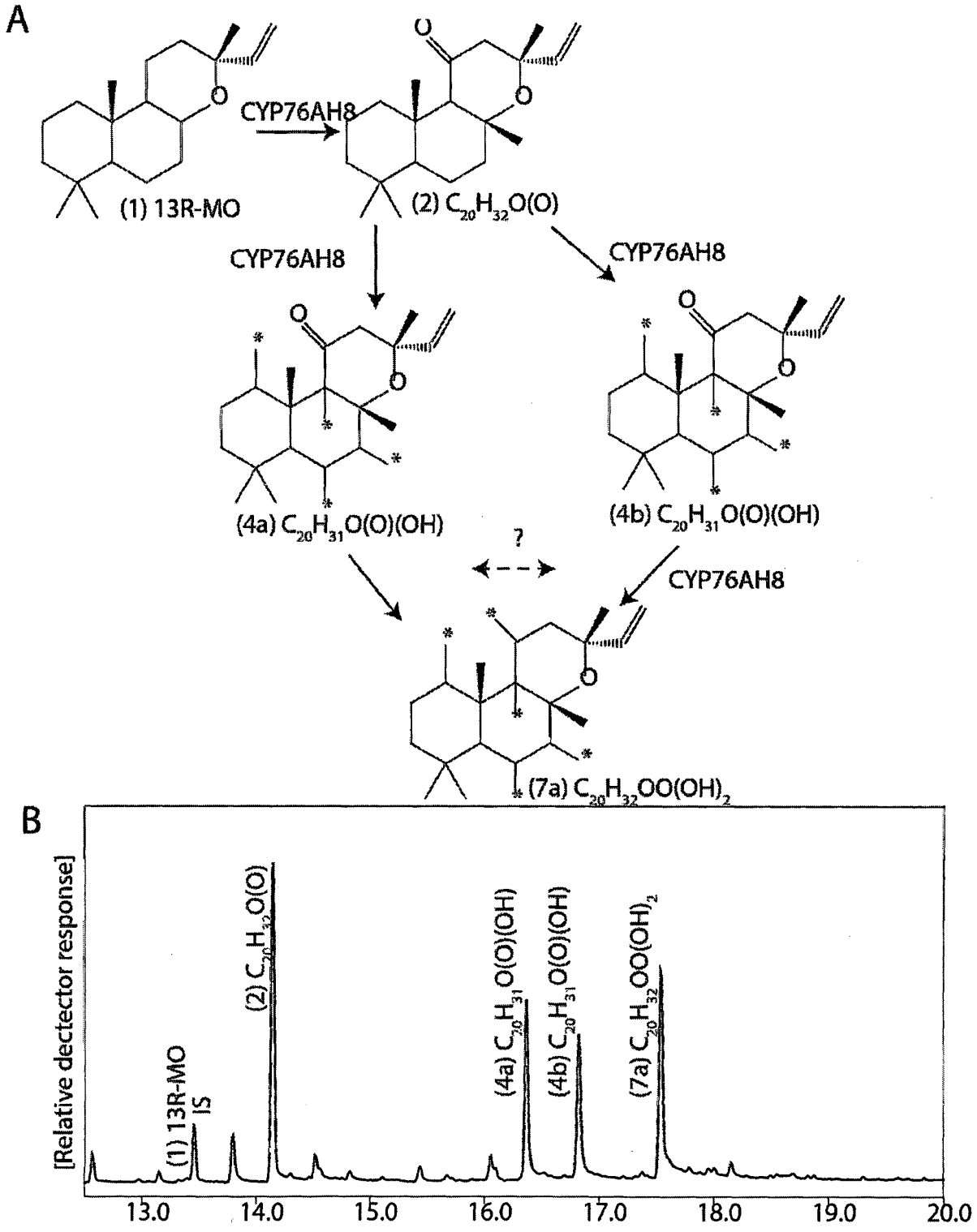

A
CYP76AH8
(1) 13R-MO
(2) $C_{20}H_{32}O(O)$
CYP76AH8
CYP76AH8
(4a) $C_{20}H_{31}O(O)(OH)$
(4b) $C_{20}H_{31}O(O)(OH)$
?
CYP76AH8
(7a) $C_{20}H_{32}OO(OH)_2$
B
[Relative dectector response]
(1) 13R-MO
IS
(2) $C_{20}H_{32}O(O)$
(4a) $C_{20}H_{31}O(O)(OH)$
(4b) $C_{20}H_{31}O(O)(OH)$
(7a) $C_{20}H_{32}OO(OH)_2$
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0

