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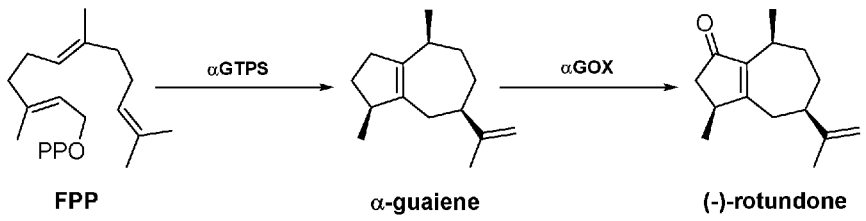
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(54) Title: MICROBIAL PRODUCTION OF ROTUNDONE

FIG. 2



(57) Abstract: The present disclosure provides methods and compositions for producing rotundone. In various aspects, the present disclosure provides enzymes, polynucleotides encoding said enzymes, and recombinant microbial host cells (or microbial host strains) for the production of rotundone. In some embodiments, the present disclosure provides microbial host cells for producing rotundone at high purity and/or yield, from either enzymatic transformation of a- guaiene, or from sugar or other carbon source. The present disclosure further provides methods of making products containing rotundone, including flavor or fragrance products, among others.

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MICROBIAL PRODUCTION OF ROTUNDONE

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/727,815, filed September 6, 2018, which is hereby incorporated by reference in its entirety.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: MAN-019PC Sequence Listing_ST25; date recorded: September 6, 2019; file size: 235,920 bytes).

BACKGROUND

Rotundone is an oxygenated sesquiterpene (sesquiterpenoid) that is responsible for a pleasing spicy, 'peppery' aroma in various plants, including grapes (especially syrah or shiraz, mourvèdre, durif, vespolina, and grüner veltliner varietals), and a large number of herbs and spices, such as, e.g., black and white pepper, oregano, basil, thyme, marjoram, and rosemary. Given its aroma, rotundone is an attractive molecule for applications in fragrances and flavors.

α-Guaiene is the precursor to (-)-rotundone. α-Guaiene is a sesquiterpene hydrocarbon found in oil extracts from various plants, and is converted to (-)-rotundone by aerial oxidation or enzymatic transformation.

Given the commercial value of rotundone, cost effective, scalable, and/or sustainable processes for its production are needed.

SUMMARY

In various aspects, the present disclosure provides methods and compositions for producing rotundone. In various aspects, the present disclosure provides enzymes, polynucleotides encoding said enzymes, and recombinant microbial host cells (or microbial host strains) for the production of rotundone. In some embodiments, the present disclosure provides microbial host cells for producing rotundone at high purity

and/or yield, from either enzymatic transformation of α-guaiene, or from sugar or other carbon source. The present disclosure further provides methods of making products containing rotundone, including flavor or fragrance products, among others.

In some embodiments, the present disclosure provides a microbial host cell expressing an enzyme pathway catalyzing the conversion of farnesyl diphosphate (FPP) to rotundone, the enzymatic pathway comprising an α-guaiene terpene synthase enzyme (αGTPS) and an α-guaiene oxidase (αGOX) enzyme. In these embodiments, the microbial cells can synthesize rotundone product from any suitable carbon source. In some embodiments, the specificity of the α-guaiene synthase enzyme enables production of rotundone at high yield with fewer terpenoid byproducts. In some embodiments, the αGOX produces rotundone as the predominant oxygenated product.

In some embodiments, the microbial host cell further expresses or overexpresses one or more enzymes in the methylerythritol phosphate (MEP) and/or the mevalonic acid (MVA) pathway to catalyze the conversion of glucose or other carbon sources to isopentenyl pyrophosphate (IPP) and/or dimethylallyl pyrophosphate (DMAPP). In some embodiments, the microbial host cell further expresses an enzyme catalyzing the conversion of IPP and/or DMAPP to farnesyl diphosphate (FPP), allowing for rotundone to be produced from sugar or other carbon sources (carbon substrates such as C1, C2, C3, C4, C5, and/or C6 carbon substrates). In some embodiments, the host cell is a bacteria engineered to increase carbon flux through the MEP pathway.

In some embodiments, the microbial host cell expresses an α-guaiene oxidase (αGOX) enzyme, which may be a P450 enzyme, non-heme iron oxygenase (NHIO), or laccase providing for biotransformation of α-guaiene substrate. α-Guaiene substrate can be added to whole cell or cellular extracts or purified enzyme. In some embodiments, the cell further expresses at least one cytochrome P450 reductase to support P450 enzyme activity for whole cell bioconversion processes. In some embodiments, the αGOX produces rotundone as the predominant oxygenated product.

In some embodiments, the microbial host cell further expresses one or more alcohol dehydrogenases. In some embodiments, the alcohol dehydrogenase converts one or more alcohol intermediates, produced by the reaction of α-guaiene with αGOX, to rotundone.

In some embodiments, the microbial host cell is prokaryotic or eukaryotic, and may be a bacteria or yeast.

Other aspects and embodiments of the invention will be apparent from the following detailed disclosure.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the chemical structure of rotundone.

FIG. 2 illustrates a biosynthetic pathway for the production of rotundone. Farnesyl diphosphate is converted to α-guaiene by an α-guaiene terpene synthase (αGTPS) enzyme; and α-guaiene is converted to (-)-rotundone by an α-guaiene oxidase (αGOX) enzyme.

FIG. 3 illustrates that the production of (-)-rotundone from α-guaiene can proceed directly from the sesquiterpene precursor, or can involve the production of one or both alcohol intermediates which are subsequently converted to the ketone by an enzyme with alcohol dehydrogenase activity.

FIG. 4 shows results from the screening of amino acid substitutions in an exemplary α-guaiene terpene synthase (AcDGuaS3, SEQ ID NO: 8). Derivatives were tested for α-guaiene production in *E. coli*. The figure shows fold improvement in α-guaiene production.

FIG. 5 lists the altered profile toward production of α-guaiene in *E. coli* for several amino acid substitutions in AcDGuaS3. Fold improvement of α-guaiene as a % of the total products is listed.

FIG. 6 shows production of α-guaiene with expression of wild-type AcDGuaS3 (α-GS) and mutant AcDGuaS3 having an F406L mutation (α-GS1) in *E. coli*.

FIGS. 7A and 7B show production of rotundol and rotundone in *E. coli* expressing α-GS1 and an exemplary CYP450 system (engineered kaurene oxidase; KOeng). Expression of *Vitis vinifera* dehydrogenase (VvDH) along with α-GS1, KOeng as the α-guaiene oxidase, and *Camptotheca acuminata* cytochrome P450 reductase (CaCPR) reduces the titer of rotundol (FIG. 7A) and increases rotundone titer (FIG. 7B).

FIGS. 8A and B show Gas-Chromatography/Mass Spectrometry (GC/MS) confirmation of production of rotundone from *E. coli* strain expressing α-GS1, KOeng, CaCPR, and VvDH. FIG. 8A shows the abundance of rotundone in GC/MS and FIG. 8B shows the gas chromatogram for rotundone.

FIG. 9 shows production of rotundol and rotundone in *E. coli* expressing α-GS1 and an alternative CYP450 system. Expression of α-GS1, *Helianthus annuus* germacrene A monooxygenase engineered for expression in *E. coli* (HaGAO), and AaCPR (*Artemisia annua* cytochrome P450 reductase) produces primarily rotundone.

FIG. 10 shows a multiple sequence alignment of five kaurene oxygenases, including KOeng (SEQ ID NO: 51), which functions as α-guaiene oxidase enzyme. The homologs are HaKO (SEQ ID NO: 50), AaKO (SEQ ID NO: 49), CcKO (SEQ ID NO: 47), LsKO (SEQ ID NO: 46), and NtKO (SEQ ID NO: 45).

DETAILED DESCRIPTION

In various aspects, the present disclosure provides microbial host cells (or microbial host strains) and methods for producing rotundone and methods of making products containing rotundone, such as flavor and fragrance products, among others. In other aspects, the present invention provides enzymes and polynucleotides encoding enzymes for the production of rotundone.

In some embodiments, the present disclosure provides a microbial host cell, including bacteria and yeast, expressing an enzyme pathway catalyzing the conversion of farnesyl diphosphate (FPP) to rotundone. In various embodiments, the enzymatic pathway comprises a α-guaiene synthase enzyme (αGTPS) and an α-guaiene oxidase (αGOX) enzyme. In these embodiments, the microbial cells can synthesize rotundone product from any suitable carbon source. In some embodiments, the specificity of the α-guaiene synthase enzyme enables production of rotundone at high yield with fewer terpenoid byproducts. In some embodiments, the microbial host cell may further expresses one or more alcohol dehydrogenase enzymes (ADH). In some embodiments, the ADH converts one or more alcohol intermediates, produced by the reaction of α-guaiene with αGOX, to rotundone.

In some embodiments, the microbial host cell expresses an α-guaiene oxidase (αGOX) enzyme, providing for biotransformation of α-guaiene substrate. In some embodiments, αGOX is a P450 enzyme, non-heme iron oxygenase (NHIO), or laccase. In some embodiments, the cell may further express a cytochrome P450 reductase to support P450 activity. In some embodiments, the microbial host cell may further express one or more alcohol dehydrogenase enzymes (ADH). In some embodiments, the ADH converts one or more alcohol intermediates, produced by the reaction of α-guaiene with αGOX, to rotundone.

Rotundone comprises a guaiene carbon skeleton with a single ketone group in the carbon 2 position (see FIG. 1). α-Guaiene is the precursor to rotundone. α-Guaiene is a sesquiterpene hydrocarbon found in oil extracts from various plants. While α-guaiene can be converted to rotundone by aerial oxidation or enzymatic transformation, these processes are not efficient, in part due to the specificity of enzymes used.

A biosynthetic pathway for rotundone is shown in FIG. 2. The C15 sesquiterpene precursor substrate farnesyl diphosphate (FPP) is cyclized to α-guaiene by an αGTPS terpene synthase enzyme. The α-guaiene (i.e., cyclized FPP) is then oxidized to rotundone via an αGOX enzyme. The production of the ketone moiety in α-guaiene resulting in rotundone can proceed directly, or can alternatively proceed through alcohol intermediates, with either stereochemistry of the alcohol intermediate, i.e., (2R)-rotundol or (2S)-rotundol (see FIG. 3).

The αGTPS enzyme is a terpene synthase enzyme (TPS). TPS enzymes are responsible for the synthesis of the terpene molecules from two isomeric 5-carbon precursor building blocks, leading to 5-carbon isoprene, 10-carbon monoterpenes, 15-carbon sesquiterpenes and 20-carbon diterpenes. The structures and functions of TPS enzymes are described in Chen *et al.*, *The Plant Journal*, 66: 212-229 (2011). Tobacco 5-epi-aristolochene synthase, a terpene synthase, has been described along with structural coordinates, including key active site coordinates. These structural coordinates can be used for constructing homology models of αGTPS enzymes, which are useful for guiding the engineering of αGTPS enzymes with improved specificity and productivity. See, US 6,645,762, US 6,495,354, and US 6,645,762, which are hereby incorporated by reference in their entireties.

In some embodiments, the TPS enzyme is selected from *Vitis vinifera* GuaS (VvGuas) enzyme (SEQ ID NO: 1), patchouli synthase (PcPS) enzyme from *Pogostemon cablin* (Uniprot Q49SP3) (SEQ ID NO: 2), *Vitis vinifera* germacrene D synthase (VvGDS; NCBI Ref #XP_002282488.1) (SEQ ID NO: 21), or a variant thereof. In some embodiments, the TPS enzyme is selected from *Aquilaria crassna*, for example, AcC1 (Uniprot D0VMR5); AcC2 (Uniprot D0VMR6) (SEQ ID NO: 3); AcC3 (Uniprot D0VMR7) (SEQ ID NO: 4); or AcC4 (Uniprot D0VMR8) (SEQ ID NO: 5), or a variant thereof. In some embodiments, the *A. crassna* TPS is a mutant of AcC1, for example AcC1mut1-M42 (SEQ ID NO: 6) and AcC1mut2-M50 (SEQ ID NO: 7). Other TPS enzymes are provided herein as SEQ ID NO:8 (*Aquilaria crassna* AcDGuaS3), SEQ ID NO:9 (*Aquilaria crassna* AcDGuaS4), SEQ ID NO:10 (*Aquilaria crassna* AcDGuaS2), SEQ ID NO:11 (*Aquilaria crassna* AcDGuaS5), SEQ ID NO: 12 (*Aquilaria spp.* AmiDGuaS1), SEQ ID NO: 13 (*Aquilaria spp.* AmiDGuaS2), SEQ ID NO: 14 (*Aquilaria spp.* AmiDGuaS3), SEQ ID NO: 15 (*Aquilaria spp.* AmaDGuaS1), SEQ ID NO: 16 (*Aquilaria spp.* AmaDGuaS2), SEQ ID NO: 17 (*Aquilaria spp.* AsDGuaS1), SEQ ID NO: 18 (*Aquilaria spp.* AsDGuaS2), SEQ ID NO: 19 (*Aquilaria spp.* AsDGuaS3), and SEQ ID NO: 20 (*Aquilaria spp.* AsDGuaS4), or a variant thereof.

Terpene synthase variants include α-guaiene synthase enzymes comprising an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NOs: 1 to 21. In some embodiments, the variant comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of any one SEQ ID NOs: 1 to 21. In some embodiments, the variant includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to an amino acid sequence selected from SEQ ID NOs: 1 to 21. In some embodiments, the terpene synthase comprises a substitution to one or more of the substrate binding site or active site. In some embodiments, modifications to enzymes can be informed by construction of a homology model. In some embodiments, the amino acid modifications can be selected to improve one or more of: enzyme productivity, selectivity for the desired substrate and/or product, stability, temperature tolerance, and expression.

In some embodiments, the α-guaiene synthase enzyme comprises an amino acid sequence that has at least 50% sequence identity with any one of SEQ ID NOs: 1, 3, 4, 6 to 10, 11 to 15, or 19. In some embodiments, the α-guaiene synthase enzyme comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of any one SEQ ID NOs: 1, 3, 4, 6 to 10, 11 to 15, or 19. In some embodiments, the α-guaiene synthase enzyme includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to an amino acid sequence selected from SEQ ID NOs: 1, 3, 4, 6 to 10, 11 to 15, or 19.

In some embodiments, the α-guaiene synthase enzyme comprises an amino acid sequence that has 50% or more sequence identity with SEQ ID NO: 8. In some embodiments, the α-guaiene synthase enzyme comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of SEQ ID NO: 8. In some embodiments, the α-guaiene synthase enzyme includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to the amino acid sequence of SEQ ID NO: 8.

In some embodiments, the α-guaiene synthase may have one, two, three, four, five or more amino acid substitutions at positions corresponding to positions selected from 72, 273, 290, 368, 371, 374, 377, 381, 382, 399, 406, 419, 433, 442, 443, 454, 512, and 522 of SEQ ID NO: 8. For example, in some embodiments the α-GTPS comprises an amino acid sequence having one or more (e.g, 2, 3, 4, 5, or all) of the amino acid substitutions selected from T72I, M273L, R290K, F368M, I371L, S374A, R377V, Y381W, F382L, I399V, F406L, L419T, V433I, Y442L, I443M, E454K, F512L, and K522D relative to SEQ ID NO: 8. In some embodiments, the α-GTPS includes an amino acid substitution at the position corresponding to position 406 of SEQ ID NO: 8, and which is optionally F406L, F406A, F406I, F406V, or F406G. In some embodiments, the

α-GTPS enzyme includes an amino acid substitution at the position corresponding to position 443 of SEQ ID NO: 8, which is optionally I443M. In some embodiments, the α-GTPS enzyme includes a mutation at the position corresponding to position 512 of SEQ ID NO: 8, which is optionally F512L, F512A, F512I, F512V, or F512G.

Amino acid substitutions may be conservative or non-conservative substitutions.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, size, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the amino acid residues involved. The 20 naturally occurring amino acids can be grouped into the following six standard amino acid groups:

(1) hydrophobic: Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr; Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro; and

(6) aromatic: Trp, Tyr, Phe.

