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(71) Applicant: **THE UNIVERSITY OF MANCHESTER** [GB/GB]; Oxford Road, Manchester M13 9PL (GB).

(72) Inventors: **SCRUTTON, Nigel, Shaun**; c/o The University of Manchester, Oxford Road, Manchester Greater Manchester M13 9PL (GB). **TOOGOOD, Helen, Susan**; c/o The University of Manchester, Oxford Road, Manchester Greater Manchester M13 9PL (GB). **CURRIN, Andrew**; c/o The University of Manchester, Oxford Road, Manchester Greater Manchester M13 9PL (GB).

(74) Agent: **CLEGG, Richard** et al.; Mewburn Ellis LLP, City Tower, 40 Basinghall Street, London Greater London EC2V 5DE (GB).

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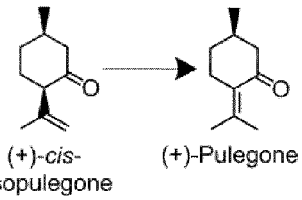

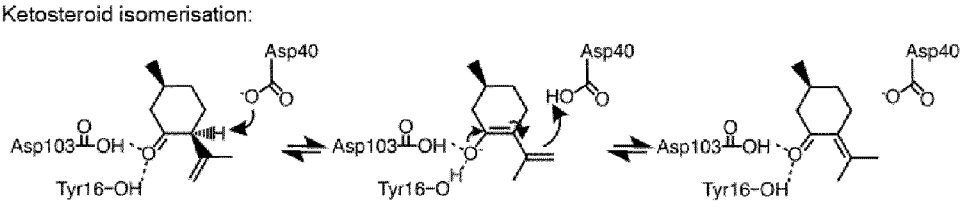

Figure 2

(57) Abstract: A method comprising catalysis of the conversion of (+)-cis-isopulegone to (R)-(+)-pulegone using a ketosteroid isomerase is disclosed. A mutated ketosteroid isomerase having (+)-cis-isopulegone to (R)-(+)-pulegone isomerase activity is also disclosed. A method comprising catalysis of the conversion of (+)-cis-isopulegone to an isopulegol isomer using a menthone reductase is also disclosed.

[Continued on next page]

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Monoterpenoid Biosynthesis

Field of the Invention

The present invention relates to the fields of molecular biology and biotechnology. More particularly, the invention relates to methods and compositions for monoterpenoid biosynthesis.

Background to the Invention

The terpenoids comprise a diverse family of over 55,000 known natural compounds, possessing diverse valuable properties (including anti-cancer, anti-microbial and anti-inflammatory effects)[1]. They are widely used commercially, from bulk commodity compounds (e.g. farnesene as a biofuel) to fine chemical pharmaceuticals (e.g. the antimalarial artemisinin), both produced by engineered microbes[2,3]. Terpenes are synthesised from 5-carbon isoprene units, the number of which defines their terpenoid class. Monoterpenoids, derived from 2 isoprene units, include limonene and limonene derivatives which have widespread commercial use as flavours, fragrances and pharmaceuticals[3,4]. Limonene isomers are the most abundant monocyclic monoterpenes in nature. D-limonene is derived from the peel of citrus fruits like oranges and lemons and has been shown to exhibit anticancer activity[5] and enhance the delivery of lipophilic agents through the skin[6]. L-limonene is abundant in herbs like *Mentha* spp., where it is the precursor for several mint fragrance compounds[7].

Of all the limonene derivatives, (1*R*,2*S*,5*R*)-(−)-menthol is one of the best known. Primarily derived from *Mentha* x *piperita* (peppermint) and *Mentha canadensis* (cornmint), it is noticeable by its characteristic cooling anaesthetic effects and aroma[8]. Additional properties include antibacterial, anticancer and anti-inflammatory activity[9–12]. Commercially, (−)-menthol is utilised both as a pure compound and in the extracted essential oil of peppermint, with an estimated 30,000 tonnes consumed annually[13]. Whilst the majority of (−)-menthol is extracted from *M. canadensis*, a proportion is generated synthetically using a variety of different precursors. For example, Takasago synthesise thousands of tonnes of (−)-menthol using a six-step process using β-pinene as the starting compound[13]. There is an increasing need for lower cost and higher yield production of (−)-menthol, but optimisation through conventional breeding methods is difficult because peppermint is a sterile hybrid plant[4,14]. Given an increasing preference for 'natural' (−)-menthol, there is much interest in production using microbes through metabolic engineering and synthetic biology approaches[14–16].

The biosynthesis of (−)-menthol from (−)-limonene involves six chemical transformations (**Figure 1B**), each of which has been partially characterised. The native enzymes for five of these reactions have been identified in *Mentha* species[4,17–23].

Croteau and Venkatachalam[22] first reported (+)-*cis*-isopulegone as the intermediate compound for synthesis of (*R*)-(+)-pulegone from (−)-isopiperitenone in *M. piperita*. However, the enzyme catalyzing the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone has long remained elusive. Isopulegone isomerase (IPGI) activity has been demonstrated in crude cell extracts of *Mentha* species[4,22], but the enzyme responsible for this activity has not been identified.

The identification of an enzyme catalyzing conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone is therefore a key obstacle to the biosynthetic production of menthol isomers from (−)-limonene.

Summary of the Invention

In a first aspect the present invention provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone using a ketosteroid isomerase.

In some embodiments the ketosteroid isomerase is ketosteroid isomerase from *Pseudomonas putida* or a fragment, variant or homologue thereof.

In some embodiments the ketosteroid isomerase comprises an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:34, 35, 36 and 37.

In some embodiments the ketosteroid isomerase comprises, or consists of, an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:1, 2, 3, 4, 5, 6, 7, 8, 9 and 38.

In some embodiments the method is a method for producing (*R*)-(+)-pulegone or a derivative thereof.

In some embodiments the method is a method for producing one or more of (*R*)-(+)-pulegone, (+)-menthofuran, (-)-menthone, (+)-isomenthone, (+)-neomenthol, (-)-menthol, (+)-isomenthol and (+)-neoisomenthol.

In some embodiments the method further comprises catalysis of the conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone using a pulegone reductase.

In some embodiments the method further comprises catalysis of the conversion of (-)-menthone to (-)-menthol and/or (+)-neomenthol using a menthone reductase.

In some embodiments the method further comprises catalysis of the conversion of (+)-isomenthone to (+)-isomenthol and/or (+)-neoisomenthol using a menthone reductase.

In some embodiments the method further comprises catalysis of the conversion of (-)-isopiperitenone to (+)-*cis*-isopulegone using a (-)-isopiperitenone reductase.

In some embodiments the method further comprises catalysis of the conversion of (-)-*trans*-isopiperitenol to (-)-isopiperitenone using an isopiperitenol dehydrogenase.

In some embodiments the method further comprises catalysis of the conversion of limonene to (-)-*trans*-isopiperitenol using a limonene hydroxylase or a cytochrome P450 reductase.

The present invention also provides a method for producing a menthol isomer, comprising:

catalysis of the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone using a ketosteroid isomerase;

catalysis of the conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone using a pulegone reductase; and

catalysis of the conversion of (-)-menthone to (-)-menthol and/or (+)-neomenthol using a menthone reductase, and/or catalysis of the conversion of (+)-isomenthone to (+)-isomenthol and/or (+)-neoisomenthol using a menthone reductase.

The present invention also provides the use of a ketosteroid isomerase to catalyse the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

The present invention also provides a composition comprising a ketosteroid isomerase and one or more of: a pulegone reductase, a menthone reductase, a (-)-isopiperitenone reductase, an isopiperitenol dehydrogenase, a limonene hydroxylase, a cytochrome P450 reductase, a limonene synthase and a geranyl diphosphate synthase.

The present invention also provides a nucleic acid, or plurality of nucleic acids, encoding a ketosteroid isomerase and one or more of: a pulegone reductase, a menthone reductase, a (-)-isopiperitenone reductase, an isopiperitenol dehydrogenase, a limonene hydroxylase, a cytochrome P450 reductase, a limonene synthase and a geranyl diphosphate synthase.

The present invention also provides a ketosteroid isomerase having (+)-*cis*-isopulegone to (*R*)-(+)-pulegone isomerase activity, and comprising an amino acid sequence comprising a substitution corresponding to one or more of the following positions relative to SEQ ID NO:1: V88, L99, Y16, Y57, D103, V20, F56, L61, V66, F86, M90, V101, M116, W120, G60 and A118.

In some embodiments the ketosteroid isomerase comprises one or more substitutions corresponding to the following amino acid substitutions relative to SEQ ID NO:1: D103S, V88I, L99I, L99V and V101A.

In some embodiments the ketosteroid isomerase comprises the amino acid substitution corresponding to D103S relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitution corresponding to V101A relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitution corresponding to L99I relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitution corresponding to L99V relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I and L99V relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitutions corresponding to L99V and D103S relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitutions corresponding to L99I and D103S relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I, L99V and D103S relative to SEQ ID NO:1.

In some embodiments the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I, L99V, V101A and D103S relative to SEQ ID NO:1.

The present invention also provides a nucleic acid encoding a ketosteroid isomerase according to the present invention.

The present invention also provides an expression vector comprising a nucleic acid, or plurality of nucleic acids according to the present invention.

The present invention also provides a cell comprising a composition, a nucleic acid, a plurality of nucleic acids, a ketosteroid isomerase or an expression vector according to the present invention.

The present invention also provides a composition comprising a composition, a nucleic acid, a plurality of nucleic acids, a ketosteroid isomerase, an expression vector or a cell according to the present invention.

The present invention also provides the use of a composition, a nucleic acid, a plurality of nucleic acids, a ketosteroid isomerase, an expression vector or a cell according to the present invention in a method for producing (*R*)-(+)-pulegone or a derivative thereof.

In some embodiments the method is a method for producing one or more of (*R*)-(+)-pulegone, (+)-menthofuran, (-)-menthone, (+)-isomenthone, (+)-neomenthol, (-)-menthol, (+)-isomenthol and (+)-neoisomenthol.

The present invention also provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone using a glutathione S-transferase.

The present invention also provides the use of a glutathione S-transferase to catalyse the conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone.

In some embodiments the glutathione S-transferase is human glutathione S-transferase A3 (GSTA3-3) or a fragment, variant or homologue thereof.

In some embodiments the glutathione S-transferase comprises, or consists, of an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:10, 11, 12, 13, 14, 15 and 16.

The present invention also provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (+)-iso-isopulegol, (+)-neo-isopulegol and/or (-)-isopulegol) using a menthone reductase.

The present invention also provides the use of a menthone reductase to catalyse the conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (+)-iso-isopulegol, (+)-neo-isopulegol and/or (-)-isopulegol).

In some embodiments, the isopulegol isomer is selected from (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and (+)-neo-isopulegol. In some embodiments, the isopulegol isomer is (+)-neoiso-isopulegol.

In some embodiments the menthone reductase is *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR) or a fragment, variant or homologue thereof.

In some embodiments the menthone reductase comprises, or consists of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:17.

Description

The present invention is based on the identification by the inventors of enzymes having (+)-*cis*-isopulegone to (*R*)-(+)-pulegone isomerase activity. Conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone is a key step in the conversion of limonene and upstream biosynthetic precursors to menthol isomers.

The inventors demonstrate that Δ^5-3-ketosteroid isomerase (KSI) from *Pseudomonas putida* can catalyse (+)-*cis*-isopulegone to (*R*)-(+)-pulegone isomerisation. The reaction exclusively yields the (*R*)-enantiomer of pulegone, which is required for downstream menthol biosynthesis (no (*S*)-(−)-pulegone was detected). Through a process of robotics-automated directed evolution the inventors moreover identify mutants of KSI capable of producing (*R*)-(+)-pulegone with an increased yield as compared to wildtype KSI. In particular, the KSI variant V88I/L99V/V101A/D103S is identified, which yields 89% (*R*)-(+)-pulegone conversion from 1 mM (+)-*cis*-isopulegone in 24 h, and displays a 5.6 fold increase in isopulegone

isomerase activity as compared to wildtype KSI. This variant is also shown to function efficiently within a biosynthesis cascade, demonstrating the feasibility of biosynthetic production of menthol isomers from limonene.

The inventors have also identified enzymes catalysing conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and *trans*-isopulegone, and enzymes catalysing conversion of (+)-*cis*-isopulegone to (+)-neoiso-isopulegol, (+)-iso-isopulegol, (+)-neo-isopulegol and/or (-)-isopulegol.

Ketosteroid isomerase

The present invention provides methods using and compositions comprising ketosteroid isomerase.

"Ketosteroid isomerase" as used herein generally refers to an enzyme capable of catalyzing the conversion of 3-oxo-Δ^5-steroid to 3-oxo-Δ^4-steroid. Ketosteroid isomerase is also referred to as "steroid Δ-isomerase", "Δ^5-ketosteroid isomerase" and "Δ^5-3-ketosteroid isomerase", and has the IUBMB Enzyme Commission number 5.3.3.1. Ketosteroid isomerase may optionally be characterised by ability to catalyze conversion of a 3-oxo-Δ^5-steroid to 3-oxo-Δ^4-steroid, e.g. conversion of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione (described e.g. in Talalay and Benson, "Δ5-3-Ketosteroid Isomerase", in Boyer, *The Enzymes* 6, (3rd Ed., Academic Press. pp. 591–618).

The structure and function of ketosteroid isomerase is described e.g. in Ha *et al.*, Curr Opin Struct Biol. (2001) 11(6):674-8, which is hereby incorporated by reference in its entirety.

Ketosteroid isomerases have been identified and characterised from a wide range of species, including bacteria *Comamonas testosteroni* (UniProt: P00947-1, v2; SE ID NO:19) and *Pseudomonas putida* (UniProt: P07445-1, v1; SE ID NO:1), *Streptomyces sp. AA4* (UniProt: D9VBV6-1, v1; SEQ ID NO:20) and mammalian species.

Ketosteroid isomerase from *Pseudomonas putida* is a 131 amino acid polypeptide consisting of the sequence of SEQ ID NO:1 (UniProt: P07445-1, v1). In this specification "ketosteroid isomerase" refers to a ketosteroid isomerase from any species and includes isoforms, fragments, variants or homologues of ketosteroid isomerase from any species. Homologues include orthologues. In some embodiments, the ketosteroid isomerase is a prokaryotic ketosteroid isomerase, e.g. a bacterial ketosteroid isomerase. In some embodiments, the ketosteroid isomerase is from, or is derived from, a *Pseudomonas* species, e.g. *Pseudomonas putida*.

As used herein, a "fragment", "variant" or "homologue" of a protein may optionally be characterised as having at least 50%, preferably one of 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of the reference protein. Fragments, variants, isoforms and homologues of a reference protein may be characterised by the ability to perform a function performed by the reference protein.

A “fragment” generally refers to a fraction of the reference protein. A “variant” generally refers to a protein having an amino acid sequence comprising one or more amino acid substitutions, insertions, deletions or other modifications relative to the amino acid sequence of the reference protein, but retaining a considerable degree of sequence identity (e.g. at least 60%) to the amino acid sequence of the reference protein. An “isoform” generally refers to a variant of the reference protein expressed by the same species as the species of the reference protein. A “homologue” generally refers to a variant of the reference protein produced by a different species as compared to the species of the reference protein. For example, *Mentha piperita* (*R*)-(+)-pulegone reductase (PulR; UniProt: Q6WAU0-1, v1) and *Nicotiana tabacum* double bond reductase (NtDBR; UniProt: Q9SLN8-1, v1) are homologues of one another.

A “fragment” of a reference protein may be of any length (by number of amino acids), although may optionally be at least 25% of the length of the reference protein (that is, the protein from which the fragment is derived) and may have a maximum length of one of 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the length of the reference protein.

A fragment of ketosteroid isomerase may have a minimum length of one of 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids, and may have a maximum length of one of 15, 20, 25, 30, 40, 50, 100, 110, 120 or 130 amino acids.

Fragments, variants, isoforms and homologues of a ketosteroid isomerase may optionally be characterised by ability to catalyze conversion of a 3-oxo-Δ^5-steroid to 3-oxo-Δ^4-steroid, e.g. conversion of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione, and/or ability to catalyze conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

In some embodiments the ketosteroid isomerase comprises a ketosteroid isomerase active site. As used herein, a “ketosteroid isomerase active site” refers to the region of a ketosteroid isomerase which contains the amino acids residues which contact the substrate and catalyse isomerisation of the substrate. The active site of ketosteroid isomerase from *P. putida* comprises positions 86 to 112 of SEQ ID NO:1, shown in SEQ ID NO:34.

In some embodiments the ketosteroid isomerase comprises an amino acid sequence corresponding to the ketosteroid isomerase active site of ketosteroid isomerase from *P. putida*, e.g. as shown in SEQ ID NO:34. The skilled person able to identify an amino acid sequence corresponding to a reference sequence by alignment of the amino acid sequence to the reference sequence, for example using publicly available computer software such as ClustalOmega (Söding, J. 2005, Bioinformatics 21, 951-960). By way of example, an alignment of the amino acid sequences of ketosteroid isomerases from different species is shown in **Figure 15**; the regions of the ketosteroid isomerases which correspond to the ketosteroid isomerase active site of ketosteroid isomerase from *P. putida* are shown in **Figure 16**.

In some embodiments, the ketosteroid isomerase of the present invention comprises an amino acid sequence shown in **Figure 16**, or an amino acid sequence having at least 70%, preferably one of 75%,

80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to an amino acid sequence shown in **Figure 16**. In some embodiments, the ketosteroid isomerase comprises an amino acid sequence having at least 70%, preferably one of 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:34 or 35.

Figure 17 shows an alignment of a subregion of the active site of ketosteroid isomerase from *P. putida* corresponding to positions 86 to 107 of SEQ ID NO:1 (shown in SEQ ID NO:36) to the corresponding region of ketosteroid isomerases from several different *Pseudomonas* species. In some embodiments, the ketosteroid isomerase of the present invention comprises an amino acid sequence shown in **Figure 17**, or an amino acid sequence having at least 70%, preferably one of 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to an amino acid sequence shown in **Figure 17**. In some embodiments, the ketosteroid isomerase comprises an amino acid sequence having at least 70%, preferably one of 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:36 or 37.

In some embodiments the ketosteroid isomerase comprises an amino acid sequence comprising one or more of the following residues numbered relative to SEQ ID NO:1:

Position	Residue
88	V, I, A, L or M
99	L, I, V, S or M
101	V, P, I, C, S, A
103	D, S, E

In some embodiments, the ketosteroid isomerase of the present invention comprises or consists of an amino acid sequence shown in **Figure 15**, or an amino acid sequence having at least 70%, preferably one of 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to an amino acid sequence shown in **Figure 15**. In some embodiments, the ketosteroid isomerase comprises an amino acid sequence having at least 70%, preferably one of 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:1 or 38.

In some embodiments the ketosteroid isomerase comprises an amino acid sequence comprising one or more of the following residues numbered relative to SEQ ID NO:1:

Position	Residue
16	Y
40	D
57	Y or F
88	V, I, A, L or M
99	L, I, V, S or M

101	V, P, I, C, S, A
103	D, S, E

In some embodiments, the ketosteroid isomerase comprises one or more amino acid substitutions relative to the amino acid sequence of a reference ketosteroid isomerase (e.g. the ketosteroid isomerase having the amino acid sequence of SEQ ID NO:1). In some embodiments, the ketosteroid isomerase comprises e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acid substitutions relative to the amino acid sequence of the reference ketosteroid isomerase. In some embodiments, the ketosteroid isomerase comprises e.g. 1-3, 1-5, 1-10, 1-15 or 1-20 amino acid substitutions relative to the amino acid sequence of the reference ketosteroid isomerase.

In particular the present invention contemplates ketosteroid isomerase comprising amino acid substitutions corresponding to one or more of the following positions numbered relative to the amino acid sequence of SEQ ID NO:1: V88, L99, Y16, Y57, D103, V20, F56, L61, V66, F86, M90, V101, M116, W120, G60 and A118. In some embodiments, the ketosteroid isomerase comprises amino acid substitutions corresponding to one or more of the following positions numbered relative to the amino acid sequence of SEQ ID NO:1: V88, L99, D103, and V101.

The skilled person is well able to identify corresponding positions to the indicated positions in ketosteroid isomerases other than ketosteroid isomerase from *Pseudomonas putida* (SEQ ID NO:1). Corresponding positions can be identified e.g. by alignment of the amino acid sequence of a given ketosteroid isomerase to the amino acid sequence of SEQ ID NO:1. Sequence alignments for such purposes can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalOmega (Söding, J. 2005, Bioinformatics 21, 951-960). By way of example, an alignment of the amino acid sequence of *Comamonas testosteroni* (UniProt: P0047; SEQ ID NO:19) with SEQ ID NO:1 is shown in **Figure 14**. It can be seen from the alignment that positions D99 and Y14 of *Comamonas testosteroni* KSI correspond respectively to positions D103 and Y16 of *Pseudomonas putida* KSI (which have been shown to be Oxyanion H-Bond Donors; see e.g. Wu et al., Science (1997), 276 (5311): 415–8). An alignment of the amino acid sequences of ketosteroid isomerases from several different species is shown in **Figure 15**.

It will similarly be appreciated that reference herein to a “corresponding” substitution refers to the same amino acid substitution at the corresponding position of the subject ketosteroid isomerase. By way of example, the substitution “D99S” in SEQ ID NO:19 is a corresponding substitution to the substitution “D103S” in SEQ ID NO:1.

In some embodiments, the substitutions are conservative substitutions, for example according to the following Table. In some embodiments, amino acids in the same block in the middle column are substituted. In some embodiments, amino acids in the same line in the rightmost column are substituted:

ALIPHATIC	Non-polar	G A P

		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

In some embodiments, the substitution(s) may be functionally conservative. That is in some embodiments the substitution may not affect (or may not substantially affect) the activity of the ketosteroid isomerase. In some embodiments, the substitution(s) may yield an enzyme having similar isopulegone isomerase activity and/or specific activity as compared to the isopulegone isomerase activity/specific activity of the equivalent ketosteroid isomerase lacking the substitution(s). For example, substitution of D103 of SEQ ID NO:1 with S is functionally conservative because the serine residue similarly comprises a side chain 'OH' group which is able to participate in H-bond formation.

Accordingly, in some embodiments D103 is substituted with an amino acid comprising a side chain comprising an 'OH' group (e.g. serine, glutamic acid, threonine or tyrosine). Y16 may also participate in H-bond formation with substrate. Accordingly, in some embodiments Y16 is substituted with an amino acid comprising a side chain comprising an 'OH' group (e.g. serine, glutamic acid, threonine or aspartic acid).

In some embodiments, a ketosteroid isomerase comprising the substitution(s) give a yield of (*R*)-(+)-pulegone which is ≥ 0.75 times and ≤ 1.25 times, e.g. ≥ 0.8 times and ≤ 1.2 times, ≥ 0.85 times and ≤ 1.15 times, ≥ 0.9 times and ≤ 1.1 times, ≥ 0.91 times and ≤ 1.09 times, ≥ 0.92 times and ≤ 1.08 times, ≥ 0.93 times and ≤ 1.07 times, ≥ 0.94 times and ≤ 1.06 times, ≥ 0.95 times and ≤ 1.05 times, ≥ 0.96 times and ≤ 1.04 times, ≥ 0.97 times and ≤ 1.03 times, ≥ 0.98 times and ≤ 1.02 times, or ≥ 0.99 times and ≤ 1.01 times the yield of (*R*)-(+)-pulegone obtained using the equivalent ketosteroid isomerase lacking the substitution(s) in a comparable assay of isopulegone isomerase activity.

In some embodiments, a ketosteroid isomerase comprising the substitution(s) produces an increased amount of (*R*)-(+)-pulegone, per unit time, per unit enzyme as compared to the reference protein. In some embodiments a ketosteroid isomerase has a specific activity for conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone (expressed e.g. in $nmol.min^{-1}.mg^{-1}$) which is ≥ 0.75 times and ≤ 1.25 times, e.g. ≥ 0.8 times and ≤ 1.2 times, ≥ 0.85 times and ≤ 1.15 times, ≥ 0.9 times and ≤ 1.1 times, ≥ 0.91 times and ≤ 1.09 times, ≥ 0.92 times and ≤ 1.08 times, ≥ 0.93 times and ≤ 1.07 times, ≥ 0.94 times and ≤ 1.06 times, ≥ 0.95 times and ≤ 1.05 times, ≥ 0.96 times and ≤ 1.04 times, ≥ 0.97 times and ≤ 1.03 times, ≥ 0.98 times and ≤ 1.02 times, or ≥ 0.99 times and ≤ 1.01 times the specific activity of the equivalent ketosteroid isomerase lacking the substitution(s) in a comparable assay.

In some embodiments, the substitution(s) may affect the activity of the ketosteroid isomerase. In some embodiments, the substitution(s) may increase isopulegone isomerase activity and/or specific activity (i.e. as compared to the isopulegone isomerase activity/specific activity of the equivalent ketosteroid isomerase lacking the substitution(s)).

In some embodiments, a ketosteroid isomerase comprising the substitution(s) give a yield of (*R*)-(+)-pulegone which is more than 1 times, e.g. more than 1.1 times, 1.2 times, 1.3 times, 1.4 times, 1.5 times, 1.6 times, 1.7 times, 1.8 times, 1.9 times, 2.0 times, 2.1 times, 2.2 times, 2.3 times, 2.4 times, 2.5 times, 2.6 times, 2.7 times, 2.8 times, 2.9 times, 3.0 times, 3.5 times, 4.0 times, 5.0 times, 6.0 times, 7.0 times, 8.0 times, 9.0 times, or more than 10.0 times the yield of (*R*)-(+)-pulegone obtained using the equivalent ketosteroid isomerase lacking the substitution(s) in a comparable assay of isopulegone isomerase activity.

In some embodiments, a ketosteroid isomerase comprising the substitution(s) produces an increased amount of (*R*)-(+)-pulegone, per unit time, per unit enzyme as compared to the reference protein. In some embodiments a ketosteroid isomerase has a specific activity for conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone (expressed e.g. in $nmol.min^{-1}.mg^{-1}$) which is more than 1 times, e.g. more than 1.1 times, 1.2 times, 1.3 times, 1.4 times, 1.5 times, 1.6 times, 1.7 times, 1.8 times, 1.9 times, 2.0 times, 2.1 times, 2.2 times, 2.3 times, 2.4 times, 2.5 times, 2.6 times, 2.7 times, 2.8 times, 2.9 times, 3.0 times, 3.5 times, 4.0 times, 5.0 times, 6.0 times, 7.0 times, 8.0 times, 9.0 times, or more than 10.0 times the specific activity of the equivalent ketosteroid isomerase lacking the substitution(s) in a comparable assay.

By way of illustration, a ketosteroid isomerase comprising the amino acid sequence of SEQ ID NO:1 and comprising the amino acid substitutions D103S, V88I, L99V and V101A is demonstrated in the experimental examples of the present disclosure to have a specific activity for conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone (expressed in µM (*R*)-(+)-pulegone per µM KSI, per 24 hours) which is ~4.5 times greater than the specific activity of the equivalent ketosteroid isomerase lacking the substitutions – see e.g. **Figure 3A**, column 6.

In some embodiments, the ketosteroid isomerase of the present invention comprises one or more amino acid substitutions corresponding to the following substitutions numbered relative to SEQ ID NO:1: D103S, V88I, L99I, L99V and V101A. In some embodiments, the ketosteroid isomerase comprises amino acid substitutions corresponding to 1, 2, 3 or 4 of the substitutions.

In particular, ketosteroid isomerase comprising substitutions/combinations of substitutions corresponding to the following substitutions/combinations of substitutions are contemplated: D103S; V88I; L99I; L99V; V101A; D103S and V88I; D103S and L99I; D103S and L99V; D103S and V101A; V88I and L99I; V88I and L99V; V88I and V101A; L99I and V101A; L99V and V101A; D103S, V88I and L99I; D103S, V88I and L99V; D103S, V88I and V101A; D103S, L99I and V101A; D103S, L99V and V101A; V88I, L99I and V101A; V88I, L99V and V101A; D103S, V88I, L99I and V101A; and D103S, V88I, L99V and V101A.