Fig. 1

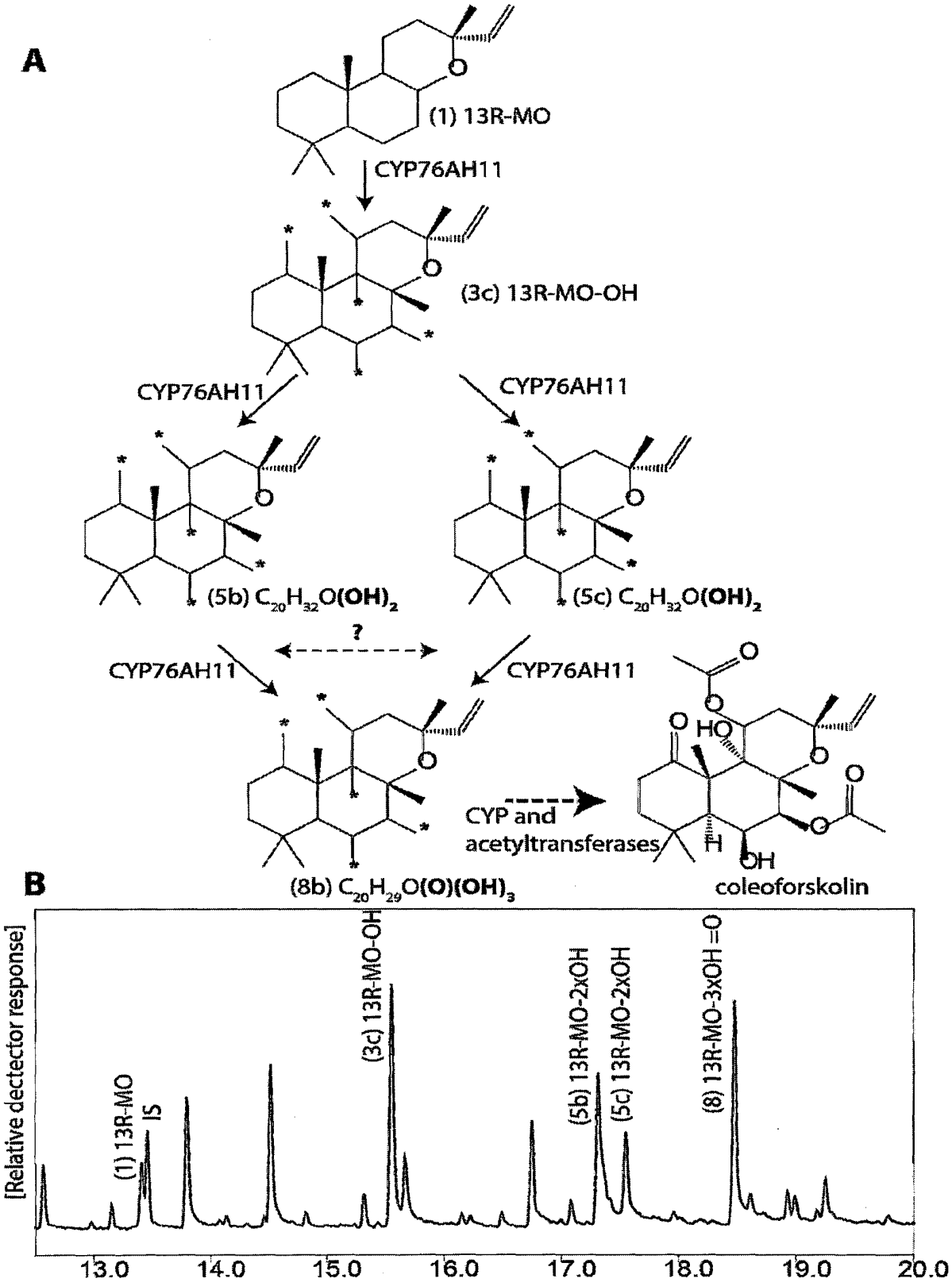

A
(1) 13R-MO
CYP76AH11
(3c) 13R-MO-OH
CYP76AH11
CYP76AH11
(5b) C20H32O(OH)2
(5c) C20H32O(OH)2
?
CYP76AH11
CYP76AH11
CYP and
acetyltransferases
(8b) C20H29O(O)(OH)3
coleoforskolin
B
[Relative dectector response]
(1) 13R-MO
IS
(3c) 13R-MO-OH
(5b) 13R-MO-2xOH
(5c) 13R-MO-2xOH
(8) 13R-MO-3xOH =O
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0

Fig. 2

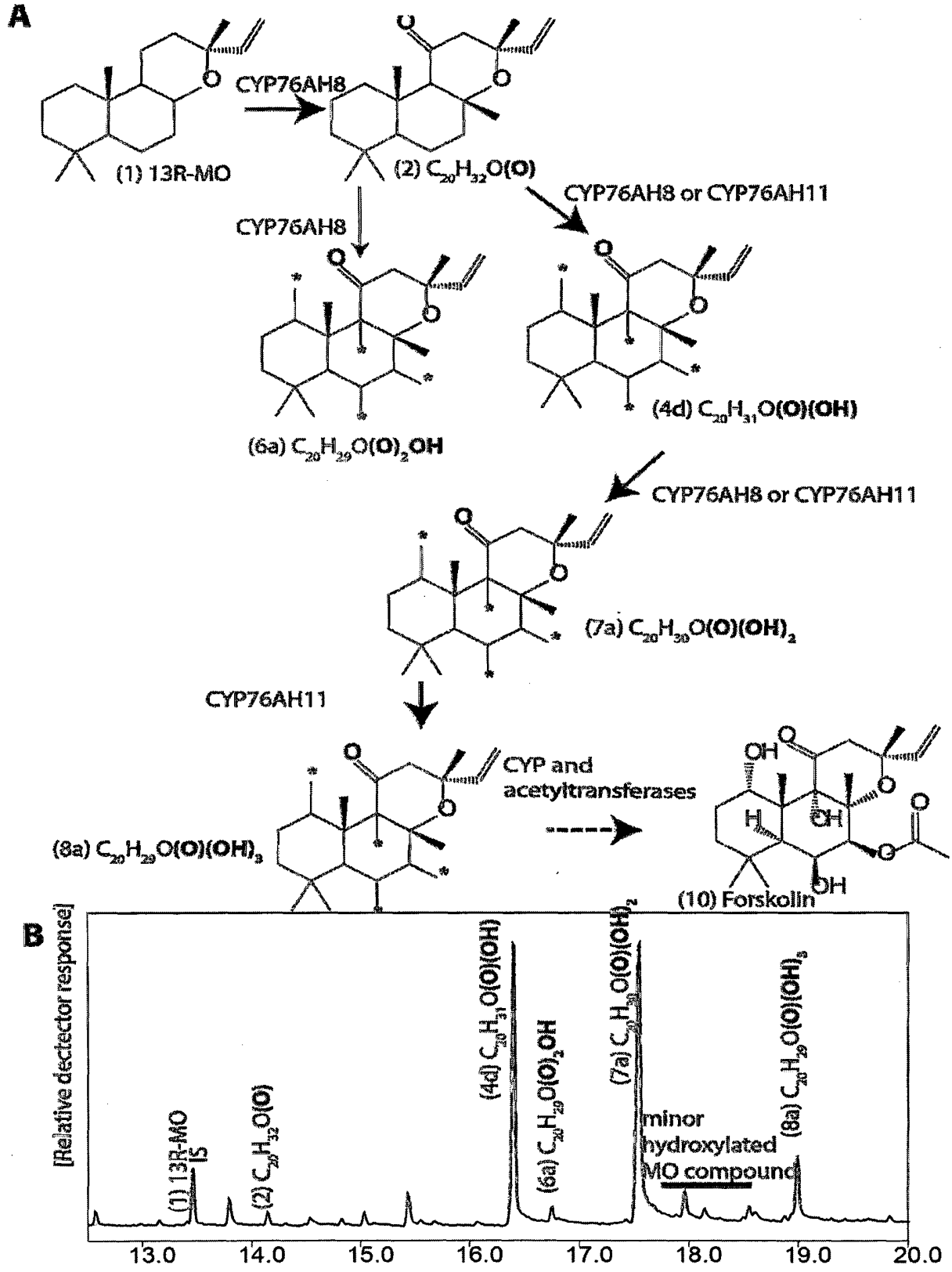

A
CYP76AH8
(1) 13R-MO
(2) $C_{20}H_{32}O(O)$
CYP76AH8 or CYP76AH11
CYP76AH8
(6a) $C_{20}H_{29}O(O)_2OH$
(4d) $C_{20}H_{31}O(O)(OH)$
CYP76AH8 or CYP76AH11
(7a) $C_{20}H_{30}O(O)(OH)_2$
CYP76AH11
(8a) $C_{20}H_{29}O(O)(OH)_3$
CYP and acetyltransferases
(10) Forskolin
B
[Relative dectector response]
(1) 13R-MO
IS
(2) $C_{20}H_{32}O(O)$
(4d) $C_{20}H_{31}O(O)(OH)$
(6a) $C_{20}H_{29}O(O)_2OH$
(7a) $C_{20}H_{30}O(O)(OH)_2$
minor hydroxylated MO compound
(8a) $C_{20}H_{29}O(O)(OH)_3$
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0