As used herein, "conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed within the same group of the six standard amino acid groups shown above. For example, the exchange of Asp by Glu retains one negative charge in the so modified polypeptide. In addition, glycine and proline may be substituted for one another based on their ability to disrupt a-helices. Some preferred conservative substitutions within the above six groups are exchanges within the following sub-groups: (i) Ala, Val, Leu and Ile; (ii) Ser and Thr; (ii) Asn and Gln; (iv) Lys and Arg; and (v) Tyr and Phe.

As used herein, "non-conservative substitutions" or "non-conservative amino acid exchanges" are defined as exchanges of an amino acid by another amino acid listed in a different group of the six standard amino acid groups (1) to (6) shown above.

Mutations in α-GTPS enzymes can be guided by homology models using molecular structures/models of sesquiterpene synthase disclosed in Drew et al., J. of Exp. Botany, Vol. 67, No. 3, pp. 799-808 (2015) and/or Kumeta et al., Plant Physiology, Vol. 154, pp. 1998-2007 (2010), which are hereby incorporated by reference in its entirety.

The similarity of nucleotide and amino acid sequences, i.e. the percentage of sequence identity, can be determined via sequence alignments. Such alignments can be carried out with several art-known algorithms, such as with the mathematical algorithm of Karlin and Altschul (Karlin & Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877), with hmmalign (HMMER package, http://hmmer.wustl.edu/) or with the CLUSTAL algorithm (Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673-80). The grade of sequence identity (sequence matching) may be calculated using e.g. BLAST, BLAT or BlastZ (or BlastX). A similar algorithm is incorporated into the BLASTN and BLASTP programs of Altschul et al (1990) J. Mol. Biol. 215: 403-410. BLAST protein searches may be performed with the BLASTP program, score=50, word length=3. To obtain gapped alignments for comparative purposes, Gapped BLAST is utilized as described in Altschul et al (1997) Nucleic Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs are used. Sequence matching analysis may be supplemented by established homology mapping techniques like Shuffle-LAGAN (Brudno M., Bioinformatics 2003b, 19 Suppl 1:154-162) or Markov random fields.

TPS enzymes can generate multiple products with the guaiene skeleton from FPP with varied amounts of α-guaiene produced by different TPS enzymes. In some embodiments, the α-guaiene synthase (or engineered variant) produces predominantly α-guaiene (e.g., greater than 50%) as the product from FPP substrate. In some embodiments, the α-guaiene synthase produces greater than about 75%, or greater than about 80%, or greater than about 85%, or greater than about 90%, or greater than about 95% α-guaiene as the product from FPP. Enzyme specificity can be determined in host microbial cells producing FPP and expressing the α-guaiene synthase, followed by chemical analysis of total terpenoid products. In some embodiments, the α-guaiene produced in the αGTPS reaction is oxidized to rotundone. In some embodiments, an αGOX enzyme oxidizes α-guaiene to rotundone. In some embodiments, the αGOX oxidizes at least one portion of the α-guaiene to a ketone. In some embodiments, the

oxidation of α-guaiene by αGOX results in the production of one or more alcohol intermediates. In some embodiments, the alcohol intermediates are converted to rotundone by one or more alcohol dehydrogenases.

In some embodiments, the αGOX enzyme is a cytochrome P450 (CYP450) enzyme. CYP450 enzymes are involved in the formation (synthesis) and breakdown (metabolism) of various molecules and chemicals within cells. CYP450 enzymes have been identified in all kingdoms of life (i.e., animals, plants, fungi, protists, bacteria, archaea, and even in viruses). Illustrative structure and function of CYP450 enzymes are described in Uracher *et al.*, *TRENDS in Biotechnology*, 24(7): 324-330 (2006). In some embodiments, the P450 enzymes are engineered to have a deletion of all or part of the wild type N-terminal transmembrane region, and the addition of a transmembrane domain derived from an *E. coli* inner membrane cytoplasmic C-terminus protein. In various embodiments, the transmembrane domain is a single-pass transmembrane domain. In various embodiments, the transmembrane domain (or "N-terminal anchor") is derived from an *E. coli* gene selected from waaA, ypfN, yhcB, yhbM, yhhm, zipA, ycgG, djlA, sohB, lpxK, F11O, motA, htpx, pgaC, ygdD, hemr, and ycls. These genes were identified as inner membrane cytoplasmic C-terminus proteins through bioinformatic prediction as well as experimental validation. The invention may employ an N-terminal anchor sequence that is a derivative of the *E. coli* wild-type transmembrane domain, that is, having one or more mutations (e.g., amino acid substitutions) with respect to the wild-type sequence. Methods of making such engineered P450 enzymes as well as engineered P450 enzymes are described in U.S. Patent Publication No. 2018/0251738, which is hereby incorporated by reference in its entirety.

In some embodiments, the CYP450 is selected from the *V. vinifera* VvSTO2 (CYP71BE5; Uniprot F6I534) (SEQ ID NO: 22); *Bacillus subtilis* CYP152A1 (Uniprot O31440) (SEQ ID NO: 23); *B. subtilis* CYP107K1 (Uniprot A5HNX5) (SEQ ID NO: 24); *Bacillus cereus* CYP106 (Uniprot Q737I9) (SEQ ID NO: 25); and *B. cereus* CYP107 (Uniprot Q737F2) (SEQ ID NO: 26); or a variant thereof.

αGOX variants include enzymes comprising an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NOS: 22 to 26. In some embodiments, the variant comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence

identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of any one SEQ ID NOS: 22 to 26. In some embodiments, the variant includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to an amino acid sequence selected from SEQ ID NO: 22 to 26. In some embodiments, the oxygenase comprises a substitution to one or more of the substrate binding site or active site. In some embodiments, modifications to enzymes can be informed by construction of a homology model. In some embodiments, selection and modification of enzymes is informed by assaying activity on α-guaiene substrate. In some embodiments, the amino acid modifications can be selected to improve one or more of: enzyme productivity, selectivity for the desired substrate and/or product, stability, temperature tolerance, and expression.

In some embodiments, the αGOX enzyme is a non-heme iron oxygenase (NHIO) or a laccase. In some embodiments, the laccase is derived from bacteria or fungi (including filamentous fungi and yeasts). By way of example, in some embodiments, the laccase is from a species selected from *Aspergillus, Neurospora* (e.g., *N. crassa*), *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Lentinus*, *Pleurotus*, *Trametes*, *Rhizoctonia* (e.g., *R. solani*), *Coprinus* (e.g., *C. plicatilis*), *Psatyrella*, *Myceliophtera* (e.g., *M. thermophila*), *Schytalidium*, and *Polyporus*, (e.g., *P. pinsitus*), *Phiebia*, and *Coriolus*, or is a derivative thereof.

In some embodiments, the CYP450 (αGOX) comprises an amino acid sequence that has at least 50% identity to SEQ ID NO: 51. In some embodiments, the CYP450 enzyme comprises an amino acid sequence that is at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least 98%, or at least 99% identical to SEQ ID NO: 51. For example, the CYP450 enzyme may comprise an amino acid sequence having from 1 to 20 or from 1 to 10 amino acid modifications with respect to SEQ ID NO: 51, the amino acid modifications being independently selected from amino acid substitutions, deletions, and insertions with respect to corresponding positions in SEQ ID NO: 51.

In some embodiments, the CYP450 comprises an amino acid sequence that has at least 50% identity to SEQ ID NO: 52. In some embodiments, the CYP450 enzyme comprises an amino acid sequence that is at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least 98%, or at least 99% identical to SEQ ID NO: 52. For example, the CYP450 enzyme may comprise an amino acid sequence having from 1 to 20 or from 1 to 10 amino acid modifications with respect to SEQ ID NO: 52, the amino acid modifications being independently selected from amino acid substitutions, deletions, and insertions with respect to corresponding positions in SEQ ID NO: 52.

In some embodiments, the CYP450 comprises an amino acid sequence that has at least 50% identity to SEQ ID NOs: 54, 55, or 56. In some embodiments, the CYP450 enzyme comprises an amino acid sequence that is at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least 98%, or at least 99% identical to SEQ ID NOs: 54, 55, or 56. For example, the CYP450 enzyme may comprise an amino acid sequence having from 1 to 20 or from 1 to 10 amino acid modifications with respect to SEQ ID NO: 54, 55, or 56, the amino acid modifications being independently selected from amino acid substitutions, deletions, and insertions with respect to corresponding positions in SEQ ID NO: 54, 55, or 56.

Amino acid modification to CYP450 enzymes can be guided by available structures, including those described in Pallan *et al.*, "Structural and kinetic basis of steroid 17α, 20-lyase activity in teleost fish cytochrome P450 17A1 and its absence in cytochrome P450 17A2," *Journal of Biological Chemistry* 290.6 (2015): 3248-3268, which is hereby incorporated by reference in its entirety. Pallan et al. describe a Zebra fish cytochrome P450 17A2 along with structural coordinates, including key active site coordinates. These structural coordinates can be used for constructing homology models of CYP450 enzymes, which are useful for guiding the engineering of CYP450 enzymes with improved specificity and productivity.

In some embodiments, the CYP450 enzyme requires the presence of an electron transfer protein capable of transferring electrons to the CYP450 protein. In some embodiments, this electron transfer protein is a cytochrome P450 reductase (CPR), which

can be expressed by the microbial host cell. Various reductases that may be used are described in U.S. Patent Publication No. 2018/0135081, which is hereby incorporated by reference in its entirety.

Exemplary P450 reductase enzymes include those shown herein as SEQ ID NOs: 27 to 34 or 53, or a variant thereof. Variants generally include enzymes comprising an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NOs: 27 to 34 or 53. In some embodiments, the variant comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of any one SEQ ID NOs: 27 to 34 or 53. In some embodiments, the variant includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to an amino acid sequence selected from SEQ ID NOs: 27 to 34 or 53.

In some embodiments, the CPR comprises an amino acid sequence that has at least 50% identity to SEQ ID NO: 53 (CaCPR). In some embodiments, the CPR enzyme comprises an amino acid sequence that is at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least 98%, or at least 99% identical to SEQ ID NO: 53. For example, the CPR enzyme may comprise an amino acid sequence having from 1 to 20 or from 1 to 10 amino acid modifications with respect to SEQ ID NO: 53, the amino acid modifications being independently selected from amino acid substitutions, deletions, and insertions with respect to corresponding positions in SEQ ID NO: 53.

In some embodiments, the αGOX reaction results in hydroxylation of α-guaiene, thereby producing one or more alcohol intermediates, e.g., (2R)-rotundol or (2S)-rotundol (see FIG. 3). In some embodiments, the αGOX further oxidizes at least a portion of the α-guaiene to a ketone. In some embodiments, the alcohol intermediates (e.g., (2R)-rotundol or (2S)-rotundol) are converted to rotundone by one or more alcohol dehydrogenases (ADHs). Thus, in some embodiments, the microbial host cell expresses one or more alcohol dehydrogenases (ADH). By way of example, in some embodiments,

the ADH is selected from an enzyme comprising an amino acid sequence selected from SEQ ID NOs: 35 to 44, or a variant thereof. Variants generally include enzymes comprising an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NOs: 35 to 44. In some embodiments, the variant comprises an amino acid sequence that has at least about 60% sequence identity, or at least about 70% sequence identity, or at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity, or at least about 98% sequence identity, or at least about 99% sequence identity with the amino acid sequence of any one SEQ ID NOS: 35 to 44. In some embodiments, the variant includes from 1 to about 20, or from 1 to about 10, or from 1 to about 5 amino acid modifications independently selected from substitutions, deletions, and insertions to an amino acid sequence selected from SEQ ID NO: 35 to 44. In some embodiments, the amino acid modifications can be selected to improve one or more of: enzyme productivity, selectivity for the desired substrate and/or product, stability, temperature tolerance, and expression.

In some embodiments, the ADH comprises an amino acid sequence that has at least 50% identity to SEQ ID NO: 43 (VvDH). In some embodiments, the ADH enzyme comprises an amino acid sequence that is at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least 98%, or at least 99% identical to SEQ ID NO: 43. For example, the ADH enzyme may comprise an amino acid sequence having from 1 to 20 or from 1 to 10 amino acid modifications with respect to SEQ ID NO: 43, the amino acid modifications being independently selected from amino acid substitutions, deletions, and insertions with respect to corresponding positions in SEQ ID NO: 43.

In some embodiments, the microbial cell expresses an αGOX, and produces predominately rotundone (e.g., at least 75% of the oxygenated product is rotundone), without expression of an ADH enzyme.

In various embodiments, the αGTPS and αGOX are expressed together in an operon, or are expressed individually. The enzymes may be expressed from extrachromosomal elements such as plasmids, or bacterial artificial chromosomes, or may be chromosomally integrated.

In some embodiments, the cell does not express an αGTPS, but expresses an α-guaiene oxidase (αGOX), allowing enzymatic biotransformation of α-guaiene, which can take place with whole cells or whole or partially purified extracts of cells.

In some embodiments, the αGOX and/or the ADH are provided in a purified recombinant form for production of rotundone from α-guaiene, or (2R)-rotundol or (2S)-rotundol, in a cell free system.

In some embodiments, the microbial host cell is also engineered to express or overexpress one or more enzymes in the methylerythritol phosphate (MEP) and/or the mevalonic acid (MVA) pathway to catalyze isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) from glucose or other carbon source.

In some embodiments, the microbial host cell is engineered to express or overexpress one or more enzymes of the MEP pathway. In some embodiments, the MEP pathway is increased and balanced with downstream pathways by providing duplicate copies of certain rate-limiting enzymes. The MEP (2-C-methyl-D-erythritol 4-phosphate) pathway, also called the MEP/DOXP (2-C-methyl-D-erythritol 4-phosphate/l-deoxy-D-xylulose 5-phosphate) pathway or the non-mevalonate pathway or the mevalonic acid-independent pathway refers to the pathway that converts glyceraldehyde-3-phosphate and pyruvate to IPP and DMAPP. The pathway typically involves action of the following enzymes: 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (IspC), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (IspG), and isopentenyl diphosphate isomerase (IspH). The MEP pathway, and the genes and enzymes that make up the MEP pathway, are described in US 8,512,988, which is hereby incorporated by reference in its entirety. For example, genes that make up the MEP pathway include *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, *idi*, and *ispA*. In some embodiments, the microbial host cell expresses or overexpresses of one or more of dxs, ispC, ispD, ispE, ispF, ispG, ispH, idi, ispA, or modified variants thereof, which results in the increased production of IPP and DMAPP. In some embodiments, rotundone is produced at least in part by metabolic flux through an MEP pathway, and wherein the microbial host cell has at least one additional

gene copy of one or more of *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, *idi*, *ispA*, or modified variants thereof.

In some embodiments, the microbial host cell is engineered to express or overexpress one or more enzymes of the MVA pathway. The MVA pathway refers to the biosynthetic pathway that converts acetyl-CoA to IPP. The mevalonate pathway typically comprises enzymes that catalyze the following steps: (a) condensing two molecules of acetyl-CoA to acetoacetyl-CoA (e.g., by action of acetoacetyl-CoA thiolase); (b) condensing acetoacetyl-CoA with acetyl-CoA to form hydroxymethylglutaryl-CoenzymeA (HMG-CoA) (e.g., by action of HMG-CoA synthase (HMGS)); (c) converting HMG-CoA to mevalonate (e.g., by action of HMG-CoA reductase (HMGR)); (d) phosphorylating mevalonate to mevalonate 5-phosphate (e.g., by action of mevalonate kinase (MK)); (e) converting mevalonate 5-phosphate to mevalonate 5-pyrophosphate (e.g., by action of phosphomevalonate kinase (PMK)); and (f) converting mevalonate 5-pyrophosphate to isopentenyl pyrophosphate (e.g., by action of mevalonate pyrophosphate decarboxylase (MPD)). The MVA pathway, and the genes and enzymes that make up the MVA pathway, are described in US 7,667,017, which is hereby incorporated by reference in its entirety. In some embodiments, the microbial host cell expresses or overexpresses one or more of acetoacetyl-CoA thiolase, HMGS, HMGR, MK, PMK, and MPD or modified variants thereof, which results in the increased production of IPP and DMAPP. In some embodiments, rotundone is produced at least in part by metabolic flux through an MVA pathway, and wherein the microbial host cell has at least one additional gene copy of one or more of acetoacetyl-CoA thiolase, HMGS, HMGR, MK, PMK, MPD, or modified variants thereof.