In some embodiments, the ketosteroid isomerase comprises, or consists of, the amino acid sequence of SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8 or 9, or an amino acid sequence having at least 70%, preferably one of 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8 or 9.

The ketosteroid isomerase of the present invention displays isopulegone isomerase activity. Ketosteroid isomerase having isopulegone isomerase activity can be determined by means known to the skilled person. For example, ketosteroid isomerase can be evaluated for isopulegone isomerase activity by analysis of the ability of the enzyme to catalyse conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone. Such assays can be performed e.g. as described in Example 1 herein. Products of reactions can be analysed e.g. by gas chromatography mass spectrometry (GC-MS) as described herein.

In some embodiments the ketosteroid isomerase has increased isopulegone isomerase activity as compared to the isopulegone isomerase activity of a reference protein, e.g. *Pseudomonas putida* KSI (SEQ ID NO:1).

Increased isopulegone isomerase activity can be determined by the detection of an increased yield of (*R*)-(+)-pulegone at the end of an assay for such activity as compared to the yield of (*R*)-(+)-pulegone obtained using the reference protein in a comparable assay. In some embodiments a ketosteroid isomerase having increased isopulegone isomerase activity gives a yield of (*R*)-(+)-pulegone which is more than 1 times, e.g. more than 1.1 times, 1.2 times, 1.3 times, 1.4 times, 1.5 times, 1.6 times, 1.7 times, 1.8 times, 1.9 times, 2.0 times, 2.1 times, 2.2 times, 2.3 times, 2.4 times, 2.5 times, 2.6 times, 2.7 times, 2.8 times, 2.9 times, 3.0 times, 3.5 times, 4.0 times, 5.0 times, 6.0 times, 7.0 times, 8.0 times, 9.0 times, or more than 10.0 times the yield of (*R*)-(+)-pulegone obtained using the reference protein in a comparable assay of isopulegone isomerase activity.

In some embodiments, (*R*)-(+)-pulegone yield may be calculated as the percentage of (+)-*cis*-isopulegone converted to (*R*)-(+)-pulegone. In some embodiments, (*R*)-(+)-pulegone yield may be the percentage of (+)-*cis*-isopulegone converted to (*R*)-(+)-pulegone per unit of ketosteroid isomerase. In some embodiments, (*R*)-(+)-pulegone yield may be the amount of (*R*)-(+)-pulegone per unit of ketosteroid isomerase. In some embodiments, (*R*)-(+)-pulegone yield may be the amount of (*R*)-(+)-pulegone produced per unit of ketosteroid isomerase per unit of time.

In some embodiments, the ketosteroid isomerase has increased specific activity as compared to a reference protein, e.g. *Pseudomonas putida* KSI. That is, in some embodiments the ketosteroid isomerase of the invention produces an increased amount of (*R*)-(+)-pulegone, per unit time, per unit enzyme as compared to the reference protein. In some embodiments a ketosteroid isomerase has a specific activity for conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone (expressed e.g. in $nmol.min^{-1}.mg^{-1}$) which is more than 1 times, e.g. more than 1.1 times, 1.2 times, 1.3 times, 1.4 times, 1.5 times, 1.6 times, 1.7 times, 1.8 times, 1.9 times, 2.0 times, 2.1 times, 2.2 times, 2.3 times, 2.4 times, 2.5 times, 2.6 times, 2.7 times, 2.8 times, 2.9 times, 3.0 times, 3.5 times, 4.0 times, 5.0 times, 6.0 times, 7.0 times, 8.0 times, 9.0 times, or more than 10.0 times the specific activity of the reference protein in a comparable assay.

Monoterpenoid biosynthesis

The present invention provides methods comprising the use of ketosteroid isomerase as described herein in monoterpenoid biosynthesis.

In this specification, "catalysis" is used to mean acceleration of a reaction. Accordingly, "catalysis" of the conversion of a substrate to a product by an enzyme refers to an increase in the rate of conversion of the substrate to the product as compared to the rate of conversion in the absence of the enzyme. The rate of conversion may be expressed e.g. as the amount of product formed per unit time. An enzyme which is capable of catalyzing a given reaction is capable of increasing the rate of conversion to more than 1 times, e.g. more than 2 times, 3 times, 4 times, 5 times, 10 times, 20 times, 30 times, 40 times, 50 times, 100 times, 500 times, 10,00 times, 10,000 times or more than the rate of conversion of the substrate to the product in the absence of the enzyme (under comparable conditions).

In accordance with various aspects of the present invention it may be necessary or desirable to detect and/or quantify one or more organic molecules, e.g. monoterpenoids. Detection and/or quantification of can be performed by any suitable analytical chemistry method e.g. gas chromatography mass spectrometry (GC-MS) as described in Example 4 of the present application.

In one aspect the invention provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone using a ketosteroid isomerase. Ketosteroid isomerase-catalysed conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone may be employed in any method involving or requiring conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

In some embodiments, the method may be a method for producing (*R*)-(+)-pulegone. In some embodiments, the method may be a method for producing a derivative of (*R*)-(+)-pulegone.

As used herein, a "derivative" of a reference molecule (e.g. a reference monoterpenoid) refers to a product derived from the reference molecule. Derivatives include metabolites. In some embodiments a derivative is a molecule formed by a chemical reaction of the reference molecule. In some embodiments a derivative is a molecule formed by conversion of the reference molecule e.g. by catalysis of the conversion of the reference molecule by an enzyme described herein. In some embodiments a derivative of a reference molecule may be a molecule formed by conversion of a derivative of the reference molecule, e.g. by catalysis of the conversion by an enzyme described herein. By way of illustration, derivatives of (*R*)-(+)-pulegone include (+)-menthofuran, (-)-menthone, (+)-isomenthone, (+)-neomenthol, (-)-menthol, (+)-isomenthol, and (+)-neoisomenthol.

In some embodiments, the method may be a method for producing a menthone isomer (e.g. (-)-menthone and/or (+)-isomenthone). In some embodiments, the method may be a method for producing (+)-isomenthone. In some embodiments, the method may be a method for producing menthofuran.

In some embodiments, the method may be a method for producing a menthol isomer. In some embodiments, the method may be a method for producing menthol isomer selected from the group consisting of menthol, neoisomenthol, neomenthol and isomenthol.

In some embodiments menthol isomer is selected from (-)-menthol, (+)-neomenthol, (+)-isomenthol, (+)-neoisomenthol, (+)-menthol, (-)-neomenthol, (-)-isomenthol and (-)-neoisomenthol. In some embodiments menthol isomer is selected from (-)-menthol, (+)-neomenthol, (+)-isomenthol and (+)-neoisomenthol.

In some embodiments the methods involve contacting (+)-*cis*-isopulegone with a ketosteroid isomerase as described herein under conditions suitable for the conversion of the (+)-*cis*-isopulegone to (*R*)-(+)-pulegone. The skilled person is readily able to determine such suitable conditions (e.g. concentrations, temperatures, pH, etc.), with reference e.g. to the experimental examples of the present application.

In some embodiments the methods comprise one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone. In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone. In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone, and one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

In some embodiments, the methods comprise converting (*R*)-(+)-pulegone to (-)-menthone. In some embodiments, the methods comprise converting (*R*)-(+)-pulegone to (+)-isomenthone. In some embodiments, the methods comprise catalyzing conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone, e.g. using a pulegone reductase. In some embodiments, the methods comprise contacting (*R*)-(+)-pulegone with a pulegone reductase under conditions suitable for conversion of the (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone. Conditions suitable for pulegone reductase-catalyzed conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone can be readily determined by the skilled person, with reference e.g. to Ringer et al., Arch Biochem Biophys (2003) 418(1):80-92 and Toogood et al., ACS Synth Biol. (2015) 4(10):1112-23, both of which are hereby incorporated by reference in their entirety. The pulegone reductase may be e.g. *Mentha piperita* pulegone reductase (UniProt: Q6WAU0-1, v1) *Nicotiana tabacum* double bond reductase (UniProt: Q9SLN8-1, v1), or a fragment, variant or homologue of one of these enzymes capable of catalyzing conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (*R*)-(+)-pulegone to (+)-isomenthone.

In some embodiments, the methods comprise converting (*R*)-(+)-pulegone to (+)-menthofuran. In some embodiments, the methods comprise catalyzing conversion of (*R*)-(+)-pulegone to (+)-menthofuran, e.g. using a menthofuran synthase or a cytochrome P450 reductase. In some embodiments, the methods comprise contacting (*R*)-(+)-pulegone with a menthofuran synthase or a cytochrome P450 reductase under conditions suitable for conversion of the (*R*)-(+)-pulegone to (+)-menthofuran. Conditions suitable for menthofuran synthase/cytochrome P450 reductase-catalyzed conversion of (*R*)-(+)-pulegone to (-)-menthofuran can be readily determined by the skilled person, with reference e.g. to Bertea et al. Arch

Biochem Biophys (2001) 390(2):279-86, which is hereby incorporated by reference in its entirety. The menthofuran synthase/cytochrome P450 reductase may be e.g. *Mentha piperita* menthofuran synthase (UniProt: Q947B7-1, v1), or a fragment, variant or homologue thereof capable of catalyzing conversion of (*R*)-(+)-pulegone to (+)-menthofuran.

In some embodiments, the methods comprise converting (-)-menthone to (-)-menthol. In some embodiments, the methods comprise catalyzing conversion of (-)-menthone to (-)-menthol, e.g. using a menthone reductase. In some embodiments, the methods comprise contacting (-)-menthone with a menthone reductase under conditions suitable for conversion of the (-)-menthone to (-)-menthol. In some embodiments, the methods comprise converting (+)-isomenthone to (+)-neoisomenthol. In some embodiments, the methods comprise catalyzing conversion of (+)-isomenthone to (+)-neoisomenthol, e.g. using a menthone reductase. In some embodiments, the methods comprise contacting (+)-isomenthone with a menthone reductase under conditions suitable for conversion of the (+)-isomenthone to (+)-neoisomenthol. Conditions suitable for menthone reductase-catalyzed conversion of (-)-menthone to (-)-menthol, and/or conversion of (+)-isomenthone to (+)-neoisomenthol can be readily determined by the skilled person, with reference e.g. to Davis et al., Plant Physiol. (2005) 137(3): 873–881, which is hereby incorporated by reference in its entirety. The menthone reductase may be e.g. *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR; Q5CAF4-1, v1), or a fragment, variant or homologue thereof capable of catalyzing conversion of (-)-menthone to (-)-menthol and/or (+)-isomenthone to (+)-neoisomenthol.

In some embodiments, the methods comprise converting (-)-menthone to (+)-neomenthol. In some embodiments, the methods comprise catalyzing conversion of (-)-menthone to (+)-neomenthol, e.g. using a menthone reductase. In some embodiments, the methods comprise contacting (-)-menthone with a menthone reductase under conditions suitable for conversion of the (-)-menthone to (+)-neomenthol. In some embodiments, the methods comprise converting (+)-isomenthone to (+)-isomenthol. In some embodiments, the methods comprise catalyzing conversion of (+)-isomenthone to (+)-isomenthol, e.g. using a menthone reductase. In some embodiments, the methods comprise contacting (+)-isomenthone with a menthone reductase under conditions suitable for conversion of the (+)-isomenthone to (+)-isomenthol. Conditions suitable for menthone reductase-catalyzed conversion of (-)-menthone to (+)-neomenthol, and/or conversion of (+)-isomenthone to (+)-isomenthol can be readily determined by the skilled person, with reference e.g. to Davis et al., Plant Physiol. (2005) 137(3): 873–881 or Toogood et al., *ACS Synth. Biol.* 2015, *4* (10), 1112–1123, both of which are hereby incorporated by reference in their entirety. The menthone reductase may be e.g. *Mentha piperita* (-)-menthone:(+)-neomenthol reductase (MNMR; Q06ZW2-1, v1), or a fragment, variant or homologue thereof capable of catalyzing conversion of (-)-menthone to (+)-neomenthol and/or (+)-isomenthone to (+)-isomenthol. Menthone reductase activity of MNMR (Q06ZW2-1, v1) is described e.g. in Toogood et al.,*ACS Synth. Biol.* 2015, *4* (10), 1112–1123.

In some embodiments, the methods comprise converting limonene to (-)-*trans*-isopiperitenol. In some embodiments, the methods comprise catalyzing conversion of limonene to (-)-*trans*-isopiperitenol, e.g. using a limonene hydroxylase or a cytochrome P450 reductase. In some embodiments, the methods

comprise contacting limonene with a limonene hydroxylase or a cytochrome P450 reductase under conditions suitable for conversion of the limonene to (-)-*trans*-isopiperitenol. Conditions suitable for limonene hydroxylase/cytochrome P450 reductase-catalyzed conversion of limonene to (-)-*trans*-isopiperitenol can be readily determined by the skilled person, with reference e.g. to Haudenschild et al., Arch. Biochem. Biophys. (2000) 379:127-136, which is hereby incorporated by reference in its entirety. The limonene hydroxylase may be e.g. *Mentha piperita* limonene-3-hydroxylase (UniProt: Q9XHE6-1, v1) or *Mentha spicata* limonene-6-hydroxylase variant F363I (UniProt: Q9XHE8-1, v1), or a fragment, variant or homologue of one of these enzymes capable of catalyzing conversion of limonene to (-)-*trans*-isopiperitenol. The cytochrome P450 reductase may be e.g. *Pseudomonas putida* Cytochrome P450cam variant Y96F/V247L/C334A (UniProt: M5B4L7-1, v1), or a fragment, variant or homologue capable of catalyzing conversion of limonene to (-)-*trans*-isopiperitenol.

Mentha piperita limonene-3-hydroxylase and *Mentha spicata* limonene-6-hydroxylase variant F363I may be employed in conjunction with one or more electron transfer partner proteins, such as *Arabidopsis thaliana* Cytochrome P450 reductase (UniProt: Q9SB48-1, v1), and *Salvia miltiorrhiza* Cytochrome P450 reductase (UniProt: S4URU2-1, v1) – see e.g. Kuo et al., The Journal of Pharmacy and Pharmacology (2006) 58:521-527. *Pseudomonas putida* Cytochrome P450cam variant Y96F/V247L/C334A may be employed in conjunction with one or more electron transfer partner proteins, such as *Pseudomonas putida* NADH-putidaredoxin reductase (UniProt: M5AXR7-1, v1) or *Pseudomonas putida* putidaredoxin (UniProt: P00259-1, v3) – see e.g. Peterson et al., Journal of Biological Chemistry (1990) 265:6066-6073.

In some embodiments, the methods comprise converting (-)-*trans*-isopiperitenol to (-)-isopiperitenone. In some embodiments, the methods comprise catalyzing conversion of (-)-*trans*-isopiperitenol to (-)-isopiperitenone, e.g. using an isopiperitenol dehydrogenase. In some embodiments, the methods comprise contacting (-)-*trans*-isopiperitenol with an isopiperitenol dehydrogenase under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to (-)-isopiperitenone. Conditions suitable for isopiperitenol dehydrogenase-catalyzed conversion of (-)-*trans*-isopiperitenol to (-)-isopiperitenone can be readily determined by the skilled person, with reference e.g. to Ringer et al., Plant Physiol. (2005) 137(3): 863–872, which is hereby incorporated by reference in its entirety. The isopiperitenol dehydrogenase may be e.g. *Mentha piperita* (-)-Isopiperitenol/(-)-carveol dehydrogenase (UniProt: Q5C9I9-1, v1), *Rhodococcus erythropolis* (4*R*,6*S*)-carveol dehydrogenase (UniProt: Q9RA05-1, v1), or a fragment, variant or homologue of one of these enzymes capable of catalyzing conversion of (-)-*trans*-isopiperitenol to (-)-isopiperitenone.

In some embodiments, the methods comprise converting (-)-isopiperitenone to (+)-*cis*-isopulegone. In some embodiments, the methods comprise catalyzing conversion of (-)-isopiperitenone to (+)-*cis*-isopulegone, e.g. using an (-)-isopiperitenone reductase. In some embodiments, the methods comprise contacting (-)-isopiperitenone with an (-)-isopiperitenone reductase under conditions suitable for conversion of the (-)-isopiperitenone to (+)-*cis*-isopulegone. Conditions suitable for (-)-isopiperitenone reductase-catalyzed conversion of (-)-isopiperitenone to (+)-*cis*-isopulegone can be readily determined by the skilled person, with reference e.g. to Ringer et al., Arch. Biochem. Biophys. (2003) 418:80-92, which

is incorporated by reference herein. The (-)-isopiperitenone reductase may be e.g. *Mentha piperita* (-)-isopiperitenone reductase (UniProt: Q6WAU1-1, v1), or a fragment, variant or homologue thereof capable of catalyzing conversion of (-)-isopiperitenone to (+)-*cis*-isopulegone.

In some embodiments, the methods comprise synthesis/preparation of limonene. In some embodiments, the methods comprise preparation of limonene from a monoterpenoid precursor such as geranyl diphosphate. In some embodiments the methods comprise converting geranyl diphosphate to limonene. In some embodiments, the methods comprise catalyzing conversion of geranyl diphosphate to limonene, e.g. using a limonene synthase. In some embodiments, the methods comprise contacting geranyl diphosphate with a limonene synthase under conditions suitable for conversion of the geranyl diphosphate to limonene. Conditions suitable for limonene synthase-catalyzed conversion of geranyl diphosphate to limonene can be readily determined by the skilled person, with reference e.g. to Bohlmann et al., J. Biol. Chem. (1997) 272 (35): 21784–92, which is hereby incorporated by reference in its entirety. The limonene synthase may be e.g. *Mentha spicata* 4S-limonene synthase (UniProt: Q40322-1, v1), or a fragment, variant or homologue thereof capable of catalyzing conversion of geranyl diphosphate to limonene.

In some embodiments, the methods comprise synthesis/preparation of geranyl diphosphate. In some embodiments, the methods comprise preparation of geranyl diphosphate from metabolic precursors of geranyl diphosphate such as glyceraldehyde-3-phosphate and pyruvate, acetyl-CoA, dimethylallyl diphosphate or isopentyl diphosphate. Geranyl diphosphate may be generated from glyceraldehyde-3-phosphate and pyruvate via the non-mevalonate pathway (i.e. the MEP pathway) and/or from acetyl-CoA via the mevalonate pathway. In some embodiments the methods comprise conversion of a metabolic precursor of geranyl diphosphate to geranyl diphosphate. In some embodiments the methods comprise conversion of dimethylallyl diphosphate and/or isopentyl diphosphate to a geranyl diphosphate, e.g. using a geranyl diphosphate synthase. In some embodiments, the methods comprise contacting dimethylallyl diphosphate and/or isopentyl diphosphate with a geranyl diphosphate synthase under conditions suitable for conversion of the dimethylallyl diphosphate/isopentyl diphosphate to geranyl diphosphate. Suitable conditions can be readily determined by the skilled person, with reference e.g. to Burke et al., Proc Natl Acad Sci U S A. (1999) 96(23): 13062–13067, which is hereby incorporated by reference in its entirety. The geranyl diphosphate synthase may be e.g. *Mentha piperita* geranyl diphosphate synthase (UniProt: Q9SBR3-1, v1), *Abies grandis* geranyl diphosphate synthase (UniProt: Q8LKJ2-1, v1), or a fragment, variant or homologue of one of these enzymes capable of catalyzing conversion of geranyl diphosphate to limonene.

The present invention also provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) using a menthone reductase. Menthone reductase-catalysed conversion of (+)-*cis*-isopulegone to an isopulegol isomer may be employed in any method involving or requiring conversion of (+)-*cis*-isopulegone to an isopulegol isomer.

In some embodiments, the isopulegol isomer is (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol). In some embodiments, the isopulegol isomer is (+)-neoiso-isopulegol and/or (-)-isopulegol.

In some embodiments, the method may be a method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol). In some embodiments, the method may be a method for producing a derivative of an isopulegol isomer. In some embodiments, the method may be a method for producing a menthol isomer. In some embodiments, the method may be a method for producing menthol isomer selected from the group consisting of menthol, neoisomenthol, neomenthol and isomenthol (e.g. (-)-menthol, (+)-neomenthol, (+)-isomenthol, (+)-neoisomenthol, (+)-menthol, (-)-neomenthol, (-)-isomenthol and (-)-neoisomenthol).

In some embodiments the methods involve contacting (+)-*cis*-isopulegone with a menthone reductase as described herein under conditions suitable for the conversion of the (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol). The skilled person is readily able to determine such suitable conditions (e.g. concentrations, temperatures, pH, etc.), with reference e.g. to the experimental examples of the present application.

In some embodiments the menthone reductase is *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR; UniProt: Q5CAF4-1, v1; SEQ ID NO:17) or a fragment, variant or homologue thereof capable of catalyzing conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol). A fragment of menthone reductase may have a minimum length of one of 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids, and may have a maximum length of one of 15, 20, 25, 30, 40, 50, 100, 150, 200, 250 or 300 amino acids.

A menthone reductase can be evaluated for the ability to catalyse conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) e.g. as described in Example 3 herein. Products of reactions can be analysed e.g. by gas chromatography and/or mass spectrometry.

In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol), e.g. as described hereinabove. In some embodiments the methods comprise one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to an isopulegol isomer. In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. as described hereinabove), and one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to an isopulegol isomer.

Isopulegol isomers may be converted to menthol isomers by hydrogenation, e.g. as described in US 20100191021 A1, which is hereby incorporated by reference in its entirety. For example, (+)-neoiso-

isopulegol can be converted by hydrogenation to (+)-neoisomenthol, (+)-iso-isopulegol can be converted by hydrogenation to (+)-isomenthol, (+)-neo-isopulegol can be converted by hydrogenation to (+)-neomenthol and (-)-isopulegol can be converted by hydrogenation to (-)-menthol. Accordingly, in some embodiments the methods of the present invention comprise conversion of one or more isopulegol isomers to one or more menthol isomers.

The present invention also provides a method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone using a glutathione S-transferase. Glutathione S-transferase-catalysed conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone may be employed in any method involving or requiring conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

In some embodiments, the method may be a method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone. In some embodiments, the method may be a method for producing a derivative of (*S*)-(-)-pulegone and/or *trans*-isopulegone.

In some embodiments the methods involve contacting (+)-*cis*-isopulegone with a glutathione S-transferase as described herein under conditions suitable for the conversion of the (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone. The skilled person is readily able to determine such suitable conditions (e.g. concentrations, temperatures, pH, etc.), with reference e.g. to the experimental examples of the present application.

In some embodiments the glutathione S-transferase is Human Glutathione S-transferase A3 (UniProt: Q16772-1, v3; SEQ ID NO:10) or a fragment, variant or homologue thereof capable of catalyzing conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone. In some embodiments the glutathione S-transferase is comprises, or consists of, the amino acid sequence of SEQ ID NO:10, 11, 12, 13, 14, 15 or 16, or a fragment, variant or homologue thereof capable of catalyzing conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone. A fragment of glutathione S-transferase may have a minimum length of one of 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids, and may have a maximum length of one of 15, 20, 25, 30, 40, 50, 100, 110, 120 or 130, 140, 150, 160, 170, 180, 190, 200, 210 or 220 amino acids.

A glutathione S-transferase can be evaluated for the ability to catalyse conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone e.g. as described in Example 6 herein. Products of reactions can be analysed e.g. by gas chromatography and/or mass spectrometry.

In some embodiments, the glutathione S-transferase comprises one or more amino acid substitutions relative to the amino acid sequence of a glutathione S-transferase (e.g. the glutathione S-transferase having the amino acid sequence of SEQ ID NO:10). In some embodiments, the glutathione S-transferase comprises e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acid substitutions relative to the amino acid sequence of the reference glutathione S-transferase. In some

embodiments, the glutathione S-transferase comprises e.g. 1-3, 1-5, 1-10, 1-15 or 1-20 amino acid substitutions relative to the amino acid sequence of the reference glutathione S-transferase.

In particular the present invention contemplates glutathione S-transferase comprising amino acid substitutions corresponding to one or more of the following positions numbered relative to the amino acid sequence of SEQ ID NO:10: A280, A216, L111, F222, L72 and S159.

In some embodiments, the glutathione S-transferase of the present invention comprises one or more amino acid substitutions corresponding to the following substitutions numbered relative to SEQ ID NO:10: A208F, A216L, L111D, F222W, L72R and S159Y. In some embodiments, the glutathione S-transferase comprises amino acid substitutions corresponding to 1, 2, 3, 4, 5 or 6 of the substitutions.

In particular, glutathione S-transferase comprising substitutions/any combination of substitutions corresponding to the following substitutions/combinations of substitutions are contemplated: A208F; A216L; A208F/A216L; L111D; F222W; and L72R/S159Y.

In some embodiments, the glutathione S-transferase comprises, or consists of, the amino acid sequence of SEQ ID NO:10, 11, 12, 13, 14, 15 or 16, or an amino acid sequence having at least 70%, preferably one of 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of SEQ ID NO:10, 11, 12, 13, 14, or 15.

In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone, e.g. as described hereinabove. In some embodiments the methods comprise one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone. In some embodiments the methods comprise one or more additional method steps preceding conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone (e.g. as described hereinabove), and one or more additional method steps subsequent to conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

In some embodiments, the methods comprise providing substrate for a conversion. The method may comprise adding the substrate or a metabolic precursor to a vessel containing the enzyme. In some embodiments the methods additionally comprise recovering the product of a conversion. In some embodiments the product may be recovered to be used as substrate in a conversion in accordance with a subsequent method step. In some embodiments the product is recovered as the final product of the method. Recovered product may be isolated/purified.

It will be appreciated that where the enzymes act sequentially in a metabolic pathway, the methods may comprise providing substrate for the first step and the enzymes for catalysing the relevant conversions.

By way of illustration, Examples 3 and 4 and **Figure 8** demonstrate production of menthol isomers from (+)-*cis*-isopulegone, by a method comprising providing (+)-*cis*-isopulegone to a composition comprising a ketosteroid isomerase, a pulegone reductase and a menthone reductase and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to menthol isomers.

It will further be appreciated that "a method comprising providing (+)-*cis*-isopulegone to a composition comprising a ketosteroid isomerase, a pulegone reductase and a menthone reductase and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to menthol isomers" implicitly includes: contacting (+)-*cis*-isopulegone with a ketosteroid isomerase under conditions suitable for conversion of the (+)-*cis*-isopulegone to (*R*)-(+)-pulegone; contacting the (*R*)-(+)-pulegone with a pulegone reductase under conditions suitable for conversion of the (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone; and contacting the (-)-menthone and/or (+)-isomenthone with a menthone reductase under conditions suitable for conversion of the (-)-menthone and/or (+)-isomenthone to a menthol isomer.

Further specific exemplary embodiments of methods of the present invention are recited below:

(1) A method for producing a menthol isomer comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to a menthol isomer.

(2) A method for producing a menthol isomer comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to a menthol isomer.

(3) A method for producing a menthol isomer comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the limonene to a menthol isomer.

(4) A method for producing a menthol isomer comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to a menthol isomer.

(5) A method for producing a menthol isomer comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase, a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to a menthol isomer.

(6) A method for producing a menthol isomer comprising providing (+)-*cis*-isopulegone, providing a ketosteroid isomerase, a pulegone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to a menthol isomer.

(7) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a pulegone reductase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to (-)-menthone and/or (+)-isomenthone.

(8) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a pulegone reductase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to (-)-menthone and/or (+)-isomenthone.

(9) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a pulegone reductase, and maintaining the composition under conditions suitable for conversion of the limonene to (-)-menthone and/or (+)-isomenthone.

(10) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a pulegone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to (-)-menthone and/or (+)-isomenthone.

(11) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase, a ketosteroid isomerase and a pulegone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to (-)-menthone and/or (+)-isomenthone.

(12) A method for producing (-)-menthone and/or (+)-isomenthone comprising providing (+)-*cis*-isopulegone, providing a ketosteroid isomerase and a pulegone reductase, and maintaining the

composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to (-)-menthone and/or (+)-isomenthone.

(13) A method for producing (+)-menthofuran comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to (+)-menthofuran.

(14) A method for producing (+)-menthofuran comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to (+)-menthofuran.

