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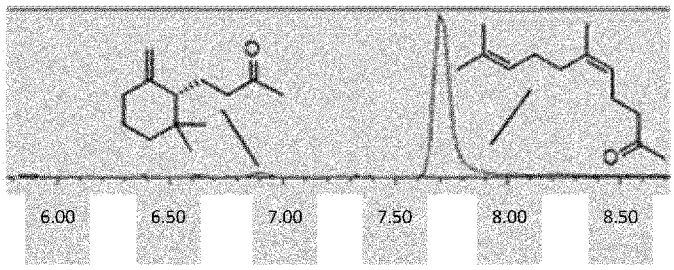
(54) Title: ENZYMATIC MONOCYCLIZATION OF ACYCLIC MONOTERPENOIDS

Figure:



(57) Abstract: Enzyme mutant with squalene-hopene-cyclase activity, selected from mutants of a wild-type enzyme comprising an amino acid sequence selected from SEQ-ID No: 1 to 3 or an amino acid sequence derived therefrom with a degree of sequence identity in the range of from 60 to 99,9 % of SEQ-ID No. 1 to 3, wherein the mutant catalyzes a one-step monocyclization reaction to produce products such as gamma-dihydroionone and/or alpha-dihydroionone.

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Enzymatic monocyclization of acyclic monoterpenoids

[0001] The present invention relates to novel enzyme mutants with cyclase activity and to a process for the monocyclization of acyclic monoterpenoids using the novel enzyme mutants.

[0002] Further embodiments of the present invention relate to nucleic acid sequences encoding for the novel enzyme mutants, to expression cassettes comprising such nucleic acid sequences and to a recombinant vector comprising, under the control of a regulative element, at least one nucleic acid sequence in accordance with the present invention or at least one expression cassette in accordance with the present invention.

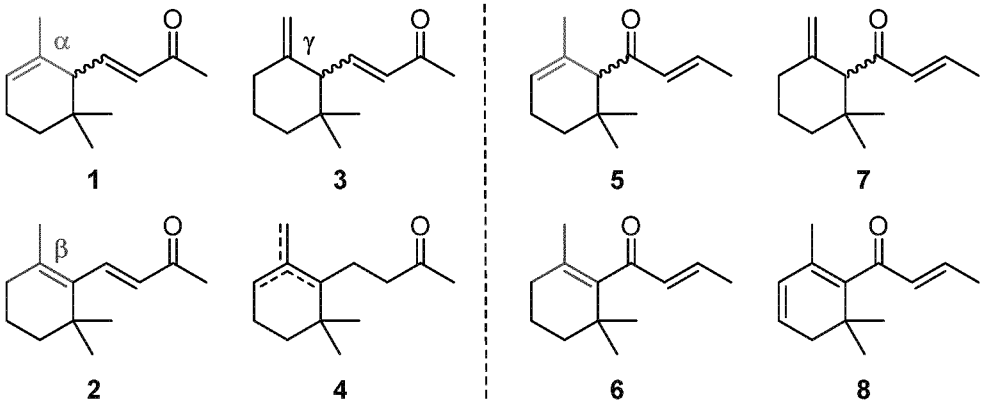
[0003] Furthermore, the present invention relates to a recombinant microorganism comprising at least one nucleic acid sequence in accordance with the present invention or at least one expression cassette in accordance with the present invention or at least one recombinant vector in accordance with the present invention.

[0004] The cyclization of terpenes with specific cyclases is known per se. As an example, squalene is cyclized to the pentacyclic compound hopene with a squalene-hopene cyclase in a process occurring in nature.

[0005] Apocarotenoids are naturally occurring isoprenoid structures derived from oxidative cleavage of several C40-carotenoids. The shorter analogues of those, mainly consisting of less than 15 carbon atoms are often used in the flavor and fragrances industry for personal care products or food flavouring agents and are therefore of high economic interest.

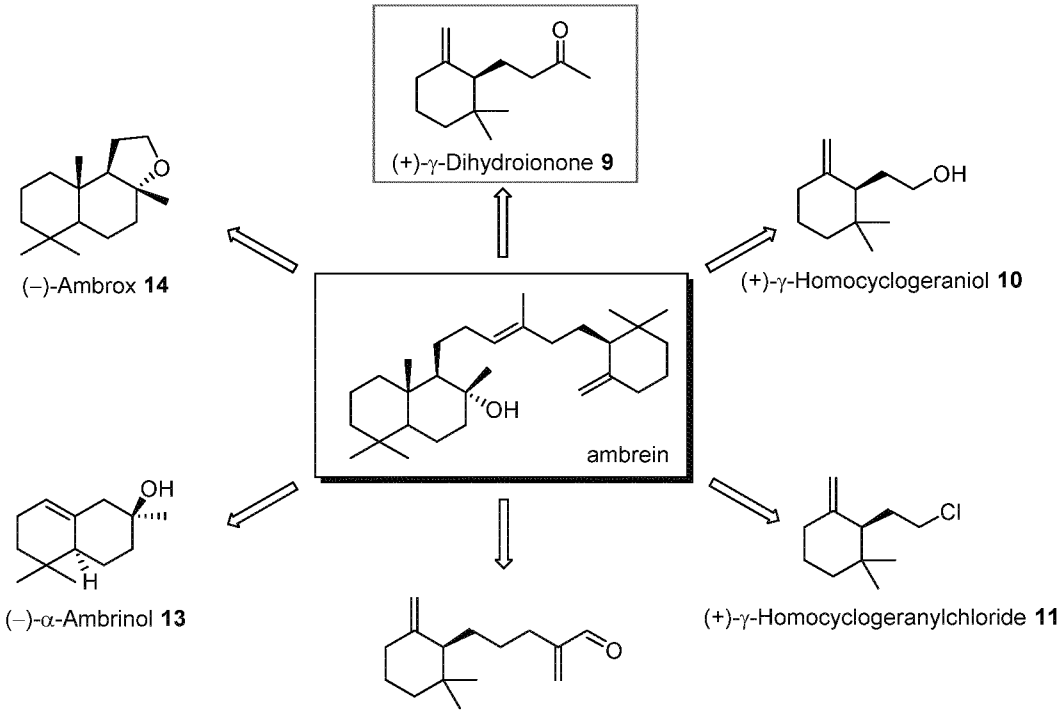
[0006] Rose ketones like Damascones **5-7**, Damascenones **8** or violet-type odor molecules like Ionones **1-3** and Dihydroionones **4** have an outstanding

position in this field due to their extremely low odor threshold.



[0007] This property together with the odor itself is closely related to the overall structure of the molecule and therefore an easy accessibility by chemical synthesis is highly desirable. The ionones **1-3** as the longest known and most studied apocarotenoids are either extracted as a mixture of isomers from plants, derived from oxidative cleavage of higher molecular carotenoids or synthesized by acid-catalyzed cyclization of their linear precursor. Hereby the choice of the acid determines the resulting product α-**1**, β-**2** or γ-ionone **3** using phosphoric, sulfuric or a lewis acid e.g. trifluoro boric acid. All of them differ in their olfactory properties whereby the (*S*)-γ-derivative has the most powerful and pleasant odor. In order to obtain the (*S*)-γ-ionone **3** a stereoselective cyclization would be preferable, but is still a challenge in classical organic chemistry and as a result multistep synthesis and lipase-catalyzed resolution approaches are commonly used.

[0008] The corresponding dihydroionones (**4**) are comparable in their properties to those of the ionones (**1-3**). Subsequently the most prominent derivative here is also the γ-derivative **9**. It is a photooxidative degradation product of ambrein, which is a main component of ambergris, also known as “floating gold” due to their high value on the market.



[0009]

[0010] Compound (+)-9 can act as a precursor to chemically synthesize 10, 11, 12 in three steps and enriched 13 in a one-step cyclization. In order to obtain (+)-9 the precursor ionone must be chemically reduced with hydrogen in presence of Raney-Nickel or otherwise irradiated with a high-pressure Hg-lamps. Direct cyclization approaches from commercially available geranyl acetone (16t) suffer from inevitably required epoxy-derivatives, protection groups or end up in bicyclic or multicyclic products. As a result (+)-9 is basically unavailable on the market due to cost-intensive and tedious synthesis.

[0011] In general, two approaches for the generation of apocarotenoids have been described in the literature: On the one hand there is the chemical approach which is divided into heterogeneous and homogeneous catalysis based on acidic cyclization and on the other hand there are some biotechnological approaches based on carotenoid cleaving enzymes.