In some embodiments, the microbial host cell is engineered to increase production of IPP and DMAPP from glucose as described in PCT Application Nos. PCT/US2018/016848 and PCT/US2018/015527, the contents of which are hereby incorporated by reference in their entireties. For example, in some embodiments the microbial host cell overexpresses MEP pathway enzymes, with balanced expression to push/pull carbon flux to IPP and DMAPP. In some embodiments, the microbial host cell is engineered to increase the availability or activity of Fe-S cluster proteins, so as to support higher activity of IspG and IspH, which are Fe-S enzymes. In some embodiments, the host cell is engineered to overexpress IspG and IspH, so as to provide

increased carbon flux to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP) intermediate, but with balanced expression to prevent accumulation of HMBPP at an amount that reduces cell growth or viability, or at an amount that inhibits MEP pathway flux.

Conversion of IPP and DMAPP precursors to farnesyl diphosphate (FPP) is typically through the action of a farnesyl diphosphate synthase (FPPS). Exemplary FPPS enzymes are disclosed in US 2018/0135081, which is hereby incorporated by reference in its entirety.

In some embodiments, the host cell is engineered to downregulate the ubiquinone biosynthesis pathway, e.g., by reducing the expression or activity of IspB, which uses IPP and FPP substrate.

In some embodiments, the microbial host cell is a bacteria selected from *Escherichia spp.*, *Bacillus spp.*, *Corynebacterium spp.*, *Rhodobacter spp.*, *Zymomonas spp.*, *Vibrio spp.*, and *Pseudomonas spp.* For example, in some embodiments, the bacterial host cell is a species selected from *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Zymomonas mobilis*, *Vibrio natriegens*, or *Pseudomonas putida*. In some embodiments, the bacterial host cell is *E. coli*.

In some embodiments, the microbial host cell is a species of *Saccharomyces*, *Pichia*, or *Yarrowia*, including, but not limited to, *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*.

In another aspect, the present disclosure provides a method for making rotundone. The method comprises providing a microbial host cell (or microbial host strain) as disclosed herein. The microbial host cell expresses an αGOX enzyme, and optionally an αGTPS enzyme as described herein. Cells expressing an αGOX enzyme can be used for bioconversion of α-guaiene using whole cells or cell extracts. Cells expressing an αGOX enzyme and an αGTPS enzyme can produce rotundone from a carbon source.

In some embodiments, the microbial host cell further expresses one or more alcohol dehydrogenases (ADHs) disclosed herein. Cells expressing ADHs can convert alcohol intermediates produced by the αGOX reaction into rotundone.

In some embodiments, the host cell is cultured to produce rotundone. In some embodiments, microbial cells are cultured with carbon substrates (sources) such as C1, C2, C3, C4, C5, and/or C6 carbon substrates. In exemplary embodiments, the carbon source is glucose, sucrose, fructose, xylose, and/or glycerol. Culture conditions are generally selected from aerobic, microaerobic, and anerobic.

In various embodiments, the host cell is cultured at a temperature between 22° C and 37° C. While commercial biosynthesis in bacteria such as *E. coli* can be limited by the temperature at which overexpressed and/or foreign enzymes (e.g., enzymes derived from plants) are stable, recombinant enzymes (including the terpenoid synthase) may be engineered to allow for cultures to be maintained at higher temperatures, resulting in higher yields and higher overall productivity. In some embodiments, the host cell is a bacterial host cell, and culturing is conducted at about 22° C or greater, about 23° C or greater, about 24° C or greater, about 25° C or greater, about 26° C or greater, about 27° C or greater, about 28° C or greater, about 29° C or greater, about 30° C or greater, about 31° C or greater, about 32° C or greater, about 33° C or greater, about 34° C or greater, about 35° C or greater, about 36° C or greater, or about 37° C.

Rotundone can be extracted from media and/or whole cells, and recovered. In some embodiments, the oxygenated rotundone product is recovered and optionally enriched by fractionation (e.g. fractional distillation). The oxygenated product can be recovered by any suitable process, including partitioning the desired product into an organic phase. The production of the desired product can be determined and/or quantified, for example, by gas chromatography (e.g., GC-MS). The desired product can be produced in batch or continuous bioreactor systems. Production of product, recovery, and/or analysis of the product can be done as described in US 2012/0246767, which is hereby incorporated by reference in its entirety. For example, in some embodiments, oxidized oil is extracted from aqueous reaction medium, which may be done by partitioning into an organic phase, followed by fractional distillation. Sesquiterpene and sesquiterpenoid components of fractions may be measured quantitatively by GC/MS, followed by blending of the fractions.

In some embodiments, the microbial host cells and methods disclosed herein are suitable for commercial production of rotundone, that is, the microbial host cells and methods are productive at commercial scale. In some embodiments, the size of the culture

is at least about 100 L, at least about 200 L, at least about 500 L, at least about 1,000 L, at least about 10,000 L, at least about 100,000 L, or at least about 1,000,000 L. In some embodiment, the culturing may be conducted in batch culture, continuous culture, or semi-continuous culture.

In some aspects, the present disclosure provides methods for making a product comprising rotundone, including flavor and fragrance compositions or products. In some embodiments, the method comprises producing rotundone as described herein through microbial culture, recovering the rotundone, and incorporating the rotundone into the flavor or fragrance composition, or a consumable product (e.g., a food product).

As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. For example, reference to "a cell" includes a combination of two or more cells, and the like.

As used herein, the term "about" in reference to a number is generally taken to include numbers that fall within a range of 10% in either direction (greater than or less than) of the number.

EXAMPLES

Rotundone is a bicyclic sesquiterpene (FIG. 1) and is responsible for pepper aromas in grapes and wine and in herbs and spices, especially black and white pepper, where it has a high odor activity value (OAV). The biosynthesis of rotundone involves cyclization of the C15 sesquiterpene precursor substrate farnesyl diphosphate (FPP) to α-guaiene by an α-GTPS terpene synthase (FIG. 2). Enzymatic oxygenation of α-guaiene can produce rotundone, and may proceed through an alcohol intermediate (FIGS. 2 and 3). For example, α-guaiene may be converted to (2S)-rotundol or (2R)-rotundol by the action of αGOX, and the alcohol intermediate(s) (rotundol) can be converted to rotundone by the action of the αGOX or an alcohol dehydrogenase.

Example 1: Engineering α-Guaiene Synthase to Improve α-Guaiene Production

The α-guaiene precursor, rotundol, or rotundone can be produced by biosynthetic fermentation processes, using microbial strains that produce high levels of MEP pathway products, along with heterologous expression of rotundone biosynthesis enzymes, including, enzymes that catalyze 1) cyclization of FPP to α-guaiene; 2) oxidation of α-

guaiene to rotundone, which can include 3) dehydrogenation of rotundol to rotundone. For example, in bacteria such as *E. coli*, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) can be produced from glucose or other carbon source, and which can be converted to farnesyl diphosphate (FPP) by recombinant farnesyl diphosphate synthase (FPPS). FPP is converted to α-guaiene by α-guaiene synthase (αGTPS) by cyclization. The α-guaiene is converted to rotundol or rotundone by oxygenation reaction catalyzed by an α-guaiene oxidase (αGOX). In instances where the αGOX enzyme catalyzes the production of (2S)-rotundol or (2R)-rotundol from α-guaiene, the conversion of rotundol to rotundone may be catalyzed by a dehydrogenase.

Using an *E. coli* background strain that produces high levels of the MEP pathway products IPP and DMAPP (see US 2018/0245103 and US 2018/0216137, which are hereby incorporated by reference), candidate αGTPS enzymes were screened by co-expression with FPPS. Fermentation was performed in 96 well plates for 48 hours. The following synthase enzymes demonstrated production of α-guaiene in *E. coli*: AcC1mut1_M42, AcC1mut2_M50, AcC2, AcC3, AcDGuaS2, AcDGuaS3, AcDGuaS4, AcDGuaS5, AmaDGuaS1, AmiDGuaS1, AmiDGuaS2, AmiDGuaS3, AsDGuaS3, PcPS, and VvGuaS. In addition to the desired α-guaiene product, active enzymes had varying product profiles. For example, all active Aquilaria enzymes showed α-bulnesene as a major product with α-guaiene. VvGuaS accumulated α-bulnesene and globulol in similar levels to α-guaiene. AcDGuaS3 was selected for subsequent studies based on its productivity and product profile.

A panel of amino acid substitutions to the AcDGuaS3 sequence were screened for their ability to convert FPP to α-guaiene in *E. coli*. The fermentation was conducted in 96 well plates for 48 hours. FIG. 4 shows several mutants (i.e., amino acid substitutions) and the associated fold-improvement in α-guaiene production. For example, F406L substitution in AcDGuaS3 demonstrated a significantly improved titer of α-guaiene (1.71 fold higher than wild-type). Amino acid substitutions were further evaluated for substitutions that shift the product profile toward α-guaiene. See FIG. 5. As shown, a single substitution in wild-type AcDGuaS3 (I443M) shows a 2.4 fold improvement in % α-guaiene relative to other products. Similarly, a F406L substitution shows a 2.12 fold improvement in % α-guaiene relative to other products. A F512 mutation demonstrated a 1.23 fold improvement in % α-guaiene relative to other

products. FIG. 6 shows the fold improvement in titer of α-guaiene, based on expression of a variant having an F406L substitution in AcDGuaS3 (α-GS1) as compared to the parent enzyme.

Example 2: Production of Rotundone

Candidate α-guaiene oxidase enzymes where screened by co-expression in *E. coli* with FPPS and α-GS1. Production of rotundol and rotundone were observed with expression of an engineered Kaurene Oxidase (KOeng). See, US 2018/0135081, which is hereby incorporated by reference in its entirety. Co-expression of *Vitis vinifera* dehydrogenase (VvDH) along with α-GS1, KOeng, and *Camptotheca acuminata* cytochrome P450 reductase (CaCPR) reduces the titer of rotundol (FIG. 7A) and increases rotundone titer (FIG. 7B). Rotundone derived from oxidation of α-guaiene by cytochrome P450 was confirmed by GC/MS (FIG. 8A and 8B). The KOeng can be further engineered to improve specificity for the α-guaiene substrate. An alignment with wild-type kaurene oxidase enzymes is shown in Example 10, which can assist this engineering.

Figure 9 shows *in vivo* production of rotundol and rotundone using an alternative CYP450 system, based on expression of *Helianthus annuus* germacrene A monooxygenase (HaGAO). The *E. coli* strain included expression of α-GS1, engineered HaGAO for expression in *E. coli* (SEQ ID NO:52), and AaCPR (*Artemisia annua* cytochrome P450 reductase; SEQ ID NO: 33). The fermentation was conducted in 96 well plates for 48 hours. As shown in FIG. 9, the oxygenated product was substantially rotundone, with only minor amounts of the rotundol intermediate.

SEQUENCES

Terpene Synthase

SEQ ID NO:1

Vitis vinifera VvGuaS

MSVPLSVSVTPILSQRIDPEVARHEATYHPNFWGDRFLHYNPDDDFCGTHACKEQQIQ
ELKEEVRKSLEATAGNTSQLLKLIDSIQRLGLAYHFEREIEEALKAMYQTYTLVDDND
HLTTVSLLFRLLRQEGYHIPSDVFKKFMDEGGNFKESLVGDLPGMLALYEAAHLMVHG
EDILDEALGFTTAHLQSMAIDSDNPLTKQVIRALKRPIRKGLPRVEARHYITIYQEDD
SHNESLLKLAKLDYNMLQSLHRKELSEITKWWKGLDFATKLPFARDRIVEGYFWILGV
YFEPQYYLARRILMKVFGVLSIVDDIYDAYGTFEELKLFTEAIERWDASSIDQLPDYM
KVCYQALLDVYEEMEEEMTKQGKLYRVHYAQAALKRQVQAYLLEAKWLKQEYIPTMEE
YMSNALVTSACSMLTTTSFVGMGDMVTKEAFDWVFSDPKMIRASNVICRLMDDIVSHE
FEQKRGHVASAVECYMKQYGVSKEEAYDEFKKQVESAWKDNNEEVLQPTAVPVPLLTR
VLNFSRMVDVLYKDEDEYTLVGPLMKDLVAGMLIDPVPM

SEQ ID NO:2

Pogostemon cablin PcPS (Q49SP3)

MELYAQSVGVGAASRPLANFHPCVWGDKFIVYNPQSCQAGEREEAEELKVELKRELKE
ASDNYMRQLKMVDAIQRLGIDYLFVEDVDEALKNLFEMFDAFCKNNHDMHATALSFRL
LRQHGYRVSCEVFEKFKDGKDGFKVPNEDGAVAVLEFFEATHLRVHGEDVLDNAFDFT
RNYLESVYATLNDPTAKQVHNALNEFSFRRGLPRVEARKYISIYEQYASHHKGLLKLA
KLDFNLVQALHRRELSEDSRWWKTLQVPTKLSFVRDRLVESYFWASGSYFEPNYSVAR
MILAKGLAVLSLMDDVYDAYGTFEELQMFTDAIERWDASCLDKLPDYMKIVYKALLDV
FEEVDEELIKLGAPYRAYYGKEAMKYAARAYMEEAQWREQKHKPTTKEYMKLATKTCG
YITLIILSCLGVEEGIVTKEAFDWVFSRPPFIEATLIIARLVNDITGHEFEKKREHVR
TAVECYMEEHKVGKQEVVSEFYNQMESAWKDINEGFLRPVEFPIPLLYLILNSVRTLE
VIYKEGDSYTHVGPAMQNIIKQLYLHPVPY

SEQ ID NO:3

Aquilaria crassna AcC2 (D0VMR6)

MSSAKLGSASEDVNRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQRELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVEASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREFGVSEEQACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:4

Aquilaria crassna AcC3 (D0VMR7)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSIPSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKRL
EALLPKLSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEQTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IVGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYIGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREFGVSEEQACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:5

Aquilaria crassna AcC4 (D0VMR8)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSDFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDDIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL

EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQRELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYIGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYDVSEEEACKKMREMIEIEWKRLNKTTLEADEVSSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:6

Aquilaria crassna AcC1mut1-M42

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDAWTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:7

Aquilaria crassna AcC1mut2-M50

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC

YMREFGVSEEQACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:8

Aquilaria crassna AcDGuaS3 (F6LJD3)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQRELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREFGVSEEQACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:9

Aquilaria crassna AcDGuaS4 (F6LJD4)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKRL
EALLPKLSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEQTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYIGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYDVSEEEACKKMREMIEIEWKRLNKTTLEADEVSSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:10

Aquilaria crassna AcDGuaS2 (F6LJD2)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETIDLPSKIQLTDEIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHLNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:11

Aquilaria crassna AcDGuaS5 (F6LJD5)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKRL
EALLPKLSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEQTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYIGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKQTLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYDVSEEEACKKMREMIEIEWKRLNKTTLEADEVSSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:12

Aquilaria spp. AmiDGuaS1 (A0A0U3ACM2)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVRNLL
VVETSDLPSKIQLTDKIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR

IIGITSLIDDTYDVYGTLEEVTLFTKAVERWDIEAVQDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEKRGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKKLNKTTLEANEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:13

Aquilaria spp. AmiDGuaS2 (A0A0U3A773)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVTNLL
VVETSDLPSKIQLTDEIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKGRIAALLRHAIEI

SEQ ID NO:14

Aquilaria spp. AmiDGuaS3 (A0A023J8Z5

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNHSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDKIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:15

Aquilaria spp. AmaDGuaS1 (A0A1B0U478)

MSSAKLGSAPEDVSRRDANYHPTVWGDFFLTHSSNFLENNHSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDKIIRLGVGYHFEMEIKAQLEKLQDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYIPQYDAELTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIATLLRHAIEI

SEQ ID NO:16

Aquilaria spp. AmaDGuaS2 (A0A0U2YQ77)

MSSAKLGSASEDVSRRDADYHPTVWGDFFLTHSSNFLENNHSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDKIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTSWCLYEATHLRVDGEDILEEAIQFSRKKL
EALLPELSFPLNECVRDALHIPYHRNVQRLAARQYISQYDAELTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRAEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIA

SEQ ID NO:17

Aquilaria spp. AsDGuaS1 (K9MQ67)

MSSAKLGSTSEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFKTEDARTLWCLYEATHLRVDGEDVLEEAIQFSRKKL
EALLPELSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDSIAEGYYWMMGAHFEPKFSLSRKFLNR

IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEKRGETVSAVRC
YMREYGVSEEEACKKMKEMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:18

Aquilaria spp. AsDGuaS2 (K9MNV6)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFKTEDARTLWCLYEATHLRVDGEDVLEEAIQFSRKKL
EALLPELSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEKRGETVSAVRC
YMREYGVSEEEACKKMKEMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:19

Aquilaria spp. AsDGuaS3 (K9MPP8)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDSILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFETEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFKTEDARTLWCLYEATHLRVDGEDVLEEAIQFSRKKL
EALLPELSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEPTKIESLSLFAKIDFN
MLQALHQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREYGVSEEEACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:20

Aquilaria spp. AsDGuaS4 (M9SVT6)

MSSAKLGSASEDVSRRDANYHPTVWGDFFLTHSSNFLENNDNILEKHEELKQEVRNLL
VVETSDLPSKIQLTDEIIRLGVGYHFEMEIKAQLEKLHDHQLHLNFDLLTTSVWFRLL
RGHGFSISSDVFKRFKNTKGEFETEDARTLWCLYEATHLRVDGEDILEEAIQFSRKRL
EALLPKLSFPLSECVRDALHIPYHRNVQRLAARQYIPQYDAEQTKIESLSLFAKIDFN
MLQALRQSELREASRWWKEFDFPSKLPYARDRIAEGYYWMMGAHFEPKFSLSRKFLNR
IIGITSLIDDTYDVYGTLEEVTLFTEAVERWDIEAVKDIPKYMQVIYTGMLGIFEDFK
DNLINARGKDYCIDYAIEVFKEIVRSYQREAEYFHTGYVPSYDEYMENSIISGGYKMF
IILMLIGRGEFELKETLDWASTIPEMVKASSLIARYIDDLQTYKAEEERGETVSAVRC
YMREFGVSEEQACKKMREMIEIEWKRLNKTTLEADEISSSVVIPSLNFTRVLEVMYDK
GDGYSDSQGVTKDRIAALLRHAIEI

SEQ ID NO:21

V. vinifera germacrene D synthase (VvGDS)

MSVPLSVSVTPILSQRIDPEVARHEATYHPNFWGDRFLHYNPDDDFCGTHACKEQQIQ
ELKEEVRKSLEATAGNTSQLLKLIDSIQRLGLAYHFEREIEEALKAMYQTYTLVDDND
HLTTVSLLFRLLRQEGYHIPSDVFKKFMDEGGNFKESLVGDLPGMLALYEAAHLMVHG
EDILDEALGFTTAHLQSMAIDSDNPLTKQVIRALKRPIRKGLPRVEARHYITIYQEDD
SHNESLLKLAKLDYNMLQSLHRKELSEITKWWKGLDFATKLPFARDRIVEGYFWILGV
YFEPQYYLARRILMKVFGVLSIVDDIYDAYGTFEELKLFTEAIERWDASSIDQLPDYM
KVCYQALLDVYEEMEEEMTKQGKLYRVHYAQAALKRQVQAYLLEAKWLKQEYIPRMDE
YMSNALVSSACSMLTTTSFVGMGDIVTKEAFDWVFSDPKMIRASNVICRLMDDIVSHE
FEQKRGHVASAVECYMKQYGVSKEEAYDEFKKQVESAWKDNNEEFLQPTAVPVPLLTR
VLNFSRMMDVLYKDEDEYTLVGPLMKDLVAGMLIDPVPM

α-Guaiene Oxidase

SEQ ID NO:22

V. vinifera VvSTO2 (F6I534; Engineered CYP71BE5 α-guaiene 2-oxidase)

MELQFSFFPILCTFLLFIYLLKRLGKPSRTNHPAPKLPPGPWKLPIIGNMHQLVGSLP
HRSLRSLAKKHGPLMHLQLGEVSAIVVSSREMAKEVMKTHDIIFSQRPCILAASIVSY
DCTDIAFAPYGGYWRQIRKISVLELLSAKRVQSFRSVREEEVLNLVRSVSLQEGVLIN
LTKSIFSLTFSIISRTAFGKKCKDQEAFSVTLDKFADSAGGFTIADVFPSIKLLHVVS
GMRRKLEKVHKKLDRILGNIINEHKARSAAKETCEAEVDDDLVDVLLKVQKQGDLEFP
LTMDNIKAVLLDLFVAGTETSSTAVEWAMAEMLKNPRVMAKAQAEVRDIFSRKGNADE
TVVRELKFLKLVIKETLRLHPPVPLLIPRESRERCAINGYEIPVKTRVIINAWAIARD
PKYWTDAESFNPERFLDSSIDYQGTNFEYIPFGAGRRMCPGILFGMANVELALAQLLY
HFDWKLPNGARHEELDMTEGFRTSTKRKQDLYLIPITYRPLPVE

SEQ ID NO:23

B. subtilis BsAGOX1 (O31440; cypC CYP152A1)

MNEQIPHDKSLDNSLTLLKEGYLFIKNRTERYNSDLFQARLLGKNFICMTGAEAAKVF
YDTDRFQRQNALPKRVQKSLFGVNAIQGMDGSAHIHRKMLFLSLMTPPHQKRLAELMT
EEWKAAVTRWEKADEVVLFEEAKEILCRVACYWAGVPLKETEVKERADDFIDMVDAFG
AVGPRHWKGRRARPRAEEWIEVMIEDARAGLLKTTSGTALHEMAFHTQEDGSQLDSRM
AAIELINVLRPIVAISYFLVFSALALHEHPKYKEWLRSGNSREREMFVQEVRRYYPFG
PFLGALVKKDFVWNNCEFKKGTSVLLDLYGTNHDPRLWDHPDEFRPERFAEREENLFD
MIPQGGGHAEKGHRCPGEGITIEVMKASLDFLVHQIEYDVPEQSLHYSLARMPSLPES
GFVMSGIRRKS

SEQ ID NO:24

B. subtilis BsAGOX1 (A5HNX5; pksS CYP107K1)

MQMEKLMFHPHGKEFHHNPFSVLGRFREEEPIHRFELKRFGATYPAWLITRYDDCMAF
LKDNRITRDVKNVMNQEQIKMLNVSEDIDFVSDHMLAKDTPDHTRLRSLVHQAFTPRT
IENLRGSIEQIAEQLLDEMEKENKADIMKSFASPLPFIVISELMGIPKEDRSQFQIWT
NAMVDTSEGNRELTNQALREFKDYIAKLIHDRRIKPKDDLISKLVHAEENGSKLSEKE
LYSMLFLLVVAGLETTVNLLGSGTLALLQHKKECEKLKQQPEMIATAVEELLRYTSPV
VMMANRWAIEDFTYKGHSIKRGDMIFIGIGSANRDPNFFENPEILNINRSPNRHISFG
FGIHFCLGAPLARLEGHIAFKALLKRFPDIELAVAPDDIQWRKNVFLRGLESLPVSLS
K

SEQ ID NO:25

B. cereus BcAGOX1 (Q737I9; BCE_2659 CYP106)

MASPENVILVHEISKLKTKEELWNPYEWYQFMRDNHPVHYDEEQDVWNVFLYEDVNRV
LSDYRLFSSRRERRQFSIPPLETRININSTDPPEHRNVRSIVSKAFTPRSLEQWKPRI
QAIANELVQHIGKYSEVNIVEEFAAPLPVTVISDLLGVPTTDRKKIKAWSDILFMPYS
KEKFNDLDVEKGIALNEFKAYLLPIVQEKRYHLTDDIISDLIRAEYEGERLTDEEIVT
FSLGLLAAGNETTTNLIINSFYCFLVDSPGTYKELREEPTLISKAIEEVLRYRFPITL
ARRITEDTNIFGPLMKKDQMVVAWVSAANLDEKKFSQASKFNIHRIGNEKHLTFGKGP
HFCLGAPLARLEAEIALTTFINAFEKIALSPSFNLEQCILENEQTLKFLPICLKTQ

SEQ ID NO:26

B. cereus BcAGOX2 (Q737F3; cypA BCE_2696 CYP107)

MKKLTFNDLNSPETMRNPIMFYKNLMEQKERFFHIDDFYGMGGAWVVFHYDDVVAILK
DSRFIKDLRKFTPPHYKQNPIEENTAVSKLFEWLMNMPNMLTVDPPDHTRLRRLVSKS
FTPRMIEDLRPRIQQIADELLDVVQEQRKMEIIADFAYPLPIIVISEMLGIPATDRNQ
FRAWTQELMKASVDPGQGTTVTATLEKFINYIEILFNEKHLNPSDDLISALVQAKEQE
DKLSKNELLSTIWLLIIAGHETTVNLISNGVLALLQHPEQMNLLRQDPSLLASAVDEL
LRYAGPIMFSSRFASEDVTIHGNRIRKGELVLLSLTAANIDPNIFPYPEELNISREEN
NHLAFGAGIHQCLGAPLARLEGQIALDTLLKRLPNLRLAIEADQLIYNHSKIRSLASL
PVIF

SEQ ID NO:45

Nicotiana tomentosiformis NtKO

MDAILNLQTVPLGTALTIGGPAVALGGISLWFLKEYVNDQKRKSSNFLPPLPEVPGLP
VIGNLLQLTEKKPHKTFTNWAETYGPIYSIKTGANTIVVLNTNELAKEAMVTRYSAIS
TRKLTNALKILTCDKSIVAISDYDEFHKTVKRHVLTSVLGPNAQKRHRIHRDTLIENV
SKQLHDLVRKYPNEAVNLRKIFQSELFGLALKQALGKDIESIYVEGLDATLPREDVLK
TLVLDIMEGAIDVDWRDFFPYLKWVPNKSFENRIQRKHLRREAVMKALIMEQRKRINS
GEKLNSYIDYLSSEANTLTEKQILMLLWEAIIETSDTTVVSTEWAMYELAKDPKRQEQ
LFLEIQNVCGSNKITEEKLCQLPYLCAVFHETLRKHSPVPIVPLRYVHEDTQLGGYHI
PKGAEIAINIYGCNRDKKVWESPEEWKPERFLDGKYDPVELQKTMAFGGGKRVCAGAL
QAMTITCTTIARLIQEFEWSLKDGEEENVATMGLTTHKLHPMQAHIKPRK

SEQ ID NO:46

Lactuca sativa LsKO

MDGVIDMQTIPLRTAIAIGGTAVALVVALYFWFLRSYASPSHHSNHLPPVPEVPGVPV
LGNLLQLKEKKPYMTFTKWAEMYGPIYSIRTGATSMVVVSSNEIAKEVVVTRFPSIST
RKLSYALKVLTEDKSMVAMSDYHDYHKTVKRHILTAVLGPNAQKKFRAHRDTMMENVS
NELHAFFEKNPNQEVNLRKIFQSQLFGLAMKQALGKDVESIYVKDLETTMKREEIFEV
LVVDPMMGAIEVDWRDFFPYLKWVPNKSFENIIHRMYTRREAVMKALIQEHKKRIASG
ENLNSYIDYLLSEAQTLTDKQLLMSLWEPIIESSDTTMVTTEWAMYELAKNPNMQDRL
YEEIQSVCGSEKITEENLSQLPYLYAVFQETLRKHCPVPIMPLRYVHENTVLGGYHVP
AGTEVAINIYGCNMDKKVWENPEEWNPERFLSEKESMDLYKTMAFGGGKRVCAGSLQA
MVISCIGIGRLVQDFEWKLKDDAEEDVNTLGLTTQKLHPLLALINPRK

SEQ ID NO:47

Cynara cardunculus var. scolymus CcKO

MDMQSIPAIAIGSTAVAIALGLFFWFFRRHVPDHIDHPNHLPSVPEVPGIPVLGNLLQ
LKEKKPYMTFTKWAETYGPIYSIRTGAISMVVVSSNAIAKEALVTRFPSISTRKLSKA
LEVLTADKTMVAMSDYNDYHKTVKRHILTAVLGPNAQKKHRVHRDIMMQNLSNQLHTF
VQNSPQEEVNLRKVFQSELFGLAMRQTMGKDVESIYVEDLGTTMNRDEIFQVLVVDPL
MGAIEVDWRDFFPYLKWIPNRNFENTIQQMYIRREAVMKALIQEHRKRIASGENLNSY
IDYLLSEAQTLSEKQLXMSLWEPIIESSDTTMVTTEWAMYELAKNPKIQDRLYREIQG
VCGSDKIXEENLGQLPYLSAIFNETLRRHGPVPIIPLRYVHEDTELGGYHIPAGTQIA
VNIYGCNMEKAVWENPEEWNPERFFEVEGDQKTMAFGGGKRVCAGSLQAMLIACIGIG
RMVQEFEWKLKDEAAQEDVNTLGLTTQKLRPLHAIIYPRKENDAKVWKC

SEQ ID NO:49

Artemisia annua AaKO

MDALTDMLQIPPATPITVAITTVTIAVAIFLYIKSHASNHSRRSTHLPPVPEVPGVPV
LGNLLQLKEKKPYLTFTRWAQTYGAIYSIRTGATSMVVVSSSEIAKEAMVTRFSSIST
RNLSKALTILTADKTMVAMSDYNDYHRTVKRHILTAMLGPNAQRKQRVHRDFMIENIS
KQLHAFVENSPKEEVDLRKIFQSELFGLAMKQAVGKDVESLNVEDLGVTMKRDEIFQV
LVVDPMMGAIEVDWRDFFPYLKWVPNKKFENTIQQMYIRRKAVMKALIKEHKKRIASG

ENLNSYIDYLLSEAQTFTDEQLIMSLWEPIIESSDTTMVTTEWAMYELAKNPKMQDRL
YRDIQSVCGSDKITEENLSQLPYLSAIFHETLRRHSPVPIIPLRHVHEDTVLGGYHVP
AGTELAVNIYGCNMEKNVWENPEEYNPDRFMKENETIDMQRTMAFGGGKRVCAGSLQA
MLISCIGIGRMVQEFEWRFKDKAEEDINTLGLTTQRLNPLRAIIKPRN

SEQ ID NO:50

Helianthus annuus HaKO

MDALTGMLPIPPATALAIGGTAIALAVAISFWFLRSYTSGESNRLPRVPEVPGVPVLG
NLLQLKEKKPYMTFTRWAETYGPIYSIRTGATSMVVVSSNEIAKEAFVTRFESISTRN
LSKALKILTDDKTMVAMSDYNDYHKTVKRHILTAMLGPNAQKKHRIQRDIMMENLSNR
LHAFVKTSTEQEEVDLREIFQSELFGLAMRQTMGKDVESIYVEDLKITMKRDEIFQVL
VVDPMMGAIDVDWRDFFPYLKWVPNKKFENTIQQMYIRREAVMKALIKQHKERIASGE
KLNSYIDYLLSEAQSLTDRQLLMSVWEPIIESSDTTMVTTEWAIYELAKNPHIQDRLY
RDIQSVCGSDIIKEEHLSQLPFITAIFHETLRRHSPVPIIPLRYVHEDTVLGGYHVPA
GTELAINIYGCNMEKSVWENPEEWNPERFMKENETIDFQKTMAFGGGKRVCAGSLQAM
LISCVGIGRMVQEFKWELKNKAQEEVNTIGLTTQMLRPLRAIIKPRN

SEQ ID NO:51

Engineered Kaurene Oxidase (KOeng)

MAWEYALIGLVVGIIIGAVAMRWYLKSYTSARRSQSNHLPRVPEVPGVPLLGNLLQLK
EKKPYMTFTKWAATYGPIYSIKTGATSVVVVSSNEIAKEALVTRFQSISTRNLSKALK
VLTADKQMVAMSDYDDYHKTVKRHILTAVLGPNAQKKHRIHRDIMMDNISTQLHEFVK
NNPEQEEVDLRKIFQSELFGLAMRQALGKDVESLYVEDLKITMNRDEILQVLVVDPMM
GAIDVDWRDFFPYLKWVPNKKFENTIQQMYIRREAVMKSLIKEQKKRIASGEKLNSYI
DYLLSEAQTLTDQQLLMSLWEPIIESSDTTMVTTEWAMYELAKNPKLQDRLYRDIKSV
CGSEKITEEHLSQLPYITAIFHETLRKHSPVPILPLRHVHEDTVLGGYHVPAGTELAV
NIYGCNMDKNVWENPEEWNPERFMKENETIDFQKTMAFGGGKRVCAGSLQALLIASIG
IGRMVQEFEWKLKDMTQEEVNTIGLTNQMLRPLRAIIKPRI