(15) A method for producing (+)-menthofuran comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the limonene to (+)-menthofuran.

(16) A method for producing (+)-menthofuran comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase, a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to (+)-menthofuran.

(17) A method for producing (+)-menthofuran comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase, a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to (+)-menthofuran.

(18) A method for producing (+)-menthofuran comprising providing (+)-*cis*-isopulegone, providing a ketosteroid isomerase and a (+)-menthofuran synthase or a cytochrome P450 reductase, and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to (+)-menthofuran.

(19) A method for producing (*R*)-(+)-pulegone comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to (*R*)-(+)-pulegone.

(20) A method for producing (*R*)-(+)-pulegone comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to (*R*)-(+)-pulegone.

(21) A method for producing (*R*)-(+)-pulegone comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the limonene to (*R*)-(+)-pulegone.

(22) A method for producing (*R*)-(+)-pulegone comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to (*R*)-(+)-pulegone.

(23) A method for producing (*R*)-(+)-pulegone comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase and a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to (*R*)-(+)-pulegone.

(24) A method for producing (*R*)-(+)-pulegone comprising providing (+)-*cis*-isopulegone, providing a ketosteroid isomerase, and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

(25) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(26) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(27) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the limonene to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(28) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(29) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase and a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(30) A method for producing (*S*)-(-)-pulegone and/or *trans*-isopulegone comprising providing (+)-*cis*-isopulegone, providing a glutathione S-transferase, and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to (*S*)-(-)-pulegone and/or *trans*-isopulegone.

(31) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing dimethylallyl diphosphate and/or isopentyl diphosphate, providing a geranyl diphosphate synthase, a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the dimethylallyl diphosphate and/or isopentyl diphosphate to an isopulegol isomer.

(32) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing geranyl diphosphate, providing a limonene synthase, a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the geranyl diphosphate to an isopulegol isomer.

(33) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing limonene, providing a limonene hydroxylase or a cytochrome P450 reductase, an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the limonene to an isopulegol isomer.

(34) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing (-)-*trans*-isopiperitenol, providing an isopiperitenol dehydrogenase, a (-)-isopiperitenone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-*trans*-isopiperitenol to an isopulegol isomer.

(35) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing (-)-isopiperitenone, providing a (-)-isopiperitenone reductase and a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (-)-isopiperitenone to an isopulegol isomer.

(36) A method for producing an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol) comprising providing (+)-*cis*-isopulegone, providing a menthone reductase, and maintaining the composition under conditions suitable for conversion of the (+)-*cis*-isopulegone to an isopulegol isomer.

In some embodiments the enzymes are provided sequentially. In some embodiments the enzymes are provided simultaneously (i.e. together). In some embodiments the enzymes are provided simultaneously by sequentially adding enzyme/enzymes (i.e. one after another).

Factors relevant to the conditions suitable for the relevant conversion or conversions in accordance with the methods of the present invention include the enzyme(s), the substrate(s), the activity of the enzyme(s), the concentration of the enzyme(s), the concentration of the substrate(s), enzyme co-factor concentration/availability, temperature, salinity, pH, agitation, carbon dioxide levels, oxygen levels, nutrient availability, reaction volume, etc.

Suitable conditions for a given conversion or combination of conversions in accordance with the methods of the present invention can be readily determined by the skilled person with reference e.g. to the experimental examples of the present application and the references identified herein, as appropriate to the desired reaction products.

For example, enzymes may be provided at different ratios to favor certain activities and/or desired reaction products. By way of illustration, in Example 3 of the present application the inventors use a molar excess of ketosteroid isomerase relative to menthone reductase to favor conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone over conversion of (+)-*cis*-isopulegone to an isopulegol isomer (e.g. (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and/or (+)-neo-isopulegol).

In some embodiments, one or more co-factors may be provided to the reaction(s). In some embodiments, one or more sources of co-factors are provided. In some embodiments systems for producing/recycling one or more co-factors or sources of co-factors are provided. In some embodiments the co-factor is selected from NADH, NAD+, NADPH, NADP+ and/or glutathione.

In some embodiments the recycling system comprises a carbohydrate and a dehydrogenase capable of catalysing the removal of hydrogen [2H] from the carbohydrate. In some embodiments the cofactor recycling system comprises glucose and a glucose dehydrogenase.

In some embodiments, the methods of the present invention are performed using isolated/purified enzyme(s). In some embodiments the enzyme(s) are obtained from a commercial source. In some embodiments the enzyme(s) may be, or may have been, expressed recombinantly and subsequently isolated/purified, e.g. as described herein. In some embodiments the enzyme(s) may be obtained from an organism (e.g. a microorganism) or cells, tissue or organs of a multicellular organism expressing the enzyme(s). In some embodiments, the enzyme(s) may be obtained from an organism or cells, tissue or organs of a multicellular organism expressing the enzyme(s) as a consequence of expression of nucleic acid endogenous to the organism. In some embodiments, the enzyme(s) may be obtained from an organism expressing the enzyme(s) as a consequence of expression of heterologous nucleic acid that is non-endogenous to the organism (e.g. a nucleic acid/expression vector according to the present invention). In some embodiments, the organism or cells, tissue or organs of a multicellular organism expressing the enzyme(s) may secrete the enzyme(s). In some embodiments, obtaining the enzyme(s) may comprise isolating/purifying the enzyme(s) from an organism (e.g. a microorganism) or cells, tissue or organs of a multicellular organism expressing the enzyme(s), or from secreted products thereof.

In some embodiments, the methods of the present invention are performed using extract(s) of an organism (e.g. a microorganism) or cells, tissue or organs of a multicellular organism expressing the enzyme(s). Extracts are prepared such that the enzyme(s) retain the relevant activity. In some embodiments, the extract(s) may be prepared from organism or cells, tissue or organs of a multicellular organism expressing the enzyme(s) as a consequence of expression of nucleic acid endogenous to the organism. In some embodiments, the extract(s) may be prepared from organism or cells, tissue or organs of a multicellular organism expressing the enzyme(s) as a consequence of expression of heterologous nucleic acid that is non-endogenous to the organism (e.g. a nucleic acid/expression vector according to the present invention). Preparation of extracts may include one or more of: homogenising the organ/tissue/cells, lysing the cells (e.g. with a lysis buffer), removing cell debris, etc.

In some embodiments the methods of the present invention are performed using live or whole cells (e.g. intact respiring cells). In some embodiments, the enzyme(s) and substrate(s) may contact one another inside a cell. In some embodiments the substrate may be produced by the cell. In some embodiments the substrate/a precursor thereof may diffuse into the cell. In some embodiments the substrate/a precursor thereof may be taken up by the cell, e.g. by active transport across the cell membrane. In some embodiments the enzyme(s) may be secreted from live cells, and the enzyme(s) and substrate(s) may contact one another outside of a cell.

Nucleic acids, expression vectors, cells and compositions

The present invention also provides a nucleic acid, or a plurality of nucleic acids, encoding a ketosteroid isomerase and one or more of: a pulegone reductase, a menthone reductase, a (-)-isopiperitenone reductase, an isopiperitenol dehydrogenase, a limonene hydroxylase, a cytochrome P450 reductase, a limonene synthase and a geranyl diphosphate synthase.

Also provided is nucleic acid encoding a ketosteroid isomerase according to the present invention. Also provided is nucleic acid encoding a glutathione S-transferase according to the present invention. Also provided is nucleic acid encoding a menthone reductase according to the present invention.

In some embodiments the nucleic acid is DNA. In some embodiments the nucleic acid is RNA. The nucleic acid may be single-stranded or double-stranded. The nucleic acid may be provided in isolated/purified form, or within a host cell.

In general, short polynucleotides can be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art. Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. In some embodiments this will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the gene which it is desired to clone, bringing the primers into contact with DNA, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA.

The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector. Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.,* 2001 , Molecular Cloning: a laboratory manual, 3rd edition, Cold Harbour Laboratory Press. Alternatively, InFusion cloning (described e.g. in Throop and LaBear, Curr Protoc Mol Biol. (2015) 110: 3.20.1–3.20.23) or other cloning techniques may be used, such as Gibson Assembly (Gibson et al., Nat. Methods 2009; 6, 343-345), CRISPR/Cas9-based methods (Wang et al., (2015) BioTechniques 58:161-170), Sequence and Ligation Independent Cloning (SLIC; Nucleic Acids Res. 2012, 40: e55) and Modular Overlap-Directed Assembly with Linkers (MODAL; Nucleic Acids Res. (2014) 42.1: e7-e7).

The present invention further provides a vector, particularly an expression vector, comprising a nucleic acid or plurality of nucleic acids according to the present invention. The vector may be used to replicate the nucleic acid in a compatible host cell. Therefore, nucleic acids according to the present invention can be produced by introducing a polynucleotide into a replicable vector, introducing the vector into a compatible host cell and growing the host cell under conditions that bring about replication of the vector.

A “vector” as used herein is an oligonucleotide molecule (DNA or RNA) used as a vehicle to transfer foreign genetic material into a cell. The vector may be an expression vector for expression of the foreign genetic material in the cell. Such vectors may include a promoter and/or a ribosome binding site (RBS) sequence operably linked to the nucleotide sequence encoding the sequence to be expressed. A vector may also include a termination codon and expression enhancers. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, RBS, enhancers and other elements, such as for example

polyadenylation signals, which may be necessary and which are positioned in the correct orientation in order to allow for protein expression.

Any suitable vectors, promoters, enhancers and termination codons known in the art may be used to express a polypeptide from a vector according to the invention. In some embodiments, the vector may be a plasmid, phage, MAC, virus, etc.

In some embodiments the vector may be a prokaryotic expression vector, e.g. a bacterial expression vector. In some embodiments the vector is a pBb vector, e.g. as described in Lee et al., J Biol Eng. (2011) 5:12, hereby incorporated by reference in its entirety. In some embodiments the vector may be a pET21b plasmid.

In some embodiments, the vector may be a eukaryotic expression vector. In some embodiments, the vector may be a eukaryotic expression vector, e.g. a vector comprising the elements necessary for expression of protein from the vector in a eukaryotic cell. In some embodiments, the vector may be a mammalian expression vector, e.g. comprising a cytomegalovirus (CMV) or SV40 promoter to drive protein expression.

Other suitable vectors would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook *et al.,* 2001, Molecular Cloning: a laboratory manual, 3rd edition, Cold Harbour Laboratory Press.

The term “operably linked” may include the situation where a selected nucleotide sequence and regulatory nucleotide sequence (e.g. promoter and/or enhancer) are covalently linked in such a way as to place the expression of the nucleotide sequence under the influence or control of the regulatory sequence (thereby forming an expression cassette). Thus a regulatory sequence is operably linked to the selected nucleotide sequence if the regulatory sequence is capable of effecting transcription of the nucleotide sequence. The resulting transcript may then be translated into a desired peptide or polypeptide. The promoter may be a T7 promoter.

In some embodiments, the vector may comprise element for facilitating translation of encoded protein from mRNA transcribed from the construct. For example, the construct may comprise a ribosomal binding site (RBS) such as a Shine-Delgarno (SD) sequence upstream of the start codon.

In some embodiments, RBS sequences may be designed to provide for different levels of expression of the encoded proteins. For example, in Example 4 of the present disclosure the inventors fine-tune relative expression of proteins encoded by the same vector through design of RBS sites predicted (according to the methodology of Salis *et al.*[31,32]) to have different translation initiation rates (TIRs).

In some embodiments, the vector may encode one or more response elements for modulating expression of the encoded protein(s). In some embodiments, the response element is an element that causes

upregulation of gene or protein expression in response to treatment with a particular agent. For example, the agent may induce transcription of DNA encoding the protein(s) from a vector including a response element for the agent. In some embodiments the agent may be isopropyl β-D-1-thiogalactopyranoside (IPTG), and the vector may comprise a *lac* operator. Other induction agent/response element combinations are known in the art.

In some embodiments, the vector may encode one or more response elements for constitutive expression of the encoded protein(s), such that no induction is necessary.

In some embodiments the vector may comprise a transcription terminator sequence downstream of the sequences encoding to the protein or proteins of interest. In some embodiments the terminator may be a T7 terminator sequence. In some embodiments the vector may comprise a sequence encoding a detectable marker in-frame with the sequence encoding the protein of interest to facilitate detection of expression of the protein, and/or purification or isolation of the protein (e.g. a His, (e.g. 6XHis), Myc, GST, MBP, FLAG, HA, E, or Biotin tag, optionally at the N- or C- terminus).

Also provided by the present invention is a cell comprising a ketosteroid isomerase, a nucleic acid or plurality of nucleic acids, or an expression vector according to the present invention.

The nucleic acids/expression vectors can be introduced into a cell by any suitable means, which are well known to the skilled person. In some embodiments the nucleic acids/expression vectors are introduced into a cell by transformation, transduction, conjugation, transfection or electroporation.

A cell comprising a ketosteroid isomerase according to the present invention may do so through expression from a nucleic acid/expression vector according to the present invention that has been introduced into the cell.

Cells contemplated for use with the present invention include prokaryotic and eukaryotic cells. For example, the prokaryotic cell may be a bacteria or archaea, and the eukaryotic microorganism may be a fungi, protist, or microscopic animal or microscopic plant organism.

Microorganisms commonly used in commercial and industrial processes are contemplated, including microorganisms used for the commercial or industrial production of chemicals, enzymes or other biological molecules. In some embodiments the bacteria may be Gram-negative bacteria. Gram-negative bacteria may be defined as a class of bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation, making positive identification possible. Gram-negative bacteria include proteobacteria or bacteria of the family *Enterobacteriaceae*, such as *Escherichia coli*, *Salmonella* sp, *Shigella* sp, or bacteria selected from the genus *Pseudomonas*, *Helicobacter*, *Neisseria*, *Legionella*, *Klebsiella* or *Yersinia*.

In some embodiments, the bacteria may be Gram-positive bacteria. Gram-positive bacteria include bacteria from the genus *Bacillus* or *coccus*, such as bacteria from the genus *Listeria*, *Clostridium* (e.g. *C.difficile*), *Staphylococcus* (e.g. *S.aureus*), or *Streptococcus*.

In some embodiments, the fungi may blastocladiomycota, chytridiomycota, glomeromycota, microsporidia, or neocallimastigomycota. In some embodiments, the fungi may be dikarya (including deuteromycota), such as fungi of the ascomycota, including pezizomycotina, saccharomycotina, and taphrinomycotina; or basidiomycota, including agaricomycotina, pucciniomycotina, and ustilaginomycotina. In some embodiments, the fungi may be fungi of the entomophthoromycotina, kickxellomycotina, mucoromycotina, or zoopagomycotina.

In particular embodiments, *Escherichia* bacteria such as *E. coli*, *Saccharomyces* yeast such as *S. cerevisiae* and cyanobacteria are contemplated for use in the present invention. In some embodiments the polypeptides may be prepared by cell-free-protein synthesis (CFPS), e.g. according using a system described in Zemella et al. Chembiochem (2015) 16(17): 2420-2431, which is hereby incorporated by reference in its entirety.

The present invention also provides a method for producing a composition according to the invention, comprising (i) culturing a cell according to the present invention under conditions suitable for expression of encoded protein(s). In some embodiments the method further comprises (ii) isolating said expressed protein(s). The invention also encompasses the compositions produced by such methods.

The present invention also provides compositions comprising the cells, nucleic acids, expression vectors, and enzymes/combinations of enzymes according to the present invention. The compositions find use e.g. in methods for monoterpenoid biosynthesis according to the present invention.

Recombinant production of polypeptides

The polypeptides according to the present invention may be prepared according to methods for recombinant protein production known to the skilled person. Molecular biology techniques suitable for recombinant production are well known in the art, such as those set out in Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th Edition), Cold Spring Harbor Press, 2012, which is hereby incorporated by reference in its entirety.

Expression may be from a nucleic acid sequence and/or an expression vector, e.g. a nucleic acid sequence or expression vector according to the present invention. Any suitable vectors, promoters, enhancers and termination codons known in the art may be used to express a peptide or polypeptide from an expression vector according to the invention. Expression may be from a cell according to the present invention. Any cell suitable for the expression of polypeptides may be used.

Production may involve culture or fermentation of cell modified to express the relevant polypeptide(s). The culture or fermentation may be performed in a bioreactor provided with an appropriate supply of

nutrients, air/oxygen and/or growth factors. Secreted proteins can be collected by partitioning culture media/fermentation broth from the cells, extracting the protein content, and separating individual proteins to isolate secreted or expressed peptide or polypeptide. Culture, fermentation and separation techniques are well known to those of skill in the art, and are described, for example, in Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th Edition; incorporated by reference herein above).

Bioreactors include one or more vessels in which cells may be cultured. Culture in the bioreactor may occur continuously, with a continuous flow of reactants into, and a continuous flow of cultured cells from, the reactor. Alternatively, the culture may occur in batches. The bioreactor monitors and controls environmental conditions such as pH, oxygen, flow rates into and out of, and agitation within the vessel such that optimum conditions are provided for the cells being cultured.

Following culturing the cells that express the polypeptide(s) of interest may be isolated. Any suitable method for separating proteins from cells known in the art may be used. In order to isolate the polypeptide it may be necessary to separate the cells from nutrient medium.

If the polypeptide(s) are secreted from the cells, the cells may be separated from the culture media that contains the secreted polypeptide(s) of interest by centrifugation.

If the polypeptide(s) of interest collect within the cell, protein isolation may comprise centrifugation to separate cells from cell culture medium, treatment of the cell pellet with a lysis buffer, and cell disruption e.g. by sonification, rapid freeze-thaw or osmotic lysis.

It may then be desirable to isolate the polypeptide(s) of interest from the supernatant or nutrient medium, which may contain other protein and non-protein components.

One approach to separating protein components from a supernatant or culture medium is by precipitation. Proteins of different solubilities are precipitated at different concentrations of precipitating agent such as ammonium sulfate. For example, at low concentrations of precipitating agent, water soluble proteins are extracted. Thus, by adding different increasing concentrations of precipitating agent, proteins of different solubilities may be distinguished. Dialysis may be subsequently used to remove ammonium sulfate from the separated proteins. Other methods for separating protein components include ion exchange chromatography and size chromatography. These may be used as an alternative to precipitation, or may be performed subsequently to precipitation.

Once the polypeptide(s) of interest have been isolated from the culture it may be desired or necessary to concentrate the peptide or polypeptide. A number of methods for concentrating proteins are known in the art, such as ultrafiltration and lyophilisation.

It will be appreciated that the polypeptides according to the present invention may be provided as components of larger polypeptides or polypeptide complexes. For example, the polypeptides described

herein may be provided as fusion polypeptides. In some embodiments the polypeptides may comprise amino acid sequence(s) to facilitate expression, folding, trafficking, processing or purification, e.g. His, (e.g. 6XHis), Myc GST, MBP, FLAG, HA, E, or Biotin tag, optionally at the N- or C- terminus.

Sequence identity

Pairwise and multiple sequence alignment for the purpose of determining percent identity between two or more amino acid or nucleic acid sequences can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalOmega (Söding, J. 2005, Bioinformatics 21, 951-960), T-coffee (Notredame et al. 2000, J. Mol. Biol. (2000) 302, 205-217), Kalign (Lassmann and Sonnhammer 2005, BMC Bioinformatics, 6(298)) and MAFFT (Katoh and Standley 2013, Molecular Biology and Evolution, 30(4) 772–780 software. When using such software, the default parameters, e.g. for gap penalty and extension penalty, are preferably used.

Sequences

SEQ ID NO:	DESCRIPTION	SEQUENCE
1	Ketosteroid isomerase from *Pseudomonas putida* (UniProt: P07445-1, v1)	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCALDVIDVMRFDEHGRIQTMQAYWSEV NLSVREPQ
2	Ketosteroid isomerase variant D103S	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCALDVISVMRFDEHGRIQTMQAYWSEV NLSVREPQ
3	Ketosteroid isomerase variant L99I	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCAIDVIDVMRFDEHGRIQTMQAYWSEV NLSVREPQ
4	Ketosteroid isomerase variant L99V	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCAVDVIDVMRFDEHGRIQTMQAYWSEV NLSVREPQ
5	Ketosteroid isomerase variant V88I-L99V	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRIEMVWNGQPCAVDVIDVMRFDEHGRIQTMQAYWSEV NLSVREPQ
6	Ketosteroid isomerase variant V88I-L99V-D103S	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRIEMVWNGQPCAVDVISVMRFDEHGRIQTMQAYWSEV NLSVREPQ
7	Ketosteroid isomerase variant L99V-D103S	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCAVDVISVMRFDEHGRIQTMQAYWSEV NLSVREPQ
8	Ketosteroid isomerase variant L99I-D103S	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCAIDVISVMRFDEHGRIQTMQAYWSEV NLSVREPQ
9	Ketosteroid isomerase variant V88I-L99V-V101A-D103S	MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQGLGG GKVRACLTGPVRASHNGCGAMPFRIEMVWNGQPCAVDAISVMRFDEHGRIQTMQAYWSEV NLSVREPQ
10	Human Glutathione S-transferase A3 (UniProt: Q16772-1, v3)	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPADAKALEEARKIFRF
11	Human Glutathione S-transferase A3 variant A208F	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPFDAKALEEARKIFRF
12	Human Glutathione S-transferase A3	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT

	variant A216L	VKKFLQPGSPRKPPADAKALEELRKIFRF
13	Human Glutathione S-transferase A3 variant A208F/A216L	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPFDAKALEELRKIFRF
14	Human Glutathione S-transferase A3 variant L111D	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPDCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPADAKALEEARKIFRF
15	Human Glutathione S-transferase A3 variant F222W	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAILNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADISLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPADAKALEEARKIFRW
16	Human Glutathione S-transferase A3 variant L72R/S159Y	MAGKPKLHYFNGRGRMEPIRWLLAAAGVEFEEKFIGSAEDLGKLRNDGSLMFQQVPMVEIDG MKLVQTRAIRNYIASKYNLYGKDIKERALIDMYTEGMADLNEMILLLPLCRPEEKDAKIALIKEKT KSRYFPAFEKVLQSHGQDYLVGNKLSRADIYLVELLYYVEELDSSLISNFPLLKALKTRISNLPT VKKFLQPGSPRKPPADAKALEEARKIFRF
17	*Mentha piperita* (-)-menthone:(-)menthol reductase (MMR; UniProt: Q5CAF4-1, v1)	MADTFTQRYALVTGANKGIGFEICRQLASKGMKVILASRNEKRGIEARERLLKESRSISDDDVV FHQLDVADPASAVAVAHFIETKFGRLDILVNNAGFTGVAIEGDISVYQECLEANIIAAQGGQAHP FHPKTTGRLIETLEGSKECIETNYYGTKRITETLIPLLQKSDSPTIVNVSSTFSTLLLQPNEWAKG VFSSNSLNEGKVEEVLHEFLKDFIDGKLQQNHWPPNFAAYKVSKAAVNAYTRIIARKYPSFCIN SVCPGFVRTDICYNLGVLSEAEGAEAPVKLALLPDGGPSGSFFSREEALSLY
18	*Mentha piperita* (*R*)-(+)-pulegone reductase (PulR; UniProt: Q6WAU0-1, v1	MVMNKQIVLNNYINGSLKQSDLALRTSTICMEIPDGCNGAILVKNLYLSVNPYLILRMGKLDIPQ FDSILPGSTIVSYGVSKVLDSTHPSYEKGELIWGSQAGWEEYTLIQNPYNLFKIQDKDVPLSYY VGILGMPGMTAYAGFFEICSPKKGETVFVTAAAGSVGQLVGQFAKMFGCYVVGSAGSKEKVD LLKNKFGFDDAFNYKEESDYDTALKRHFPEGIDIYFDNVGGKMLEAVINNMRVHGRIAVCGMV SQYSLKQPEGVHNLLKLIPKQIRMQGFVVVDYYHLYPKFLEMVLPRIKEGKVTYVEDISEGLES APSALLGVYVGRNVGNQVVAVSRE
19	Ketosteroid isomerase from *Comamonas testosteroni* (UniProt: P00947-1, v2)	MNTPEHMTAVVQRYVAALNAGDLDGIVALFADDATVEDPVGSEPRSGTAAIREFYANSLKLPL AVELTQEVRAVANEAAFAFTVSFEYQGRKTVVAPIDHFRFNGAGKVVSMRALFGEKNIHAGA
20	*Streptomyces sp. AA4* (UniProt: D9VBV6-1, v1)	MTAAPHPARTASQALHAAVRRKDKEAWLELFAADAVVEDPVGPSPFDPEGRGHRGRDAISA FWDRAIAPNESIEFLFDDSFACGDEVAFTGQVRSQHDGQVLEAEGVFVYRVDKSGQIVAVRA FWEVERTLRTARPA
21	KSIt11F	AAGGAGATATACATATGAATCTGCCGACCGCCCAGGAAGTGCAGGGTCTGATGGCGCGT TATATTGAACTGGTGG
22	KSIt12R	TCCACGGTCGCATCATCCGCATACATCTGCACAATCGCTTCAATATCGCCCACATCCACC AGTTCAATATAACGC
23	KSIt13F	TGATGCGACCGTGGAAGATCCGTTTGGCCAGCCTCCGATTCACGGCCGCGAACAGATCG CGGCGTTTTACCGCCA
24	KSIt14R	TATGGCTGGCACGCACCGGACCGGTCAGACACGCGCGCACTTTACCGCCGCCCAGGCC CTGGCGGTAAAACGCC
25	KSIt15F	TGCGTGCCAGCCATAATGGCTGCGGCGCGATGCCGTTTCGCGTGGAAATGGTGTGGAAT GGTCAACCATGTGCGCT
26	KSIt16R	ACGCTTGCATGGTTTGAATGCGGCCATGTTCATCAAAGCGCATCACATCAATCACATCCA GCGCACATGGTTGAC
27	KSIt17F	ATTCAAACCATGCAAGCGTATTGGAGCGAAGTGAATCTGAGCGTGCGCGAACCGCAGCT CGAGCACCACCACC
28	KSIt18R	GGTGGTGGTGCTCGA
29	KSI RBS sequence	TTTGTTTAACTTTAAGAAGGAGA
30	PgPGR RBS sequence	TTTGTTTAACTTTAAGAAGGAGA
31	MMR RBS sequence 1	CCCATTAAAATTTAAGCTTTATATAC
32	MMR RBS sequence 2	GACTCATCAATAAAGGTTACTTCCT
33	MMR RBS sequence 3	ATTTTCACATACGTCTACTT
34	Active site of	FRVEMVWNGQPCALDVIDVMRFDEHGR

	Ketosteroid isomerase from *Pseudomonas putida*	
35	Ketosteroid isomerase active site consensus	FRVTVTAGGHRMVIEPIDVMVFDEDGK
36	Active site subregion of Ketosteroid isomerase from *Pseudomonas putida*	FRVEMVWNGQPCALDVIDVMRF
37	Ketosteroid isomerase active site subregion consensus	FRVDMEWGGQPCSLHVIDVMEF
38	Ketosteroid isomerase consensus	MSAEAITATVNRYIELVAKGDADDIVXLYADDATVEDPVGGEVHIGRQAIRGFYSAVEGLQREC ELLTLRVAGNEAAFQFRVTVTAGGHRMVIEPIDVMVFDEDGKIASMKAYWSPADVTQL wherein 'X' can be any amino acid
39	*Pseudomonas putida* Ketosteroid isomerase gene sequence	ATGAATCTGCCGACCGCCCAGGAAGTGCAGGGTCTGATGGCGCGTTATATTGAACTGGT GGATGTGGGCGATATTGAAGCGATTGTGCAGATGTATGCGGATGATGCGACCGTGGAAG ATCCGTTTGGCCAGCCTCCGATTCACGGCCGCGAACAGATCGCGGCGTTTTACCGCCAG GGCCTGGGCGGCGGTAAAGTGCGCGCGTGTCTGACCGGTCCGGTGCGTGCCAGCCATA ATGGCTGCGGCGCGATGCCGTTTCGCGTGGAAATGGTGTGGAATGGTCAACCATGTGCG CTGGATGTGATTGATGTGATGCGCTTTGATGAACATGGCCGCATTCAAACCATGCAAGCG TATTGGAGCGAAGTGAATCTGAGCGTGCGCGAACCGCAG

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The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word "comprise," and variations such as "comprises" and "comprising," will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent "about," it will be understood that the particular value forms another embodiment.