Fig. 3

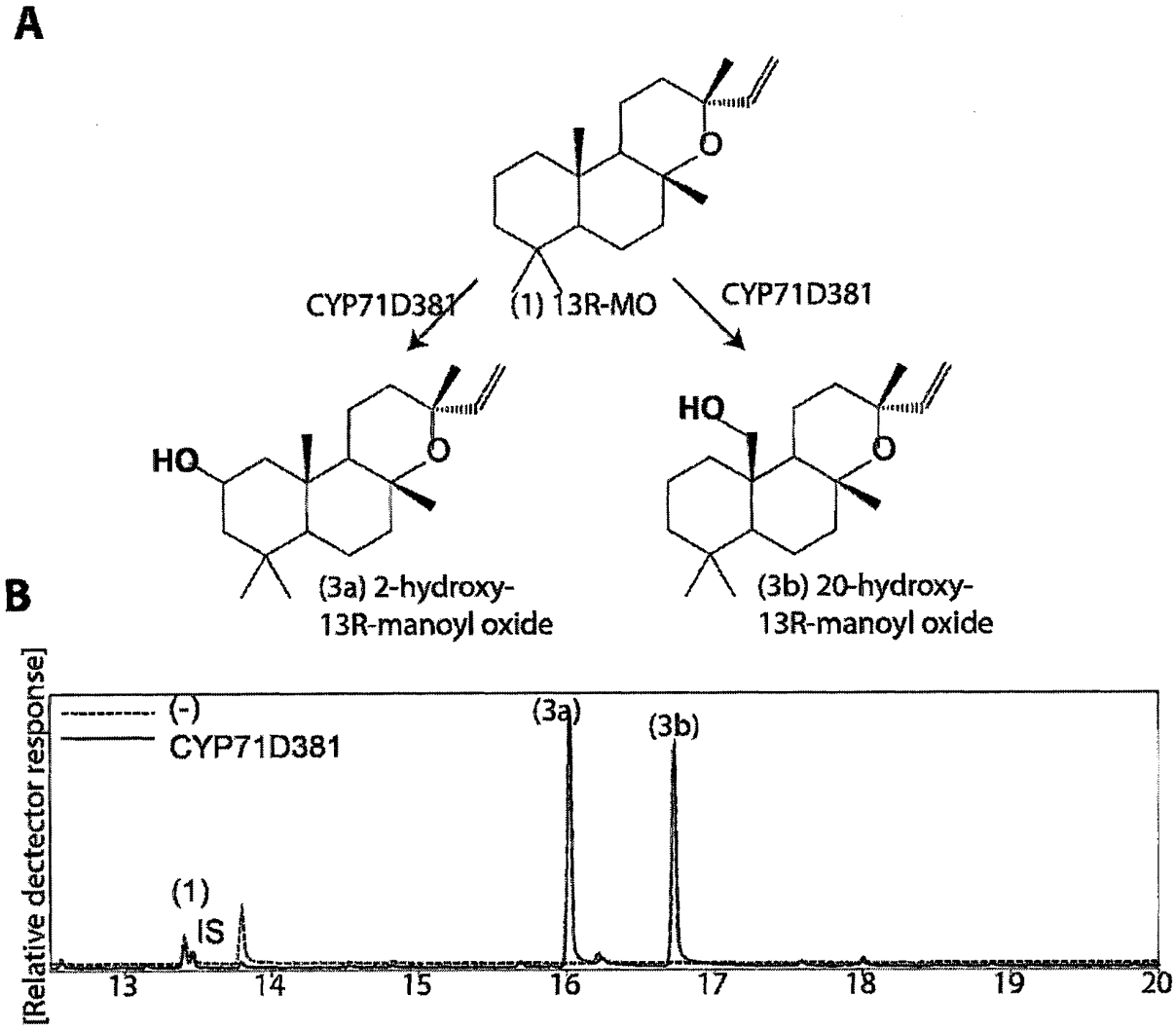

A
CYP71D381
(1) 13R-MO
CYP71D381
HO
HO
(3a) 2-hydroxy-
13R-manoyl oxide
(3b) 20-hydroxy-
13R-manoyl oxide
B
[Relative dectector response]
(-)
CYP71D381
(1)
IS
(3a)
(3b)
13
14
15
16
17
18
19
20