[0012] Chemical approaches

[0013] The earliest preparation method for the Dihydroionones (4) known in literature is the reduction of the corresponding precursor Ionone. Francke et al. described the hydrogenation of (+)-1 to the reduced compound (+)-α-

Dihydroionone with a poor *ee* of 18% (Francke, W., Schulz, S., Sinnwell, V., König, W. A., & Roisin, Y, Epoxytetrahydroedulan, a new terpenoid from the hairpencils of Euploea (Lep.: Danainae) butterflies. *Liebigs Annalen der Chemie* **1989.12**, 1195-1201 (1989)). In 2000 Fuganti et al. (Fuganti, C., Serra, S. & Zenoni, A. Synthesis and olfactory evaluation of (+)- and (-)-γ-ionone. *Helv. Chim. Acta* **83**, 2761–2768 (2000)) first evaluated the olfactory properties of (+/–)-γ-Ionone **3** and (+/–)-γ-Dihydroionone **9** by synthesizing racemic **3** over four steps, perform crystallization and lipase-catalyzed resolution and finally reduce the enantiomerically pure ionone via Palladium catalysis to get enantiopure (+)-**9** with 6% overall yield.

[0014] Tsangarakis et al. (Tsangarakis, C. & Stratakis, M. Biomimetic cyclization of small terpenoids promoted by zeolite NaY: Tandem formation of α-ambrinol from geranyl acetone. *Adv. Synth. Catal.* **347**, 1280–1284 (2005)) investigated the NaY promoted cyclization of the small terpenoids Geraniol, Geranyl acetate, Farnesyl acetate and Geranyl acetone **16t.** The catalyst was completely unselective and no yields for α-Dihydroionone are given as this compound was not present in more than insignificant amounts in the reaction mixture.

[0015] In 2008 Justicia (Justicia, J. *et al.* Titanium-catalyzed enantioselective synthesis of α-ambrinol. *Adv. Synth. Catal.* **350**, 571–576 (2008)) and co-workers presented the first enantioselective synthesis of (–)-α-Ambrinol **13** starting from Geranyl acetone **16t.** The overall sequence resulted in 18% yield over 8 chemical steps.

[0016] Another method for synthesis of regioisomerically enriched γ-Dihydroionone **9** was described by Serra, S., Fuganti, C. & Brenna, E. Two easy photochemical methods for the conversion of commercial ionone alpha into regioisomerically enriched γ-ionone and γ-dihydroionone. *Flavour Fragr. J.* **22**, 505–511 (2007). The authors used commercial α-Ionone **1**, reduced and acetylated this substrate and finally irradiated the dihydro-α-ionol acetate to get enriched **9.** The method needs 6-9 steps and lacks enantioselectivity.

[0017] In Serra, S. “An expedient preparation of enantio-enriched ambergris odorants starting from commercial ionone alpha”, *Flavour Fragr. J.* **28**, 46–52 (2013). racemic α-Ionone **1** again was used as a starting agent and was transformed in either 4 steps to enriched (+)-γ-Ionone **3** or 5 steps to enriched (+)-**9**. The overall yield of the synthesis sequence was about 16%.

[0018] Biotechnological approaches

[0019] The only biotechnological synthesis of Dihydroionones **4** was reported by Sánchez-Contreras, A., Jiménez, M. & Sanchez, S. Bioconversion of lutein to products with aroma. *Appl. Microbiol. Biotechnol.* **54**, 528–534 (2000). A colony from marigold flower dehydration mud which was capable of degrading lutein was isolated. Two microorganisms were assigned to catalyze the reaction: *Trichosporon asahii* for the carotenoid cleavage of lutein and *Paenibacillus amylolyticus* for the reduction of the resulting cleavage products.[18] The product mixture contained besides β-Ionone **2**, 3-hydroxy- β-Ionone and Dihydro-β-Ionol also traces of Dihydro-β-ionone with around 3% relative conversion related to lutein.

[0020] The squalene-hopene cyclases (SHCs) belong to the family of triterpene cyclases, which catalyze the polycyclization of linear terpenes and terpenoids. Together with the oxidosqualene cyclases (OSCs) and the diterpene cyclases, it belongs to the protonase superfamily. They initiate the reaction by protonating a prenyl group, which makes them class II terpene cyclases. The natural substrate of the bacterial SHC from Alicyclobacillus acidocaldarius is the triterpene squalene, which after protonation undergoes a cascade-like, concerted polycyclization to give pentacyclic hopene or hopanol. The complexity of this one-step reaction in the formation of nine stereocenters and twelve new bonds in the pentacyclic framework of hopene or hopanol in a 5:1 ratio. This reaction is promoted, among other things, by the pre-folding of linear squalene into an all-pre-chair conformation in the active site. On the other hand, the intermediary carbocation is stabilized by aromatic amino acids. The substrate scope of this enzyme ranges from elongated C35 terpenes to

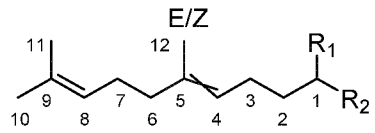
the small terpenoid geraniol. Besides isoprenyl protonation, also carbonyls and epoxides can be activated and other reaction types like isomerizations, Prins reactions and Friedel-Crafts alkylations can be catalyzed.

[0021] There still exists a need to efficient and economically feasible synthesis routes to monocyclize apocarotenoids to obtain e.g. γ-Dihydroionone **9** and other monocyclic compounds which can be used as flavors and fragrances.

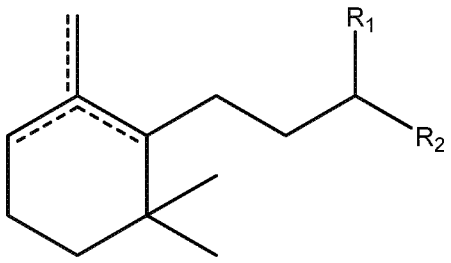
[0022] This object is achieved with enzyme mutants in accordance with claim 1.

[0023] Preferred embodiments of the invention are set forth in the dependent claims and the detailed specification hereinafter.

[0024] In the first embodiment, the present invention relates to enzyme mutants with Squalene-Hopene cyclase activity, selected from mutants of a wild-type enzyme comprising an amino acid sequence selected from SEQ-ID No. 1 to 3 or a partial sequence thereof or an amino acid sequence derived from SEQ-ID No. 2 to 3 with a degree of sequence identity in the range of from 60 to 99.9 %, preferably in the range of from 70 to 99.9% to SEQ-ID No. 2 to 3, wherein the mutant catalyzes at least the one-step monocyclization of a substrate of general formula (I)

[0025] 

[0026] to a monocyclic compound of formula (II)



[0027] wherein at least one of substituents R^1 und R^2 is selected from the group consisting of oxo, -OH, thiol, amino, ester, halogen, nitro or nitrile groups

and wherein at least one of substituents R^1 und R^2 is selected from hydrogen, alkyl or alkylene groups.

[0028] Alkyl groups preferably comprise from 1 to 10, more preferably from 1 to 8 and most preferably from 1 to 6 carbon atoms. Representative examples are methyl, ethyl, propyl, 1-methylethyl, butyl, 1-mathylpropyl, 2-methylpropyl, 1,1-dimethylethyl, pentyl, 1-methylbutyl, 2-methylbutyl, 3-methylbutyl, 2,2-dimethylpropyl, 1-ethylproypyl, hexyl, 1,1-dimethylpropyl, 1,2-dimethylpropyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, 1,1-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, 3,3-dimethylbutyl, 1-ethylbutyl, 2-ethylbutyl, 1,1,2-trimethylpropyl, 1,2,2-trimethylpropyl, 1-ethyl-1-methylpropyl and 1-ethyl-2-methylpropyl.

[0029] Alkenyl groups preferably comprise from 2 to 20, more preferably from 2 to 10 and even more preferably from 2 to 8 carbon atoms and represent linear or branched hydrocarbon residues with one or more double bonds. Representative examples are ethenyl, 1-propenyl, 2-propenyl, 1-methylethenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1-methyl-1-propenyl, 2-methy1-1-propenyl, 1-methy1-2-propenyl, 2-methy1-2-propenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1-methyl-1-butenyl, 2-methyl-1-butenyl, 3-methyl-1-butenyl, methy1-2-butenyl, 2-methy1-2-butenyl, 3-methy1-2-butenyl, 1-methy1-3-butenyl, 2-methy1-3-butenyl, 3-methy1-3-butenyl, 1,1-dimethyl-2-propenyl, 1,2-dimethyl-1-propenyl, 1,2-dimethyl-2-propenyl, 1-ethyl-1-propenyl, 1-ethyl-2-propenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl, 5-hexenyl, 1-methyl-1-pentenyl, 2-methyl-1-pentenyl, 3-methyl-1-pentenyl, 4-methyl-1-pentenyl, 1-methyl-2-pentenyl, 2-methyl-2-pentenyl, 3-methyl-2-pentenyl, 4-methyl-2-pentenyl, 1-methyl-3-pentenyl, 2-methyl-3-pentenyl, 3-methyl-3-pentenyl, 4-methyl-3-pentenyl, 1-methyl-4-pentenyl, 2-methyl-4-pentenyl, 3-methyl-4-pentenyl, 4-methyl-4-pentenyl, 1-methyl-4-pentenyl, 2-methyl-4-pentenyl, 3-methyl-4-pentenyl, 4-methyl-4-pentenyl, 1,1-dimethyl-2-butenyl, 1,1-dimethyl-3-butenyl, 1,2-dimethyl-1-butenyl, 1,2-dimethyl-3-butenyl, 1,3-dimethyl-1-butenyl, 1,3-dimethyl-2-butenyl, 1,3-dimethyl-3-butenyl,

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[0030] The term oxo defines a substituent forming a keto group together with the carbon atom to which it is bound.