Where a nucleic acid sequence is disclosed herein, the reverse complement thereof is also expressly contemplated.

Brief Description of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures.

Figures 1A and 1B. Schematics showing pathways for monoterpenoid biosynthesis. IDI = isopentenyl-diphosphate Delta-isomerase; GPPS = geranyl diphosphate synthase; LimS = (-)-limonene synthase; L3H = (-)-limonene-3-hydroxylase; CPR = cytochrome P450 reductase; IPDH = (-)-*trans*-isopiperitenol dehydrogenase; IPR = (-)-isopiperitenone reductase; IPGI = (+)-*cis*-isopulegone isomerase; PGR = (*R*)-(+)-pulegone reductase; MMR = (-)-menthone:(-)menthol reductase; MNMR = menthone:(+)-neomenthol reductase; MFS = (+)-menthofuran synthase; L6H = (-)-limonene-6-hydroxylase; CDH = (-)-*trans*-carveol dehydrogenase.

Figure 2. Schematic showing the proposed mechanism of ketosteroid isomerisation of (+)-*cis*-isopulegone.

Figures 3A and 3B. Table and graphs relating to conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone by wildtype KSI and KSI variants. (**3A**) Table summarising the results of analysis of Δ^5-3-ketosteroid isomerase (KSI) catalyzed conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone by wildtype KSI and KSI variants comprising the indicated amino acid substitutions. (**3B**) Graphs showing enantiomeric identity of pulegone product produced by conversion of (+)-*cis*-isopulegone with wildtype KSI and variant KSI as determined by chiral GC.

Figure 4. Table summarising ambiguous codons used to generate KSI variants.

Figure 5. Schematic summarising the robotics protocols enabling the automated selection of KSI variants having improved (+)-*cis*-isopulegone to (*R*)-(+)-pulegone isomerase activity.

Figures 6A and 6B. Schematic and bar chart relating to key active site positions implicated in improving KSI activity towards (+)-*cis*-isopulegone. (**6A**) Residues located in the equilenin-binding region of wild type KSI from *P. putida* (PDB: 1OH0)[35]. The residues and equilenin are shown as atom sticks of carbons. Interactions are shown as dotted lines. The backbone is shown as a ribbon structure. (**6B**) Bar chart showing comparative specific activity (SA) of wildtype KSI and KSI variants comprising the indicated amino acid substitutions for conversion (+)-*cis*-isopulegone to (*R*)-(+)-pulegone. Reactions (100 µL) were composed of 50 mM Tris pH 7.0 containing 1 mM (+)-cis-isopulegone. Absorbance was monitored at 260 nm for 1 h at 20 °C.

Figures 7A to 7F. Overlay of structures, schematics and tables relating to structural analysis of wildtype *P. putida* KSI and variants. (**7A** and **7B**) Overlay of energy minimised structure and

representative structure from MD simulations for (**7A**) WT and (**7B**) V88I/L99V/V101A/D103S variant of *P. putida* KSI with bound (+)-*cis*-isopulegone. The solvent accessible surface areas for the substrate and residues 88, 99 and 101 from the representative and minimised structures, respectively are shown as transparent surfaces, with dotted lines to illustrate potential steric clashes between the substrate and enzyme. The conformation of the β-hairpin over the course of the simulation is shown using a backbone trace of residues 90-97 every 2 ns after thermalisation. (**7C**) Schematic showing the relative arrangement of the indicated positions and substitutions in the structure of KSI. (**7D**) KSI data collection and refinement statistics for the crystal structures of the variants of *P. putida* KSI. (**7E**) Superimposition of the crystal structures of wildtype KSI and D103S, V88I/L99V, V88I/L99V/D103S and V88I/L99V/V101A/D103S. (**7F**) Distances restrained during MD simulations of wildtype KSI. The energy-minimised structure is shown, with key residues as atom sticks of carbons. The bound (+)-*cis*-isopulegone is shown as balls and sticks.

Figures 8A and 8B. Table and graph relating to analysis of conversion of the indicated substrates to the indicated monoterpenoids, by different enzymes and combinations of enzymes. (**8A**) Table showing results of conversion of the indicated substrates to the indicated monoterpenoids, by different enzymes and combinations of enzymes. Reactions (200 µL) were performed in 50 mM Tris pH 7.0 containing 1 mM monoterpenoid substrate, enzyme(s) and cofactor recycling system (10 U Sigma glucose dehydrogenase, 10 µM NADP+ and 15 mM D-glucose). After a 24 h incubation at 30°C (180 rpm), reactions were extracted with 180 µL ethyl acetate containing 0.01% *sec*-butylbenzene and dried with anhydrous $MgSO_4$. Products were analysed by GC-MS using a DB-WAX column. Reactions with individual enzymes had enzyme concentrations of 10 µM. The *in vitro* cascading reaction had enzyme concentrations of 10, 2 and 0.3 µM for KSI variant, MpPGR and MMR, respectively. The cell extract and whole cell slurry volumes in the cascading reactions were 50 µL. [1]KSI variant = V88I/L99V/V101A/D103S; [2]Additional products were detected that were not quantified (e.g. trans-isopulegone and (+)-neoiso-isopulegol). Substrates are shown in bold. N/A = not applicable. (**8B**) Graphs showing separation of (-)-menthol and precursors by GC-MS. Analysis of monoterpenoids was performed by GC-MS using a DB-WAX column.

Figures 9A and 9B. Graph and schematic relating to conversion of (+)-*cis*-isopulegone by *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR) to isopulegol isomers. (**9A**) Graph showing the results of GC-MS analysis of products of conversion of (+)-*cis*-isopulegone to (+)-neoiso-isopulegol and (-)-isopulegol by *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR). (**9B**) Schematic showing proposed mechanism of action of the NADPH-dependent MMR-catalysed reduction of (+)-*cis*-isopulegone to (+)-neoiso-isopulegol (adapted from the proposed SDR ketoreductase mechanism of MNMR with (–)-menthone[58]).

Figure 10. Schematic representation of the construct comprising DNA encoding the KSI variant V88I/L99V/V101A/D103S, *Mentha piperita* pulegone reductase (MpPGR) and *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR) in pET21b.

Figure 11. Table showing the ribosome binding sites (RBSs) used for the different enzymes, in different constructs, and the predicted translation initiation rates (TIR) for the different RBSs.

Figure 12. Table summarising the analysis of conversion of isopiperitenone to the indicated monoterpenoids by wildtype human glutathione S-transferase A3 (GSTA3-3) and variants comprising the indicated amino acid substitutions.

Figure 13. Table summarising the analysis of conversion of (+)-*cis*-isopulegone to the indicated monoterpenoids by (-)-isopiperitenone reductase and wildtype human glutathione S-transferase A3 (GSTA3-3) and variants comprising the indicated amino acid substitutions.

Figure 14. Amino acid sequence alignment of Δ^5-3-ketosteroid isomerase from *Comamonas testosteroni* (UniProt: P00947-1, v2; SEQ ID NO:19; Ctes) and Δ^5-3-ketosteroid isomerase from *Pseudomonas putida* (UniProt: P07445-1, v1; SEQ ID NO:1; Pput).

Figure 15. Amino acid sequence alignment of ketosteroid isomerases from different species. The ketosteroid isomerases are identified by UniProt accession number.

Figure 16. Amino acid sequence alignment of the active site of ketosteroid isomerases from different species. The ketosteroid isomerases are identified by UniProt accession number.

Figure 17. Amino acid sequence alignment of a subregion of the active site of ketosteroid isomerases from different *Pseudomonas* species. The ketosteroid isomerases are identified by UniProt accession number.

Examples

In the following Examples, the inventors demonstrate that Δ^5-3-ketosteroid isomerase (KSI) from *Pseudomonas putida* can perform isomerization of (+)-*cis*-isopulegone, exclusively producing the (*R*)-(+)-pulegone enantiomer required for downstream production of (−)-menthol. Through a robotics-driven directed evolution strategy a KSI variant having four active site mutations was obtained, which confer a 5.6-fold increase in activity over the wildtype KSI and producing a total (*R*)-(+)-pulegone yield of 89%. The inventors show that this variant can perform efficiently within a cascade catalysis reaction and using cell extracts, producing 14% and 11% total yield of (−)-menthol, respectively.

The inventors provide the first identification and characterization of a non-*Mentha* (+)-*cis*-isopulegone isomerase, and variants engineered to function efficiently within a biosynthetic pathway for the production of (−)-menthol using one-pot cell extract, whole cell and cascade catalysis approaches.

Example 1: Identification of KSI as (+)-*cis*-isopulegone isomerase

The inventors hypothesized that the native enzyme for conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone might utilise a mechanism similar to ketosteroid isomerases, where proton abstraction causes a

dienolate intermediate stabilised by H-bonding in the oxyanion hole, followed by reprotonation of the substrate to form a conjugated system (see **Figure 2**).

The inventors investigated two bacterial ketosteroid isomerases, one from *Streptomyces* sp. AA4 and one from *Pseudomonas putida*, to test for isopulegone isomerase activity.

(+)-*cis*-Isopulegone substrate was synthesised using a combination of chemical and enzyme transformation steps. Roughly 15% (*R*)-(+)-pulegone was present in all substrate samples, owing to its more stable bond configuration.

Following expression and purification in *E. coli*, steady-state enzyme assays were performed using 1 mM (+)-*cis*-isopulegone substrate.

Briefly, purified enzyme eluate was buffer exchanged in 50 mM Tris buffer (pH 7.0) and concentrated using Vivaspin 500 (Sartorius) prior to use in the assay. Reactions (200 µL) were performed in 50 mM Tris pH 7.0 containing 1 mM (+)-*cis*-isopulegone and 10 µM KSI and incubated at 30 °C for 24 h (180 rpm). Samples were then prepared for GC-MS analysis by solvent extraction with 180 µL ethyl acetate containing 0.01% sec-butylbenzene (Sigma) as the internal standard, and dried with anhydrous $MgSO_4$. Products were analysed by GC-MS using a DB-WAX column. Yield show in column 5 is the amount of (*R*)-(+)-pulegone in µM produced per µM of KSI in 24 hours.

Chiral product analysis was performed by analysing reactions by GC using an Agilent Technologies 7890A GC system with an FID detector and a Chirasil-DEX-CB column (Agilent; 25 m, 0.32 mm, 0.25 µm).

Because substrate stock contained ~15% (*R*)-(+)-pulegone, readings were corrected for the amount of (*R*)-(+)-pulegone in the substrate stock, to provide the enzyme yield.

Assays (100 µL) monitored by UV detection were set up as above, within a UV-Star microplate (Greiner Bio-One) and covered with a ClearVue sealing sheet (Molecular Dimensions). Reactions were monitored for (*R*)-(+)-pulegone production by measuring absorption at 260 nm using a CLARIOstar microplate reader (BMG Labtech).

Δ^5-3-ketosteroid isomerase (KSI) from *Pseudomonas putida* was found to be capable of catalysing production of (*R*)-(+)-pulegone (see **Figure 3A**, row 1; 'Wildtype').

(*R*)-(+)-pulegone is the pulegone isomer required for downstream menthol biosynthesis (that is, (*R*)-(+)-pulegone is the substrate for pulegone reductase). No (*S*)-(−)-pulegone was detected (see e.g. **Figure 3B**).

Without wishing to be bound by any theory, the position of the abstracted proton and formation of the conjugated system may be similar for both the ketosteroid and (+)-*cis*-isopulegone substrates[24–26]. The proposed mechanism for KSI activity to (+)-*cis*-isopulegone is supported by the fact that mutation of the catalytic base to a residue that cannot partake in proton abstraction (the variant D40A was tested in this case), effectively abolished activity (data not shown).

The total yield from this reaction – 35% (*R*)-(+)-pulegone from 1 mM of substrate – was modest. The inventors therefore employed a directed evolution strategy to engineer KSI for improved conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

Example 2: Directed evolution of KSI for (*R*)-(+)-pulegone biosynthesis

First round of mutagenesis: active site residue mutagenesis

The inventors hypothesised that, given the structural differences between the Δ^5-3-ketosteroid and (+)-*cis*-isopulegone substrates, it might be possible to improve yield might be improving the affinity of binding of (+)-*cis*-isopulegone substrate to KSI.

The inventors therefore mutated every amino acid known to contact KSI's natural substrate. Using a crystal structure of KSI with the reaction intermediate analogue equilenin bound (PDB 1OH0, Kim et al.[27]; see **Figure 6A**), the inventors selected every amino acid with a side-chain within 5Å of the ligand.

Mutated sequences were created by gene synthesis using ambiguous codons (**Figure 4**). The ambiguous codons used were determined by the amino acid present in the wild type (WT) sequence, such that general physicochemical properties were conserved, and designed using the online tool CodonGenie[28]. Given the generally apolar nature of the KSI active site and of (+)-*cis*-isopulegone, the inventors reasoned that maintaining this environment would help to identify optimised mutations whilst minimising the size of the variant libraries and reducing the amount of screening required. Reducing the number of screening assays was important because of the limited availability of the semisynthetic (+)-*cis*-isopulegone substrate.

Design and synthesis of variant libraries was performed as follows. The KSI gene sequence was designed using the GeneGenie online tool[33] using the wild-type amino acid sequence (Uniprot ID: P07445). Mutagenic oligonucleotides encoding ambiguous codons were designed using CodonGenie, by specifying the desired amino acid mutations and the expression organism (SEQ ID NOs:21 to 28)[28]. Eight overlapping DNA oligonucleotides (up to 80nt in length, synthesised by Integrated DNA Technologies) were used to assemble the full length gene (SEQ ID NO:39) using the SpeedyGenes gene synthesis method[34,35]. Briefly, oligonucleotides one and eight were utilised as forward and reverse primers, respectively (600nM each), whilst the remaining oligonucleotides were mixed together to form the template (30nM). The reaction also contained 0.2mM dNTPs, Q5 reaction buffer and 0.02 U μL^{-1} Q5 hot-start polymerase (New England Biolabs) in a 50 µL volume. The reaction constituted an initial denaturation at 98°C for 90 s, followed by 35 cycles of 98°C for 20 s, 60°C for 20 s and 72°C for 30 s.

Full-length genes were then purified by electrophoresis, followed by gel extraction and purification (Macherey-Nagel).

Purified genes were ligated into a linearised pET21b backbone (Novagen) to generate a C-terminally His_6-tagged enzyme by In-Fusion cloning (Clontech), according to the manufacturers protocol. using the In-Fusion cloning kit (Clontech, using the manufacturers protocol). Cloning products were transformed into T7 Express competent *E. coli* cells (New England Biolabs) and grown on LB agar plates (100 $\mu g.ml^{-1}$ ampicillin) followed by incubation overnight at 37°C.

A total of 16 amino acids were selected for mutagenesis (see **Figure 4**, column 1, 'Selected amino acid'). Tyr16 and Tyr57 are both known to partake in H-bonding with the substrate ketone, and so these residues were mutated combinatorially, using an ambiguous codon that encodes other residues capable of H-bonding (the WVC codon encoding Ser, Tyr, Cys, Thr and Asn). Val88 and Leu99 were also selected for combinatorial mutation (using an ambiguous codon encoding other hydrophobic residues; DTK encoding Phe, Leu, Ile, Met and Val), given their close proximity to the substrate (using the equilenin ligand-bound structure, PDB 1OH0). The remaining 12 residues were mutated individually. In total, 158 variants were created and screened for improvement in (*R*)-(+)-pulegone yield, using a robotics-driven screening workflow (represented schematically in **Figure 5**). Robotics enabled the automated selection of *E. coli* colonies, cell culture, protein expression induction and protein purification.

Briefly, the Hamilton Star platform (Hamilton Robotics) was used to automate the picking and inoculation of colonies into 1 mL autoinduction media (Formedium, 100 $\mu g.mL^{-1}$ ampicillin) in deep-well plates. Plates were covered using a Breathseal sealer (Greiner Bio-One) and incubated at 30°C, 1000 rpm for 24 h. The plate was duplicated by inoculating 10 µL culture into fresh LB media (100 $ug.mL^{-1}$ ampicillin) and frozen in 25% glycerol. For purification, cells were pelleted by centrifugation prior to suspension in lysis buffer containing 50% Bugbuster (Novagen), 0.1 $mg.mL^{-1}$ lysozyme (Sigma), protease inhibitors cocktail (Roche), 0.1 $mg.mL^{-1}$ DNase (Sigma) and 50mM TrisHCl (pH 8.0). Following shaking at 30°C (1000 rpm for 20 min), insoluble material was pelleted by centrifugation and the soluble fraction was added to 50 µL Ni-NTA resin (Qiagen). Resin with protein bound was then washed using 50 mM Tris, 250 mM NaCl and 10mM imidazole (pH 7.5) and eluted using the same buffer, but containing 250 mM imidazole. Eluates were quantified using the Bradford assay (Bio-Rad) following the manufacturers protocol.

Steady-state enzyme assays were then performed as described in Example 1, and the yield of (*R*)-(+)-pulegone was assessed by GC-MS.

KSI variants were produced and purified as follows. Samples with improved yield were cultured in LB medium, containing 100 $\mu g.mL^{-1}$ ampicillin, using a frozen glycerol stock as the inoculum. Recombinant plasmids were extracted and purified, according to the manufacturer's instructions (Macherey-Nagel). Each plasmid was subject to Sanger sequencing (GATC) to ascertain the variant sequence.

Individual variants were cultured in 500 mL antibiotic-selective LB media (100 μg.mL^{-1} ampicillin), grown at 37°C (180 rpm) to an OD of 0.6-0.8 (600 nm), followed by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Cultures were incubated overnight at 25°C (180 rpm) and the cell harvested by centrifugation (3,000 x *g* for 5 min). Cell pellets were suspended in 4 mL lysis buffer (50mM Tris HCl pH 8.0 containing 250 mM NaCl, 0.1 mg.mL^{-1} lysozyme, protease inhibitors cocktail, 0.1 mg/mL DNase and 10 mM imidazole). Following 30 min incubation on ice, samples were sonicated (20% amplitude for 2 min) and the soluble fraction isolated by centrifugation (21,000 x *g*, 4°C). Protein purification was performed with 0.5 mL Ni-NTA resin slurry (Qiagen) using the robotic purification buffers.

Four enzyme variants were found to yield an increase in (*R*)-(+)-pulegone as compared to the yield obtained using wildtype KSI – see **Figure 3A**, rows 2 to 5. Three of these mutants encoded mutation of L99 – KSI variants designated 'L99I', 'L99V' and 'V88I/L99V'. Interestingly, both L99I and L99V involve mutation to a smaller side chain, suggesting that this may be a factor in improving yield. Furthermore, the fourth selected variant D103S was also a mutation to a residue with smaller side chain. Given that the serine hydroxyl group can participate in H-bonding, this residue could maintain H-bonding to the substrate ketone in a similar way to Asp.

Enzyme kinetic characterization of the improved mutants was performed using UV measurement, detecting production of (*R*)-(+)-pulegone through absorbance at 260 nm as described in Example 1. This showed that the KSI variant D103S exhibited the greatest increase in specific activity, with a 1.6-fold increase compared to the WT (**Figure 6B**).

Second round: compiling mutations

To test whether any of the first round mutations could be combined to further improve product yield, the three variants with L99 mutations were combined with D103S. Each variant was purified and tested for improvement both total yield (by GC-MS) and turnover rate (by monitoring absorbance at 260nm) as described in Example 1.

Each variant exhibited a modest further improvement in (*R*)-(+)-pulegone yield (see **Figure 3A**, rows 6 to 8), with the best KSI variant V88I/L99V/D103S exhibiting a 1.9-fold improvement in yield over the wildtype (65.3% total yield (*R*)-(+)-pulegone), as the result of a 2.5-fold increase in specific activity (**Figure 6B**).

Third round: structure-guided mutation

A high-resolution crystal structures were obtained for purified KSI variants. D103S, V88I/L99V, V88I/L99V/D103S and V88I/L99V/V101A/D103S were concentrated using Vivaspin 500 (Sartorius) columns to 10 mg.mL^{-1} and crystallised using a Mosquito robot (TTP Labtech) and the following crystallisation solutions: *i*) 10-25% PEG3350 with 0.2 M $MgCl_2$; *ii*) 10-25% PEG3350 with 0.2 M ammonium acetate (pH 4.6) and *iii*) 10-25% PEG3350 with 0.2 M ammonium acetate (pH 5.5). Crystals were flash frozen in 30% glycerol and data were collected at Diamond Light Source, Oxford (beamlines IO4 and IO3). Reflections were merged and scaled with Xia2[45]. All mutant structures were solved by molecular replacement using Phaser-MR (CCP4)[46] and wild type KSI (PDB code: 1OPY) as a search

model. Initial model building was done with AutoBuild (PHENIX)[47] followed by iterative cycles of manual model building and refinement in COOT[48] and Phenix.refine[49] respectively. Mutations could be clearly identified in *Fo-Fc* difference maps and were modelled accordingly. The final data collection and refinement statistics are shown in **Figure 7D**. The atomic coordinates and structure factors were deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ.

DFT modelling was performed using Gaussian09 revision D.01[50]. An active site model was created from the crystal structure of KSI with bound androstenedione (PDB ID 1OHS) including the side-chains and α-carbons of the 15 first-shell residues shown in **Figure 7E**, with *cis*-(+)-isopulegone built in using the coordinates of the corresponding androstenedione atoms as the starting point. Energy minimisation was carried out using the B3LYP hybrid functional and 6-31G(d) basis sets[51] with the D3 version of Grimme's dispersion[52]. Since the active site is solvent exposed, a continuum solvent model to mimic the shielding effect of water. Molecular dynamics simulations were carried out using Gromacs 4.6.1 with the Gromos 53A6 force field and periodic boundary conditions[53-54]. For wildtype KSI, the crystal structure of KSI with bound androstenedione (PDB: 1OHS) was used, with (+)-*cis*-isopulegone built in using the coordinates of the corresponding androstenedione atoms as the starting point. For KSI variant V88I/L99V/V101A/D103S, (+)-*cis*-isopulegone was placed in the active site of the crystal structure (PDB: 1OPY) by aligning the protein to the wildtype KSI model.

The enzyme was placed in a solvation box of minimum 10 Å around the protein. The simulation protocol was as follows: after energy minimization the system was thermalized 300 K for 100 ps using NVT dynamics, and the pressure was then equilibrated for 100 ps using NPT dynamics; the protein and substrates were constrained during these steps. All constraints and pressure couplings were then switched off and harmonic constraints of 100 kJ/mol/Å^2 were applied to keep the (+)-*cis*-isopulegone near a reactive conformation, as defined by the distances in the energy minimised DFT model for the variant: 2.0 Å for the distance between (+)-*cis*-isopulegone carbonyl oxygen and the carboxylic/hydroxyl H of D/S103 and 3.5 Å for the distance between substrate C and carboxylate O of D40 (**Figure 7F**). The system was then relaxed at 250 K, 280 K, 290 K and 300 K for 1 ns each, before the 50 ns production MD runs at 300 K. Representative structures for illustrative purposes were selected as those with the lowest RMSD for the substrate and residues 40 and 103 relative to the average, following structural alignment to the backbone.

The high-resolution crystal structure for KSI V88I/L99V/D103S confirmed the close proximity of the three mutations in the active site (shown in **Figures 7A** to **7C**). Through inspecting the spatial arrangement of these positions the inventors identified that V101 is positioned directly between L99V and D103S. Given that each of the V88I/L99V/D103S mutations resulted in a shortening of the amino acid side chain, the inventors reasoned that mutagenesis of V101 to an amino acid with a shorter side chain might also improve conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

Addition of the mutation V101A to form the KSI variant V88I/L99V/V101A/D103S was found to significantly improve product yield, resulting in a total yield of 89% (*R*)-(+)-pulegone (see **Figure 3A**, row 9). Kinetic characterisation of this variant demonstrated a 5.6-fold increase in specific activity as compared to the wildtype KSI (6.43 $nmol.min^{-1}.mg^{-1}$ compared to 1.16 $nmol.min^{-1}.mg^{-1}$; see **Figure 6B**).

Example 3: Performance of optimized KSI V88I/L99V/V101A/D103S for (−)-menthol production by cascade biocatalysis

Having obtained KSI variants with improved activity for (+)-*cis*-isopulegone, the inventors next investigated their use in the production of (−)-menthol using a cascade biocatalysis reaction with purified enzymes.

For the first downstream reaction, pulegone reductase from *M. piperita* ($MpPGR^{21}$) was selected. This enzyme expressed and purified roughly equal yields of (−)-menthone and (+)-isomenthone products from the (*R*)-(+)-pulegone substrate (**Figure 8**, row 'MpPGR'). This is a higher yield compared to the corresponding enzyme from *Nicotiana tabacum,* NtDBR, used previously under the same reaction conditions[29].

For the second downstream reaction, menthone reductase (MMR) from *M. piperita* was selected. This enzyme exhibits efficient production of (−)-menthol from (−)-menthone *in vitro* (**Figure 8**, row 'MMR'). During characterization MMR was found to exhibit isopulegone reductase activity, wherein incubation of purified MMR with (+)-*cis*-isopulegone yields isomers of isopulegol, principally (+)-neoiso-isopulegol, and also (-)-isopulegol (**Figure 9A**). This was confirmed by performing GC-MS analysis of the reaction containing MMR and (+)-*cis*-isopulegone as substrate, using a DB-5 column (20 m by 0.1 mm [internal diameter]; film thickness, 0.1 m) using the conditions described in Siedenburg *et al.* Appl. Environ. Microbiol. (2012) 78(4):1055-1062.

In initial assays, MMR isopulegone reductase activity was found to dominate over the activity of KSI. To overcome this issue, a 30-fold molar excess of KSI was used in reactions with MMR, allowing KSI activity to predominate.

Using this approach the inventors successfully synthesised (−)-menthone and (−)-menthol from (+)-*cis*-isopulegone in a three-enzyme cascade reaction.

Briefly, codon optimised genes for MpPGR (Uniprot ID: Q6WAU0) and MMR (ID: Q5CAF4) were synthesised (GeneArt) and ligated into a pET21b plasmid. Protein expression and purification was performed as described above, except that overnight cultures were incubated at 20°C (180 rpm). Cascade reactions (200 µL) were performed in 50 mM Tris pH 7.0 containing 10 µM KSI, 1 µM MpPGR, 0.3 µM MMR with 1 mM (+)-*cis*-isopulegone. Additionally, a cofactor recycling system (glucose dehydrogenase (10U, Sigma), 10 µM NADP+ (Fisher) and 15 mM D-glucose (Fisher)) was added to ensure sufficient NADPH was available for both MpPGR and MMR activity. Reactions were incubated and analysed by GC/GC-MS.

The results of the cascade biocatalysis reactions are shown in **Figure 8**, row 'Cascade'. Roughly 33% of detectable products after 24 h consisted of intermediates of the menthol biosynthesis pathway, of which 44% consisted of (−)-menthol (63.4 µM yield, 14.1% conversion). Interestingly, despite the ability of MpPGR to produce both (−)-menthone and (+)-isomenthone, cascade catalysis using KSI V88I/L99V/V101A/D103S yielded primarily (−)-menthone (and subsequently also (−)-menthol).

Example 4: Performance of optimized KSI variant V88I/L99V/V101A/D103S for (−)-menthol production using cell extracts and whole cells

Synthesis of menthol isomers (+)-neomenthol, (−)-menthol, (+)-isomenthol and (+)-neoisomenthol from (*R*)-(+)-pulegone, using a one-pot cell extract approach has been reported previously[30]. The inventors wished to extend the methodology for synthesis of (−)-menthol from (+)-*cis*-isopulegone using a similar approach.

A single expression construct was created for the same three-enzymes used for the cascade reaction approach, with each gene and its respective ribosome-binding site (RBS) under the control of one T7 promoter (**Figure 10**). Each gene was amplified by PCR and ligated into the pET21b backbone using the In-Fusion cloning kit, following the manufacturer's instructions. Following transformation into competent *E. coli* NEB5α cells (New England Biolabs), cell culture and plasmid extraction (as above), correct constructs were identified by DNA sequencing.