SEQ ID NO:52

Helianthus annuus germacrene A monooxygenase; Engineered HaGAO

MAKPPLFFIVIIGLIVVAASFLYKLLTRPTSSKNRLPEPWRLPIIGHMHHLIGTMPHR
GVMDLARKYGSLMHLQLGEVSAIVVSSPKWAKEILTTYDIPFANRPETLTGEIIAYHN
TDIVLAPYGEYWRQLRKLCTLELLSVKKVKSFQSLREEECWNLVQEIKASGSGTPFNL
SEGIFKVIATVLSRAAFGKGIKDQKQFTEIVKEILRETGGFDVADIFPSKKFLHHLSG
KRGRLTSIHNKLDSLINNLVAEHTVSKSSKVNETLLDVLLRLKNSEEFPLTADNVKAI
ILDMFGAGTDTSSATVEWAISELIRCPRAMEKVQAELRQALNGKERIKEEEIQDLPYL
NLVIRETLRLHPPLPLVMPRECRQAMNLAGYDVANKTKLIVNVFAINRDPEYWKDAES
FNPERFENSNTTIMGADYEYLPFGAGRRMCPGSALGLANVQLPLANILYYFKWKLPNG
ASHDQLDMTESFGATVQRKTELMLVPSF

SEQ ID NO:54

Alpha-humulene 10-hydroxylase; Engineered CYP71BA1

MAQDLRLILIIVGAIAIIALLVHGFLLIKRSSRSSVHKQQVLLASLPPSPPRLPLIGN
IHQLVGGNPHRILLQLARTHGPLICLRLGQVDQVVASSVEAVEEIIKRHDLKFADRPR
DLTFSRIFFYDGNAVVMTPYGGEWKQMRKIYAMELLNSRRVKSFAAIREDVARKLTGE
IAHKAFAQTPVINLSEMVMSMINAIVIRVAFGDKCKQQAYFLHLVKEAMSYVSSFSVA
DMYPSLKFLDTLTGLKSKLEGVHGKLDKVFDEIIAQRQAALAAEQAEEDLIIDVLLKL
KDEGNQEFPITYTSVKAIVMEIFLAGTETSSSVIDWVMSELIKNPKAMEKVQKEMREA
MQGKTKLEESDIPKFSYLNLVIKETLRLHPPGPLLFPRECRETCEVMGYRVPAGARLL
INAFALSRDEKYWGSDAESFKPERFEGISVDFKGSNFEFMPFGAGRRICPGMTFGISS
VEVALAHLLFHFDWQLPQGMKIEDLDMMEVSGMSATRRSPLLVLAKLIIPLP

SEQ ID NO:55

Ent-isokaurene C2-hydroxylase; Engineered CYP71Z18

MAQDLRLILIIVGAIAIIALLVHGFLKSAVTKPKLNLPPGPWTLPLIGSIHHIVSNPL
PYRAMRELAHKHGPLMMLWLGEVPTLVVSSPEAAQAITKTHDVSFADRHINSTVDILT
FNGMDMVFGSYGEQWRQLRKLSVLELLSAARVQSFQRIREEEVARFMRSLAASASAGA
TVDLSKMISSFINDTFVRESIGSRCKYQDEYLAALDTAIRVAAELSVGNIFPSSRVLQ
SLSTARRKAIASRDEMARILGQIIRETKESMDQGDKTSNESMISVLLRLQKDAGLPIE
LTDNVVMALMFDLFGAGSDTSSTTLTWCMTELVRYPATMAKAQAEVREAFKGKTTITE
DDLSTANLRYLKLVVKEALRLHCPVPLLLPRKCREACQVMGYDIPKGTCVFVNVWAIC

RDPRYWEDAEEFKPERFENSNLDYKGTYYEYLPFGSGRRMCPGANLGVANLELALASL
LYHFDWKLPSGQEPKDVDVWEAAGLVAKKNIGLVLHPVSHIAPVNA

SEQ ID NO:56

Premnaspirodiene oxygenase; Engineered
CYP71D55_V482I/A484I

MAQDLRLILIIVGAIAIIALLVHGFFLLRKWKNSNSQSKKLPPGPWKLPLLGSMLHMV
GGLPHHVLRDLAKKYGPLMHLQLGEVSAVVVTSPDMAKEVLKTHDIAFASRPKLLAPE
IVCYNRSDIAFCPYGDYWRQMRKICVLEVLSAKNVRSFSSIRRDEVLRLVNFVRSSTS
EPVNFTERLFLFTSSMTCRSAFGKVFKEQETFIQLIKEVIGLAGGFDVADIFPSLKFL
HVLTGMEGKIMKAHHKVDAIVEDVINEHKKNLAMGKTNGALGGEDLIDVLLRLMNDGG
LQFPITNDNIKAIIFDMFAAGTETSSSTLVWAMVQMMRNPTILAKAQAEVREAFKGKE
TFDENDVEELKYLKLVIKETLRLHPPVPLLVPRECREETEINGYTIPVKTKVMVNVWA
LGRDPKYWDDADNFKPERFEQCSVDFIGNNFEYLPFGGGRRICPGISFGLANVYLPLA
QLLYHFDWKLPTGMEPKDLDLTELVGITIARKSDLMLVATPYQPSRE

Cytochrome P450 Reductase

SEQ ID NO:27

Stevia rebaudiana SrCPR

MAQSDSVKVSPFDLVSAAMNGKAMEKLNASESEDPTTLPALKMLVENRELLTLFTTSF
AVLIGCLVFLMWRRSSSKKLVQDPVPQVIVVKKKEKESEVDDGKKKVSIFYGTQTGTA
EGFAKALVEEAKVRYEKTSFKVIDLDDYAADDDEYEEKLKKESLAFFFLATYGDGEPT
DNAANFYKWFTEGDDKGEWLKKLQYGVFGLGNRQYEHFNKIAIVVDDKLTEMGAKRLV
PVGLGDDDQCIEDDFTAWKELVWPELDQLLRDEDDTSVTTPYTAAVLEYRVVYHDKPA
DSYAEDQTHTNGHVVHDAQHPSRSNVAFKKELHTSQSDRSCTHLEFDISHTGLSYETG
DHVGVYSENLSEVVDEALKLLGLSPDTYFSVHADKEDGTPIGGASLPPPFPPCTLRDA
LTRYADVLSSPKKVALLALAAHASDPSEADRLKFLASPAGKDEYAQWIVANQRSLLEV
MQSFPSAKPPLGVFFAAVAPRLQPRYYSISSSPKMSPNRIHVTCALVYETTPAGRIHR
GLCSTWMKNAVPLTESPDCSQASIFVRTSNFRLPVDPKVPVIMIGPGTGLAPFRGFLQ
ERLALKESGTELGSSIFFFGCRNRKVDFIYEDELNNFVETGALSELIVAFSREGTAKE
YVQHKMSQKASDIWKLLSEGAYLYVCGDAKGMAKDVHRTLHTIVQEQGSLDSSKAELY
VKNLQMSGRYLRDVW

SEQ ID NO:28

Arabidopsis thaliana AtCPR1

MATSALYASDLFKQLKSIMGTDSLSDDVVLVIATTSLALVAGFVVLLWKKTTADRSGE
LKPLMIPKSLMAKDEDDDLDLGSGKTRVSIFFGTQTGTAEGFAKALSEEIKARYEKAA
VKVIDLDDYAADDDQYEEKLKKETLAFFCVATYGDGEPTDNAARFYKWFTEENERDIK
LQQLAYGVFALGNRQYEHFNKIGIVLDEELCKKGAKRLIEVGLGDDDQSIEDDFNAWK
ESLWSELDKLLKDEDDKSVATPYTAVIPEYRVVTHDPRFTTQKSMESNVANGNTTIDI
HHPCRVDVAVQKELHTHESDRSCIHLEFDISRTGITYETGDHVGVYAENHVEIVEEAG
KLLGHSLDLVFSIHADKEDGSPLESAVPPPFPGPCTLGTGLARYADLLNPPRKSALVA
LAAYATEPSEAEKLKHLTSPDGKDEYSQWIVASQRSLLEVMAAFPSAKPPLGVFFAAI
APRLQPRYYSISSSPRLAPSRVHVTSALVYGPTPTGRIHKGVCSTWMKNAVPAEKSHE
CSGAPIFIRASNFKLPSNPSTPIVMVGPGTGLAPFRGFLQERMALKEDGEELGSSLLF
FGCRNRQMDFIYEDELNNFVDQGVISELIMAFSREGAQKEYVQHKMMEKAAQVWDLIK
EEGYLYVCGDAKGMARDVHRTLHTIVQEQEGVSSSEAEAIVKKLQTEGRYLRDVW

SEQ ID NO:29

A. thaliana AtCPR2

MASSSSSSSTSMIDLMAAIIKGEPVIVSDPANASAYESVAAELSSMLIENRQFAMIVT
TSIAVLIGCIVMLVWRRSGSGNSKRVEPLKPLVIKPREEEIDDGRKKVTIFFGTQTGT
AEGFAKALGEEAKARYEKTRFKIVDLDDYAADDDEYEEKLKKEDVAFFFLATYGDGEP
TDNAARFYKWFTEGNDRGEWLKNLKYGVFGLGNRQYEHFNKVAKVVDDILVEQGAQRL
VQVGLGDDDQCIEDDFTAWREALWPELDTILREEGDTAVATPYTAAVLEYRVSIHDSE
DAKFNDINMANGNGYTVFDAQHPYKANVAVKRELHTPESDRSCIHLEFDIAGSGLTYE
TGDHVGVLCDNLSETVDEALRLLDMSPDTYFSLHAEKEDGTPISSSLPPPFPPCNLRT
ALTRYACLLSSPKKSALVALAAHASDPTEAERLKHLASPAGKDEYSKWVVESQRSLLE
VMAEFPSAKPPLGVFFAGVAPRLQPRFYSISSSPKIAETRIHVTCALVYEKMPTGRIH
KGVCSTWMKNAVPYEKSENCSSAPIFVRQSNFKLPSDSKVPIIMIGPGTGLAPFRGFL
QERLALVESGVELGPSVLFFGCRNRRMDFIYEEELQRFVESGALAELSVAFSREGPTK
EYVQHKMMDKASDIWNMISQGAYLYVCGDAKGMARDVHRSLHTIAQEQGSMDSTKAEG
FVKNLQTSGRYLRDVW

SEQ ID NO:30

A. thaliana eATR2

MASSSSSSSTSMIDLMAAIIKGEPVIVSDPANASAYESVAAELSSMLIENRQFAMIVT
TSIAVLIGCIVMLVWRRSGSGNSKRVEPLKPLVIKPREEEIDDGRKKVTIFFGTQTGT
AEGFAKALGEEAKARYEKTRFKIVDLDDYAADDDEYEEKLKKEDVAFFFLATYGDGEP
TDNAARFYKWFTEGNDRGEWLKNLKYGVFGLGNRQYEHFNKVAKVVDDILVEQGAQRL
VQVGLGDDDQCIEDDFTAWREALWPELDTILREEGDTAVATPYTAAVLEYRVSIHDSE
DAKFNDITLANGNGYTVFDAQHPYKANVAVKRELHTPESDRSCIHLEFDIAGSGLTMK
LGDHVGVLCDNLSETVDEALRLLDMSPDTYFSLHAEKEDGTPISSSLPPPFPPCNLRT
ALTRYACLLSSPKKSALVALAAHASDPTEAERLKHLASPAGKDEYSKWVVESQRSLLE
VMAEFPSAKPPLGVFFAGVAPRLQPRFYSISSSPKIAETRIHVTCALVYEKMPTGRIH
KGVCSTWMKNAVPYEKSEKLFLGRPIFVRQSNFKLPSDSKVPIIMIGPGTGLAPFRGF
LQERLALVESGVELGPSVLFFGCRNRRMDFIYEEELQRFVESGALAELSVAFSREGPT
KEYVQHKMMDKASDIWNMISQGAYLYVCGDAKGMARDVHRSLHTIAQEQGSMDSTKAE
GFVKNLQTSGRYLRDVW

SEQ ID NO:32

S. rebaudiana SrCPR3

MAQSNSVKISPLDLVTALFSGKVLDTSNASESGESAMLPTIAMIMENRELLMILTTSV
AVLIGCVVVLVWRRSSTKKSALEPPVIVVPKRVQEEEVDDGKKKVTVFFGTQTGTAEG
FAKALVEEAKARYEKAVFKVIDLDDYAADDDEYEEKLKKESLAFFFLATYGDGEPTDN
AARFYKWFTEGDAKGEWLNKLQYGVFGLGNRQYEHFNKIAKVVDDGLVEQGAKRLVPV
GLGDDDQCIEDDFTAWKELVWPELDQLLRDEDDTTVATPYTAAVAEYRVVFHEKPDAL
SEDYSYTNGHAVHDAQHPCRSNVAVKKELHSPESDRSCTHLEFDISNTGLSYETGDHV
GVYCENLSEVVNDAERLVGLPPDTYFSIHTDSEDGSPLGGASLPPPFPPCTLRKALTC
YADVLSSPKKSALLALAAHATDPSEADRLKFLASPAGKDEYSQWIVASQRSLLEVMEA
FPSAKPSLGVFFASVAPRLQPRYYSISSSPKMAPDRIHVTCALVYEKTPAGRIHKGVC
STWMKNAVPMTESQDCSWAPIYVRTSNFRLPSDPKVPVIMIGPGTGLAPFRGFLQERL
ALKEAGTDLGLSILFFGCRNRKVDFIYENELNNFVETGALSELIVAFSREGPTKEYVQ
HKMSEKASDIWNLLSEGAYLYVCGDAKGMAKDVHRTLHTIVQEQGSLDSSKAELYVKN
LQMSGRYLRDVW

SEQ ID NO:33

Artemisia annua AaCPR

MAQSTTSVKLSPFDLMTALLNGKVSFDTSNTSDTNIPLAVFMENRELLMILTTSVAVL
IGCVVVLVWRRSSSAAKKAAESPVIVVPKKVTEDEVDDGRKKVTVFFGTQTGTAEGFA
KALVEEAKARYEKAVFKVIDLDDYAAEDDEYEEKLKKESLAFFFLATYGDGEPTDNAA
RFYKWFTEGEEKGEWLDKLQYAVFGLGNRQYEHFNKIAKVVDEKLVEQGAKRLVPVGM
GDDDQCIEDDFTAWKELVWPELDQLLRDEDDTSVATPYTAAVAEYRVVFHDKPETYDQ
DQLTNGHAVHDAQHPCRSNVAVKKELHSPLSDRSCTHLEFDISNTGLSYETGDHVGVY
VENLSEVVDEAEKLIGLPPHTYFSVHADNEDGTPLGGASLPPPFPPCTLRKALASYAD
VLSSPKKSALLALAAHATDSTEADRLKFLASPAGKDEYAQWIVASHRSLLEVMEAFPS
AKPPLGVFFASVAPRLQPRYYSISSSPRFAPNRIHVTCALVYEQTPSGRVHKGVCSTW
MKNAVPMTESQDCSWAPIYVRTSNFRLPSDPKVPVIMIGPGTGLAPFRGFLQERLAQK
EAGTELGTAILFFGCRNRKVDFIYEDELNNFVETGALSELVTAFSREGATKEYVQHKM
TQKASDIWNLLSEGAYLYVCGDAKGMAKDVHRTLHTIVQEQGSLDSSKAELYVKNLQM
AGRYLRDVW