Fig. 4

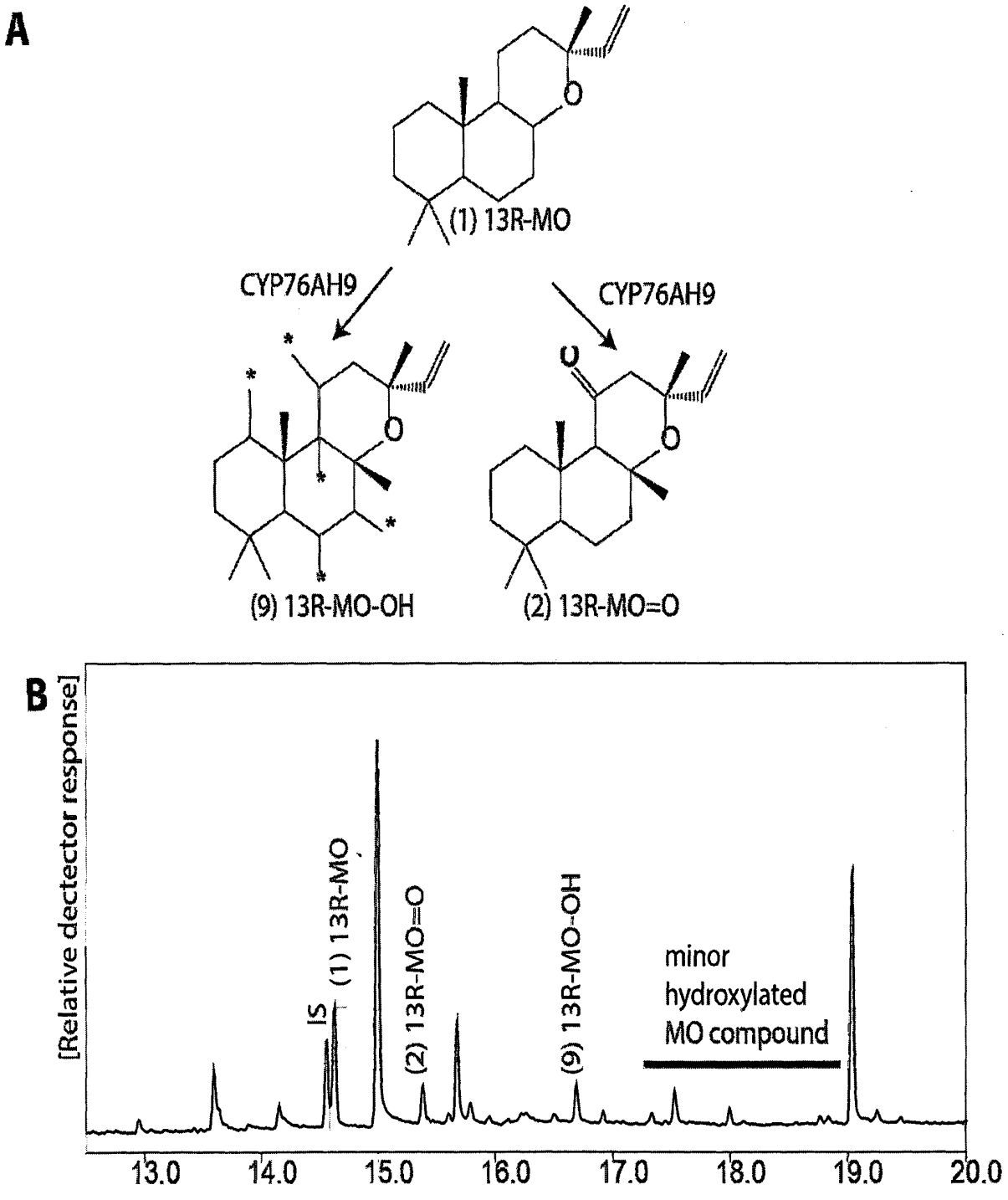
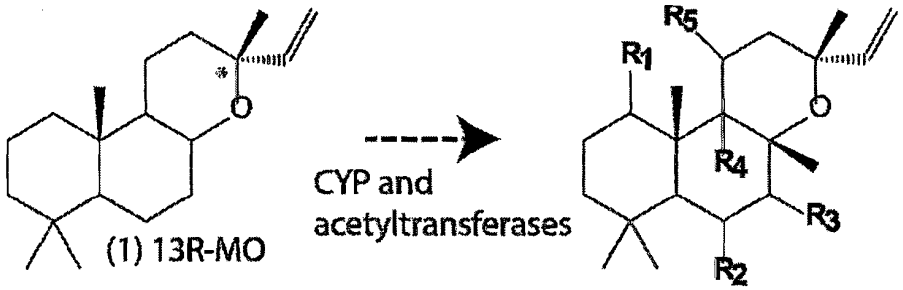

A
(1) 13R-MO
CYP76AH9
CYP76AH9
(9) 13R-MO-OH
(2) 13R-MO=O
B
[Relative dectector response]
IS
(1) 13R-MO
(2) 13R-MO=O
(9) 13R-MO-OH
minor
hydroxylated
MO compound
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0

Fig 5



Pharmacologically active MO derivatives					
R_1	R_2	R_3	R_4	R_5	**Compound**
OH	OH	OAc	OH	O	forskolin
OH	OAc	OH	OH	O	iso-forskolin
OH	OH	OH	OH	O	forskolin B
OH	OH	OAc	OH	O	forskolin D
H	OH	OAc	H	O	9-deoxyforskolin
H	OH	OAc	H	O	1,9-dideoxyforskolin
O	OH	OAc	OH	OAc	coleoforskolin