Consequently, the expression of all three genes could be induced by addition of IPTG.

GC/GC-MS analysis of *Mentha* monoterpenoids was performed as follows. GC-MS achiral quantitative analysis was conducted on a 7890B GC coupled to a 5975 series MSD quadrupole mass spectrometer and equipped with a 7693 autosampler (Agilent, Technologies, UK). The sample (1µL) was injected onto a DB-WAX column (30 m x 0.320 mm x 0.25 µm; Agilent Technologies) with an inlet temperature of 240°C and a split ratio of 20:1. Helium was used as the carrier gas with a flow rate of 1.5 mL/min and a pressure of 1.5603 psi. The chromatography was programmed to begin at 40°C with a hold time of 1 minute, followed by an increase to 150°C at a rate of 10°C/min, then a subsequent increase to 210°C at a rate of 80°C/min and a final hold time of 1 min. The total run time for the analysis was 13.75 min. The MS was equipped with an electron impact ion source using 70eV ionisation and a fixed emission of 35 µA. The mass spectrum was collected for the range 35-550 mz with a scan speed of 3,125 (N=1).

Chiral product analysis was performed by analysing reactions by GC using an Agilent Technologies 7890A GC system with an FID detector and a Chirasil-DEX-CB column (Agilent; 25 m, 0.32 mm, 0.25 µm). In this method the injector temperature was at 180°C with a split-less injection. The carrier gas was helium with a flow rate of 1 $mL.min^{-1}$ and a pressure of 5.8 psi. The program began at 70°C with an increase of temperature to 150°C at a rate of 20°C/minute and a hold for 3 min. This was followed by an increase of temperature to 190°C at a rate of 2°C/minute and a hold for 3 min.

For all GC-MS analyses, *sec*-butylbenzene was used as an internal standard to allow for accurate quantification. 0.01% *sec*-butylbenzene was added to all samples and the quantification of members of the menthol pathway was calculated relative to the peak area of this internal standard. Calibration curves were constructed for accurate quantification and calibration standards using the same methods as the reaction extracts. Vendor binary files were converted to open mzXML data format36 using ProteoWizard msConvert[37]. Automated peak profiling and quantification was conducted using in-house scripts written in R.

As expected, overexpression of all three enzymes together primarily yielded isopulegol isomers using cell extracts, much like the cascade biocatalysis *in vitro* where equal concentrations of enzyme were added, due to the reductase activity of MMR.

Both KSI and MpPGR encoded the RBS from the parental pET21b plasmid, which, according to the Salis calculator, have a high predicted TIR (6600 and 11000, respectively). A significantly reduced level of MMR expression was desired, so three new RBS' were designed, with a predicted TIR of 1000, 500 and 200. Each of these sequences were cloned upstream of the MMR gene using the In-Fusion kit.

Given that dilution of MMR in the cascade biocatalysis reactions had enabled synthesis of (−)-menthol, the inventors reasoned that placing an RBS with a low translation rate (using the RBS calculator developed by Salis *et al.*[31,32]) in front of MMR could mimic this effect *in vivo* and reduce the unwanted side reaction. The initial pathway design encoded an MMR RBS with a predicted translation initiation rate (TIR) of 4100; consequently three alternative constructs were designed using MMR RBSs with a predicted TIR of ~1000, ~500 and ~200 (**Figure 11**).

Plasmids were transformed into competent *E. coli* NiCo21 (DE3) cells and grown on LB agar (100 μg.mL^{-1} ampicillin). Single colonies were selected and grown in 200 mL phosphate buffered Terrific Broth pH 7.0 (Formedium, containing additional 0.4% glycerol and 100 μg.mL^{-1} ampicillin). Expression was induced using 0.1 mM IPTG, and cultures were incubated overnight at 20°C. Cell pellets were suspended in 3 mL lysis buffer, containing 50 mM Tris pH 7.0, 10% glycerol, protease inhibitor cocktail, 1 mM $MgCl_2$, 0.1 mg.mL^{-1} DNase, 0.1 mg.mL^{-1} lysozyme and 1 mM dithiothreitol. Lysis and centrifugation was performed as above to obtain the cell extract. Reactions were set up using the same components as the cascade catalysis reaction, with 50 μL cell extract used in a 200 μL reaction volume.

Two designs did not demonstrate detectable MMR activity (RBS TIR 1000 and 200); however, the RBS with a predicted TIR of 500 did show activity by production of both (−)-menthol and isopulegol isomers (**Figure 8**, row 'Cell extract'). This construct yielded 52.8 μM (−)-menthol, similar to that of the cascade catalysis approach, though roughly 59% of substrate was still converted to isopulegol isomer(s).

Finally, the inventors investigated conversion of (+)-*cis*-isopulegone to menthol isomers (and intermediates) using whole *E. coli* cells engineered to express KSI, MpPGR and MMR (i.e. without treatment to lyse the cells). The results are shown in **Figure 8**, row 'Whole cells'. The enzymes were

found to perform well, and to produce (−)-menthol with a higher yield as compared to the yield obtained by cascade catalysis, or using cell extracts (158.8 μM, 27% total yield).

Example 5: Conclusions

The inventors successfully identify KSI from *Pseudomonas putida* as an isopulegone isomerase capable of catalyzing conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

The native enzyme that catalyses this reaction in *M. piperita* enzyme remains unknown, this is the first enzyme identified to be capable of performing this reaction.

Through three rounds of directed evolution the inventors obtained an optimized KSI variant V88I/L99V/V101A/D103S, capable of producing almost 90% yield of (*R*)-(+)-pulegone after 24 h, driven by a 5.6-fold increase in activity over the wildtype KSI. This activity enabled the production of (−)-menthol through cell extract, whole cell and cascade catalysis approaches, when used together with the reductases MpPGR and MMR.

The identification and engineering of KSI as an isopulegone isomerase completes the enzymatic route from (−)-limonene to (−)-menthol, enabling for first time the biosynthetic production of menthol by synthetic biology in engineered microbes.

Integrating the KSI-MpPGR-MMR construct into larger biosynthetic cascades enables the production of menthol isomers from inexpensive precursors, providing a sustainable and renewable production platform to meet the growing demand for natural menthol products.

Example 6: Conversion of (+)-*cis*-isopulegone to (*S*)-(–)-pulegone and *trans*-isopulegone by glutathione-S-transferase

The inventors also investigated double bond isomerisation of (+)-*cis*-isopulegone by human glutathione S-transferase A3.

Wildtype human glutathione S-transferase A3 (GSTA3-3) was expressed in and purified from *E. coli*.

GSTA3-3 variants comprising the following substitutions were also prepared: A208F, A216L, A208F/A216L, L111D, F222W, L72R/S159Y.

Purified wildtype GSTA3-3 and variants of GSTA3-3 were reacted with (+)-*cis*-isopulegone (via IPR/isopiperitenone) to determine the identification and enantiomeric identity of the products.

Biotransformations of isopiperitenone were performed in 1 mL reactions in PBS buffer pH 7.3 containing isopiperitenone (5 mM), enzyme (10 μM), glutathione (0.5 mM), IPR (10 μM), GDH (10 U), NADP+ (15 μM). The reactions were agitated at 30°C for 6-24 h at 180 rpm. Monoterpenoids were extracted with 0.9

mL ethyl acetate containing 0.1 % *sec*-butylbenzene internal standard. Product yields were determined by GC analysis using a DB-WAX column.

The product yields are shown in **Figure 12**. In each case, GSTA3-3 generated both (*S*)-pulegone and *trans*-isopulegone from (+)-*cis*-isopulegone, which are isomers of each other.

Purified wildtype GSTA3-3 and variants of GSTA3-3 were then reacted with (+)-*cis*-isopulegone to determine the identification and enantiomeric identity of the products.

Biotransformations of (+)-*cis*-isopulegone were performed in 1 mL reactions in PBS buffer pH 7.3 containing (+)-*cis*-isopulegone (2.5 mM), enzyme (10 µM) and glutathione (0.5 mM). The reactions were agitated at 30 °C for 18 h at 180 rpm. Monoterpenoids were extracted with 0.9 mL ethyl acetate containing 0.1 % *sec*-butylbenzene internal standard. Product yields were determined by GC analysis using a DB-WAX column.

The product yields are shown in **Figure 13**. In each case, GSTA3-3 generated both (*S*)-pulegone and *trans*-isopulegone from (+)-*cis*-isopulegone. Different variants produced different ratios of these products, but none produced (*R*)-pulegone.

Example 7: Identification of KSI homologues and conserved positions

The inventors performed a BLASTP search using KSI from *P. putida* (UniProt: P07445-1, v1; SEQ ID NO:1) as the query sequence in order to identify homologues of KSI from *Pseudomonas putida*.

An alignment of KSI from *P. putida* (UniProt: P07445-1, v1; SEQ ID NO:1) and 249 other sequences is shown in **Figure 15**. A consensus sequence derived from the alignment is shown in SEQ ID NO:38.

The inventors next performed an alignment of the active site region of the KSI sequences (corresponding to positions 86 to 112 of SEQ ID NO:1), shown in **Figure 16**. A consensus sequence derived from the alignment is shown in SEQ ID NO:35.

Finally, the inventors performed an alignment of a subregion of the active site region of KSI sequences from *Pseudomonas* species (corresponding to positions 86 to 107 of SEQ ID NO:1), shown in **Figure 17**. A consensus sequence derived from the alignment is shown in SEQ ID NO:37.

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Claims:

1. A method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone using a ketosteroid isomerase.

2. The method according to claim 1, wherein the ketosteroid isomerase is ketosteroid isomerase from *Pseudomonas putida* or a fragment, variant or homologue thereof.

3. The method according to claim 1 or claim 2, wherein the ketosteroid isomerase comprises an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:34, 35, 36 or 37.

4. The method according to any one of claims 1 to 3, wherein the ketosteroid isomerase comprises, or consists of, an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:1, 2, 3, 4, 5, 6, 7, 8, 9 and 38.

5. The method according to any one of claims 1 to 4, wherein the method is a method for producing (*R*)-(+)-pulegone or a derivative thereof.

6. The method according to any one of claims 1 to 5, wherein the method is a method for producing one or more of (*R*)-(+)-pulegone, (+)-menthofuran, (-)-menthone, (+)-isomenthone, (+)-neomenthol, (-)-menthol, (+)-isomenthol and (+)-neoisomenthol.

7. The method according to any one of claims 1 to 6, wherein the method further comprises catalysis of the conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone using a pulegone reductase.

8. The method according to claim 7, wherein the method further comprises catalysis of the conversion of (-)-menthone to (-)-menthol and/or (+)-neomenthol using a menthone reductase.

9. The method according to claim 7, wherein the method further comprises catalysis of the conversion of (+)-isomenthone to (+)-isomenthol and/or (+)-neoisomenthol using a menthone reductase.

10. The method according to any one of claims 1 to 9, wherein the method further comprises catalysis of the conversion of (-)-isopiperitenone to (+)-*cis*-isopulegone using a (-)-isopiperitenone reductase.

11. The method according to claim 10, wherein the method further comprises catalysis of the conversion of (-)-*trans*-isopiperitenol to (-)-isopiperitenone using an isopiperitenol dehydrogenase.

12. The method according to claim 11, wherein the method further comprises catalysis of the conversion of limonene to (-)-*trans*-isopiperitenol using a limonene hydroxylase or a cytochrome P450 reductase.

13. A method for producing a menthol isomer, comprising:

catalysis of the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone using a ketosteroid isomerase;

catalysis of the conversion of (*R*)-(+)-pulegone to (-)-menthone and/or (+)-isomenthone using a pulegone reductase; and

catalysis of the conversion of (-)-menthone to (-)-menthol and/or (+)-neomenthol using a menthone reductase, and/or catalysis of the conversion of (+)-isomenthone to (+)-isomenthol and/or (+)-neoisomenthol using a menthone reductase.

14. Use of a ketosteroid isomerase to catalyse the conversion of (+)-*cis*-isopulegone to (*R*)-(+)-pulegone.

15. A composition comprising a ketosteroid isomerase and one or more of: a pulegone reductase, a menthone reductase, a (-)-isopiperitenone reductase, an isopiperitenol dehydrogenase. a limonene hydroxylase, a cytochrome P450 reductase, a limonene synthase and a geranyl diphosphate synthase.

16. A nucleic acid, or plurality of nucleic acids, encoding a ketosteroid isomerase and one or more of: a pulegone reductase, a menthone reductase, a (-)-isopiperitenone reductase, an isopiperitenol dehydrogenase, a limonene hydroxylase, a cytochrome P450 reductase, a limonene synthase and a geranyl diphosphate synthase.

17. A ketosteroid isomerase having (+)-*cis*-isopulegone to (*R*)-(+)-pulegone isomerase activity, and comprising an amino acid sequence comprising a substitution corresponding to one or more of the following positions relative to SEQ ID NO:1: V88, L99, Y16, Y57, D103, V20, F56, L61, V66, F86, M90, V101, M116, W120, G60 and A118.

18. The ketosteroid isomerase according to claim 17, wherein the ketosteroid isomerase comprises one or more substitutions corresponding to the following amino acid substitutions relative to SEQ ID NO:1: D103S, V88I, L99I, L99V and V101A.

19. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitution corresponding to D103S relative to SEQ ID NO:1.

20. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitution corresponding to V101A relative to SEQ ID NO:1.

21. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitution corresponding to L99I relative to SEQ ID NO:1.

22. The ketosteroid isomerase according to claim 17 or claim 19, wherein the ketosteroid isomerase comprises the amino acid substitution corresponding to L99V relative to SEQ ID NO:1.

23. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I and L99V relative to SEQ ID NO:1.

24. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitutions corresponding to L99V and D103S relative to SEQ ID NO:1.

25. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitutions corresponding to L99I and D103S relative to SEQ ID NO:1.

26. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I, L99V and D103S relative to SEQ ID NO:1.

27. The ketosteroid isomerase according to claim 17 or claim 18, wherein the ketosteroid isomerase comprises the amino acid substitutions corresponding to V88I, L99V, V101A and D103S relative to SEQ ID NO:1.

28. A nucleic acid encoding a ketosteroid isomerase according to any one of claims 17 to 27.

29. An expression vector comprising a nucleic acid, or plurality of nucleic acids according to claim 16, or a nucleic acid according to claim 28.

30. A cell comprising a composition according to claim 15, a nucleic acid or plurality of nucleic acids according to claim 16, a ketosteroid isomerase according to any one of claims 17 to 27, a nucleic acid according to claim 28, or an expression vector according to claim 29.

31. A composition comprising a composition according to claim 15, a nucleic acid or plurality of nucleic acids according to claim 16, a ketosteroid isomerase according to any one of claims 17 to 27, a nucleic acid according to claim 28, an expression vector according to claim 29, or a cell according to claim 30.

32. Use of a composition according to claim 15, a nucleic acid or plurality of nucleic acids according to claim 16, a ketosteroid isomerase according to any one of claims 17 to 27, a nucleic acid according to claim 28, an expression vector according to claim 29, a cell according to claim 30 or a composition according to claim 31 in a method for producing (*R*)-(+)-pulegone or a derivative thereof.

33. The use according to claim 32 wherein the method is a method for producing one or more of (*R*)-(+)-pulegone, (+)-menthofuran, (-)-menthone, (+)-isomenthone, (+)-neomenthol, (-)-menthol, (+)-isomenthol and (+)-neoisomenthol.

34. A method comprising catalysis of the conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone using a glutathione S-transferase.

35. Use of a glutathione S-transferase to catalyse the conversion of (+)-*cis*-isopulegone to (*S*)-(-)-pulegone.

36. The method according to claim 34 or the use according to claim 35, wherein the glutathione S-transferase is human glutathione S-transferase A3 (GSTA3-3) or a fragment, variant or homologue thereof.

37. The method or use according to any one of claims 34 to 36, wherein the glutathione S-transferase comprises, or consists of, an amino acid sequence having at least 70% sequence identity to one or more of the amino acid sequences of SEQ ID NOs:10, 11, 12, 13, 14, 15 and 16.

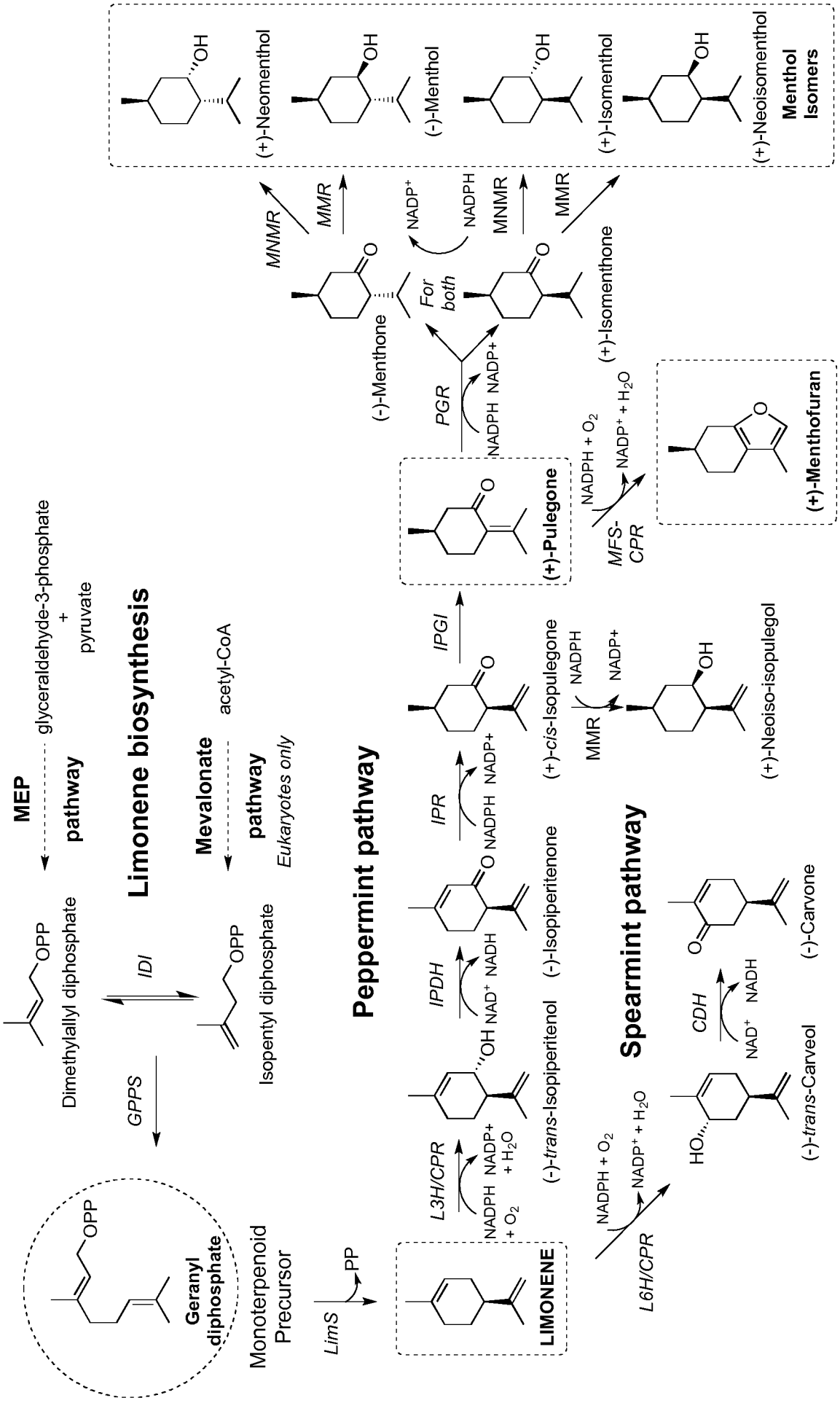
38. A method comprising catalysis of the conversion of (+)-*cis*-isopulegone to an isopulegol isomer using a menthone reductase.

39. Use of a menthone reductase to catalyse the conversion of (+)-*cis*-isopulegone to an isopulegol isomer.

40. The method according to claim 38 or the use according to claim 39, wherein the isopulegol isomer is selected from (+)-neoiso-isopulegol, (-)-isopulegol, (+)-iso-isopulegol and (+)-neo-isopulegol, optionally wherein the isopulegol isomer is (+)-neoiso-isopulegol.

41. The method or use according to any one of claims 38 to 40, wherein the menthone reductase is *Mentha piperita* (-)-menthone:(-)menthol reductase (MMR) or a fragment, variant or homologue thereof.

42. The method or use according to any one of claims 38 to 41, wherein the menthone reductase comprises, or consists of, an amino acid sequence having at least 70% sequence identity to the amino acid sequence of SEQ ID NO:17.


Limonene biosynthesis
MEP
pathway
glyceraldehyde-3-phosphate
+
pyruvate
Mevalonate
pathway
Eukaryotes only
acetyl-CoA
Dimethylallyl diphosphate
Isopentyl diphosphate
IDI
GPPS
Geranyl diphosphate
Monoterpenoid Precursor
LimS
LIMONENE
L3H/CPR
NADPH NADP+ + O2 + H2O
(-)-trans-Isopiperitenol
IPDH
NADH NAD+
(-)-Isopiperitenone
IPR
NADPH NADP+
Peppermint pathway
IPGI
(+)-Pulegone
PGR
NADPH NADP+
(-)-Menthone
For both
NADP+
NADPH
MNMR
MMR
(+)-Isomenthone
MMR
(+)-Neomenthol
(-)-Menthol
(+)-Isomenthol
(+)-Neoisomenthol
Menthol Isomers
NADPH + O2
NADP+ + H2O
MFS-CPR
(+)-Menthofuran
(+)-cis-Isopulegone
NADPH
MMR
NADP+
(+)-Neoiso-isopulegol
NADPH + O2
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L6H/CPR
Spearmint pathway
CDH
NAD+ NADH
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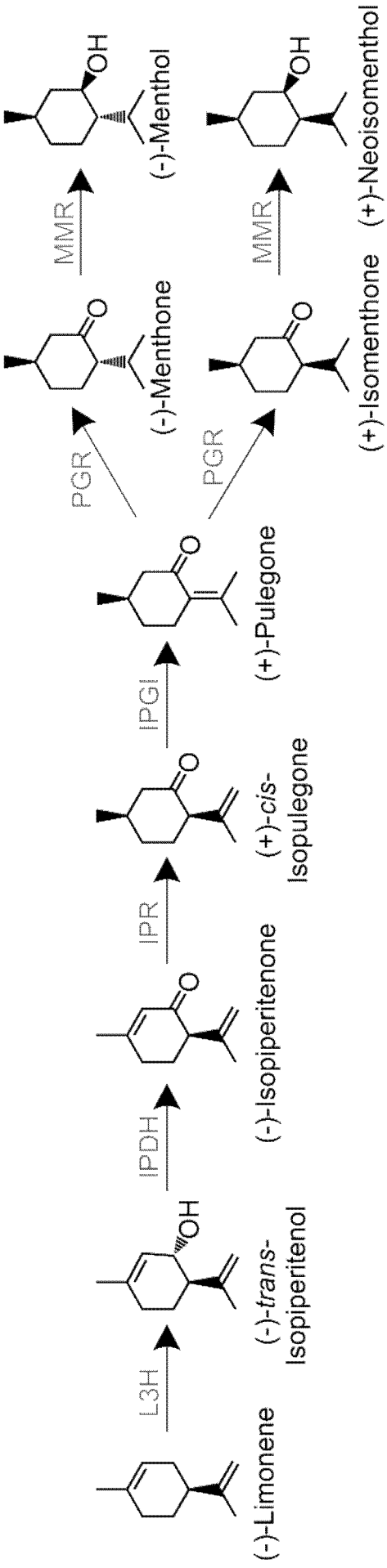
(-)-Carvone

Figure 1A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IPDH
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MMR
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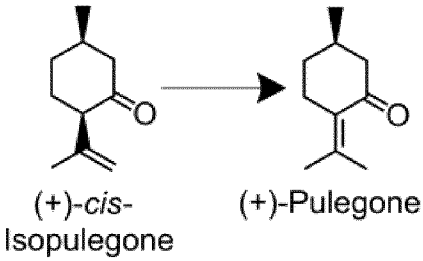
(+)-Isomenthone
MMR
(+)-Neoisomenthol

Figure 1B



Ketosteroid isomerisation:

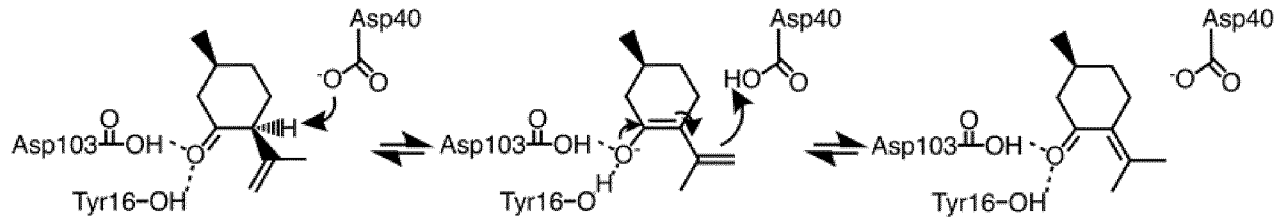


Figure 2

Round[1]	Variant	Yield[2] (%)	Yield[2] (%/μM KSI^{-1})	Yield[2] (μM.μM KSI^{-1})	Relative Activity (fold)
-	Wild type	34.8 (±0.13)	1.25 (±0.01)	13.88 (±0.08)	1.0
1	D103S	46.3 (±1.92)	1.89 (±0.10)	20.09 (±1.16)	1.4
	L99I	37.9 (±0.11)	2.13 (±0.01)	23.58 (±0.10)	1.7
	L99V	38.4 (±0.68)	1.70 (±0.04)	18.46 (±0.48)	1.3
	V88I L99V	40.0 (±0.85)	1.72 (±0.05)	19.39 (±0.57)	1.4
2	V88I L99V D103S	65.3 (±3.3)	2.31 (±0.14)	26.47 (±1.59)	1.9
	L99V D103S	55.5 (±1.41)	2.26 (±0.08)	25.51 (±0.83)	1.8
	L99I D103S	64.8 (±1.17)	2.30 (±0.05)	25.91 (±0.58)	1.9
3	V88I L99V V101A D103S	89.4 (±0.71)	5.76 (±0.06)	63.10 (±0.61)	4.5

[1]Screening round number. [2]Yield of (*R*)-(+)-pulegone. Standard deviation shown in brackets. Reactions contained 1 mM (+)-*cis*-isopulegone, 10 μM KSI, 50 mM Tris pH 7.0 in 200 μL total volume.