SEQ ID NO:34

Pelargonium graveolens PgCPR

MAQSSSGSMSPFDFMTAIIKGKMEPSNASLGAAGEVTAMILDNRELVMILTTSIAVLI
GCVVVFIWRRSSSQTPTAVQPLKPLLAKETESEVDDGKQKVTIFFGTQTGTAEGFAKA
LADEAKARYDKVTFKVVDLDDYAADDEEYEEKLKKETLAFFFLATYGDGEPTDNAARF
YKWFLEGKERGEWLQNLKFGVFGLGNRQYEHFNKIAIVVDEILAEQGGKRLISVGLGD
DDQCIEDDFTAWRESLWPELDQLLRDEDDTTVSTPYTAAVLEYRVVFHDPADAPTLEK
SYSNANGHSVVDAQHPLRANVAVRRELHTPASDRSCTHLEFDISGTGIAYETGDHVGV
YCENLAETVEEALELLGLSPDTYFSVHADKEDGTPLSGSSLPPPFPPCTLRTALTLHA
DLLSSPKKSALLALAAHASDPTEADRLRHLASPAGKDEYAQWIVASQRSLLEVMAEFP
SAKPPLGVFFASVAPRLQPRYYSISSSPRIAPSRIHVTCALVYEKTPTGRVHKGVCST
WMKNSVPSEKSDECSWAPIFVRQSNFKLPADAKVPIIMIGPGTGLAPFRGFLQERLAL
KEAGTELGPSILFFGCRNSKMDYIYEDELDNFVQNGALSELVLAFSREGPTKEYVQHK
MMEKASDIWNLISQGAYLYVCGDAKGMARDVHRTLHTIAQEQGSLDSSKAESMVKNLQ
MSGRYLRDVW

SEQ ID NO:53

Camptotheca acuminata cytochrome P450 reductase; CaCPR

MAQSSSVKVSTFDLMSAILRGRSMDQTNVSFESGESPALAMLIENRELVMILTTSVAV
LIGCFVVLLWRRSSGKSGKVTEPPKPLMVKTEPEPEVDDGKKKVSIFYGTQTGTAEGF
AKALAEEAKVRYEKASFKVIDLDDYAADDEEYEEKLKKETLTFFFLATYGDGEPTDNA
ARFYKWFMEGKERGDWLKNLHYGVFGLGNRQYEHFNRIAKVVDDTIAEQGGKRLIPVG
LGDDDQCIEDDFAAWRELLWPELDQLLQDEDGTTVATPYTAAVLEYRVVFHDSPDASL
LDKSFSKSNGHAVHDAQHPCRANVAVRRELHTPASDRSCTHLEFDISGTGLVYETGDH
VGVYCENLIEVVEEAEMLLGLSPDTFFSIHTDKEDGTPLSGSSLPPPFPPCTLRRALT
QYADLLSSPKKSSLLALAAHCSDPSEADRLRHLASPSGKDEYAQWVVASQRSLLEVMA
EFPSAKPPIGAFFAGVAPRLQPRYYSISSSPRMAPSRIHVTCALVFEKTPVGRIHKGV
CSTWMKNAVPLDESRDCSWAPIFVRQSNFKLPADTKVPVLMIGPGTGLAPFRGFLQER
LALKEAGAELGPAILFFGCRNRQMDYIYEDELNNFVETGALSELIVAFSREGPKKEYV
QHKMMEKASDIWNMISQEGYIYVCGDAKGMARDVHRTLHTIVQEQGSLDSSKTESMVK
NLQMNGRYLRDVW

Alcohol Dehydrogenase

SEQ ID NO: 35

Brachypodium distachyon BdDH

MSAAAAVSSSSSPRLEGKVALVTGGASGIGEAIVRLFRQHGAKVCIADVQDEAGQQVR
DSLGDDAGTDVLFVHCDVTVEEDVSRAVDAAAEKFGTLDIMVNNAGITGDKVTDIRNL
DFAEVRKVFDINVHGMLLGMKHAARVMIPGKKGSIVSLASVASVMGGMGPHAYTASKH
AVVGLTKSVALELGKHGIRVNCVSPYAVPTALSMPHLPQGEHKGDAVRDFLAFVGGEA
NLKGVDLLPKDVAQAVLYLASDEARYISALNLVVDGGFTSVNPNLKAFED

SEQ ID NO:36

Citrus sinensis CsABA2

MSNSNSTDSSPAVQRLVGRVALITGGATGIGESTVRLFHKHGAKVCIADVQDNLGQQV
CQSLGGEPDTFFCHCDVTKEEDVCSAVDLTVEKFGTLDIMVNNAGISGAPCPDIREAD
LSEFEKVFDINVKGVFHGMKHAARIMIPQTKGTIISICSVAGAIGGLGPHAYTGSKHA
VLGLNKNVAAELGKYGIRVNCVSPYAVATGLALAHLPEEERTEDAMVGFRNFVARNAN
MQGTELTANDVANAVLFLASDEARYISGTNLMVDGGFTSVNHSLRVFR

SEQ ID NO:37

Citrus sinensis CsDH

MATPPISSLISQRLLGKVALVTGGASGIGEGIVRLFHRHGAKVCFVDVQDELGYRLQE
SLVGDKDSNIFYSHCDVTVEDDVRRAVDLTVTKFGTLDIMVNNAGISGTPSSDIRNVD
VSEFEKVFDINVKGVFMGMKYAASVMIPRKQGSIISLGSVGSVIGGIGPHHYISSKHA
VVGLTRSIAAELGQHGIRVNCVSPYAVPTNLAVAHLPEDERTEDMFTGFREFAKKNAN
LQGVELTVEDVANAVLFLASEDARYISGDNLIVDGGFTRVNHSFRVFR

SEQ ID NO:38

Citrus sinensis CsDH1

MSKPRLQGKVAIIMGAASGIGEATAKLFAEHGAFVIIADIQDELGNQVVSSIGPEKAS
YRHCDVRDEKQVEETVAYAIEKYGSLDIMYSNAGVAGPVGTILDLDMAQFDRTIATNL
AGSVMAVKYAARVMVANKIRGSIICTTSTASTVGGSGPHAYTISKHGLLGLVRSAASE
LGKHGIRVNCVSPFGVATPFSAGTINDVEGFVCKVANLKGIVLKAKHVAEAALFLASD
ESAYVSGHDLVVDGGFTAVTNVMSMLEGHG

SEQ ID NO:39

Citrus sinensis CsDH2

MSNPRMEGKVALITGAASGIGEAAVRLFAEHGAFVVAADVQDELGHQVAASVGTDQVC
YHHCDVRDEKQVEETVRYTLEKYGKLDVLFSNAGIMGPLTGILELDLTGFGNTMATNV
CGVAATIKHAARAMVDKNIRGSIICTTSVASSLGGTAPHAYTTSKHALVGLVRTACSE
LGAYGIRVNCISPFGVATPLSCTAYNLRPDEVEANSCALANLKGIVLKAKHIAEAALF
LASDESAYISGHNLAVDGGFTVVNHSSSSAT

SEQ ID NO:40

Citrus sinensis CsDH3

MTTAGSRDSPLVAQRLLGKVALVTGGATGIGESIVRLFHKHGAKVCVVDINDDLGQHL
CQTLGPTTRFIHGDVAIEDDVSRAVDFTVANFGTLDIMVNNAGMGGPPCPDIREFPIS
TFEKVFDINTKGTFIGMKHAARVMIPSKKGSIVSISSVTSAIGGAGPHAYTASKHAVL

GLTKSVAAELGQHGIRVNCVSPYAILTNLALAHLHEDERTDDARAGFRAFIGKNANLQ
GVDLVEDDVANAVLFLASDDARYISGDNLFVDGGFTCTNHSLRVFR

SEQ ID NO:41

Rhodococcus erythropolis ReCDH

MARVEGQVALITGAARGQGRSHAIKLAEEGADVILVDVPNDVVDIGYPLGTADELDQT
AKDVENLGRKAIVIHADVRDLESLTAEVDRAVSTLGRLDIVSANAGIASVPFLSHDIP
DNTWRQMIDINLTGVWHTAKVAVPHILAGERGGSIVLTSSAAGLKGYAQISHYSAAKH
GVVGLMRSLALELAPHRVRVNSLHPTQVNTPMIQNEGTYRIFSPDLENPTREDFEIAS
TTTNALPIPWVESVDVSNALLFLVSEDARYITGAAIPVDAGTTLK

SEQ ID NO:42

VoDH1

MSTASSGDVSLLSQRLVGKVALITGGATGIGESIARLFYRHGAKVCIVDIQDNPGQNL
CRELGTDDACFFHCDVSIEIDVIRAVDFVVNRFGKLDIMVNNAGIADPPCPDIRNTDL
SIFEKVFDVNVKGTFQCMKHAARVMVPQKKGSIISLTSVASVIGGAGPHAYTGSKHAV
LGLTKSVAAELGLHGIRVNCVSPYAVPTGMPLAHLPESEKTEDAMMGMRAFVGRNANL
QGIELTVDDVANSVVFLASDEARYVSGLNLMLDGGFSCVNHSLRVFR

SEQ ID NO:43

Vitis vinifera VvDH

MAATSIDNSPLPSQRLLGKVALVTGGATGIGESIVRLFLKQGAKVCIVDVQDDLGQKL
CDTLGGDPNVSFFHCDVTIEDDVCHAVDFTVTKFGTLDIMVNNAGMAGPPCSDIRNVE
VSMFEKVFDVNVKGVFLGMKHAARIMIPLKKGTIISLCSVSSAIAGVGPHAYTGSKCA
VAGLTQSVAAEMGGHGIRVNCISPYAIATGLALAHLPEDERTEDAMAGFRAFVGKNAN
LQGVELTVDDVAHAAVFLASDEARYISGLNLMLDGGFSCTNHSLRVFR

SEQ ID NO:44

Zingiber zerumbet ZzSDR

MRLEGKVALVTGGASGIGESIARLFIEHGAKICIVDVQDELGQQVSQRLGGDPHACYF
HCDVTVEDDVRRAVDFTAEKYGTIDIMVNNAGITGDKVIDIRDADFNEFKKVFDINVN

GVFLGMKHAARIMIPKMKGSIVSLASVSSVIAGAGPHGYTGAKHAVVGLTKSVAAELG
RHGIRVNCVSPYAVPTRLSMPYLPESEMQEDALRGFLTFVRSNANLKGVDLMPNDVAE
AVLYLATEESKYVSGLNLVIDGGFSIANHTLQVFE

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What is claimed is:

1. A microbial host cell for producing rotundone, the microbial cell expressing a heterologous α-guaiene synthase enzyme (αGTPS) and a heterologous α-guaiene oxidase (αGOX) enzyme.

2. The microbial cell of claim 1, further expressing a farnesyl diphosphate synthase.

3. The microbial cell of claim 2, wherein the αGTPS enzyme comprises an amino acid sequence of any one of SEQ ID NOs: 1 to 21, or variant thereof.

4. The microbial cell of claim 3, wherein α-guaiene synthase enzyme comprises an amino acid sequence having 50% or more sequence identity with any one of SEQ ID NOs: 1 to 21.

5. The microbial cell of claim 3, wherein the αGTPS enzyme comprises an amino acid sequence having 50% or more sequence identity to SEQ ID NO: 8.

6. The microbial cell of claim 5, wherein the αGTPS enzyme comprises one or more amino acid substitutions at positions selected from 72, 273, 290, 368, 371, 374, 377, 381, 382, 399, 406, 419, 433, 442, 443, 454, 512, and 522 relative to SEQ ID NO: 8.

7. The microbial cell of claim 6, wherein the αGTPS enzyme comprises one or more amino acid substitutions selected from T72I, M273L, R290K, F368M, I371L, S374A, R377V, Y381W, F382L, I399V, F406L, L419T, V433I, Y442L, I443M, E454K, F512L, and K522D relative to SEQ ID NO: 8.

8. The microbial cell of claim 4, wherein the α-guaiene synthase produces predominantly α-guaiene as the product from FPP substrate.

9. The microbial cell of any one of claims 1 to 8, wherein the αGOX enzyme is a cytochrome P450 (CYP450) enzyme.

10. The microbial cell of claim 9, wherein the CYP450 comprises the amino acid sequence of SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or a variant thereof.

11. The microbial cell of claim 10, wherein the CYP450 comprises an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NOs: 51, 52, or 22 to 26.

12. The microbial cell of claim 9, wherein the CYP450 comprises an amino acid sequence having 50% or more sequence identity to SEQ ID NO: 51.

13. The microbial cell of claim 9, wherein the CYP450 comprises an amino acid sequence having 50% or more sequence identity to SEQ ID NO: 52.

14. The microbial cell of any one of claims 1 to 13, wherein the microbial host cell expresses a cytochrome P450 reductase enzyme.

15. The microbial cell of any one of claims 1 to 14, wherein the αGTPS and αGOX are expressed together in an operon.

16. The microbial cell of any of claims 1 to 15, wherein the microbial host cell further expresses one or more alcohol dehydrogenases (ADHs).

17. The microbial cell of claim 16, wherein the ADH comprises an amino acid sequence of any one of SEQ ID NOs: 35-44, or a variant thereof.

18. The microbial cell of claim 11, wherein the ADH comprises an amino acid sequence having 50% or more sequence identity to SEQ ID NO: 43.

19. The microbial cell of any one of claims 1 to 18, wherein one or more enzymes are expressed from extrachromosomal elements.

20. The microbial cell of any one of claims 1 to 18, wherein one or more enzymes are expressed from genes that are chromosomally integrated.

21. The microbial cell of any one of claims 1 to 20, wherein the microbial host cell overexpresses one or more enzymes in the methylerythritol phosphate (MEP) or the mevalonic acid (MVA) pathway.

22. The microbial cell of any one of claims 1 to 21, wherein the microbial cell is a bacteria, optionally selected from *Escherichia spp.*, *Bacillus spp.*, *Corynebacterium spp.*, *Rhodobacter spp.*, *Zymomonas spp.*, *Vibrio spp.*, and *Pseudomonas spp.*

23. The microbial cell of claim 22, wherein the bacterial host cell is selected from *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Zymomonas mobilis*, *Vibrio natriegens*, or *Pseudomonas putida.*

24. The microbial cell of any one of claims 1 to 21, wherein the microbial host cell is a yeast, optionally selected from *Saccharomyces*, *Pichia*, or *Yarrowia.*

25. The microbial cell of claim 24, wherein the microbial cell is *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica.*

26. A method for making rotundone, comprising: culturing the microbial cell of any one of claims 1 to 25, and recovering the rotundone.

27. The method of claim 26, wherein the microbial cells are cultured with C1, C2, C3, C4, C5, and/or C6 carbon substrates.

28. The method of claim 27, wherein the carbon source is glucose, sucrose, fructose, xylose, and/or glycerol.

29. The method of any one of claims 26 to 28, wherein culture conditions are selected from aerobic, microaerobic, and anaerobic.

30. The method of claim 29, wherein the microbial cell is cultured at a temperature between 22° C and 37° C.

31. A method for producing rotundone, comprising feeding α-guaiene to a microbial cell expressing an α-guaiene oxidase (αGOX), or to an extract of the cell, or to a reaction vessel

comprising recombinant αGOX, wherein the αGOX optionally comprises the amino acid sequence of SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or a variant thereof.

32. The method of claim 31, wherein the αGOX is a CYP450 comprising an amino acid sequence that has 50% or more sequence identity with any one of SEQ ID NO: 51, SEQ ID NO: 52, or SEQ ID NOS: 22 to 26.

33. The method of claim 31, wherein the αGOX is a non-heme iron oxygenase (NHIO) or a laccase.

34. The method of any one of claims 31 to 33, wherein the microbial cell expresses one or more alcohol dehydrogenases.

35. The method of any one of claims 31 to 33, wherein the extract or reaction vessel further comprises one or more alcohol dehydrogenases.

36. The method of claim 33 or 34, wherein the alcohol dehydrogenase comprises an amino acid sequence selected from SEQ ID NOs: 35-44, or a variant thereof.