Fig. 6

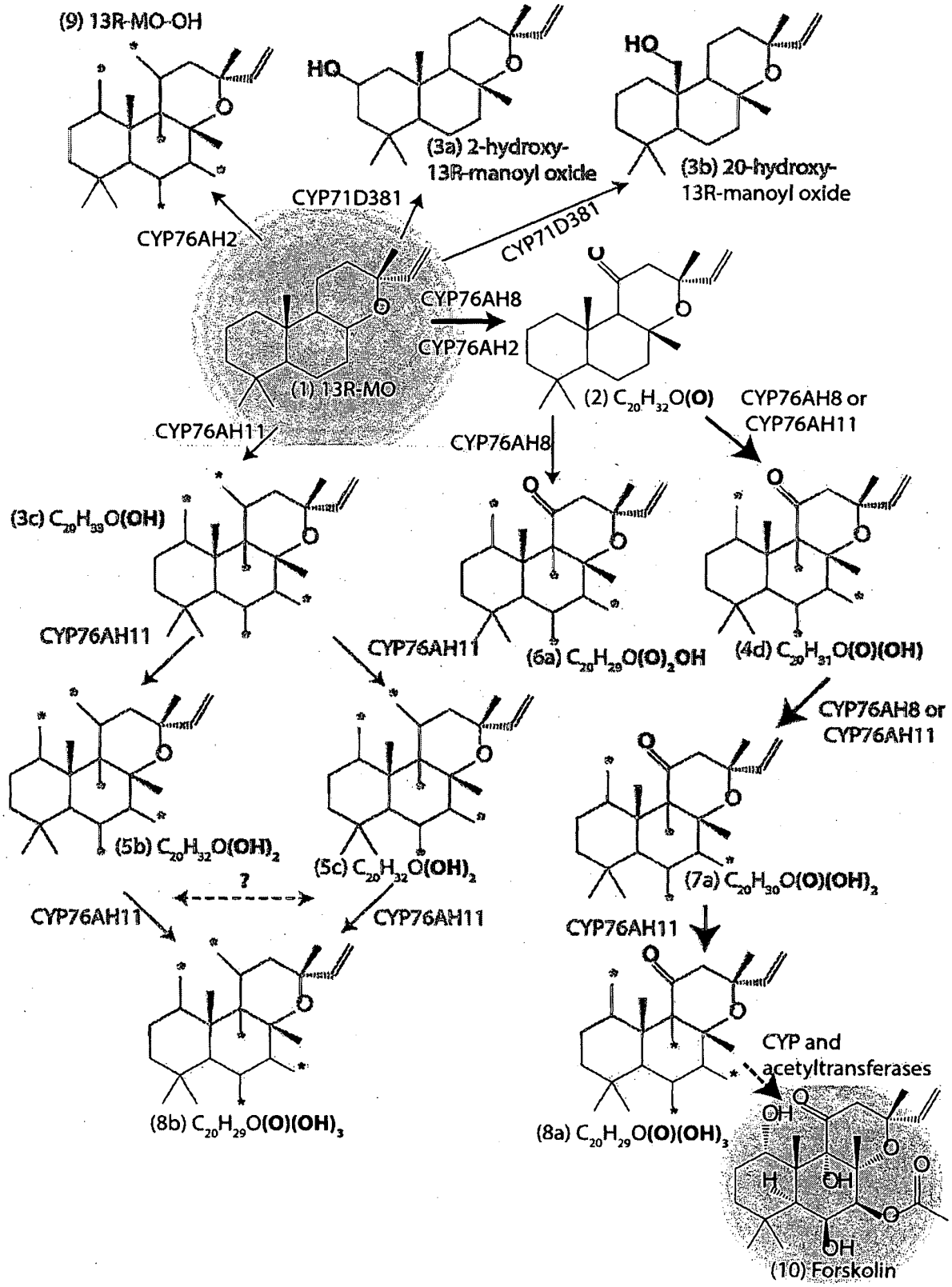

(9) 13R-MO-OH
HO
(3a) 2-hydroxy-13R-manoyl oxide
HO
(3b) 20-hydroxy-13R-manoyl oxide
CYP71D381
CYP76AH2
CYP71D381
CYP76AH8
CYP76AH2
(1) 13R-MO
(2) $C_{20}H_{32}O$(O)
CYP76AH8 or CYP76AH11
CYP76AH11
CYP76AH8
(3c) $C_{20}H_{33}O$(OH)
CYP76AH11
CYP76AH11
(6a) $C_{20}H_{29}O(O)_2OH$
(4d) $C_{20}H_{31}O(O)(OH)$
CYP76AH8 or CYP76AH11
(5b) $C_{20}H_{32}O(OH)_2$
(5c) $C_{20}H_{32}O(OH)_2$
?
(7a) $C_{20}H_{30}O(O)(OH)_2$
CYP76AH11
CYP76AH11
CYP76AH11
CYP and acetyltransferases
(8b) $C_{20}H_{29}O(O)(OH)_3$
(8a) $C_{20}H_{29}O(O)(OH)_3$
(10) Forskolin

Fig. 7

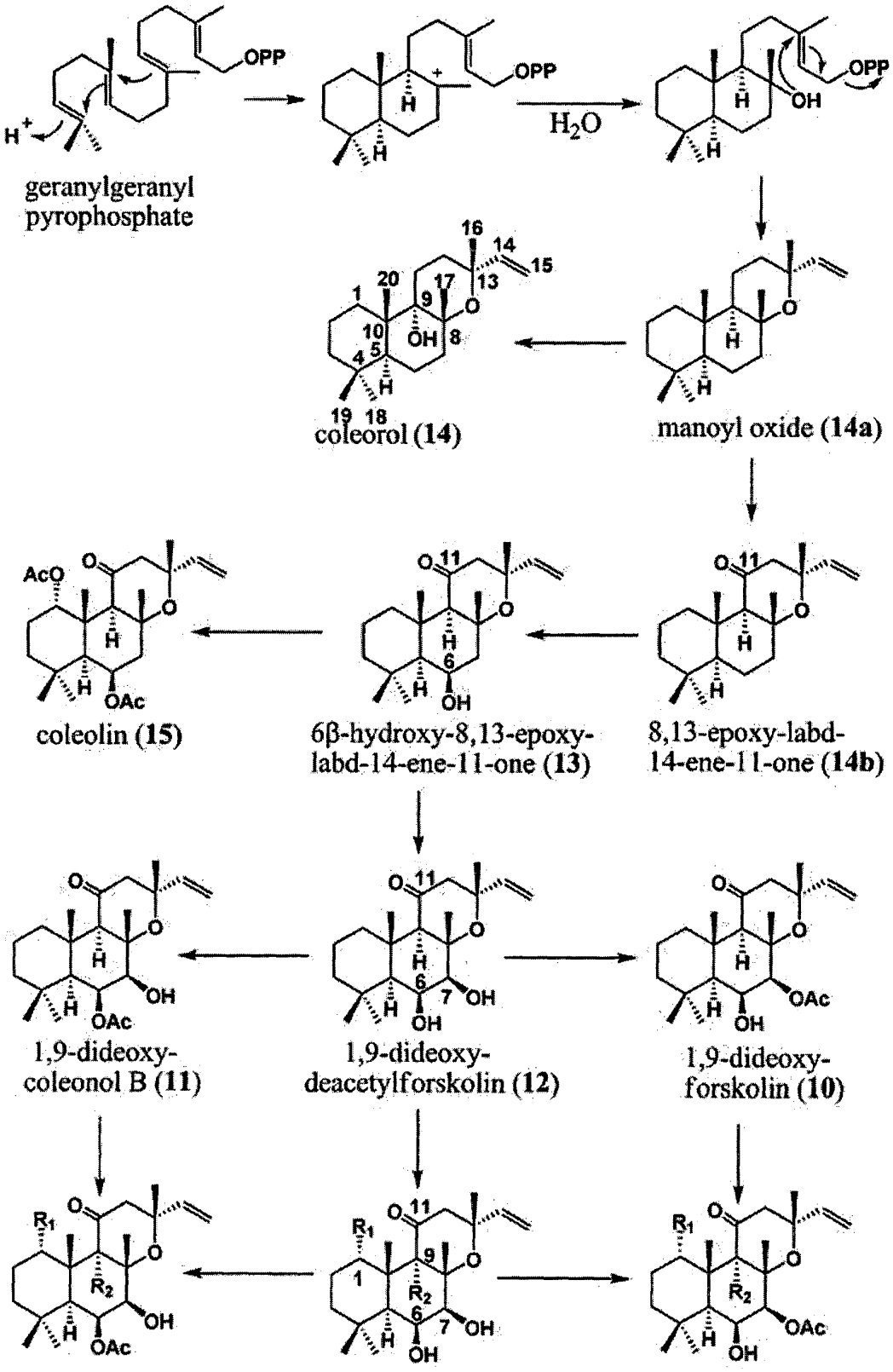

geranylgeranyl pyrophosphate
OPP
H+
H2O
OH
coleorol (14)
manoyl oxide (14a)
AcO
OAc
coleolin (15)
6β-hydroxy-8,13-epoxy-labd-14-ene-11-one (13)
8,13-epoxy-labd-14-ene-11-one (14b)
1,9-dideoxy-coleonol B (11)
1,9-dideoxy-deacetylforskolin (12)
1,9-dideoxy-forskolin (10)
R1
R2