Figure 3A

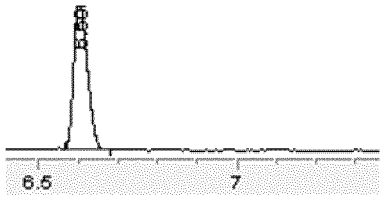
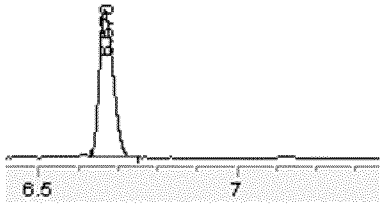
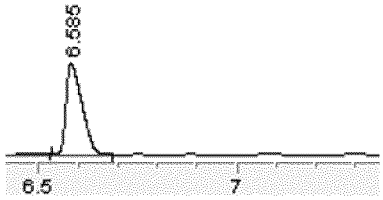
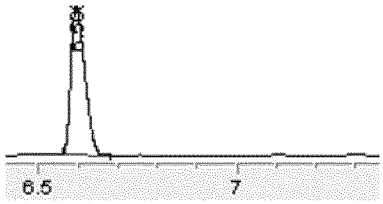
Enzyme/Standard	GC Trace	Product
(*R*)-Pulegone standard		N/A
(*S*)-Pulegone standard		N/A
Wild type KSI		(*R*)-Pulegone
KSI variant V88I/L99V/V101A/D103S		(*R*)-Pulegone

Figure 3B

Selected amino acid	Ambiguous codon used	Mixed bases	Genetic combinations	Amino acids encoded	Characteristic
V88, L99	DTK	(G,A,T) T (G,T)	6	Phe, Leu, Ile, Met, Val	Apolar
Y16, Y57, D103	WVC	(A,T) (A,C,G) C	6	Ser, Tyr, Cys, Thr, Asn	Polar/H-bonding
V20, F56, L61, V66, F86, M90, V101, M116, W120	WKS	(T,A) (T,G) (G,C)	8	Phe, Leu, Ile, Met, Cys, Trp, Ser, Arg	Larger side chains, apolar and polar
G60	RSC	(A,G) (G,C) C	4	Gly, Ala, Thr, Ser	Small side chains
A118	SYG	(G,C) (TC) G	4	Ala, Val, Leu, Pro	Small side chains, apolar

Figure 4

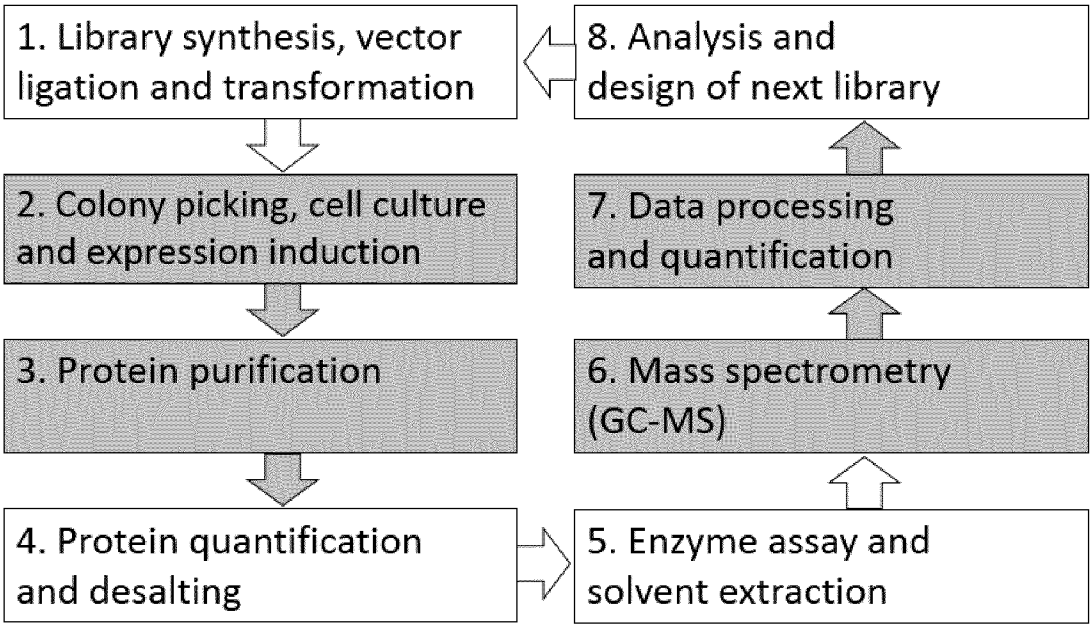


Figure 5

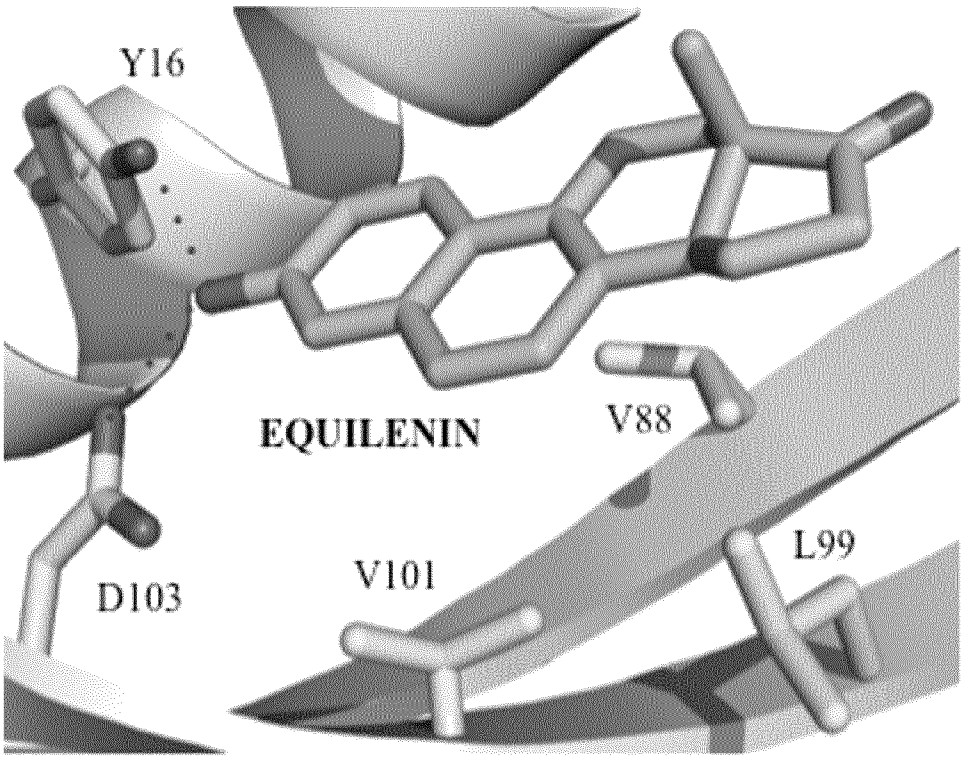


Figure 6A

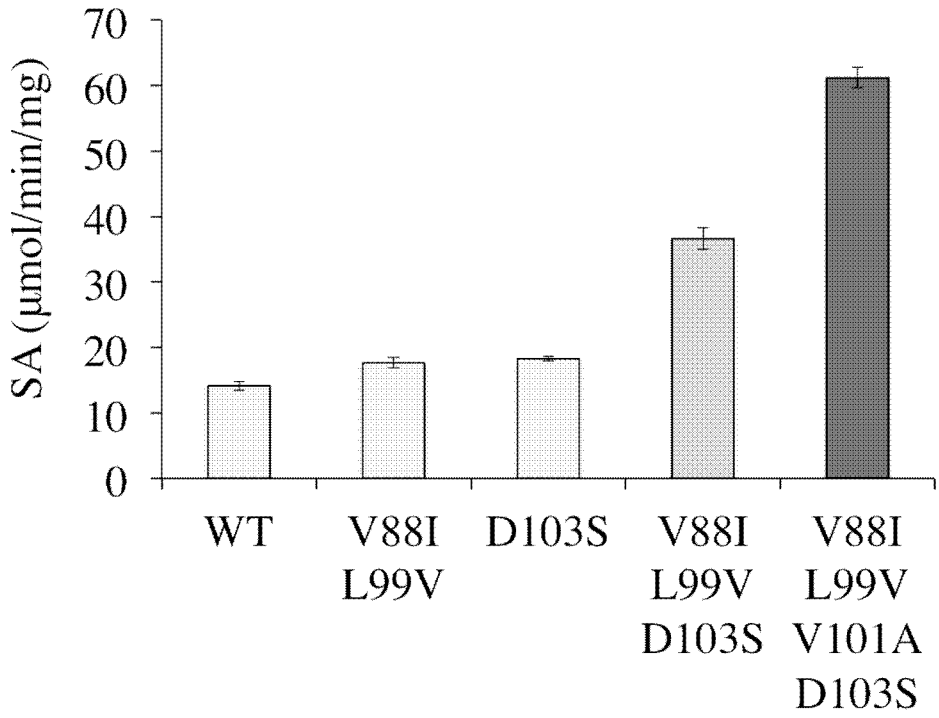


Figure 6B

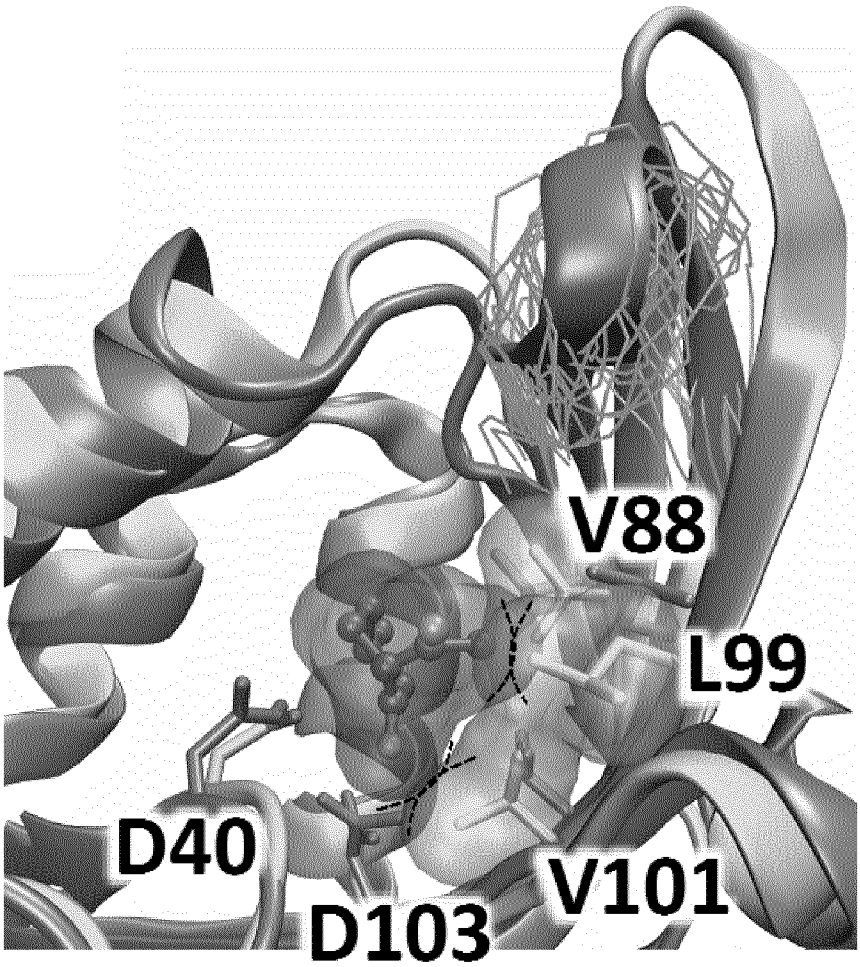


Figure 7A

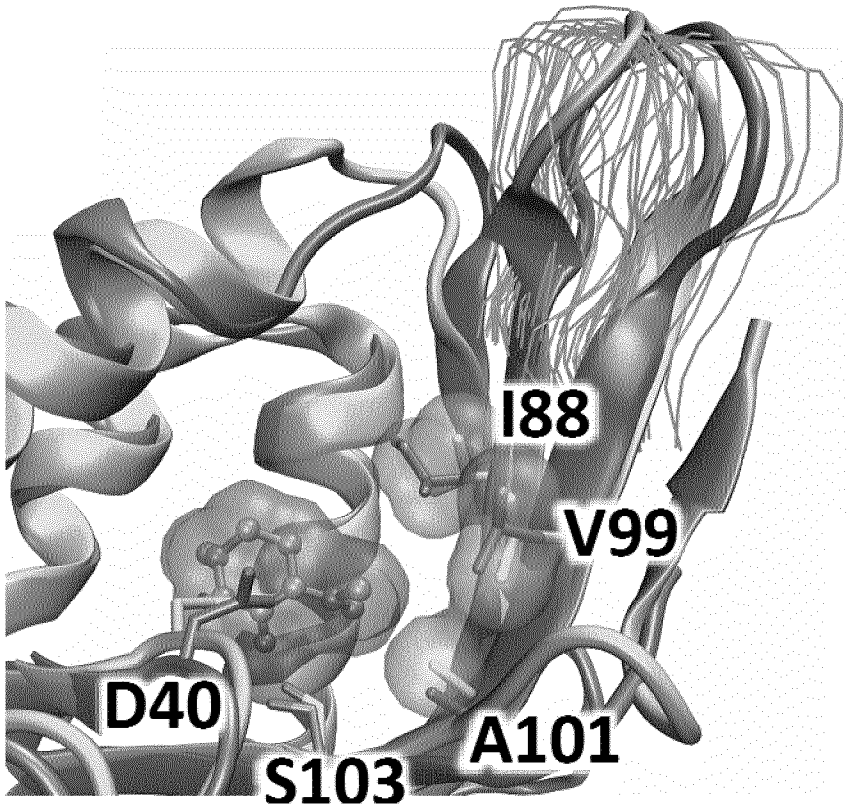


Figure 7B

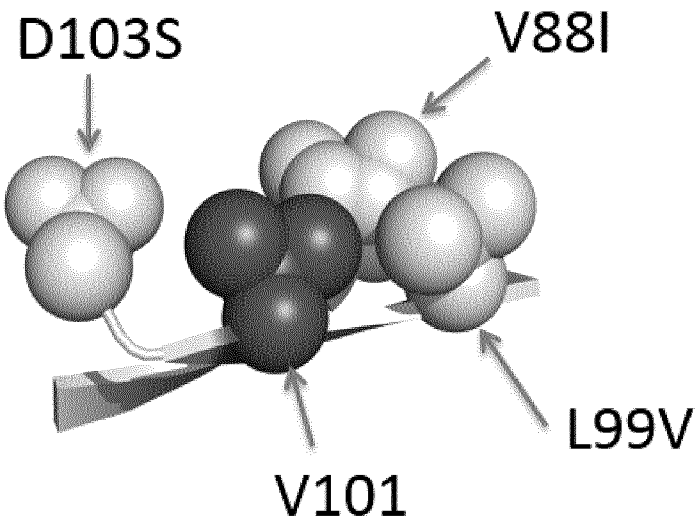


Figure 7C

	KSI Variant			
	D103S	V88I/L99V	V88I/L99V/D103S	V88I/L99V/V101A/D103S
PDB Code	<To be deposited>	<To be deposited>	<To be deposited>	<To be deposited>
Data collection				
Wavelength	1.00	1.00	1.00	1.00
Space group	**P 21 21 21**	**P 21 21 21**	**C2**	**C 2 2 21**
a, *b*, *c* (Å)	36.17 73.53 94.82	35.69 73.54 95.41	86.61 71.54 50.63	35.740 94.720 73.810
α, β, γ (°)	90.00 90.00 90.00	90.00 90.00 90.00	90.00 90.37 90.00	90.00 90.00 90.00
Resolution (Å)	47.7 (1.9)	95.4 (2.0)	43.3 (1.08)	29.1 (1.49)
R_{sym} or R_{merge}	0.74 (0.10)	0.8 (0.91)	0.93 (0.09)	0.43 (2.7)
I / σI	11.3 (2.2)	11.6 (2.1)	8.9 (1.2)	23.1 (2.7)
Completeness (%)	99.7 (97.1)	99.9 (97.98)	87.4 (42.9)	99.6 (98.9)
Redundancy	6.3 (6.4)	6.5 (6.5)	3.3 (2.7)	5.8 (4.7)
CC half	0.7	0.9	0.5	0.9
total observations	19954	17276	115237	20641
Refinement				
Resolution (Å)	1.92	95.4 (2.0)	43.3 (1.08)	29.1 (1.49)
R_{work} / R_{free}	0.204 (0.252)	0.183 (0.227)	0.18 (0.198)	0.183 (0.202)
No. atoms	2122	2088	2346	1046
R.m.s. deviations				
Bond lengths (Å)	0.01	0.009	0.006	0.007
Bond angles (°)	1.19	1.05	1.01	1.08

[a]Values in the parentheses refer to the outer resolution shell.

Figure 7D

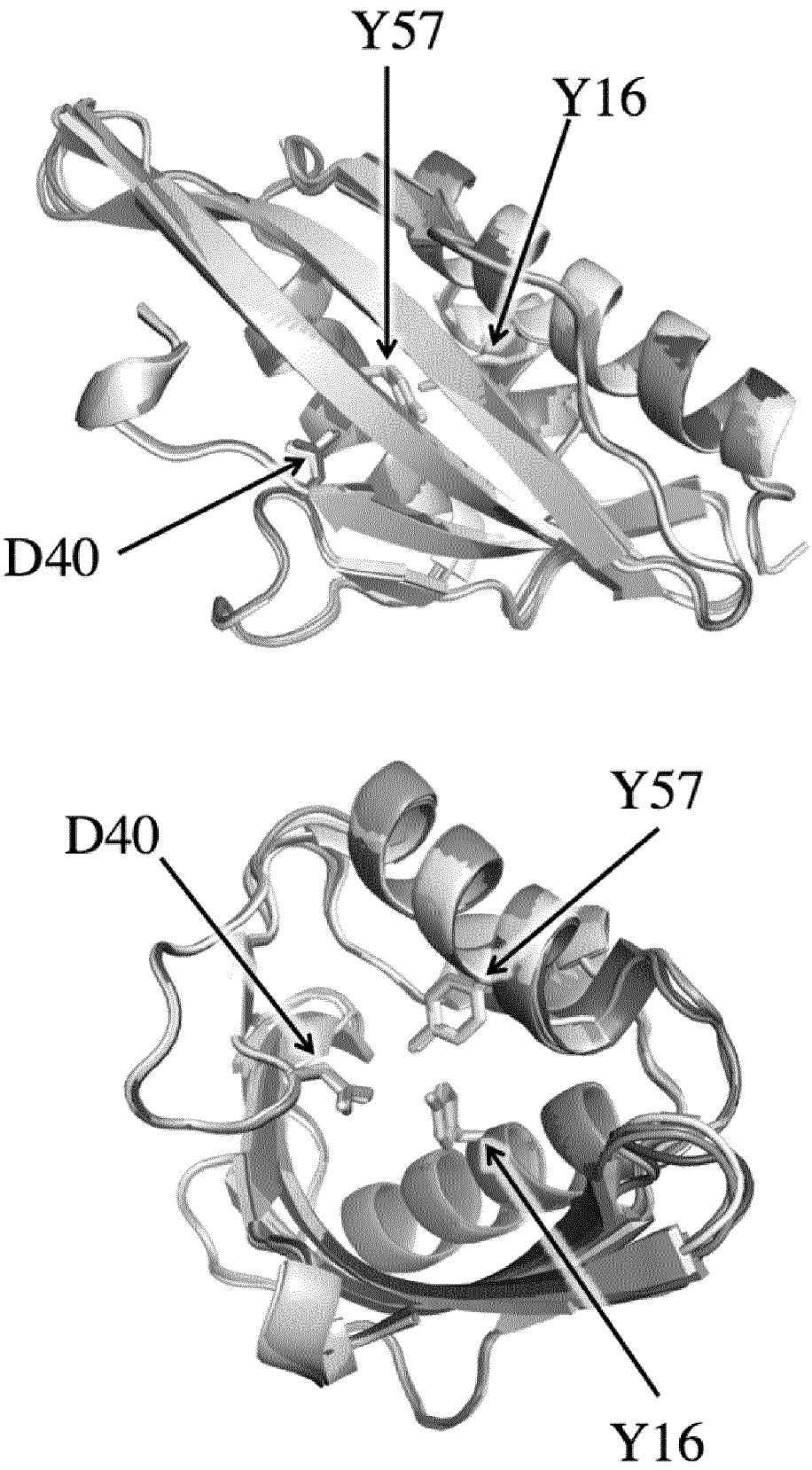


Figure 7E

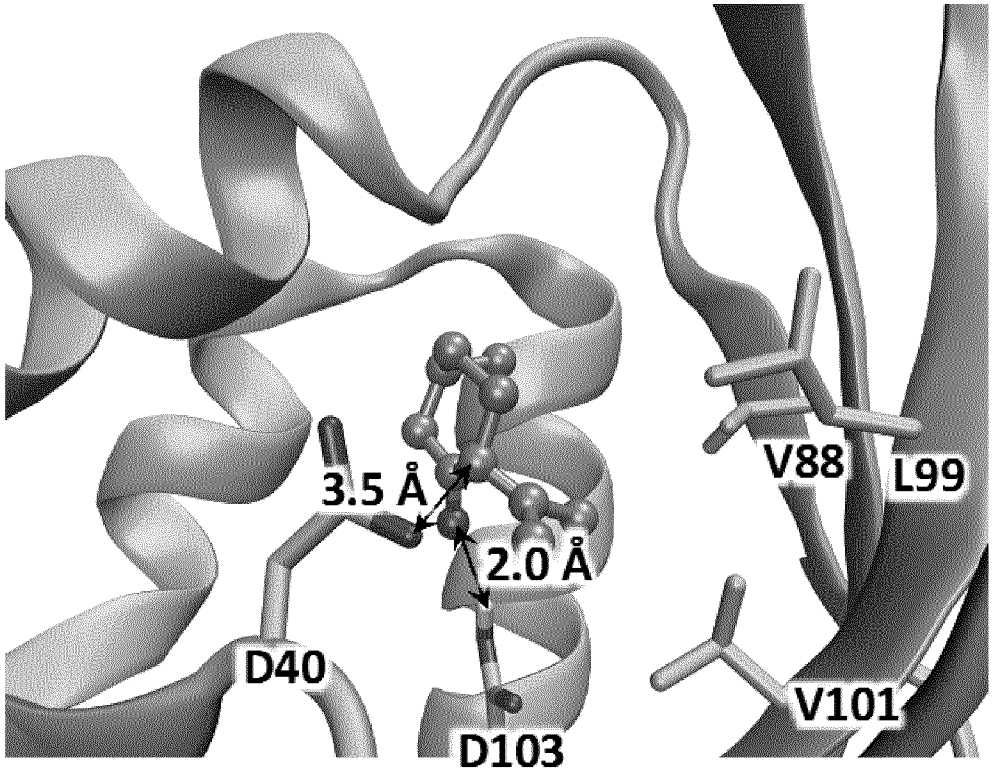


Figure 7F

Biocatalyst	Substrate remaining/Product yield (μM)					
	Isopulegone	Pulegone	Menthone	Isomenthone	Menthol	Neoisomenthol
Individual enzymes						
KSI WT	**361.7**	210.4	N/A	N/A	N/A	N/A
KSI variant[1-2]	**40.7**	436.3	N/A	N/A	N/A	N/A
MpPGR	N/A	**10.0**	153.3	160.1	N/A	N/A
MMR	N/A	N/A	**8.0**	-	280.7	-
Cascading reactions						
Cascade[2]	**16.7**	10.3	25.2	16.6	63.4	11.1
Cell extract[2]	**17.1**	11.5	60.6	34.8	52.8	18.6
Whole cells[2]	**32.5**	1.0	44.7	98.6	158.8	19.6

Figure 8A

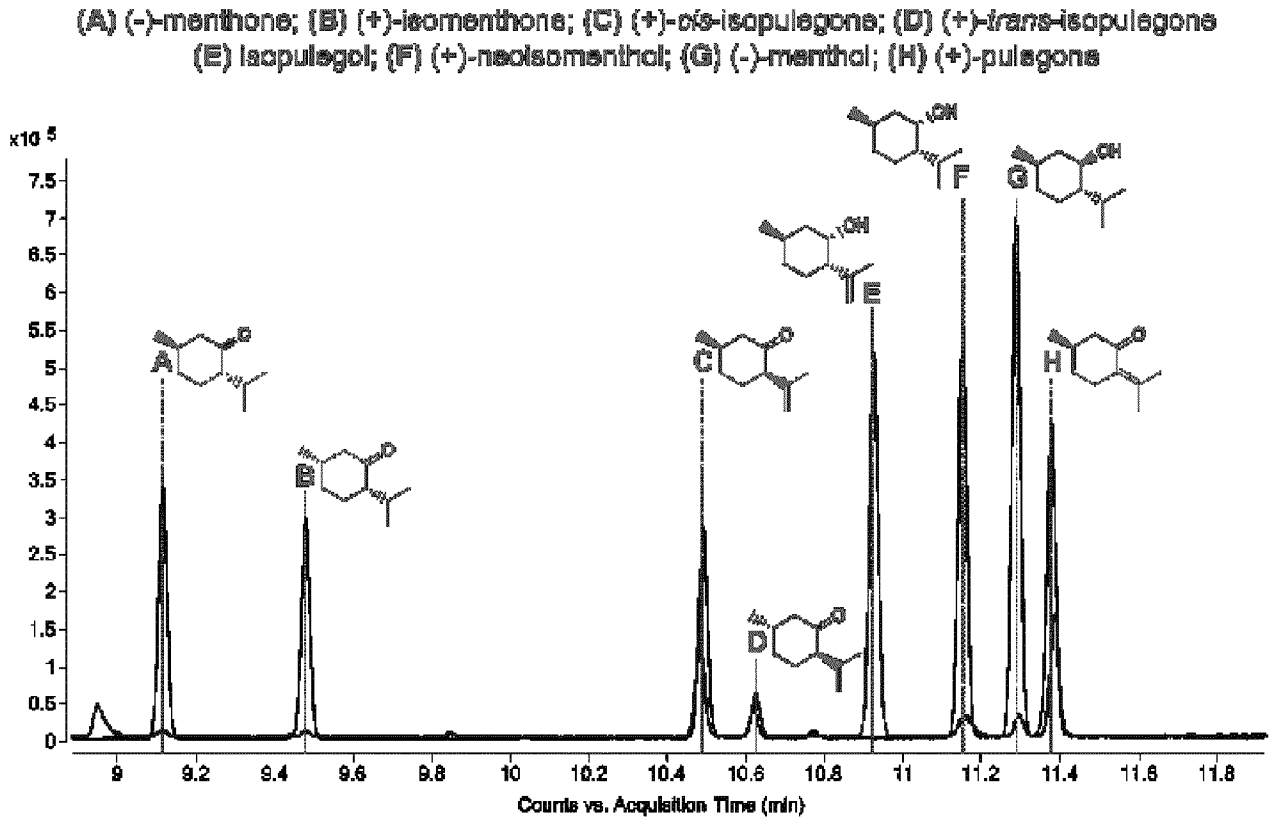


Figure 8B

(A) (-)-Isopulegol (B) (+)-Neo-isopulegol
(C) (+)-Iso-isopulegol (D) (+)-Neoiso-isopulegol