[0031] The enzyme mutant in accordance with the present invention has a squalene-hopene-cyclase activity, i.e. it catalyzes the cyclization of squalene to hopene. Squalene-hopene-cyclases (also referred to as SHC hereinafter) have been described in the literature and are classified as EC. 5.4.99.17 in the EC enzyme classification scheme.

[0032] The term cyclase-activity as used herein refers to an enzyme activity determined under standard conditions with a reference substrate which describes the formation of a cyclic product starting from a non-cyclic product. Standard conditions are substrate concentration, pH value and temperature. The measurement can be made with recombinant cyclase-expressing cells, fractions thereof or with the purified enzyme.

[0033] The compounds of formula (I) which are used as substrate in accordance with the present invention belong to the group of acyclic monoterpenoids.

[0034] These compounds are monoterpenes that do not contain a cycle.

[0035] The present invention encompasses, but is not limited to enzyme mutants with squalene-hopene-cyclase activity catalyzing the monocyclization of substrates of formula (I) and comprising an amino acid sequence SEQ-ID No. 1 to 3 or a partial sequence thereof comprising e.g. at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550 or 600 consecutive amino acid residues of one of these sequences.

[0036] The degree of homology to sequence ID Nos 1 to 3 is at least 60, more preferably at least 75 and even more preferably at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 %. Homology is determined using the algorithm of Pearson and Litman, Proc. Natl. Acad. Sci USA 85(8), 1988, 2444-2448. A

homology respectively identity of an enzyme mutant in % in accordance with the present invention in particular means a respective identity of the amino acid groups based on the entire length of the amino acid sequence.

[0037] In accordance with a first preferred embodiment, up to 40%, preferably up to 30%, more preferably up to 20 % of the amino acid groups in the enzyme mutant are modified compared to SEQ-ID No. 1 or SEQ-ID No. 2 to 3 by deletion, insertion, substitution, addition or a combination thereof.

[0038] In accordance with another preferred embodiment of the invention, the enzyme mutant comprises

a) a mutation in position G600 of SEQ-ID No. 1 or

b) a mutation in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position (referred to hereinafter also as equivalent position) of the mutation corresponds to position G600 of SEQ-ID No. 1.

[0039] The term functional mutants, as used herein, relates to enzyme mutants comprising at least one mutation in an equivalent position as defined above.

[0040] Preferred mutations in position G600 of SEQ-ID. No. 1 or in a position corresponding to position G600 in SEQ-ID. No. 1 in one of the amino acid sequences SEQ-ID. No. 2 to SEQ-ID 3 is a substitution selected from the group consisting of G600A (SEQ-ID No. 9), G600S (SEQ-ID No.10), G600C (SEQ-ID No.15), G600T (SEQ-ID No. 8), G600N (SEQ-ID No.11), G600D (SEQ-ID No.12), G600Q (SEQ-ID No.13) und G600E (SEQ-ID No.14).

[0041] The enzyme mutants in accordance with the present invention may, in addition to the mutation in position G600 of SEQ-ID No. 1 or a corresponding to position G600 in SEQ-ID. No. 1 in one of the amino acid sequences SEQ-ID. No. 2 to SEQ-ID 3 further comprise at least one mutation in one of positions Y420 or L607 of SEQ-ID. No. 1, for example enzyme mutants of SEQ-ID Nos 4 (G600T/L607A) and 5 (G600T/L607A/Y420F), or at least one mutation in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3

wherein the position of the mutation corresponds to position Y420 respectively L607 of SEQ-ID No. 1.

[0042] Further preferred enzyme mutants in accordance with the present invention comprise three mutations in positions Y420, G600 und L607 of Sequence-ID. No. 1, for example enzyme mutant of SEQ-ID No. 5, or three mutations in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position of the mutation corresponds to position Y420, G600 and L607 of SEQ-ID No. 1 or comprise four mutations in positions A306, Y420, G600 und L607 of Sequence-ID. No. 1, for example enzyme mutant of SEQ-ID No. 6 (G600T/L607A/Y420F/A306V), or four mutations in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position of the mutations correspond to positions A306, Y420, G600 and L607 of SEQ-ID No. 1 or five mutations in positions A306, Y420, D436, G600 and L607 of Sequence-ID. No. 1, for example enzyme mutant of SEQ-ID No. 7 (G600T/L607A/Y420F/A306V/D436I), or five mutations in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position of the mutations correspond to positions A306, Y420, D436, G600 and L607 of SEQ-ID No. 1.

[0043] Particularly preferred enzyme mutants comprise an amino acid sequence selected from SEQ-ID. Nos. 4 to 8.

[0044] Other aspects of the present invention relate to a nucleic acid sequence, encoding for an enzyme mutant in accordance with the present invention, to expression cassettes, comprising a nucleic acid sequence encoding for an enzyme mutant in accordance with the present invention, to a recombinant vector, comprising under the control of at least one regulative element, at least one nucleic acid sequence encoding for an enzyme mutant in accordance with the present invention or comprising at least one expression cassette, comprising a nucleic acid sequence encoding for an enzyme mutant in accordance with the present invention.

[0045] Furthermore, the present invention relates to a recombinant microorganism comprising at least one nucleic acid sequence in

accordance with the present invention or at least one expression cassette in accordance with the present invention or at least one recombinant vector in accordance with the present invention.

[0046] Sequence ID No. 1 represents the sequence of the squalene hopene cyclase from *Alicyclobacillus acidocaldarius* (hereinafter *AacSHC*), which catalyzes the polycyclization of linear C_{30} terpene squalene to pentacyclic hopene/hopanol building up nine stereocenters enantiopure.

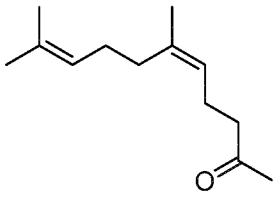
[0047] In the course of the present invention, it was found that position G600 is a hot spot position for smaller substrate conversion and the Arginine having bulky but fairly flexible properties.

[0048] Surprisingly we found that mainly small polar amino acids drive the monocyclization reaction of acyclic monoterpenoids of formula (I), e.g. geranyl acetone **16**. We assume this is facilitated due to hydrogen-bonding of the Threonine residue to the carbonyl-group of the substrate or the corresponding position in other substrates.

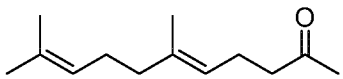
[0049] While the invention is described hereinafter by reference to geranyl acetone as one possible substrate of formula (I), the enzyme mutants in accordance with the present invention are generally capable to convert acyclic monoterpenoids of general formula (I) to monocyclic products of formula (II).

[0050] The substrates of formula (I) include all isomeric forms of the respective compounds, i.e. constitutional isomers, stereoisomers or their mixtures such as optical isomers or geometrical isomers such as E- and Z-isomers as well as any combinations thereof. If the substrate comprises more than one asymmetric center all combinations of different conformations of such asymmetric centers are possible, such as e.g. pairs of enantiomers.

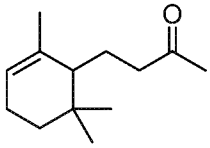
[0051] The *E*- and *Z*-isomer of geranyl acetone (**16c** and **16t**) were converted with variant G600R to bicyclic products as major products and, to a smaller extent, to the monocyclic products α-(**17**)-and γ-dihydroionone (**9**)



16c



16t



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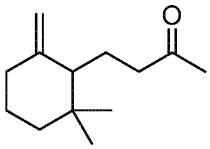


9

[0052] In the course of the present invention, it has been found that mainly small and polar amino acids drive the monocyclization reaction at position G600 and the variant G600T performed best in terms of selectivity. In particular the variants G600T (SEQ-ID No.8), G600N (SEQ-ID No.11), G600C (SEQ-ID No.15), G600S (SEQ-ID No.10), G600D (SEQ-ID No.12), G600E (SEQ-ID No. 14) and in small amounts the unpolar G600A (SEQ-ID No. 9) generated monocylic γ-dihydroionone **9** and/or α-dihydroionone **17**.