Fig. 8

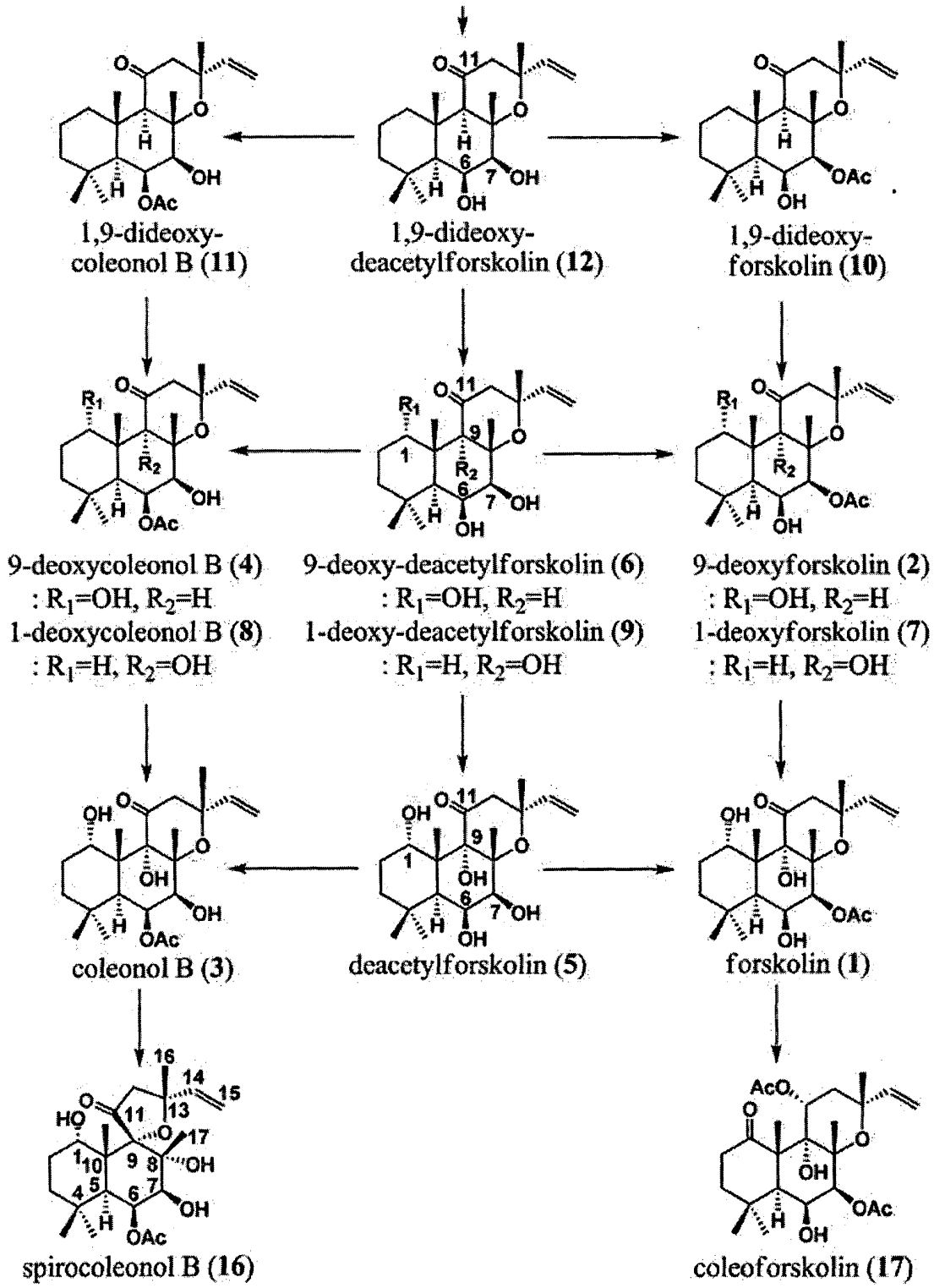


Fig. 8 (continued)