37. The method of any one of claims 31 to 36, further comprising, recovering rotundone.

FIG. 1

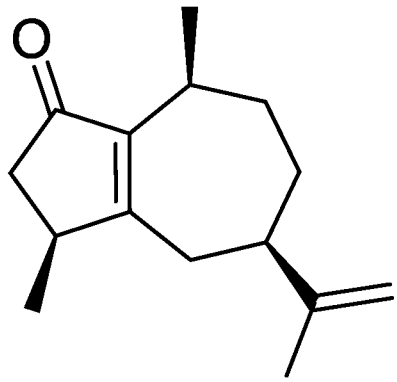


FIG. 2

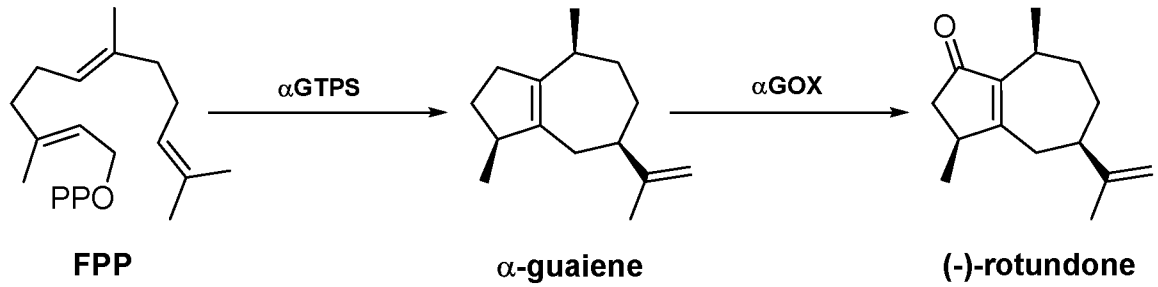


FIG. 3

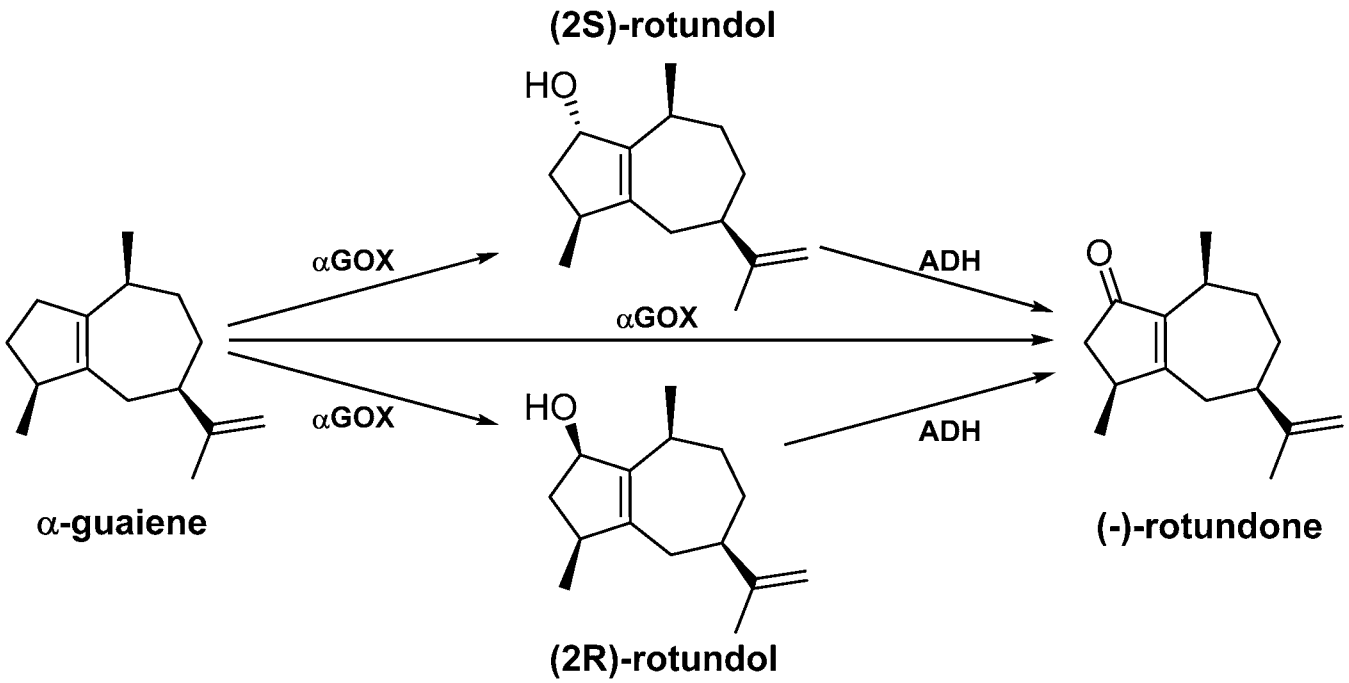

(2S)-rotundol
HO
αGOX
ADH
αGOX
O
αGOX
HO
ADH
α-guaiene
(-)-rotundone
(2R)-rotundol

FIG. 4

Mutant	Fold improvement in α-guaiene
F406L	1.71
Y442L	1.16
I371L	1.15
M273L	1.15
S374A	1.13
I399V	1.12
R377V	1.08
L419T	1.08
F382L	1.07
R290K	1.07
F512L	1.07
K522D	1.07
F368M	1.07
T72I	1.06
E454K	1.06
V433I	1.05
Y381W	1.05
I298V	1.04
L297I	1.03
G268C	1.02
I343V	1.02
V311L	1.02
L446I	1.01
Q447V	1.01
E457H	1.00
I291V	0.98
P508R	0.98
I265V	0.98
I229L	0.98
S503M	0.97
Q530G	0.97
A544P	0.95
I443M	0.94

Mutant	Fold improvement in α-guaiene
V505L	0.94
W147L	0.94
V24I	0.94
A451F	0.91
Q447K	0.89
V111L	0.89
V506L	0.88
V459A	0.88
A266V	0.87
V532K	0.87
T145G	0.86
S504P	0.86
D528H	0.86
Y270F	0.85
E517D	0.82
T448S	0.81
R414M	0.81
E453Q	0.81
S224L	0.77
S527T	0.76
G339T	0.73
T235	0.72
E431K	0.71
del_A354	0.67
L423F	0.67
M519L	0.64
S400T	0.58
R490D	0.49
S397A	0.40
T295A	0.39
Y449H	0.35
M432I	0.31

Mutant	Fold improvement in α-guaiene
G531K	0.27
G401S	0.25
Q376F	0.21
I329L	0.17
M273H	0.16
I407A	0.15
S509I	0.15
A440C	0.13
V304A	0.12
G401C	0.11
K522D	0.08
S502P	0.07
R536F	0.05
A275V	0.05
L288F	0.03
L516I	0.03
G523E	0.02
I398L	0.02
D521K	0.02
T301I	0.02
S529V	0.01
H276Y	0.01
L297V	0.01
Y375F	0.01
ins523_524_N	0.01
T533A	0.00
T533L	0.00
R463E	0.00
G339A	0.00
ins526_527_G	0.00
del_G531	0.00

FIG. 5

Mutant	Fold improvement in % α-guaiene
I443M	2.40
F406L	2.12
F512L	1.23

FIG. 6

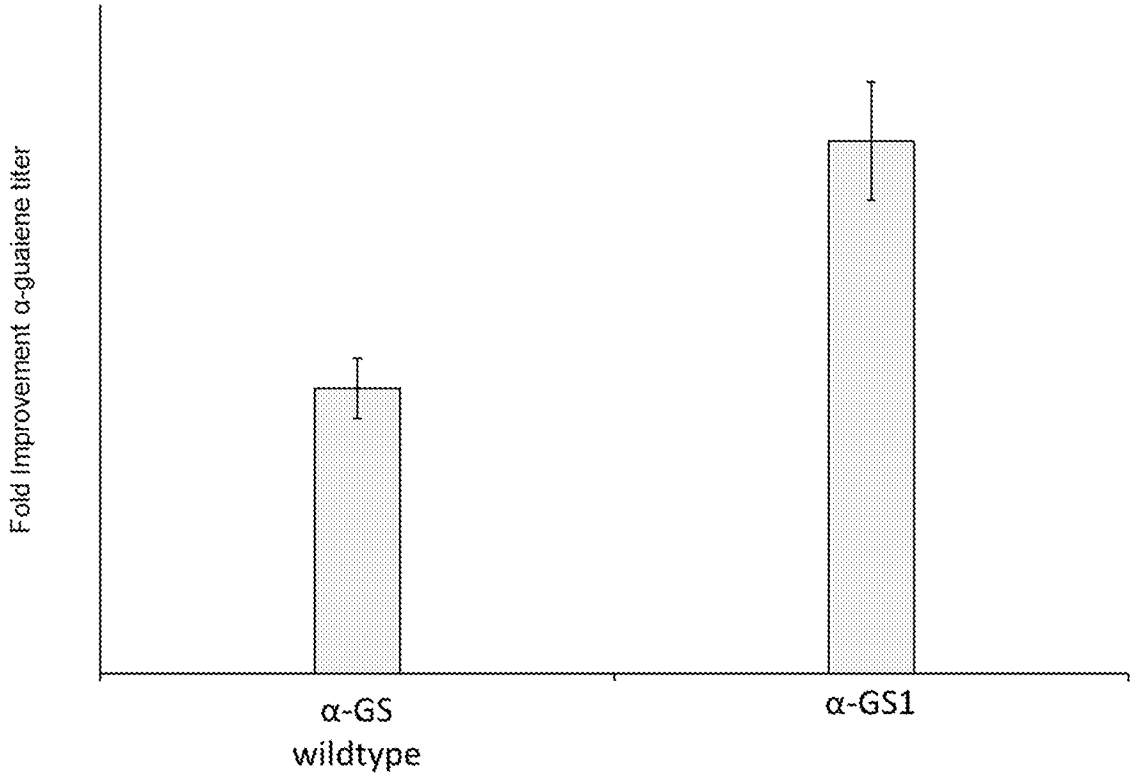


FIG. 7A

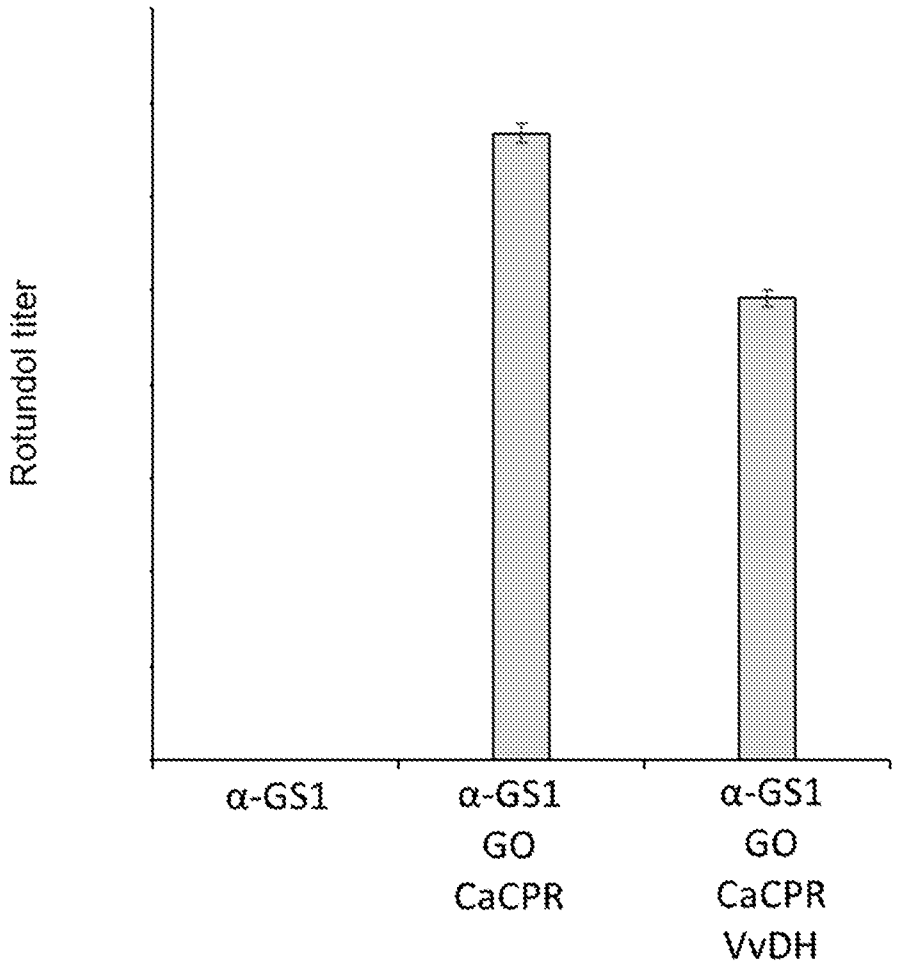


FIG. 7B

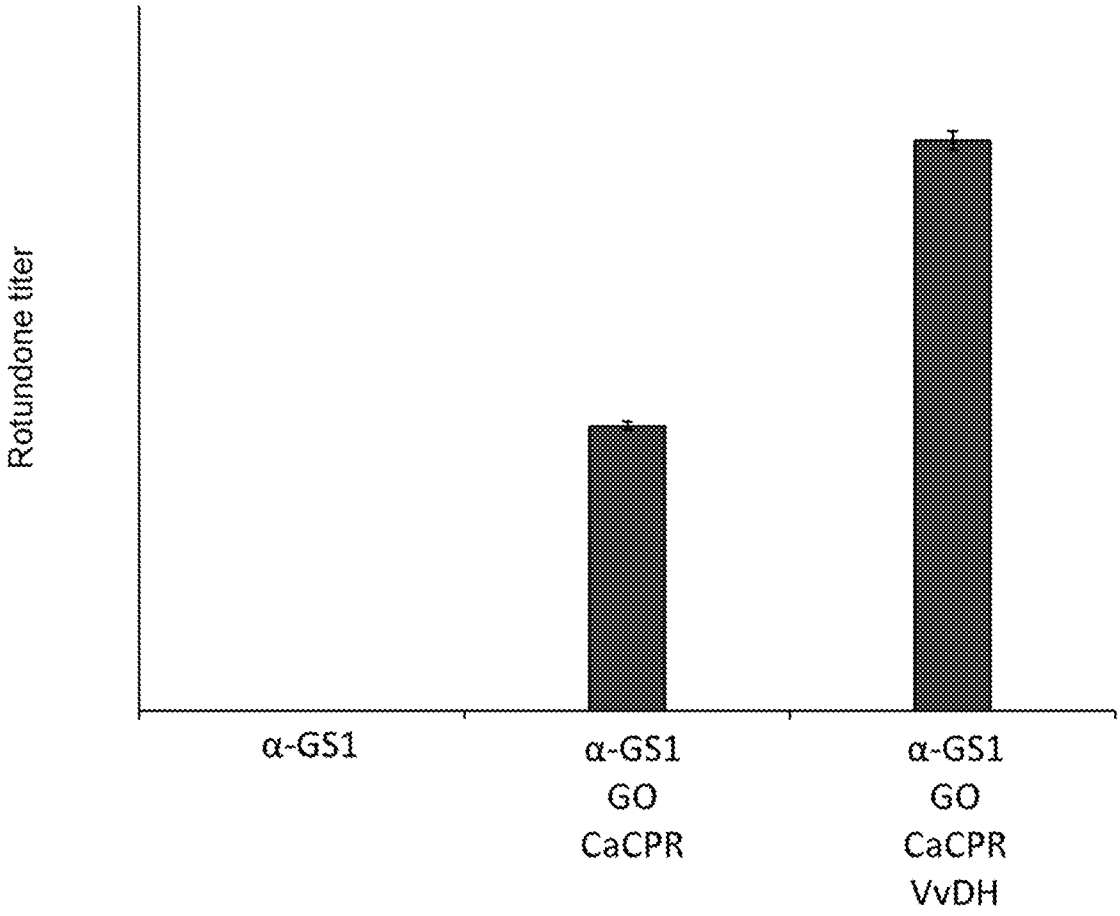


FIG. 8A

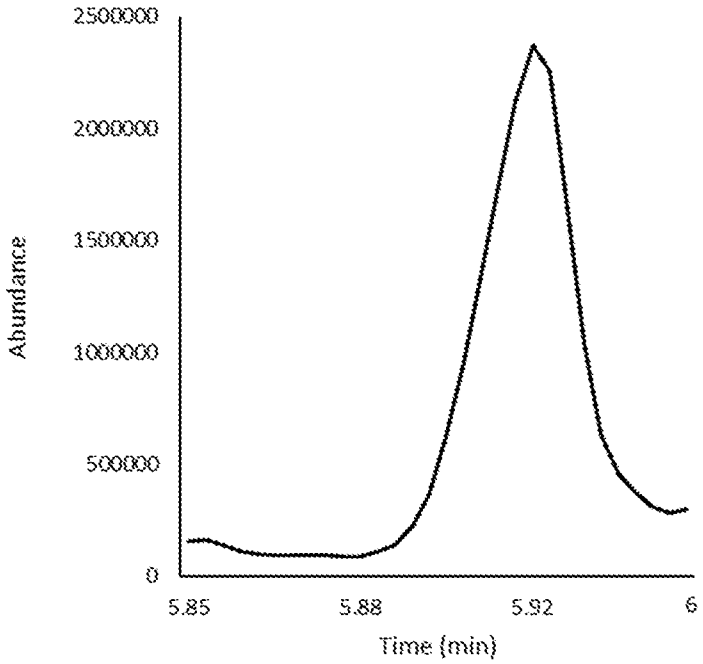

2500000
2000000
1500000
1000000
500000
0
Abundance
5.85
5.88
5.92
6
Time (min)