[0053] Docking studies of the substrate **16c** in the active site of the variant G600T (SEQ-ID No. 8) with YASARA were carried out and suggest two major pre-folding states: Pre-folding state 1 favors bicyclization due to the coordination of the carbonyl moiety by the Y420-hydroxy group. The resulting second carbocation of the cation cascade reaction may interact with one lone-pair of the oxygen and thereby form a covalent bond. Bulky amino acids at position G600 favor this pre-folding state. However, there is a turning point at the size of threonine, where polarity seems to play a more significant role. It is currently assumed that this is due to hydrogen bonding capabilities of polar residues. Pre-folding state 2 shows the carbonyl moiety hydrogen-bonded by G600T and Y609 thus the lone-pairs of the oxygen are faced away from the resulting second carbocation ultimately resulting in monocyclic products. Furthermore, steric interaction

of the C1-methyl group of the substrate **16c** and the Leucine at position 607 can be assumed in this pre-folding state.

[0054] Based on the docking results site-directed mutagenesis at the position L607 was introduced and the results revealed that smaller amino acids than leucine in this position are beneficial for the monocyclization reaction.

[0055] Site-directed mutagenesis of variant G600T (SEQ-ID No. 8) with the degenerated codon RVT (encoding only smaller acids than leucine, e.g. Alanine, Asparagine, Aspartate, Glycine, Isoleucine, Serine, Threonine, Valine at position L607) revealed variant G600T/L607A (SEQ-ID No. 4, hereinafter N1) showing almost double total turnover number to monocyclic products compared to G600T (SEQ-ID No. 8) and higher selectivity (79%) towards the desired γ-dihydroionone **9** (G600T: 50%).

[0056] Interestingly the substrates containing a hydroxy-moiety instead of a carbonyl (geranyl isopropanol and calmusol) show better conversion towards monocyclic compounds with the variant G600N/L607A. This could be due to the ability of the Asparagine at position 600 to also accept hydrogen-bonds instead of only donating them as in the case of Threonine. The monocyclization of geranyl isopropanol turned out be better with variant G600T (SEQ-ID No. 8) than G600T/L607A (SEQ-ID No.4.) As this substrate lacks the keto-methyl group it should not be dependent on increased space at position L607.

[0057] Introducing a third mutation in position Y420 yielding variant G600T/L607A/Y420F (SEQ-ID No. 5, hereinafter N2) doubled the direct turnover to monocyclic products compared to variant G600R/L607T and a selectivity of 94 % towards the desired product γ-dihydroionone **9**. Bicyclization was reduced to only 2% and the traces of the hydration product were completely eliminated.

[0058] Introducing a fourth mutation in position A306 yielded variant G600T/L607A/Y420F/A306V (SEQ-ID No. 6, hereinafter referred to as neryl acetone monocyclase or NMC) which showed a nearly 160-fold increased total turnover number compared to the wild-type and 97% selectivity towards the desired product γ-dihydroionone **9**.

[0059] Sulfuric acid-catalyzed cyclization experiments of γ-dihydroionone 12 to gain (+/–)-α-ambrinol **13** depending on the starting material revealed the enantiopure product (+)-α-ambrinol **13** and therefore enantiopure (-)-γ-dihydroionone **9**. Thus, the NMC-catalyzed reaction does not only proceed with high total turnover and product selectivity, but also enantioselectivity is tightly controlled with >99.5% *ee*.

[0060] Hydrogen-bond-knock-out-variants with the variant N2 (as the product selectivity was already very high at this point) *via* site-directed mutagenesis at positions 600 and 609 were prepared. The variant KO600 (T600 knocked out) showed almost 80% less conversion and 22% less selectivity towards the desired product **9**. Variant KO609 (Y609 knocked out) lost almost full activity and 50% of selectivity towards the monocyclization. Single-point mutation variant Y609F (T600 and Y609 knocked out, Y420 switched on) showed no conversion at all.

[0061] The mutational experiments and the computational can be explained by the following mechanism:

[0062] After the substrate **16c** enters the active site its carbonyl moiety is loosely coordinated by the Threonine at position 600. By this attractive interaction, the carbonyl moiety flips into the direction of the Tyrosine at position 609 for tight binding by a strong hydrogen bond. This hydrogen-bond mediated conformation allows the highly product- and enantioselective monocyclization of neryl acetone **16c** in one catalytic step. The increased space at position 607 (Leucine→Alanine) creates some space for the C1-methyl group of the substrate on the one hand and can possibly shorten the distance between T600 and Y609 on the other hand. Both scenarios solely or in combination favor the carbonyl moiety flip to the Tyrosine. The mediating role of the T600 is emphasized by the variant KO600 which still shows some conversion of the substrate with good selectivity. Together with the disabled hydrogen-bond donor at position 420 (Tyrosine→Phenylalanine) this would explain the residual good selectivity towards the desired monocyclic product **9**. The key role of the strong hydrogen binding Y609 is underlined by the variant KO609 and Y609F.

The variant KO609 almost lost its full activity, due to weak coordination of the carbonyl moiety by the Threonine at position 600 and the latter enzyme showed no activity towards monocyclic product at all.

[0063] The potential of the engineered NMC and some variants (G600T, G600N/L607S, N1, N2) generated on the engineering pathway was investigated. *E*/*Z*-mixtures of substrate analoga were used for the biotransformations. Neryl acetone **16c** was best converted by NMC in terms of monocyclization. The substrates containing a hydroxy-moiety instead of a carbonyl showed better conversion towards monocyclic compounds with the variant G600N/L607S (SEQ-ID No.17). This is presumably due to the fact that the asparagine´s and hydroxyl-moiety binding capabilities match better than that of threonine and a hydroxyl-moiety. The monocyclization of substrate **18** (formula (I) with R^1 being =O and R^2 being hydrogen towards compound **19** turned out be better with variant G600T (SEQ-ID No. 8) than G600T/L607A (SEQ-ID No. 4). Product **19** lacks, compared to **16**, the keto-methyl group and thus should not be dependent on less steric bulk at position L607.

[0064] The scalability of this reaction could be demonstrated by converting 2 g (2.24 ml) of neryl acetone **16c** with the engineered variant NMC with still high selectivity (95%; 1% α-product **14**) towards the desired product **12**. Isolation of the product (89% yield) and subsequent cyclization confirmed enantiopure conversion. Interestingly when using the *E*/*Z*-mixture of geranyl acetone **16** the evolved NMC converts the *Z*-isomer **16c** prior to the *E*-isomer **16t**. This is to our knowledge the first *Z*-selective type II cyclase reported in the literature.

[0065] The results of the experiments show the easy scalability, of the monocyclization of substrates of formula (I) to the desired monocyclic products by using the enzyme mutants in accordance with the present invention which provides an interesting perspective for industrial purposes to obtain products of formula (II) which are currently not easily accessible.

[0066] The results furthermore show the applicability of inducing pre-folding by attraction of the substrate´s polar functional group. At the same time they

resemble the importance of choice of the polar functional group for creating hydrogen bonds as not every hydrogen bond has the same power.

[0067] The present invention thus provides a catalytic one-step product- and enantioselective abortive cyclization of compounds of formula (I) towards compounds of formula (II) in gram-scale. This reaction is enabled by engineering polar functional groups inside the active site of *Aac*SHC, thus adding the ability to anchor polar functional groups of non-natural substrates *via* hydrogen-bonding. This novel feature of protonases allows the enzyme to induce non-natural pre-folding and result in abortive cyclizations products. These findings set the fundament for the evolution of protonases to control and abort complex non-natural cationic cyclization cascades for desired terpene products.

[0068] A further subject of the present invention are nucleic acid sequences encoding for an enzyme mutant in accordance with the present invention as defined in the claims and described in detail hereinbefore.

[0069] These nucleic acid sequences (e.g. single or double stranded DNA and RNA-sequences such as cDNA and mRNA) may be obtained as described in the literature such as e.g. by fragmental condensation of single overlapping complementary nucleic acid constituents of the double helix or by any other method described in the literature for the manufacture or isolation of nucleic acids. The chemical synthesis of oligonucleotides is known to the skilled person so that no further details need to be given here.

[0070] The invention also includes nucleic acid fragments which can be used as hybridizing probes or primers for the identification or amplification of nucleic acid sequences in accordance with the present invention.

[0071] The present invention furthermore relates to expression cassettes comprising a nucleic acid sequence in accordance with the present invention. An expression cassette is an expression unit which is functionally linked to the nucleic acid or the gene to be expressed. Thus, an expression cassette encompasses not only nucleic acid sequences

regulating transcription and translation but also nucleic acid sequences which, as a result of translation and transcription are intended to be expressed as a protein.