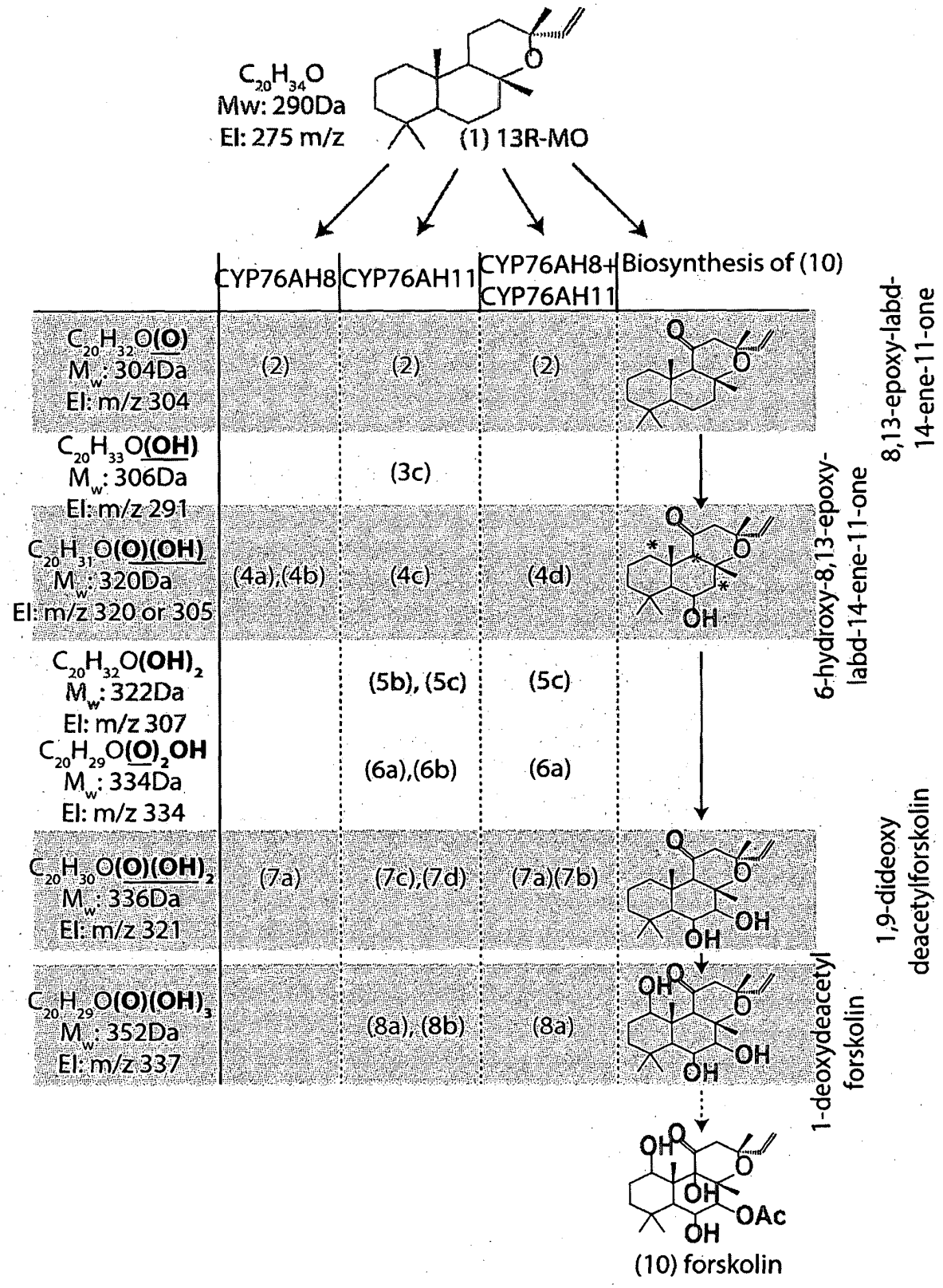

C20H34O
Mw: 290Da
EI: 275 m/z
(1) 13R-MO
CYP76AH8
CYP76AH11
CYP76AH8+CYP76AH11
Biosynthesis of (10)
C20H32O(O)
Mw: 304Da
EI: m/z 304
(2)
(2)
(2)
8,13-epoxy-labd-14-ene-11-one
C20H33O(OH)
Mw: 306Da
EI: m/z 291
(3c)
C20H31O(O)(OH)
Mw: 320Da
EI: m/z 320 or 305
(4a),(4b)
(4c)
(4d)
6-hydroxy-8,13-epoxy-labd-14-ene-11-one
C20H32O(OH)2
Mw: 322Da
EI: m/z 307
(5b), (5c)
(5c)
C20H29O(O)2OH
Mw: 334Da
EI: m/z 334
(6a),(6b)
(6a)
C20H30O(O)(OH)2
Mw: 336Da
EI: m/z 321
(7a)
(7c),(7d)
(7a)(7b)
1,9-dideoxy deacetylforskolin
C20H29O(O)(OH)3
Mw: 352Da
EI: m/z 337
(8a), (8b)
(8a)
1-deoxydeacetyl forskolin
OAc
(10) forskolin

Fig. 9

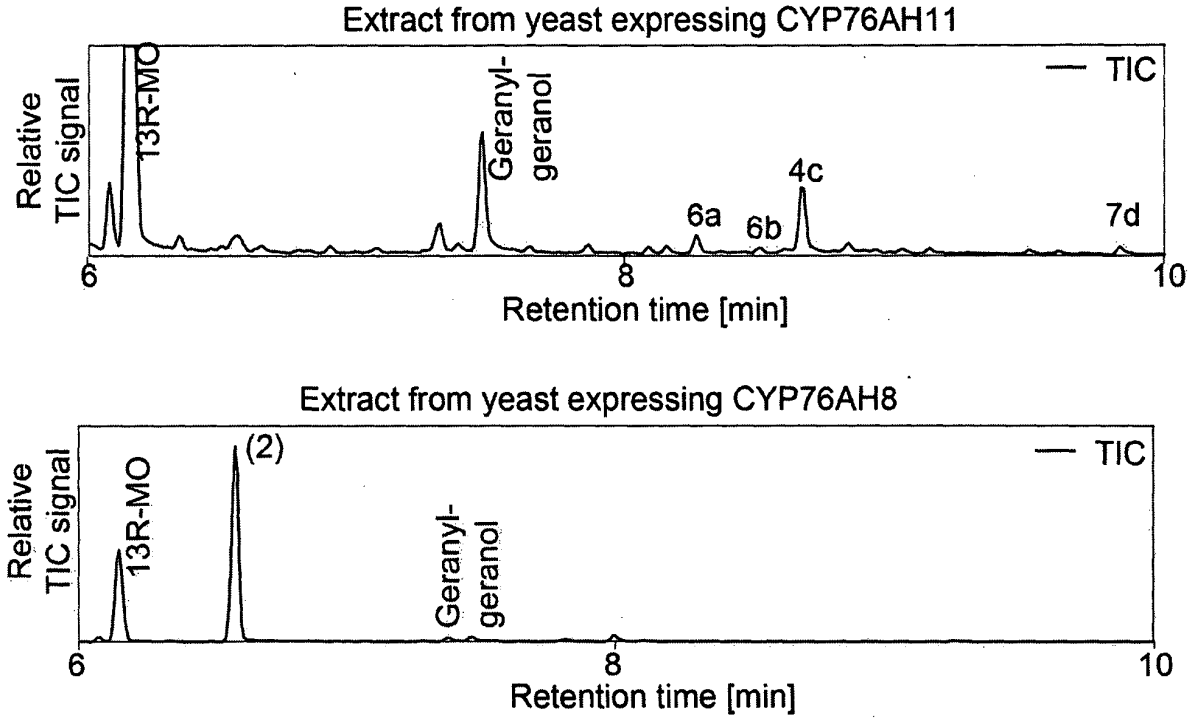

Extract from yeast expressing CYP76AH11
Relative TIC signal
13R-MO
Geranyl-geranol
6a
6b
4c
7d
TIC
6
8
10
Retention time [min]
Extract from yeast expressing CYP76AH8
Relative TIC signal
13R-MO
(2)
Geranyl-geranol
TIC
6
8
10
Retention time [min]