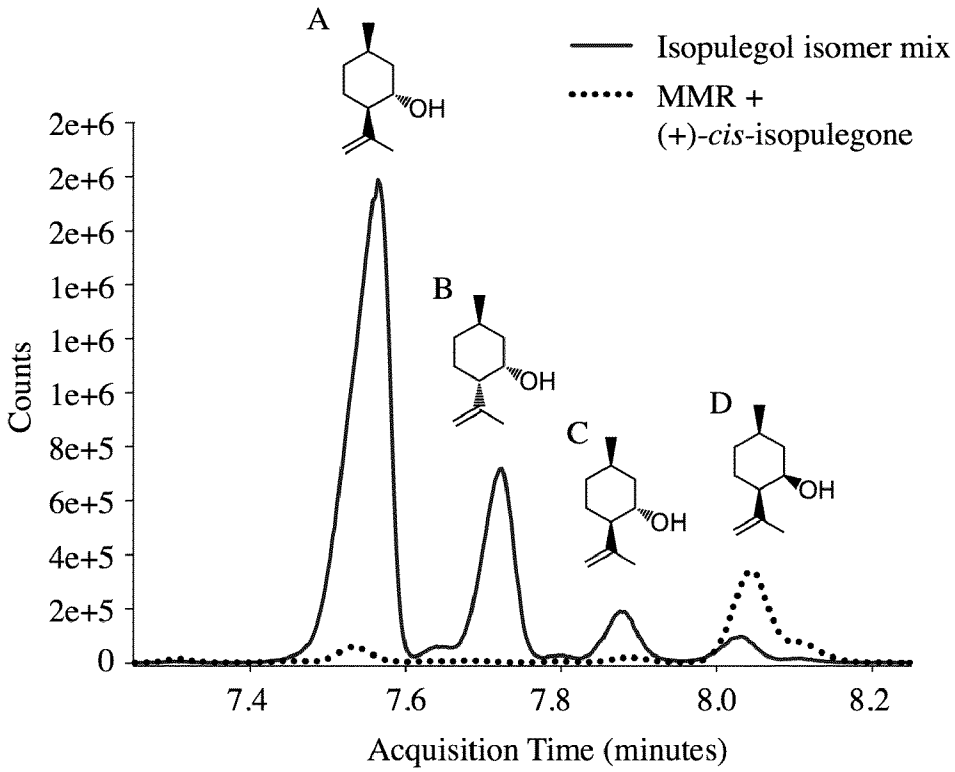


Figure 9A

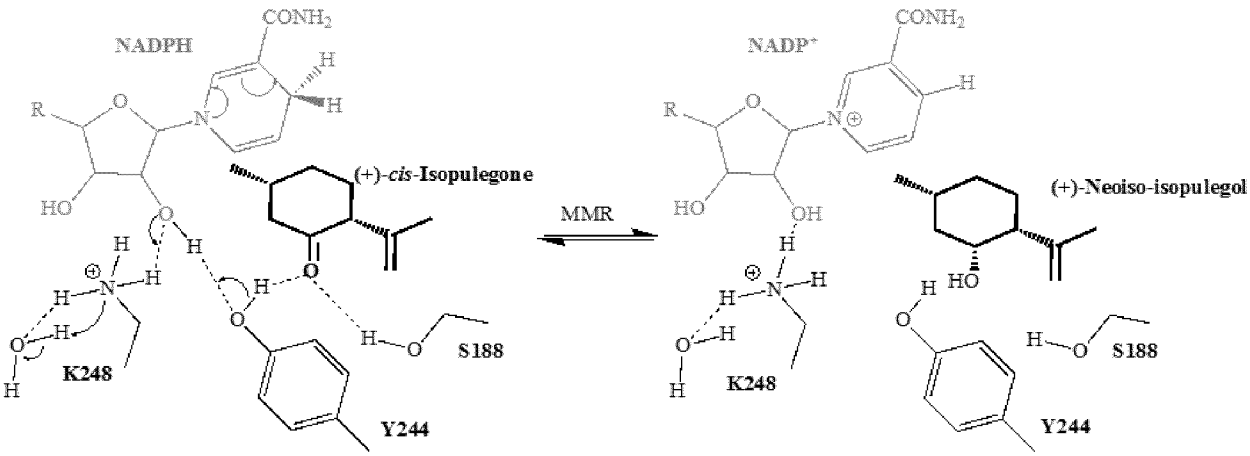


Figure 9B

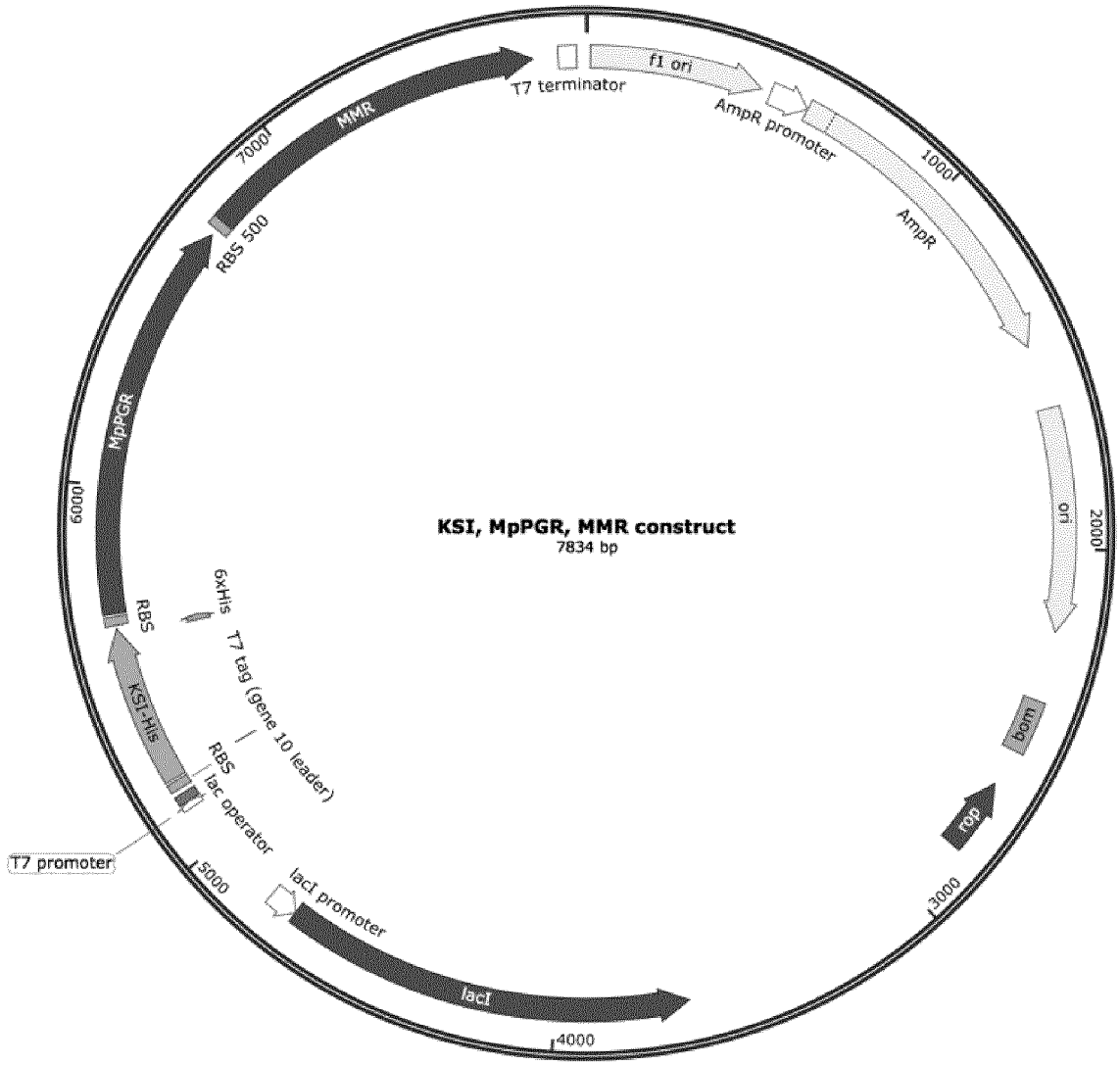


Figure 10

Gene	RBS sequence	Predicted TIR
KSI	TTTGTTTAACTTTAAGAAGGAGA	6689
PgPGR	TTTGTTTAACTTTAAGAAGGAGA	11325
MMR	CCCATTAAAATTTAAGCTTTATATAC	918
	GACTCATCAATAAAGGTTACTTCCT	535
	ATTTTCACATACGTCTACTT	199

Figure 11

Biotransformations of purified GSTA3 variants with isopiperitenone/IPR.

Conditions	Reaction time (h)	Product Yield (μM)			Ratio (***S***)-Pulegone:*trans*-Isopulegone
		cis-Isopulegone	*trans*-Isopulegone	(***S***)-Pulegone	
04/10/16					
GSTA3-3 WT	6	295.0 ± 6.7	527.5 ± 4.1	170.1 ± 0.3	0.32:1
	24	172.2 ± 8.1	313.0 ± 7.6	155.4 ± 0.1	0.50:1
GSTA3-3	6	270.5 ± 4.4	577.9 ± 21.2	169.1 ± 11.5	0.29:1
A208F	24	140.1 ± 8.2	315.5 ± 9.6	165.7 ± 0.3	0.53:1
GSTA3-3	6	283.5 ± 0.3	547.1 ± 2.4	169.8 ± 0.7	0.31:1
A216L	24	158.8 ± 3.0	320.5 ± 4.0	156.5 ± 2.9	0.49:1
11/10/16					
GSTA3-3 WT	6	144.2 ± 7.4	370.5 ± 17.9	142.5 ± 5.2	0.38:1
GSTA3-3 A208F/A216L	6	161.0 ± 3.2	392.6 ± 5.9	108.9 ± 5.6	0.28:1

Figure 12

Biotransformations of purified GSTA3 variants with *cis*-isopulegone ± NtDBR.

Conditions	Product Yield (μM)		Ratio
	trans-Isopulegone	(***S***)-Pulegone	(***S***)-Pulegone:*trans*-Isopulegone
GSTA3-3 WT	*438.2*	*251.1*	0.57:1
GSTA3-3 L111D	237.7 ± 3.9	231.4 ± 4.3	0.97:1
GSTA3-3 Y222W	250.3 ± 7.6	222.3 ± 1.0	0.89:1
GSTA3-3 L72R/S159Y	463.0 ± 15.3	315.9 ± 17.6	0.68:1

Figure 13

```
Ctes   --MNTPEHMTAVVQRYVAALNAGDLDGIVALFADDATVEDPVGSEPRSGTAAIREFYANS
Pput   MNLPTAQEVQGLMARYIELVDVGDIEAIVQMYADDATVEDPFGQPPIHGREQIAAFYRQG
         : * :.: .:: **:  ::.**::.** ::********** .*. *  *   *  ** :.

Ctes   LKLPLA-VELTQEVRAVA-NEAAFAFTVSFEYQGRKTVVAPIDHFRFNGAGKVVSMRALF
Pput   LGGGKVRACLTGPVRASHNGCGAMPFRVEMVWNGQPCALDVIDVMRFDEHGRIQTMQAYW
       *    . . **  ***   . .*: * *.: ::*:  .:  ** :**:  *:: :*:* :

Ctes   GEKNIHAGA--
Pput   SEVNLSVREPQ
       .* *: .
```

Figure 14

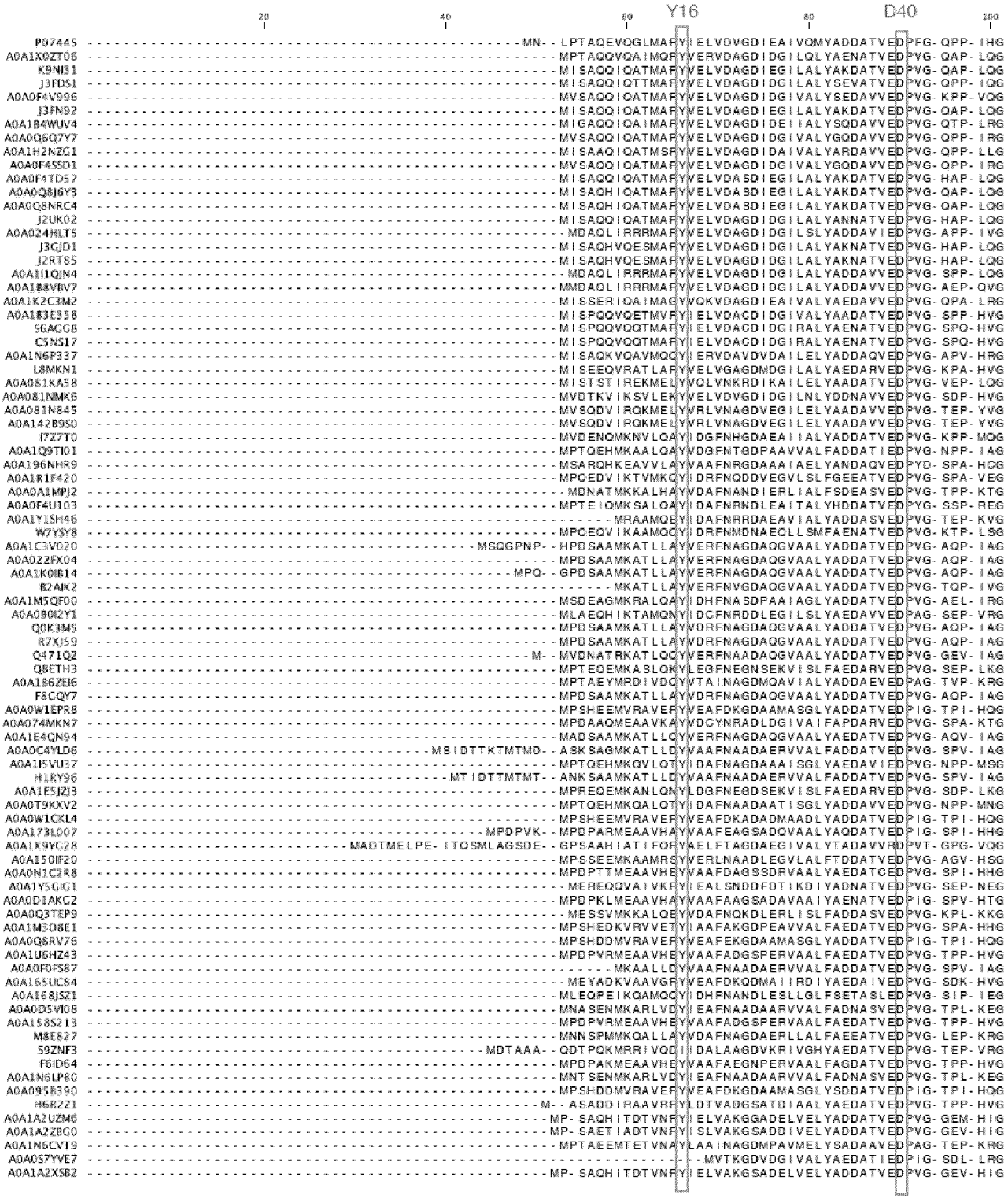


Figure 15

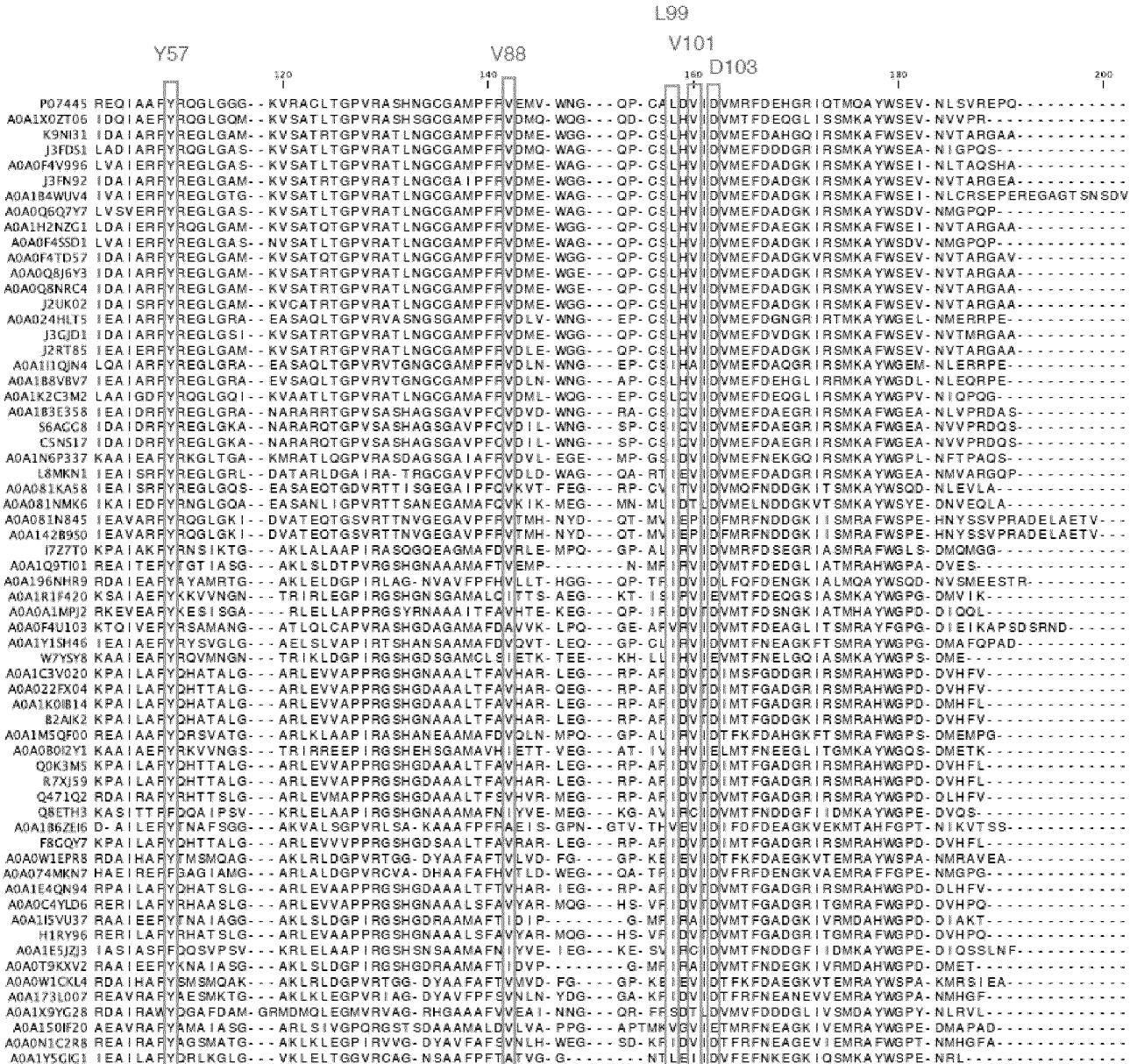


Figure 15 (cont.)

P07445 - 131
A0A1X0ZT06 - 126
K9NI31 - 129
J3FDS1 - 127
A0A0F4V996 - 129
J3FN92 - 129
A0A1B4WUV4 D 141
A0A0Q6Q7Y7 - 127
A0A1H2NZG1 - 129
A0A0F4SSD1 - 127
A0A0F4TD57 - 129
A0A0Q8J6Y3 - 129
A0A0Q8NRC4 - 129
J2UK02 - 129
A0A024HLT5 - 127
J3GJD1 - 129
J2RT85 - 129
A0A1I1QJN4 - 127
A0A1B8VBV7 - 128
A0A1K2C3M2 - 127
A0A1B3E358 - 129
S6AGG8 - 129
C5NS17 - 129
A0A1N6P337 - 128
L8MKN1 - 128
A0A081KA58 - 127
A0A081NMK6 - 128
A0A081N845 - 137
A0A142B9S0 - 137
I7Z7T0 - 126
A0A1Q9TI01 - 120
A0A196NHR9 - 128
A0A1R1F420 - 125
A0A0A1MPJ2 - 123
A0A0F4U103 - 133
A0A1Y1SH46 - 122
W7YSY8 - 123
A0A1C3V020 - 132
A0A022FX04 - 125
A0A1K0IB14 - 128
B2AIK2 - 119
A0A1M5QF00 - 126
A0A080I2Y1 - 125
Q0K3M5 - 125
R7XJS9 - 125
Q471Q2 - 126
Q8ETH3 - 124
A0A186ZEI6 - 126
F8GQY7 - 125
A0A0W1EPR8 - 125
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A0A0C4YLD6 - 137
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A0A1X9YG28 - 146
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A0A1Y5GIG1 - 117
A0A0D1AKG2 - 124
A0A0Q3TEP9 - 124
A0A1M3D8E1 - 123
A0A0Q8RV76 - 125
A0A1U6HZ43 - 125
A0A0F0FS87 - 119
A0A165UC84 - 116
A0A168JSZ1 - 125
A0A0D5VI08 - 130
A0A158S213 - 125
M8E827 - 130
S9ZNF3 - 137
F6ID64 - 125
A0A1N6LP80 - 130
A0A095B390 - 125
H6R2Z1 - 123
A0A1A2UZM6 - 123
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A0A1N6CVT9 - 126
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A0A1A2XSB2 - 123

Figure 15 (cont.)

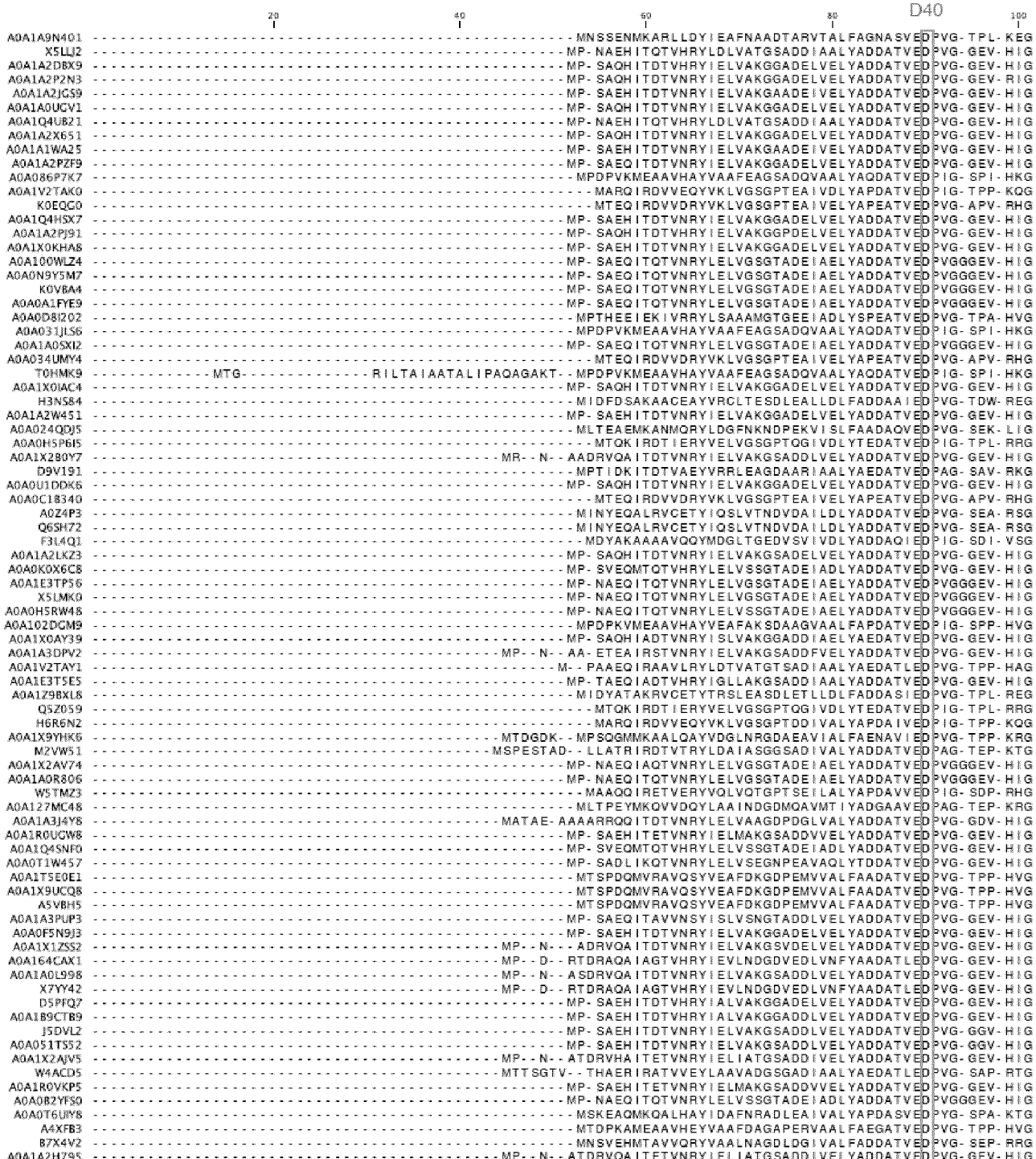

Figure 15 (cont.)

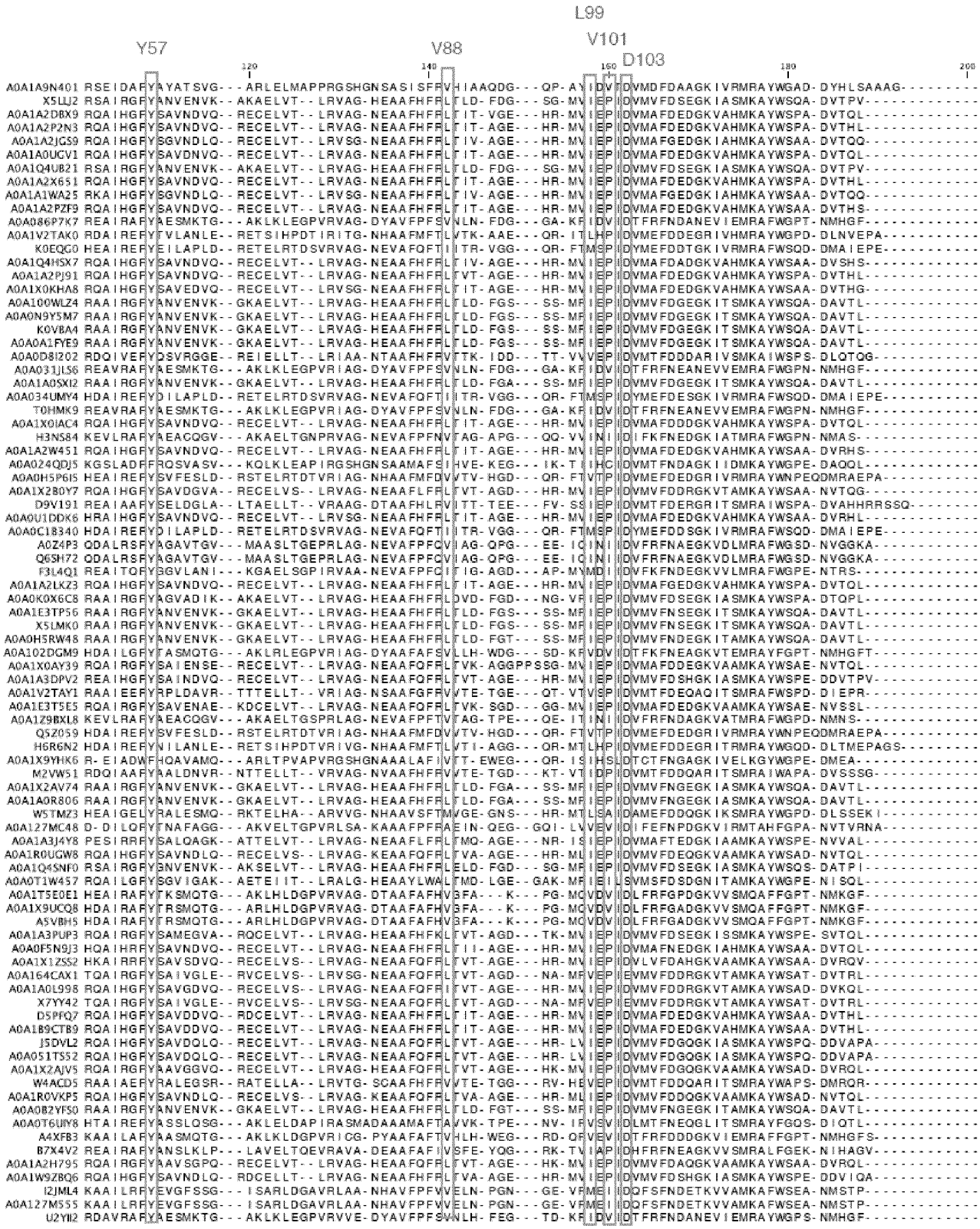

Figure 15 (cont.)

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A0A1A2P2N3 - 123
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A0A1Q4UB21 - 123
A0A1A2X651 - 123
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A0A1A2PZF9 - 123
A0A086P7K7 - 124
A0A1V2TAK0 - 125
K0EQG0 - 125
A0A1Q4HSX7 - 123
A0A1A2PJ91 - 123
A0A1X0KHA8 - 123
A0A100WLZ4 - 124
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K0VBA4 - 124
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A0A0D8I202 - 124
A0A031JLS6 - 124
A0A1A0SXI2 - 124
A0A034UMY4 - 125
T0HMK9 - 147
A0A1X0IAC4 - 123
H3NS84 - 123
A0A1A2W451 - 123
A0A024QDJ5 - 125
A0A0H5P6I5 - 126
A0A1X2B0Y7 - 127
D9V191 - 128
A0A0U1DDK6 - 123
A0A0C1B340 - 125
A0Z4P3 - 125
Q6SH7Z - 125
F3L4Q1 - 122
A0A1A2LKZ3 - 123
A0A0K0X6C8 - 123
A0A1E3TP56 - 124
X5LMK0 - 124
A0A0H5RW48 - 124
A0A102DGM9 - 125
A0A1X0AY39 - 126
A0A1A3DPV2 - 127
A0A1V2TAY1 - 123
A0A1E3T5E5 - 123
A0A1Z9BXL8 - 123
Q5Z059 - 126
H6R6N2 - 127
A0A1X9YHK6 - 129
M2VW51 - 131
A0A1X2AV74 - 124
A0A1A0R806 - 124
W5TMZ3 - 124
A0A127MC48 - 126
A0A1A3J4Y8 - 130
A0A1R0UGW8 - 123
A0A1Q4SNF0 - 123
A0A0T1W457 - 124
A0A1T5E0E1 - 122
A0A1X9UCQ8 - 122
A5VBH5 - 122
A0A1A3PUP3 - 123
A0A0F5N9J3 - 123
A0A1X1ZSS2 - 126
A0A164CAX1 - 127
A0A1A0L998 - 127
X7YY42 - 127
D5PFQ7 - 123
A0A1B9CTB9 - 123
J5DVL2 - 124
A0A051TS52 - 124
A0A1X2AJV5 - 127
W4ACD5 - 129
A0A1R0VKP5 - 123
A0A0B2YFS0 - 124
A0A0T6UIY8 - 125
A4XFB3 - 125
B7X4V2 - 125
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U2YII2 - 174

Figure 15 (cont.)