[0072] Preferably, such expression cassettes comprise a promotor in 5-direction relative to the encoding sequence and a terminator sequence in 3-direction relative to the encoding sequence, and, eventually further regulative elements functionally linked to the encoding sequence.

[0073] An expression cassette in accordance with the present invention can e.g. be obtained by fusion of a suitable promotor with a suitable nucleotide sequence with a terminator signal. Respective recombination and cloning techniques are described e.g. in T. Maniatis, E.F. Fritsch, and J. Sambrook, “Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (N.Y.), 1989.

[0074] The present invention furthermore relates to recombinant vectors, comprising under the control of at least one regulative element, at least one nucleic acid sequence in accordance with the present invention or at least one expression cassette in accordance with the present invention. The term vector, as used in the present invention, comprises plasmids and phages as well as any other vectors known to the skilled person such as viruses such as CV40, baculovirus and adenovirus, transposons, IS elements, phasmids, cosmids and linear or circular DNA and which may be replicated autonomously in the host organism or by chromosomes.

[0075] Suitable plasmids are e.g. described in WO 2012/066059 on page 37.

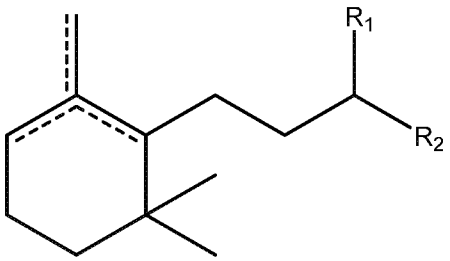
[0076] Another aspect of the present invention are recombinant microorganisms comprising at least one nucleic acid sequence in accordance with the present invention or at least one expression cassette in accordance with the present invention or at least one recombinant vector in accordance the present invention. The term microorganism encompasses wild-type microorganisms as well as genetically modified recombinant microorganisms.

[0077] As recombinant host organisms for the nucleic acid sequence or the expression cassette in accordance with the present invention any

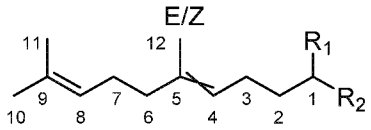
prokaryotic or eucaryotic organisms are principally suitable. Preferably bacteria, fungi or yeasts are used. Particularly preferred are gram-positive or gram-negative bacteria, more preferably bacteria of the group consisting of the families enterobacteriaceae, pseudomonaceae, rhizobiaceae, streptomycetaceae or nocardiaceae, in particular of the groups escherichia, pseudomonas, Streptomyces, nocardia, burkholderia, salmonella, agrobacterium, clostridium or rhodococcus with escherichia coli being particularly preferred.

[0078] Another subject of the present invention is a process for the manufacture of compounds of formula (II)

[0079]



[0080] wherein at least one of substituents R^1 and R^2 is selected from the group consisting of oxo, -OH, thiol, amino, ester, halogen, nitro or nitrile groups and wherein at least one of substituents R^1 und R^2 is selected from hydrogen, alkyl or alkylene groups wherein compounds of formula (I)



wherein R^1 and R^2 are as defined above, are cyclized with an enzyme mutant in accordance with the present invention or in the presence of a microorganism expressing an enzyme mutant in accordance with the present invention.

[0081] The skilled person will select the best suitable reaction conditions for the process in accordance with the present invention based on his professional knowledge so that in principle no further details need to be given here. Exemplary process conditions may be taken form the working examples, which constitute embodiments of the present invention.

[0082] The present invention provides enzyme mutants which are particularly suitable for the monocyclization of acyclic monoterpenoids such as e.g. geranyl acetone **16** to compounds of formula (II) in good yield and high purity, in particular high isomeric or enantiomeric purity. The compounds of formula (II) are of particular interest in the flavor and fragrance industry.

[0083] The present invention thus for the first time provides a process for the manufacture of the compounds of formula (II) starting from natural materials by a biotechnological process which is much simpler and faster than the conventional chemical routes known in the art. Complex multistep processes with low yield are replaced by simple one-step processes with good yield and purity of the desired products.

[0084] Further preferred embodiments are the subject-matter of the dependent claims. The invention is in some more detail described in the following examples and the accompanying figure.

[0085] The Figure shows the conversion of substrate 16 with squalene-hopene cyclase from *Thermesynechococcus elongatus* (*Tel*SHC).

[0086] **Working Examples:**

[0087] **Material**

[0088] Chemicals:

[0089] The chemicals used for syntheses, molecular biology and biochemical work have been purchased from Carl-Roth (Karlsruhe, DE), VWR (Pennsylvania, US), Sigma-Aldrich (St. Louis, US) and Alfa-Aesar (Ward Hill, US). The substrates (E/Z)-geranyl acetone from VWR (A19184.14), Calmusal from ambinter (18445-88-0) and ambrinol from Amyris. All the other subtrates for biocatalytic purposes were chemically synthesized and analyzed by ^{1}H-NMR, ^{13}C-NMR and GC/MS.

[0090] **Molecular biological kits.**

[0091] The molecular biological kits for DNA-purification (*Zymoclean DNA Clean & Concentrator Kit)*, Agarose gel-extraction (*Zymoclean Gel DNA Recovery* Kit) and plasmid isolation (*Zyppy™Plasmid Miniprep Kit*) were purchased from *ZymoResearch* (Irvine, US).

[0092] **Table 1: List of Buffers & Media**

Buffer	Ingredients
10x phosphate buffer (KP_i-buffer)	0.17 M KH_2PO_4, 0.72 M K_2HPO_4, pH=7.4
Whole cell buffer	100 mM Citric acid, 0.1 % SDS, pH=6.0
Cyclodextrin (CD) buffer	0.2 % SDS, 10mM (2-Hydroxypropyl)-β-cyclodextrin, pH=6.0
Lysis buffer	200 mM Citric acid, 0.1% EDTA, pH=6.0
Extraction buffer	100 mM Citric acid, 1% CHAPS, pH=6.0

[0093] *Table 2: List of media used*

Medium	Ingredients
Lysogeny broth	10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract
Auto-induction medium*(T-DAB)	12 g/L tryptone, 24 g/L yeast extract, 2.9 g/L glucose, 11.1 g/L Glycerol, 7.6 g/L Lactose

[0094] based on: https://www.tci.uni-hannover.de/uploads/tx_tkpublikationen/Poster_for_Wien_autoinduction_Zhaopeng_Li.pdf

[0095] Table 3: List of primers used in this work

No	Name (SEQ-ID No.)	Sequence (5´→3´) Forward/Reverse
1	*Aac*SHC_G600C (ID 36, ID 37)	CCGTATTATACCGGCACCTGCTTCCCGGGCG / CGCCCGGGAAGCAGGTGCCGGTATAATACGG
2	*Aac*SHC_G600D (ID 38, ID 39)	CCGTATTATACCGGCACCGATTTCCCGGGCG / CGCCCGGGAAATCGGTGCCGGTATAATACGG
3	*Aac*SHC_G600E	CCGTATTATACCGGCACCGAATTCCCGGGCG / CGCCCGGGAATTCGGTGCCGGTATAATACGG

	(ID 40, ID 41)	
4	*Aac*SHC_G600H (ID 42, ID 43)	CCGTATTATACCGGCACCCATTTCCCGGGCG / CGCCCGGGAAATGGGTGCCGGTATAATACGG
5	*Aac*SHC_G600I (ID 44, ID 45)	CCGTATTATACCGGCACCATTTTCCCGGGCG / CGCCCGGGAAAATGGTGCCGGTATAATACGG
6	*Aac*SHC_ G600K (ID 46, ID 47)	CCGTATTATACCGGCACCAAATTCCCGGGCG / CGCCCGGGAATTTGGTGCCGGTATAATACGG
7	*Aac*SHC_G600M (ID 48, ID 49)	CCGTATTATACCGGCACCATGTTCCCGGGCG / CGCCCGGGAACATGGTGCCGGTATAATACGG
8	*Aac*SHC_G600N (ID 50, ID 51)	CCGTATTATACCGGCACCAACTTCCCGGGCG / CGCCCGGGAAGTTGGTGCCGGTATAATACGG
9	*Aac*SHC_G600P (ID 52, ID 53)	CCGTATTATACCGGCACCCCGTTCCCGGGCG / CGCCCGGGAACGGGGTGCCGGTATAATACGG
10	*Aac*SHC_G600Q (ID 54, ID 55)	CCGTATTATACCGGCACCCAGTTCCCGGGCG / CGCCCGGGAACTGGGTGCCGGTATAATACGG
11	*Aac*SHC_ G600L (ID 56, ID 57)	CCGTATTATACCGGCACCCTATTCCCGGGCG / CGCCCGGGAATAGGGTGCCGGTATAATACGG
12	*Aac*SHC_G600S (ID 58, ID 59)	CCGTATTATACCGGCACCTCGTTCCCGGGCG / CGCCCGGGAACGAGGTGCCGGTATAATACGG
13	*Aac*SHC_G600T (ID 60, ID 61)	CCGTATTATACCGGCACCACCTTCCCGGGCG / CGCCCGGGAAGGTGGTGCCGGTATAATACGG
14	*Aac*SHC_G600V (ID 62, ID 63)	CCGTATTATACCGGCACCGTGTTCCCGGGCG / CGCCCGGGAACACGGTGCCGGTATAATACGG
15	*Aac*SHC_G600Y (ID 64, ID 65)	CCGTATTATACCGGCACCTATTTCCCGGGCG / CGCCCGGGAAATAGGTGCCGGTATAATACGG
16	*Aac*NMC_Y609F (ID 66, ID 67)	GGCGATTTTTATGCGGGCTTTACCATGTATC GCCATGTG/ CACATGGCGATACATGGTAAAGCCCGCATAAAA ATCGC
17	*Aac*SHC_Y420F (ID 68, ID 69)	CAACGGCGGCTGGGGCGCGTTTGATGTGGATA ACACCAGC/ GCTGGTGTTATCCACATCAAACGCGCCCCAGC CGCCGTTG