Fig. 10

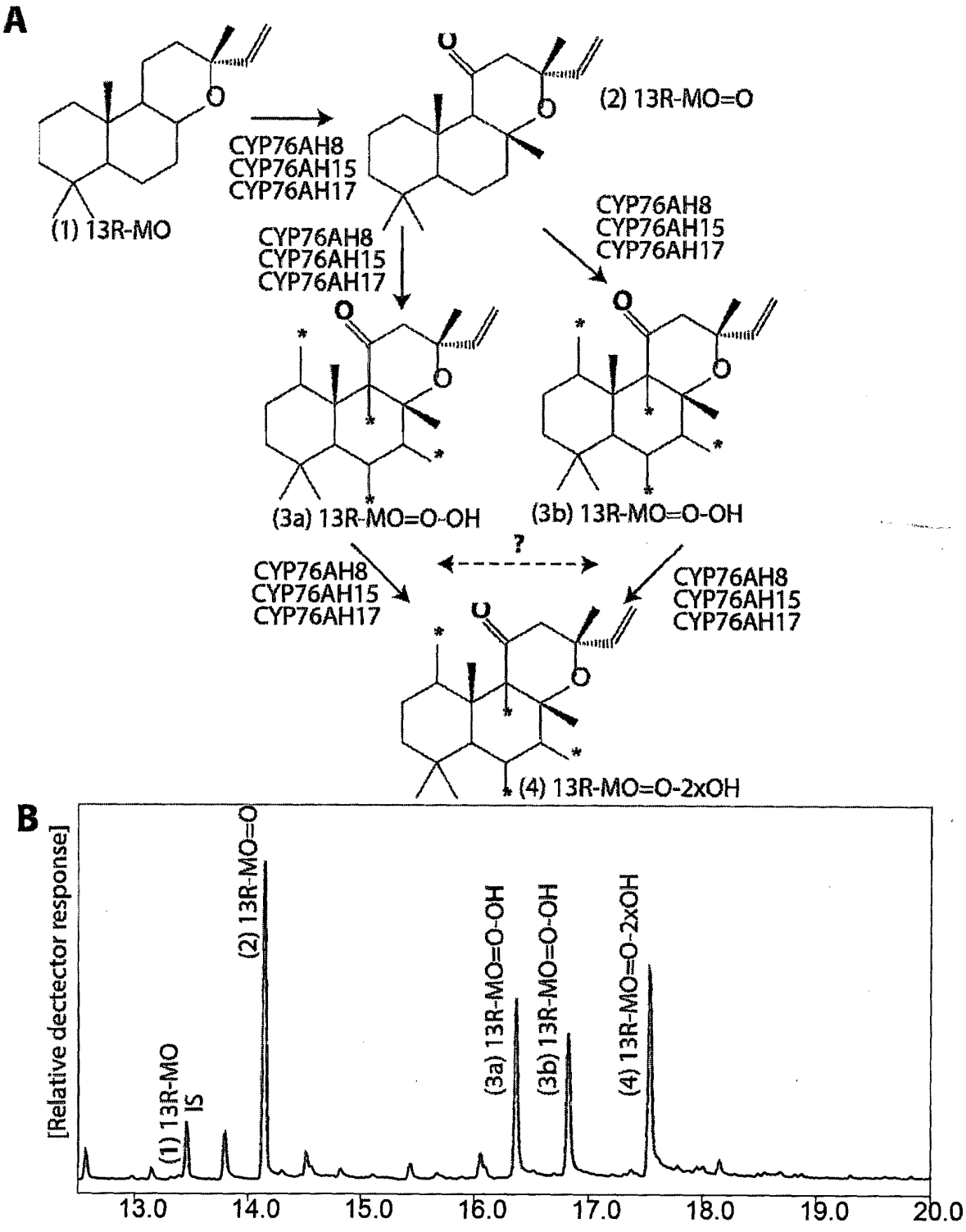

A
(1) 13R-MO
CYP76AH8
CYP76AH15
CYP76AH17
(2) 13R-MO=O
CYP76AH8
CYP76AH15
CYP76AH17
CYP76AH8
CYP76AH15
CYP76AH17
(3a) 13R-MO=O-OH
(3b) 13R-MO=O-OH
?
CYP76AH8
CYP76AH15
CYP76AH17
CYP76AH8
CYP76AH15
CYP76AH17
(4) 13R-MO=O-2xOH
B
[Relative dectector response]
(1) 13R-MO
IS
(2) 13R-MO=O
(3a) 13R-MO=O-OH
(3b) 13R-MO=O-OH
(4) 13R-MO=O-2xOH
13.0
14.0
15.0
16.0
17.0
18.0
19.0
20.0

Fig. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050020

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P5/00 C12P17/06 C12N9/02
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)
C12P C12N

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, BIOSIS, Sequence Search, 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	Johan Andersen-Ranberg: "Identification and characterization of biosynthetic parts involved in plant diterpenoid biosynthesis", Denmark, 1 January 2014 (2014-01-01), XP055183626, Retrieved from the Internet: URL:http://worldwidescience.org/topicpages/d/ditp+xtp+dxtp.html [retrieved on 2015-04-16] abstract ----- -/--	1-16, 21-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2015	30/04/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schneider, Patrick

1

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.
X	DATABASE UniProt [Online] 16 October 2013 (2013-10-16), "RecName: Full=Ferruginol synthase {ECO:0000303¦PubMed:23812755}; EC=1.14.13.190 {ECO:0000269¦PubMed:23812755}; AltName: Full=Cytochrome P450 76AH1 {ECO:0000303¦PubMed:23812755};", XP002738560, retrieved from EBI accession no. UNIPROT:S4UX02 Database accession no. S4UX02 81% identical to SEQ ID NO:1 (CYP76AH8) and 78% identical to SEQ ID NO:10 (CYP76AH17) and 74% identical to SEQ ID NO:11 (CYP76AH15); sequence -----	9-13,16, 18,20
X	Johan Andersen-Ranberg: "Identification and Characterization of Biosynthetic Parts Involved in Plant Diterpenoid Biosyntheses - Employees", , 1 January 2014 (2014-01-01), XP055183341, University of Copenhagen Retrieved from the Internet: URL:http://plen.ku.dk/english/employees/?pure=en%2Fpublications%2Fidentification-and-characterization-of-biosynthetic-parts-involved-in-plant-diterpenoid-biosyntheses(9b672fe8-15a1-4685-8204-ce711d76b041).html [retrieved on 2015-04-15] the whole document -----	1-16, 21-38
A	PHILIPP ZERBE ET AL: "Gene discovery of modular diterpene metabolism in nonmodel systems", PLANT PHYSIOLOGY, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, US , vol. 162, no. 2 1 June 2013 (2013-06-01), pages 1073-1091, XP002724041, ISSN: 0032-0889, DOI: 10.1104/PP.113.218347 Retrieved from the Internet: URL:http://www.plantphysiol.org/content/162/2/1073 [retrieved on 2013-04-23] the whole document -----	1-38

-/--

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2015/050020

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	MORTEN T NIELSEN ET AL: "Microbial synthesis of the forskolin precursor manoyl oxide in enantiomerically pure form", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 80, no. 23, 1 December 2014 (2014-12-01), pages 7258-7265, XP002735928, ISSN: 0099-2240, DOI: 10.1128/AEM.02301-14 [retrieved on 2014-09-19] the whole document ----	1-38
A,P	PATERAKI IRINI ET AL: "Manoyl oxide (13R), the biosynthetic precursor of forskolin, is synthesized in specialized root cork cells in Coleus forskohlii", PLANT PHYSIOLOGY, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, US, vol. 164, 1 March 2014 (2014-03-01), pages 1222-1236, XP002724033, ISSN: 0032-0889 the whole document ----	1-38

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