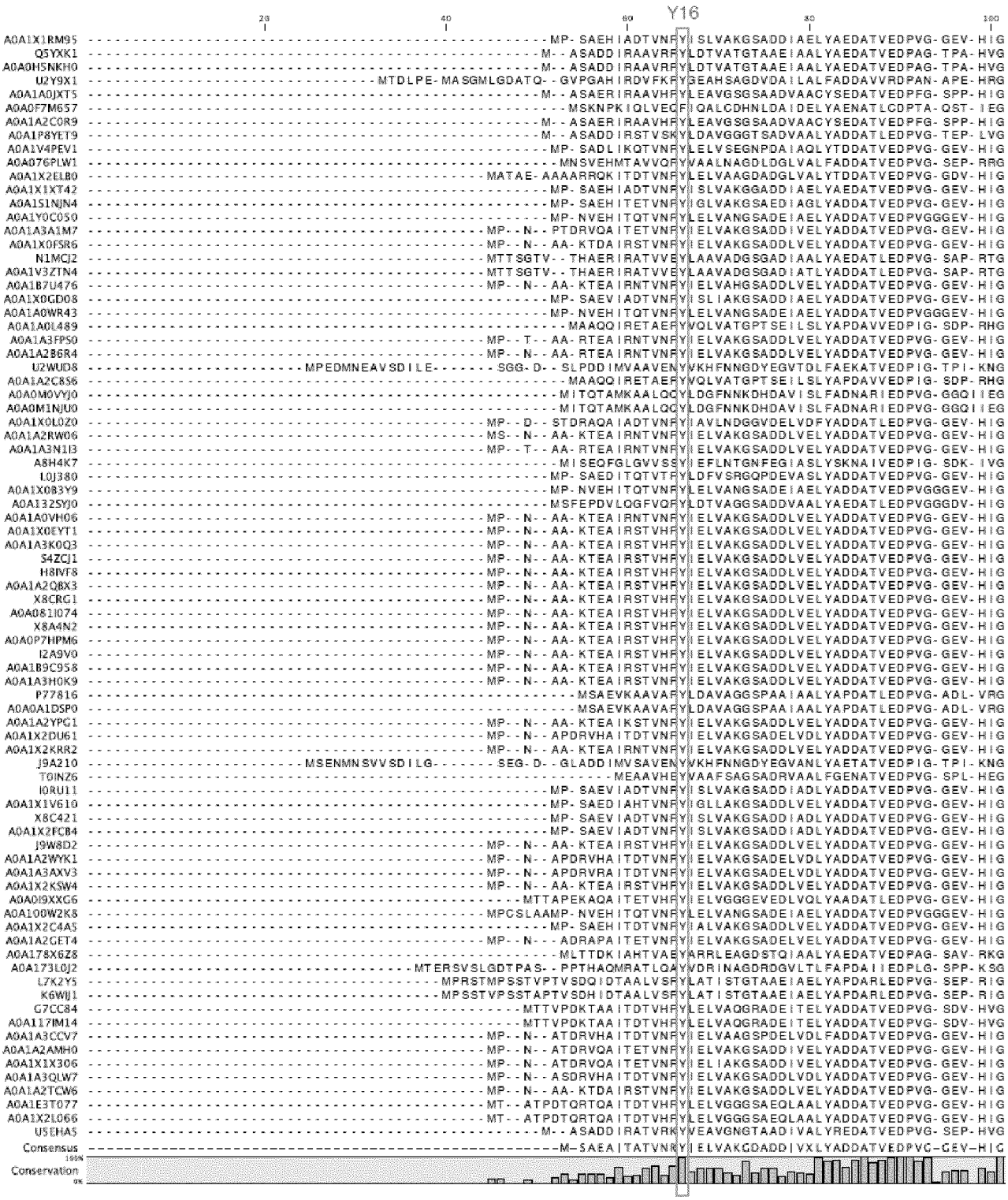


Figure 15 (cont.)

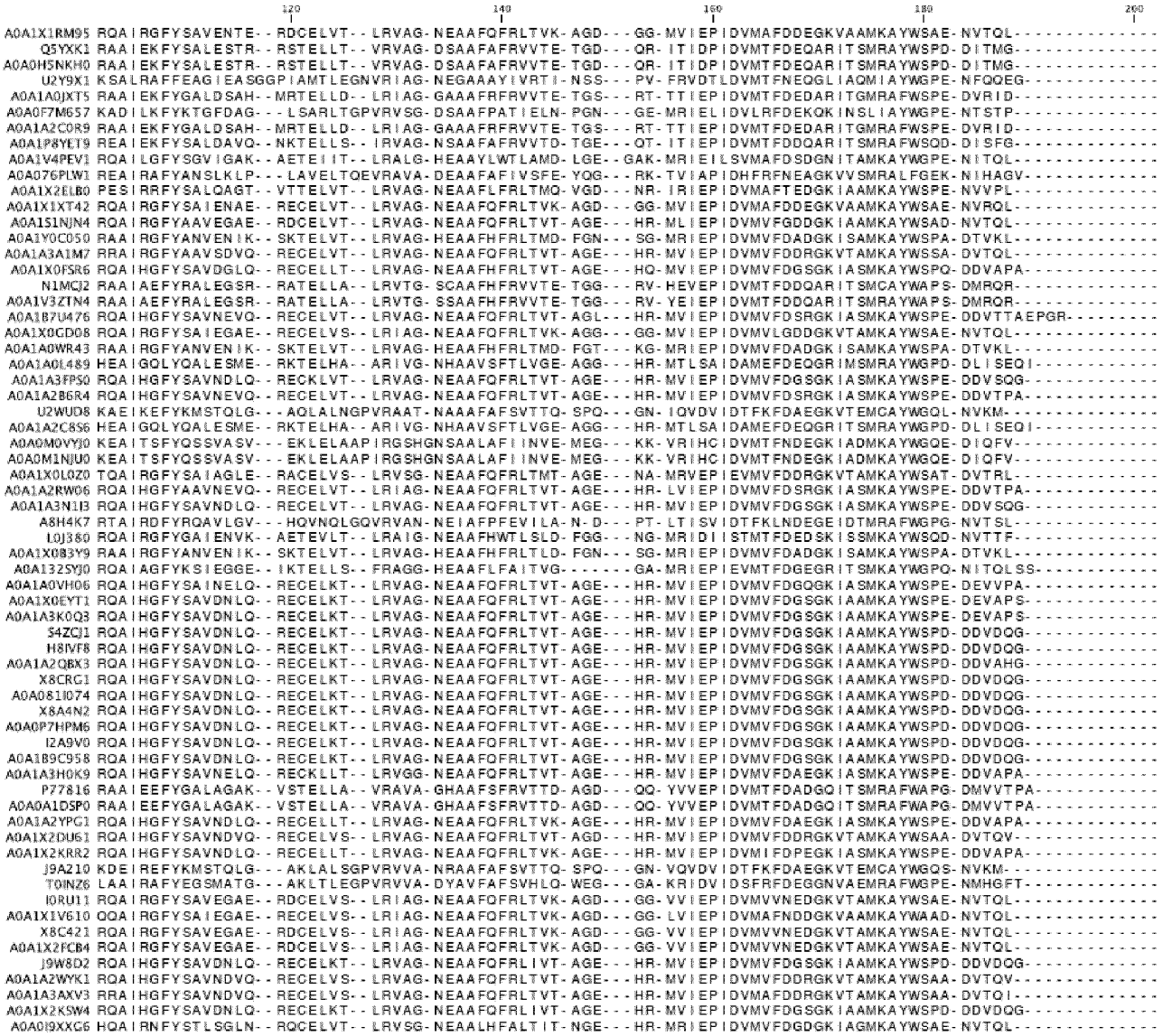
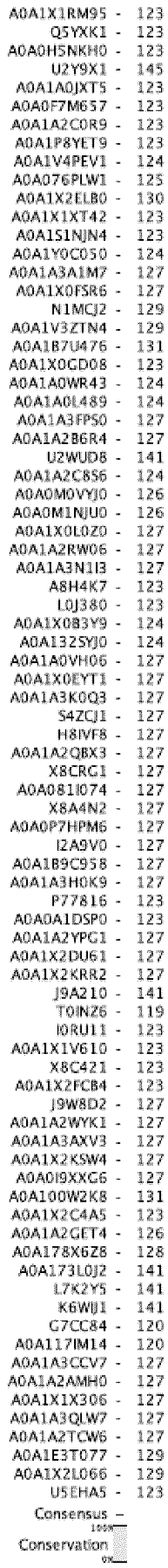


Figure 15 (cont.)



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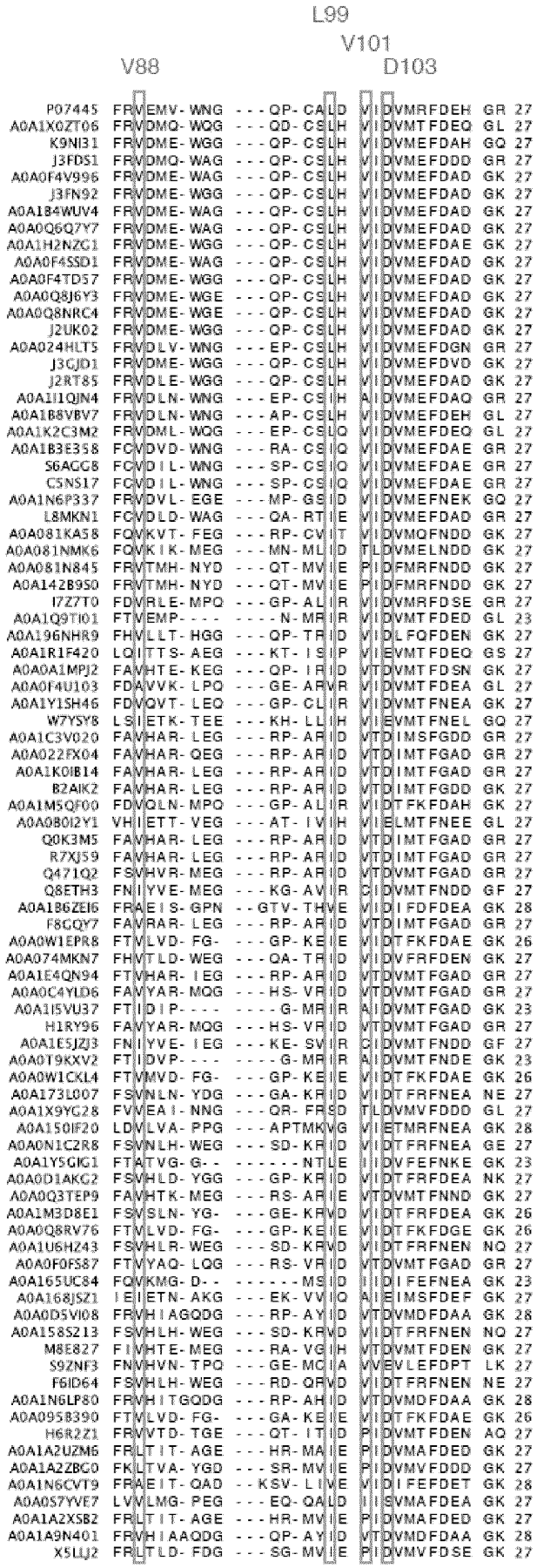


Figure 16

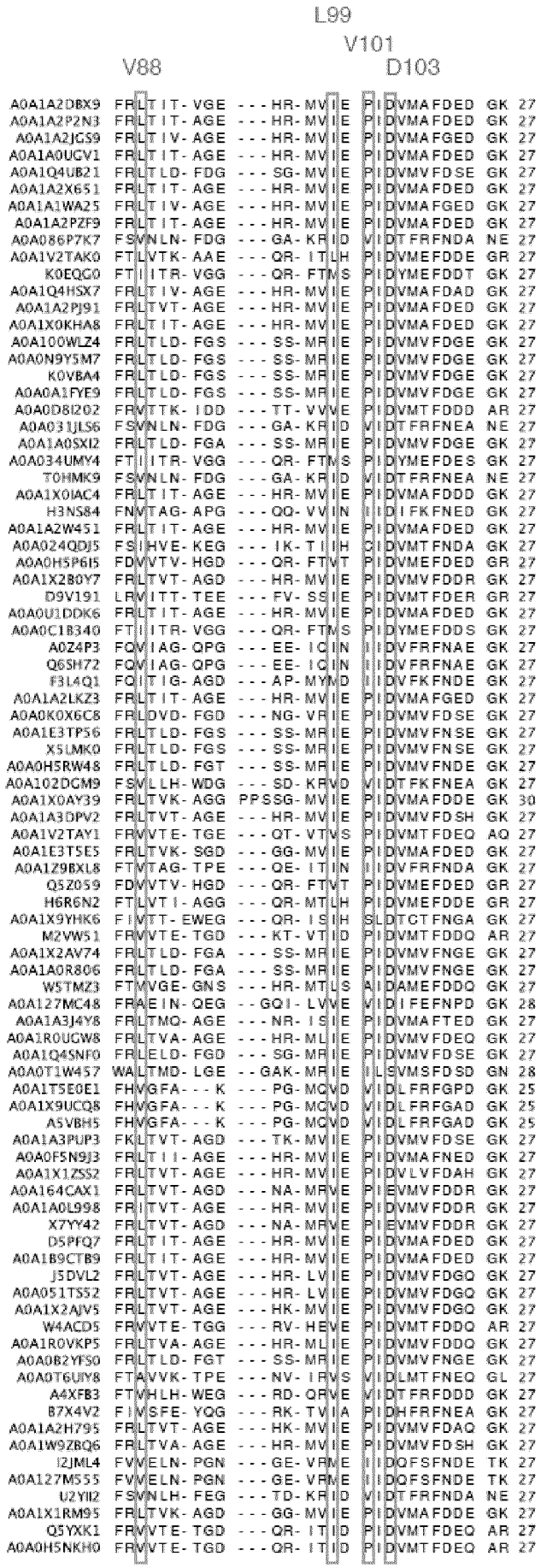


Figure 16 (cont.)

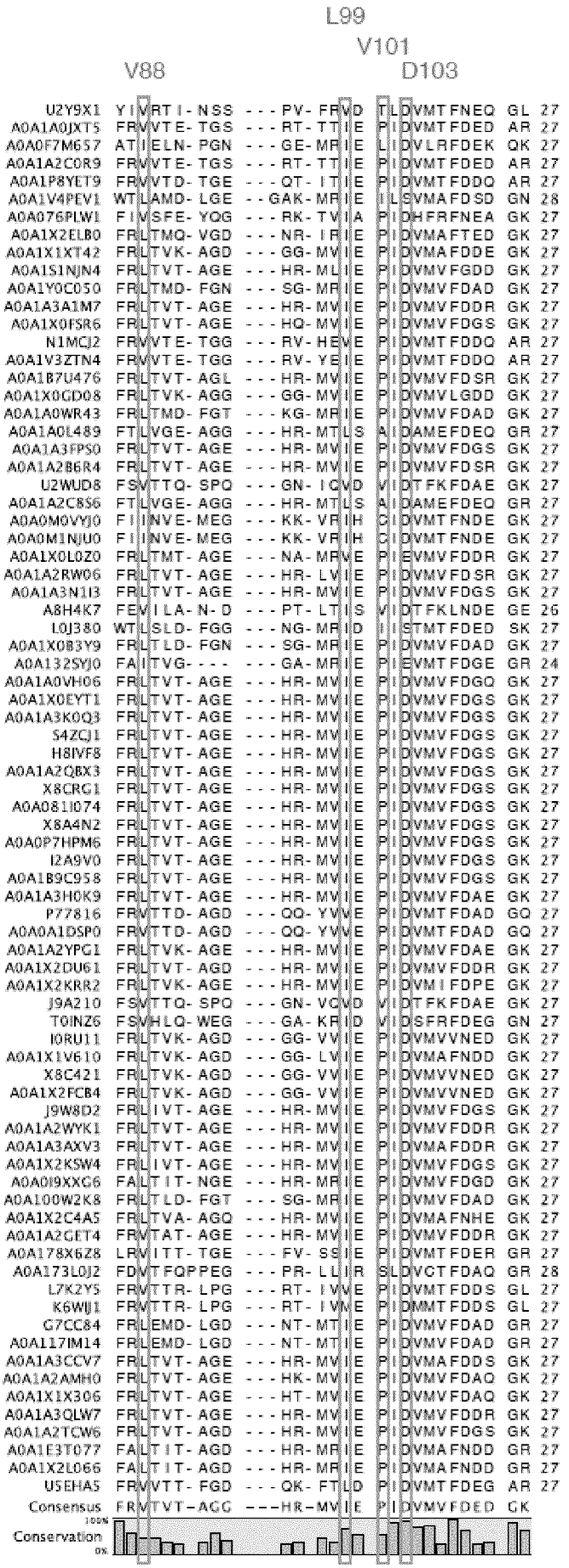
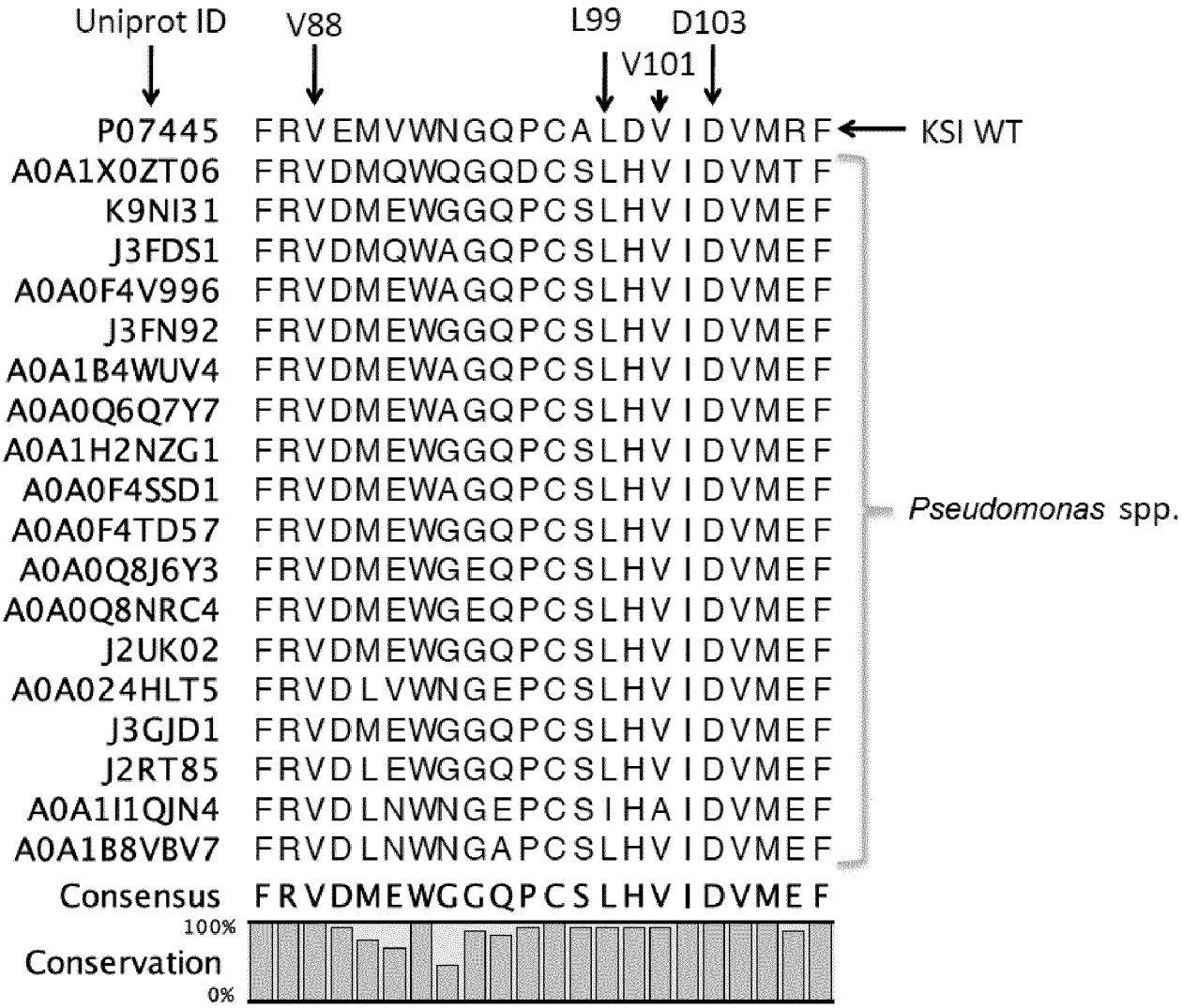


Figure 16 (cont.)


Uniprot ID
V88
L99
V101
D103
P07445 FRVEMVWNGQPCALDVIDVMRF
KSI WT
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K9NI31 FRVDMEWGGQPCSLHVIDVMEF
J3FDS1 FRVDMQWAGQPCSLHVIDVMEF
A0A0F4V996 FRVDMEWAGQPCSLHVIDVMEF
J3FN92 FRVDMEWGGQPCSLHVIDVMEF
A0A1B4WUV4 FRVDMEWAGQPCSLHVIDVMEF
A0A0Q6Q7Y7 FRVDMEWAGQPCSLHVIDVMEF
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A0A0F4SSD1 FRVDMEWAGQPCSLHVIDVMEF
A0A0F4TD57 FRVDMEWGGQPCSLHVIDVMEF
A0A0Q8J6Y3 FRVDMEWGEQPCSLHVIDVMEF
A0A0Q8NRC4 FRVDMEWGEQPCSLHVIDVMEF
J2UK02 FRVDMEWGGQPCSLHVIDVMEF
A0A024HLT5 FRVDLVWNGEPCSLHVIDVMEF
J3GJD1 FRVDMEWGGQPCSLHVIDVMEF
J2RT85 FRVDLEWGGQPCSLHVIDVMEF
A0A1I1QJN4 FRVDLNWNGEPCSIHAIDVMEF
A0A1B8VBV7 FRVDLNWNGAPCSLHVIDVMEF
Pseudomonas spp.
Consensus FRVDMEWGGQPCSLHVIDVMEF
100%
Conservation
0%

Figure 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/082377

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P5/00 C12N9/90 C07C13/18
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N C07C

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/077470 A2 (UNIV WASHINGTON [US]; BAKER DAVID [US]; ZANGHELLINI ALEXANDRE [US]; SI) 8 July 2010 (2010-07-08) abstract paragraph [0010] paragraph [0012] - paragraph [0013] paragraph [0055]; claim 1; figure 20; sequence 9	17,20, 21,28-31

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2019	01/04/2019

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

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/082377

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.:
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:

see additional sheet

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 an 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.:

1-33

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-33

   Ketosteroid isomerases, methods of catalysing the conversion of (+)-cis-isopulegone to (R)-(+)-pulegone based on said ketoisomerases, mutated ketoisomerases and the subject-matter relating thereto

   ---

2. claims: 34-37

   Methods of catalysing the conversion of (+)-cis-isopulegone to (S)-(-)-pulegone based on the use of a glutathione S-transferase and the subject-matter relating thereto.

   ---

3. claims: 38-42

   Methods of catalysing the conversion of (+)-cis-isopulegone to isopulegone based on the use of a menthone reductase and the subject-matter relating thereto.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VANDANA LAMBA ET AL: "Evaluation of the Catalytic Contribution from a Positioned General Base in Ketosteroid Isomerase", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 138, no. 31, 27 July 2016 (2016-07-27), pages 9902-9909, XP055541610, ISSN: 0002-7863, DOI: 10.1021/jacs.6b04796 abstract page 9903, column 1, paragraph 7 - column 2, paragraph 1; tables 1,2	17,28-31
X	Suhng Wook Kim ET AL: "Identification of active site residues by site-directed mutagenesis of delta5-3-ketosteroid isomerase from Pseudomonas putida biotype B", Journal of Bacteriology, 1 May 1995 (1995-05-01), pages 2602-2605, XP055545514, Washington Retrieved from the Internet: URL:https://jb.asm.org/content/jb/177/9/2602.full-text.pdf abstract; figures 2,3	17,28-31
A	US 2016/289702 A1 (SCRUTTON NIGEL SHAUN [GB] ET AL) 6 October 2016 (2016-10-06) abstract paragraph [0160]; claims 12,20; figure 2b; example 9	1-33
A	HELEN S. TOOGOOD ET AL: "Enzymatic Menthol Production: One-Pot Approach Using Engineered Escherichia coli", ACS SYNTHETIC BIOLOGY, vol. 4, no. 10, 5 June 2015 (2015-06-05), pages 1112-1123, XP055546040, Washington, DC,USA ISSN: 2161-5063, DOI: 10.1021/acssynbio.5b00092 abstract; figure 1	1-33
A	KELLY J. VINING ET AL: "Draft Genome Sequence of Mentha longifolia and Development of Resources for Mint Cultivar Improvement", MOLECULAR PLANT, vol. 10, no. 2, 1 February 2017 (2017-02-01), pages 323-339, XP055546076, GB ISSN: 1674-2052, DOI: 10.1016/j.molp.2016.10.018 abstract; table 7	1-33

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Amirhossein Ahkami ET AL: "Multiple Levels of Regulation Determine Monoterpenoid Essential Oil Compositional Variation in the Mint Family", Molecular Plant, 5 January 2015 (2015-01-05), pages 188-191, XP055546085, England DOI: 10.1016/j.molp.2014.11.009 Retrieved from the Internet: URL:https://ac.els-cdn.com/S1674205214000100/1-s2.0-S1674205214000100-main.pdf?_tid=458f05a3-2135-47f8-ad53-1bb5266ee168&acdnat=1548167274_e45299baa2730a46fa739e4376b2a0ba figure 1	1-33
A	POLLACK R M: "Enzymatic mechanisms for catalysis of enolization: ketosteroid isomerase", BIOORGANIC CHEMIS, ACADEMIC PRESS INC., NEW YORK, NY, US, vol. 32, no. 5, 1 October 2004 (2004-10-01), pages 341-353, XP004571234, ISSN: 0045-2068, DOI: 10.1016/J.BIOORG.2004.06.005 abstract	1-33
A	DATABASE UniProt [Online] 1 April 1988 (1988-04-01), "RecName: Full=Steroid Delta-isomerase; EC=5.3.3.1; AltName: Full=Delta(5)-3-ketosteroid isomerase;", XP002788237, retrieved from EBI accession no. UNIPROT:P07445 Database accession no. P07445 cited in the application the whole document	1-33
X,P	ANDREW CURRIN ET AL: "Engineering the "Missing Link" in Biosynthetic (-)-Menthol Production: Bacterial Isopulegone Isomerase", ACS CATALYSIS, vol. 8, no. 3, 24 January 2018 (2018-01-24), pages 2012-2020, XP055541608, US ISSN: 2155-5435, DOI: 10.1021/acscatal.7b04115 the whole document	1-33

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/082377

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010077470 A2	08-07-2010	US 2012100594 A1	26-04-2012
		US 2015037870 A1	05-02-2015
		WO 2010077470 A2	08-07-2010
US 2016289702 A1	06-10-2016	NONE	