	*Aac*SHC_L607M (ID 70, ID 71)	GGTTCCCGGGCGATTTTTATGCCATGTATACCA TGTATCGCC/ GGCGATACATGGTATAGCCGGCATAAAAATCGC CCGGGAACC
	*Aac*SHC_L607S (ID 72, ID 73)	GGTTCCCGGGCGATTTTTATAGCCATTATACCAT GTATCGCC/ GGCGATACATGGTATAGCCGCTATAAAAATCGCC CGGGAACC
	*Aac*SHC_L607Y (ID 74, ID 75)	GGTTCCCGGGCGATTTTTATTATGGCTATACCATG TATCGCC/ GGCGATACATGGTATAGCCATAATAAAAATCGCCC GGGAACC
	*Aac*SHC_L607V (ID 76, ID 77)	GGTTCCCGGGCGATTTTTGTCCCATGTATACCATG TATCGCC/ GGCGATACATGGTATAGCCGGGACAAAAATCGCC CGGGAACC
18	*Aac*SHC_L607RVT (ID 78, ID 79)	GGTTCCCGGGCGATTTTTATRNTGGCTATACCATG TATCGC/ ATAAAAATCGCCCGGGAACCCGGTGCCGGTATAA TACGG
19	*Aac*SHC_I261X (ID 80, ID 81, ID 82, ID 83)	GCGATGGCAGCTGGGGCGGCNDTCAGCCGCCGTG GTTTTATGC/ GCGATGGCAGCTGGGGCGGCVHGCAGCCGCCGTG GTTTTATGC/ GCGATGGCAGCTGGGGCGGCTGGCAGCCGCCGTG GTTTTATGC/ TGCCGCCCCAGCTGCCATCGCCCGCCTGGCGTTCCAG
20	*Aac*SHC_F365X (ID 84, ID 85, ID 86, ID 87)	GAAACCGGGCGGCTTTGCGNDTCAGTTTGATAACGTG TATTATCCGG/ GAAACCGGGCGGCTTTGCGVHGCAGTTTGATAACGTG TATTATCCGG / GAAACCGGGCGGCTTTGCGTGGCAGTTTGATAACGTG TATTATCCGG / CGCAAAGCCGCCCGGTTTCAGGTTCGGGCGTTTCAC
21	*Aac*SHC_L36X (ID 88, ID 89, ID 90, ID 91)	GGCTATTGGTGGGGCCCGNDTCTGAGCAACGTGAC CATG/ GGCTATTGGTGGGGCCCGVHGCTGAGCAACGTGACC ATG/

		GGCTATTGGTGGGGCCCGTGGCTGAGCAACGTGAC CATG/ CGGGCCCCACCAATAGCCTTCATCTTTCTGGCAGC TCAG
22	*Aac*SHC_S307X (ID 92, ID 93, ID 94, ID 95)	GGCTGGATGTTTCAGGCGNDTATTAGCCCGGTGT GGG/ GGCTGGATGTTTCAGGCGVHGATTAGCCCGGTGT GGG/ GGCTGGATGTTTCAGGCGTGGATTAGCCCGGTGT GGG/ CGCCTGAAACATCCAGCCGCCATAATCCAGTTCCACG
23	*Aac*SHC_A306X (ID 96, ID 97, ID 98, ID 99)	GGCGGCTGGATGTTTCAGNDTAGCATTAGCCCGGTG/ GGCGGCTGGATGTTTCAGVHGAGCATTAGCCCGGTG/ GGCGGCTGGATGTTTCAGTGGAGCATTAGCCCGGTG/ CTGAAACATCCAGCCGCCATAATCCAGTTCCACGCCA TACAG

ID represents the SEQ-ID No. in the sequence listing.

[0096] **General analytics**

[0097] **Nuclear Magnetic Resonance**

[0098] ^{1}H- und ^{13}C-NMR spectra were recorded on a Bruker Avance 500 Spectrometer at 500,15 MHz for ^{1}H- and 125 MHz for ^{13}C. The chemical shifts δ are referred to tetramethylsilane (=TMS) in ppm set to 0. All substances were dissolved in $CDCl_3$ and recorded at room temperature.

[0099] **Circular dichroism**

[00100] The specific optical rotation of the compounds was measured on a Perkin Elmer Polarimeter 241.Therefore the substance was dissolved in $CHCl_3$ (c=0.5 mg/ml) and the specific rotation was measured with a sodium and a mercury spectral lamp.

[00101] **Gas chromatography**

[00102] GC analyses were performed using an Agilent GC 7820A equipped with a mass spectrometer MSD 5977B and a HP-5MS capillary column (Agilent, 30 m x 250 µm x 0.25 µm) and helium as carrier gas with a constant pressure of 14.168 ψ. Injections (1 µL) were performed in split mode (10:1). Relative conversion rates were calculated directly from GC-MS

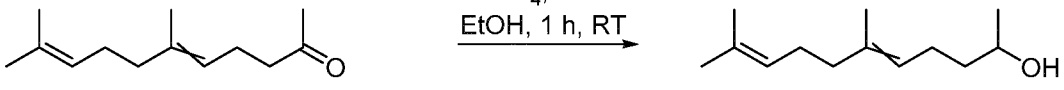
spectra by integration-quotient of substrates and products. Chiral GC analysis was performed on a Shimadzu GC-2010 equipped with a CP ChiraSil-Dex CB capillary column (Agilent, 25 m x 250 µm x 0.25 µm) and hydrogen as carrier gas with constant velocity (linear velocity: 33.1 cm/s). Injections (1 µL) were performed in split mode (5:1). Temperature programs are listed in table 4.

[00103] Table 4: Temperature programs used in this work

Name	Rate (°C/min)	Temp. (°C)	Hold (min)
Dihydroion long		120	0.1
	2	145	0.6
Dihydrion short		120	0.1
	2	137	0.6
Calmusal		110	0.1
	2	135	0.6
General		50	3
	6	120	0
	10	150	0
	15	170	0
	20	200	0
	25	250	0
	30	310	
Chiral		70	3
		140	0
	8	180	2

[00104] **Chemical Synthesis**

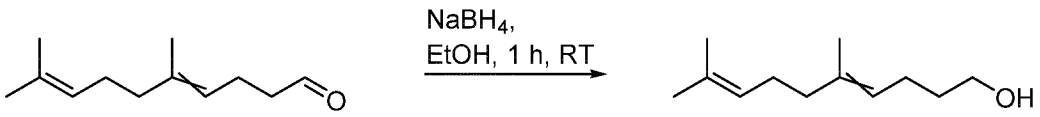
[00105] **Synthesis of geranyl isopropanol**

[00106] 

[00107] For the reduction reaction geranyl acetone (0.50 ml, 2.34 mmol 1.00 eq.) was dissolved in ethanol (10 ml). Sodium borohydride (0.088 g, 2.34 mmol, 1.00 eq.) was then added carefully and the reaction mixture was stirred at room temperature for 1 h. After the reaction was complete, the mixture was quenched with 0.5 N HCl (2 ml) and stirred again for 30 min. Then distilled water (50 ml) was added and the aqueous phase was extracted three times with DCM. The combined organic phases were dried over $CaCl_2$ and the geranyl isopropanol was obtained as a clear oil (0.49 ml, 2.04 mmol, 87%).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 1.19 (d, *J* = 2.9 Hz, 3H), 1.50 (quart, *J* = 7.7 Hz, 2H) 1.6 (s, 3H), 1.62 (s, 3H), 1.68 (s, 3H), 1.88-1.92 (t, *J* = 7.3 Hz, 2H), 2.04-2.12 (m, 4H), 3.77-3.84 (sept, *J* = 17.43 Hz, 1H), 5.05-5.10 (t, *J* = 6.7 Hz, 1H), 5.12-5.17 (t, *J* = 6.8 Hz, 1H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** δ (ppm) 16.50 (1C), 16.66 (1C), 22.44-25.63 (4C), 38.15-38.70 (2C), 66.97 (1C), 75.67 (1C), 122.22-123.24 (2C), 134.9 (1C). **MS (EI):** *m/z* (%) = 196 (0.3), 153 (32), 135 (21), 109 (58), 95 (21), 82 (19), 81 (21), 69 (100), 68 (13), 67 (44). The data are consistent with the literature[1].