FIG. 8B

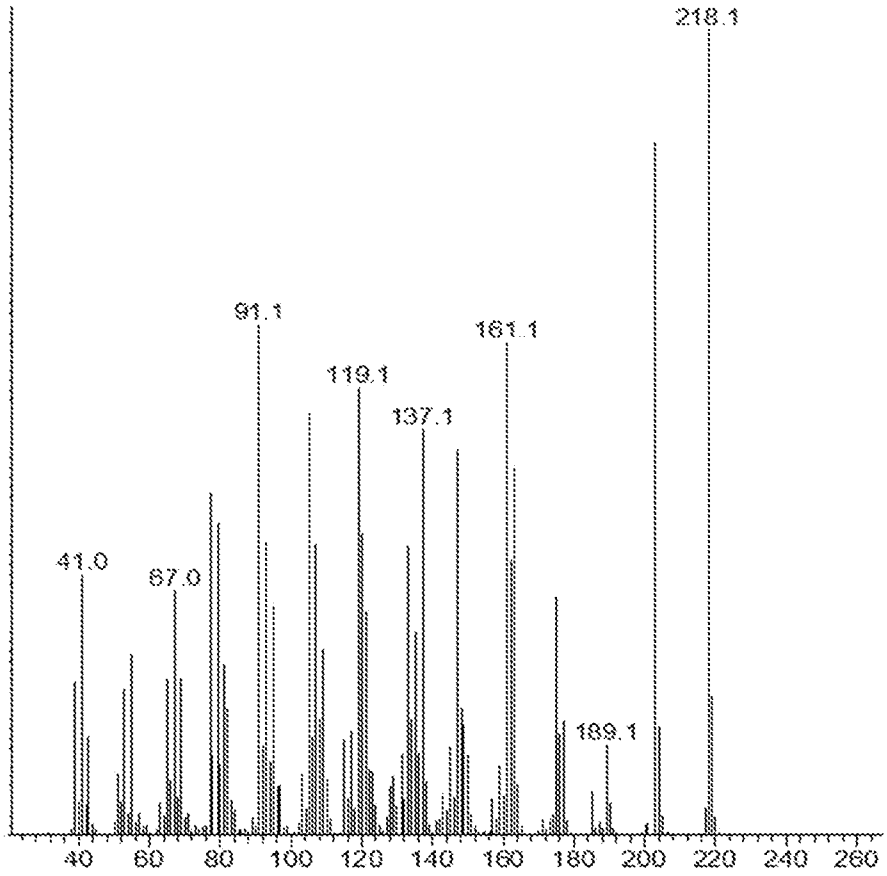

218.1
91.1
161.1
119.1
137.1
41.0
67.0
189.1
40
60
80
100
120
140
160
180
200
220
240
260

FIG. 9

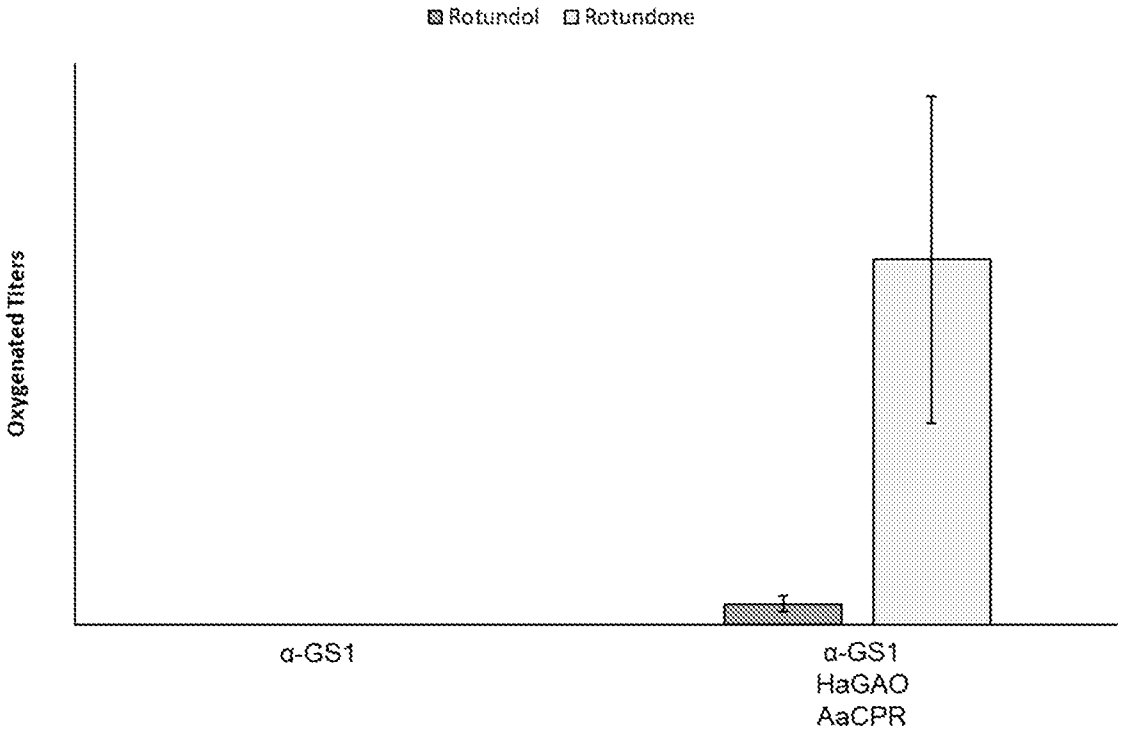

Rotundol
Rotundone
Oxygenated Titers
α-GS1
α-GS1
HaGAO
AaCPR

FIG. 10



NtKO (SEQ ID NO: 45)
LsKO (SEQ ID NO: 46)
CcKO (SEQ ID NO: 47)
KOeng (SEQ ID NO: 51)
AaKO (SEQ ID NO: 49)
HaKO (SEQ ID NO: 50)

FIG. 10 (contd.)

```
NtKO            MDAILNLQTVPLGTALTIGGPAVALG-GISLWFLKEYVNDQKRKSSNFLPPLPEVPGLPV
LsKO            MDGVIDMQTIPLRTAIAIGGTAVALVVALYFWFLRSYASP-SHH-SNHLPPVPEVPGVPV
CcKO            ----MDMQSIP---AIAIGSTAVAIALGLFFWFFRRHVPDHIDH-PNHLPSVPEVPGIPV
AaKO            MDALTDMLQIPPATPITVAITTVTIAVAI-FLYIKSHASNHSRR-STHLPPVPEVPGVPV
KOeng           --MAWEYALIGLVVGIIIGAVAMR-------WYLKSYTSARRSQ-SNHLPRVPEVPGVPL
HaKO            MDALTGMLPIPPATALAIGGTAIALAVAISFWFLRSYTSG---E-SNRLPRVPEVPGVPV
                         :     : :.  ::         ::. :.       .. ** :*****:*:
NtKO            IGNLLQLTEKKPHKTFTNWAETYGPIYSIKTGANTIVVLNTNELAKEAMVTRYSAISTRK
LsKO            LGNLLQLKEKKPYMTFTKWAEMYGPIYSIRTGATSMVVVSSNEIAKEVVVTRFPSISTRK
CcKO            LGNLLQLKEKKPYMTFTKWAETYGPIYSIRTGAISMVVVSSNAIAKEALVTRFPSISTRK
AaKO            LGNLLQLKEKKPYLTFTRWAQTYGAIYSIRTGATSMVVVSSSEIAKEAMVTRFSSISTRN
KOeng           LGNLLQLKEKKPYMTFTKWAATYGPIYSIKTGATSVVVVSSNEIAKEALVTRFQSISTRN
HaKO            LGNLLQLKEKKPYMTFTRWAETYGPIYSIRTGATSMVVVSSNEIAKEAFVTRFESISTRN
                :******.****: ***.**  **.****.*** ::**:.:. :***..***: :****:
NtKO            LTNALKILTCDKSIVAISDYDEFHKTVKRHVLTSVLGPNAQKRHRIHRDTLIENVSKQLH
LsKO            LSYALKVLTEDKSMVAMSDYHDYHKTVKRHILTAVLGPNAQKKFRAHRDTMMENVSNELH
CcKO            LSKALEVLTADKTMVAMSDYNDYHKTVKRHILTAVLGPNAQKKHRVHRDIMMQNLSNQLH
AaKO            LSKALTILTADKTMVAMSDYNDYHRTVKRHILTAMLGPNAQRKQRVHRDFMIENISKQLH
KOeng           LSKALKVLTADKQMVAMSDYDDYHKTVKRHILTAVLGPNAQKKHRIHRDIMMDNISTQLH
HaKO            LSKALKILTDDKTMVAMSDYNDYHKTVKRHILTAMLGPNAQKKHRIQRDIMMENLSNRLH
                *: ** :** ** :**:*** ::*.*****:**::******.. * :** :::*:*. **
NtKO            DLVRKYP-NEAVNLRKIFQSELFGLALKQALGKDIESIYVEGLDATLPREDVLKTLVLDI
LsKO            AFFEKNP-NQEVNLRKIFQSQLFGLAMKQALGKDVESIYVKDLETTMKREEIFEVLVVDP
CcKO            TFVQNSP-QEEVNLRKVFQSELFGLAMRQTMGKDVESIYVEDLGTTMNRDEIFQVLVVDP
AaKO            AFVENSP-KEEVDLRKIFQSELFGLAMKQAVGKDVESLNVEDLGVTMKRDEIFQVLVVDP
KOeng           EFVKNNPEQEEVDLRKIFQSELFGLAMRQALGKDVESLYVEDLKITMNRDEILQVLVVDP
HaKO            AFVKTSTEQEEVDLREIFQSELFGLAMRQTMGKDVESIYVEDLKITMKRDEIFQVLVVDP
                 :. . . :: *:**::***:*****:.*::***:**: *:.*  *: *::::::.**:*
NtKO            MEGAIDVDWRDFFPYLKWVPNKSFENRIQRKHLRREAVMKALIMEQRKRINSGEKLNSYI
LsKO            MMGAIEVDWRDFFPYLKWVPNKSFENIIHRMYTRREAVMKALIQEHKKRIASGENLNSYI
CcKO            LMGAIEVDWRDFFPYLKWIPNRNFENTIQQMYIRREAVMKALIQEHRKRIASGENLNSYI
AaKO            MMGAIEVDWRDFFPYLKWVPNKKFENTIQQMYIRRKAVMKALIKEHKKRIASGENLNSYI
KOeng           MMGAIDVDWRDFFPYLKWVPNKKFENTIQQMYIRREAVMKSLIKEQKKRIASGEKLNSYI
HaKO            MMGAIDVDWRDFFPYLKWVPNKKFENTIQQMYIRREAVMKALIKQHKERIASGEKLNSYI
                : ***:************:**..*** *:. : **:****:** ::.:** ***:*****
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/50004

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12P 5/00, 7/26; A01N 27/00; C12N 9/02 (2019.01)

CPC - C12P 5/00, 5/002, 7/26; A01N 27/00; A23L 27/13; C12N 9/0073; C12Y 114/13078

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)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	JP 2017216974 A (HASEGAWA T CO LTD) 14 December 2017; abstract; page 3, paragraph 5; page 8 paragraph 7-10	31-33 --- 1-2, 9/1-2, 10/9/1-2, 11/10/9/1-2, 12/9/1-2, 34/31-33, 35/31-33 --- 3-4, 8, 9/3-4, 9/8, 10/9/3-4, 10/9/8, 11/10/9/3-4, 11/10/9/8, 12/9/3-4, 12/9/8
Y --- A	DREW, et al. 'Two Key Polymorphisms in a Newly Discovered Allele of the Vitis vinifera TPS24 Gene are Responsible for the Production of the Rotundone Precursor α-guaiene' Journal of Experimental Botany, 2016, Vol. 67, No. 3 pp. 799–808, abstract. DOI:10.1093/jxb/erv491	1-2, 9/1-2, 10/9/1-2, 11/10/9/1-2, 12/9/1-2 --- 3-4, 8, 9/3-4, 9/8, 10/9/3-4, 10/9/8, 11/10/9/3-4, 11/10/9/8, 12/9/3-4, 12/9/8
Y	US 2018/0135081 A1 (MANUS BIOSYNTHESIS, INC.) 17 May 2018; paragraphs [0002], [0016], [0138]-[0140]	9/1-2, 10/9/1-2, 11/10/9/1-2, 12/9/1-2, 34/31-33, 35/31-33

☒ 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
"D" document cited by the applicant in the international application
"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
07 January 2020 (07.01.2020)	24 JAN 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/50004

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2015/0007368 A1 (SARAN et al.) 01 January 2015; paragraph [0021]	3-4, 8, 9/3-4, 9/8, 10/9/3-4, 10/9/8, 11/10/9/3-4, 11/10/9/8, 12/9/3-4, 12/9/8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/50004

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 14-30, 36-37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13, 31-35; SEQ ID NO: 1 (αGTPS enzyme); SEQ ID NO: 51 (CYP450 sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US19/50004

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-13, 31-35, SEQ ID NO: 1 (αGTPS enzyme), and SEQ ID NO: 51 (CYP450 sequence) are directed toward microbial host cells and methods for producing rotundone.

The host cells and methods will be searched to the extent that they encompass SEQ ID NO: 1 (first exemplary αGTPS enzyme) and SEQ ID NO: 51 (first exemplary CYP450 sequence). Applicant is invited to elect additional enzyme(s) and/or CYP450 sequence(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and available as an option within at least one searchable claim, to be searched. Additional enzyme(s) and/or CYP450 sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-2, 3-4 (each in-part), 8-12 (each in-part), and 31-35 (each in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (αGTPS enzyme) and SEQ ID NO: 51 (CYP450 sequence). Applicants must specify the searchable claims that encompass any additionally elected enzyme(s) and/or CYP450 sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (αGTPS enzyme).

No technical features are shared between the αGTPS enzymes and/or CYP450 sequences, of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a microbial host cell for producing rotundone, the microbial cell expressing a heterologous α-guaiene synthase enzyme (αGTPS) and a heterologous α-guaiene oxidase (αGOX) enzyme; a method for producing rotundone, comprising feeding α-guaiene to a microbial cell expressing an α-guaiene oxidase (αGOX), or to an extract of the cell, or to a reaction vessel comprising recombinant αGOX; these shared technical features are previously disclosed by JP 2017216974 A (HASEGAWAT CO LTD) (hereinafter 'Hasegawat') in view of the publication entitled 'Two Key Polymorphisms in a Newly Discovered Allele of the Vitis vinifera TPS24 Gene are Responsible for the Production of the Rotundone Precursor α-guaiene' by Drew, et al. (hereinafter 'Drew').

Hasegawat discloses a microbial host cell for producing rotundone (a microbial host cell for producing rotundone; abstract; page 8, paragraphs 9-10), the microbial cell expressing a heterologous α-guaiene oxidase (αGOX) enzyme (expressing cytochrome P450 (a heterologous α-guaiene oxidase (αGOX) enzyme); abstract; page 8, paragraphs 9-10); a method for producing rotundone (a method for producing rotundone; abstract), comprising feeding α-guaiene to a microbial cell expressing an α-guaiene oxidase (αGOX) (comprising culturing (feeding) α-guaiene with a microbial cell expressing an α-guaiene oxidase (αGOX); page 8 paragraph 7), or to an extract of the cell, or to a reaction vessel comprising recombinant αGOX. Hasegawat further discloses wherein the vector may further comprise a heterologous synthase gene (page 8, paragraph 13).

Hasegawat does not disclose a α-guaiene synthase enzyme (αGTPS).

Drew discloses a α-guaiene synthase enzyme (αGTPS) (VvGuaS (α-guaiene synthase enzyme); abstract).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify the disclosure of Hasegawat to include a α-guaiene synthase enzyme, as disclosed by Drew, in order to provide a more effective process for the production of rotundone.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Hasegawat and Drew references, unity of invention is lacking.