[00108] **Synthesis of 6,10-dimethylundeca-5,9-dien-2-ol (23)**

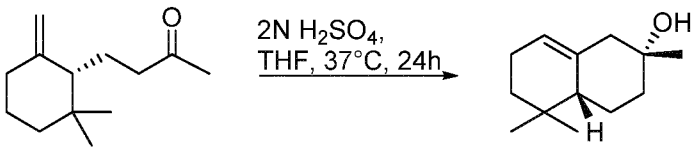


22 **23**

[00109] The reaction was carried out analog to synthesis **(1)**. The product was obtained as a clear oil (0.21 ml, 1.04mmol, 43%).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 1.19-1.34 (m, 2H), 1.55-1.59 (m, 2H) 1,61(s, 3H), 1.62-1.65 (m, 1H), 1.68 (s, 3H), 1.69-1.71 (m, 2H), 2.04-2.12 (m, 4H), 3.62-3.67 (t, *J* = 6.6 Hz, 2H), 5.03-5.19 (m, 2H). The data is consistent with the literature[2].

[00110] **Sulfuric acid catalyzed cyclization of (–)-γ-dihydroionone**



(+)-α-ambrinol

[00111] For the cyclization reaction (–)-γ-dihydroionone (400 µL, 1.8 mmol) was dissolved in THF (15 mL) in a 50 mL Schott-bottle. 2N sulfuric acid (5 mL) was then added and the reaction mixture was shaken at 37 °C for 24h. The reaction was quenched by addition of water (20 mL) and extracted with Diethylether (3 x 30 mL). The combined organic phases were dried over $MgSO_4$ and purified *via* silica chromatography (10:1, hexane: ethyl acetate) to yield the slightly yellowish liquid (+)-α-ambrinol (350 µL, 1.5 mmol, 88 % yield); $([\alpha]_D^{20}$ = +84.6; Lit. = 81.8[3]).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 0.87 (s, 3H), 0.91 (s, 3H) 1.14 (m, 1H), 1.22 (s, 3H), 1.24-1.40 (m, 3H), 1.45-1.51 (m, 2H), 1.67-1.74 (m, 2H), 1.98-2.02 (m, 2H), 2.06-2.17 (m, 2H), 5.45 (t, J = 3.84Hz, 1H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** δ (ppm) 22.6 (1C), 23.8 (1C), 25.05 (1C), 26.02 (1C), 28.07 (1C), 29.24 (1C), 31.11 (1C), 28.95(1C), 47.25 (1C), 49.82 (1C), 70.28 (1C), 122.04 (1C), 137.39 (1C). The data is consistent with the literature[4].

(+)-α-ambrinol **MS (EI):** m/z (%) = 194 (5), 176 (40), 161 (30), 136 (100), 121 (66), 120 (40), 109 (28), 105 (31), 95 (49), 93 (28).

Side product: (–)-β-ambrinol: **MS (EI):** m/z (%) = 194 (6), 176 (55), 161 (100), 136 (40), 121 (84), 107 (43), 106 (46), 105 (60), 93 (52), 91 (42).

[00112] General methods

[00113] Plasmid isolation

[00114] Isolation of the plasmid proceeded following to the standard protocol of *Zyppy™Plasmid Miniprep Kit* by *ZymoResearch*.[5] For the photometric determination of the plasmid DNA concentration, 1 µL was measured on a *Nanodrop 1000* (Agilent, Santa Clara, US) at a wavelength of 260 nm.

[00115] **Site-saturation/-directed mutagenesis**

[00116] The gene encoding for *Aac*SHC (UniProt: P33247) or a variant based on this gene was cloned into a pET-22b(+) vector system (Merck, Darmstadt, Germany). SacI and NdeI were used as restriction sites. Cloning followed the standard protocol of *Novagene´*s KOD Hot Start DNA Polymerase.[6] The composition of the PCR mixture and the temperature profile are described in Table 5 and Table 6.

[00117] *Table 5: Composition of the PCR mixture*

substance	volume [µl]	final concentration
ddH_20	29	
DMSO	2.5	
KOD Hot Start Buffer (10x)	5	1x
dNTPs (2 mM each)	5	250 µm (each)
$MgSO_4$ (25 mM)	4.5	2 mM
Template DNA	1	0,5-5 ng/ µl
Primer *forward* (10 µM)	1	0,2 µm
Primer *reverse* (10 µM)	1	0,2 µm
KOD Hot Start DNA Polymerase	1	

[00118] *Table 6: PCR temperature profile*

step	Temperature [°C]	time [s]	cycles
Initial denaturation	95	120	1
Denature	95	30	
Annealing	60	30	30
Extension	70	210	
Final extension	72	420	1

[00119] Site-saturation libraries were generated employing the “22c-trick” method.[7] PCR products were digested with 1 µL DpnI for 2h at 37 °C, purified by agarose gel electrophoresis and ligated into the pET22b(+) vector by Gibson assembly[8]. After purification using the DNA Clean & Concentrator ™-5 kit[9] the plasmids were transformed via heat-shock

method. Site-directed clones were digested and directly transformed afterwards.

[00120] **Plasmid transformation**

[00121] Chemically competent cells based on rubidium chloride were produced for the transformation of the plasmid DNA.[10] The transformation was carried out under sterile conditions. For site saturation libraries 3 µL of the purified PCR product was added to 25 µL XL1-blue competent cells and incubated for 30 min on ice, followed by a heat shock at 42 °C for 105 s with subsequent ice cooling for 3 min. After adding 500 µl of LB medium, the cells were incubated for 40min at 37 ° C and used for inoculation of a 5 mL LB medium (Ampicillin, c_{end}= 100 µg/ml) pre-culture overnight. After isolation of the plasmid, transformation into 50 µL BL21 (DE3) was performed using the heat shock method. After regeneration 150 µL were streaked out on an agar plate (Ampicillin, c_{end}= 100 µg/ml) and incubated at 37 °C overnight. For quality control the plasmid was isolated from another 150 µL and sent for sequencing. For site-directed mutants the PCR product was directly transformed into XL1-blue competent cells after digest. After regeneration 300 µL were streaked out on an agar plate for single clone picking.

[00122] **Expression of *Aac*SHC libraries in 96-DW plates**

[00123] Individual colonies were picked from generated agar plates and cultivated in 500 µL LB medium (Ampicillin, c_{end}= 100 µg/ml) for 18-20h at 37 °C, 800 rpm. Expression cultures were inoculated with 10 µL of the pre-culture into 1 mL of T-DAB autoinduction medium (Ampicillin, c_{end}= 100 µg/ml) with lactose as the inductor. The cultures were incubated for 20h at 37 °C, 800 rpm and harvested afterwards (4000 x g, 20 min).

[00124] **Expression in 24 DW-plates**

[00125] Individual colonies were picked from generated agar plates and cultivated in 2 mL LB medium (Ampicillin, c_{end}= 100 µg/ml) for 18-20h at 37 °C, 180 rpm. Expression cultures were inoculated with 40 µL of the pre-culture into 4 mL of T-DAB autoinduction medium (Ampicillin, c_{end}= 100 µg/ml) with

lactose as the inductor. The cultures were incubated for 20h at 37 °C, 600 rpm and harvested afterwards (4000 x g, 20 min).

[00126] **Thermolysis purification**[11,12]

[00127] Harvested or lyophilized cells were resuspended in 1 mL *Lysis buffer* and incubated for 60 min at 70 °C. The cell suspension was centrifuged (14000 x g, 1 min) and the supernatant was discarded. As the enzyme is membrane-bound 1 mL 1%-CHAPS buffer was added to extract it from the cell pellet by shaking at room temperature for 1d, 600 rpm. After subsequent centrifugation (14000 x g, 1 min) the supernatant containing the *Aac*SHC was transferred to a new tube followed by SDS-PAGE analysis and determination of enzyme concentration by using the *Nanodrop 1000* (Agilent, Santa Clara, US). Therefore the "Protein A280" mode was chosen with MW= 71439 Da and molar extinction coeffizient ε = 185180 as protein specific data.

[00128] **SDS-PAGE**

[00129] After protein purification and extraction 20 µl of of the enzyme preparation was mixed with 10 µl SDS loading buffer and heated to 95 ° for 10 min. Afterwards 10 µl of the preparation were loaded on the pre-prepared SDS-PAGE.

[00130] **Screening of *Aac*SHC libraries via GC-MS**

[00131] Harvested pellets were resuspended in 400 µL *whole cell buffer* and transferred to another 96-DW plate equipped with 1.2 mL glass inlets. Afterwards 4 µL substrate/DMSO stock solution (substrate c_{end}= 2mM) was added directly into the cell suspension, the plates were sealed and shaken for 20h at 30 °C, 600 rpm. In order to stop the reaction 600 µL cyclohexene/o-xylol (1:1) was added and the mixture was incubated for 10 min. The plates were centrifuged (4000 x g, 5 min), sealed using PP-sealings and a GC-MS equipped with a PAL-Sampler was used to inject directly from the organic phase. Quantification was made directly from the Total Ion Count chromatogramm by quotient $AREA_{product}$/ ($AREA_{substrate}$+$AREA_{product}$)*100. In total 90 variants per plate were

screened. Promising variants were rescreened by expression in 24 DW-plates.

[00132] **Verification of promising hits**

[00133] Promising candidates from the 96-DW screening were taken for inoculation of a 5 mL LB pre-culture. Afterwards the plasmids were isolated and transformed for single colony picking. The single colonies were expressed in 24 DW-plates and after harvesting the OD_{600} was set to 20 in *whole cell* buffer substrate was added (c_{end}=4.4 mM) The reactions were carried out at least in technical duplicates. Reactions were stopped by adding Dichloromethane. After two extraction the resulting organic phase was measured directly over GC-MS. Quantification was made directly from the Total Ion Count chromatogramm by quotient $AREA_{product}$/ ($AREA_{substrate}$+$AREA_{product}$)*100.

[00134] **Determination of Total Turnover Number**

[00135] After expression and harvesting in 24 DW-plates the cell pellets were frozen at -80°C overnight. Afterwards the frozen pellets were lyophilized in a *Christ alpha 2-4 LD plus* overnight. For the reaction setup 10 mg of the E. coli whole cells were resuspended in 1 ml *cyclodextrin* buffer and 2 µl (c_{end}=8.8 mM) of substrate was added to the suspension and the reaction was stirred for 20h at 30°C. The reaction was stopped by addition of DCM and 10 mM of 1-Undecanol was added. The reaction was extracted three times and the combined organic phases were measured over GC-MS. Quantification was made by 1-Undecanol as internal standard. The protein concentration was determined by extracting the enzyme from 10 mg for each batch in triplikates *via* thermolysis (see (6)). Verification and quality control was done by SDS-PAGE.

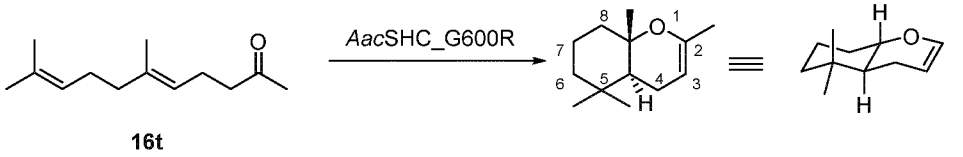
[00136] **Up-scaling reactions**

[00137] In order to isolate and determine the structure of the products upscalings of the biotransformations were performed. Therefore, the corresponding variant was expressed and the harvested cell pellets were lyophilized. Afterwards 3g of lyophilized whole cells were resuspended in 200 mL buffer (0.1% SDS, 50 mM Citric acid, 5mM (2-Hydroxypropyl)-β-

cyclodextrin) and 200 µl substrate was added. The reactions were carried out in closed 250 mL flasks at 30 °C and 250 rpm for seven days. The crude product was centrifuged to get rid of the cell debris. The aqueous phase containing the product encapsulated by cyclodextrin was extracted with diethyl ether three times, reduced under vacuum, purified over column chromatography (petroleum ether: ethyl acetate; 50:1→10:1) and evaluated via NMR and GC/MS.

[00138] For *Z*-geranyl acetone conversion 10g of lyophilized whole cells were used in 500 mL *cyclodextrin* (CD) buffer and 2 g (2.24 ml) substrate was added.

[00139] *E*-geranyl acetone **16t** with G600R (SEQ-ID No.16)

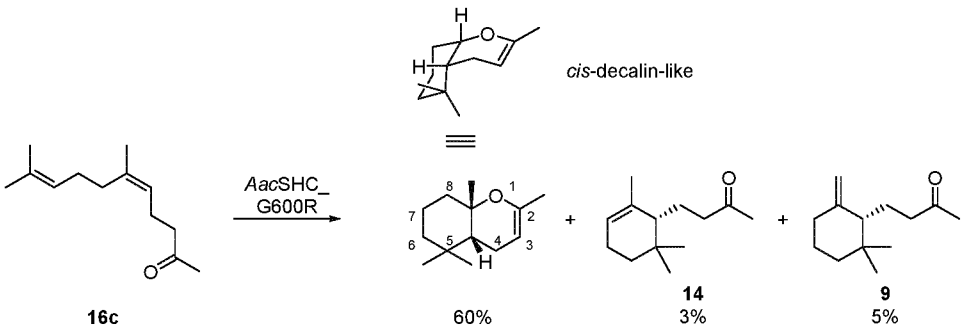


[00140]

[00141] Colorless oil, 0.167 ml, 0.77 mmol, 85 % yield. (4S,8S)-2,5,5,8-tetramethyl-4,5,6,7,8,8-hexahydro-4H-chromene **11t**: **^{1}H-NMR ($CDCl_3$, 500 MHz):** δ (ppm) 0.81 (s, 3H), 0.91 (s, 3H) 1.17 (s, 3H), 1.21-1.29 (m, 1H), 1.4-1.6 (m, 5H), 1.68 (s, 3H), 1.72-1.94 (m, 3H), 4.4-4.5 (m, 1H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** δ (ppm) 19.07 (1C), 19.21 (1C), 19.82 (1C), 20.51 (1C), 20.77 (1C) 30.31 (1C), 32.25 (1C), 39.99 (1C), 41.65 (1C), 48. 37 (1C), 76.48 (1C), 94.97 (1C), 147.97 (1C). The data is consistent with the literature.[13]

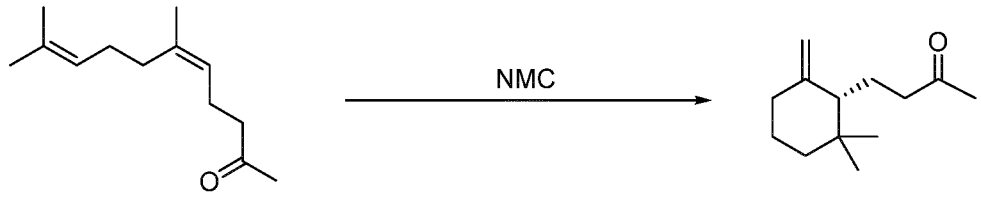
[00142] *Z*-geranyl acetone **16c** with G600R (SEQ-ID No.16)

[00143]



[00144] Colorless oil, 0.098 ml, 0.45 mmol, 49 % yield. (4R,8S)-2,5,5,8-tetramethyl-4,5,6,7,8,8-hexahydro-4H-chromene 11t: ^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 0.85 (s, 3H), 0.87 (s, 3H) 1.16 (s, 3H), 1.32-1.39 (m, 1H), 1.54 (s, 3H), 1.6-1.66 (m, 3H), 1.68 (s, 3H), 1.72-1.97 (m, 3H), 2.14-2.27 (m, 1H), 4.4-4.5 (d, *J* = 2.6 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ (ppm) 18.13 (1C), 19.79 (1C), 20.54 (1C), 21.19 (1C), 26.50 (1C) 32.46 (1C), 33.73 (1C), 39.66 (1C), 41.99 (1C), 44.00 (1C), 74.71 (1C), 94.56 (1C), 148.76 (1C).

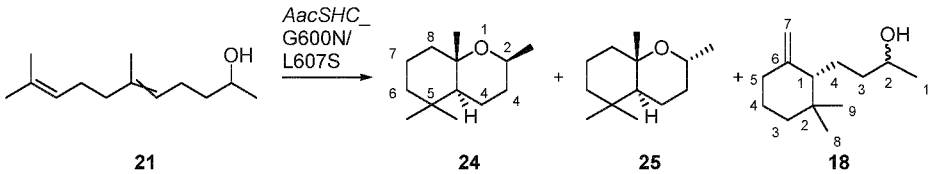
[00145] *Z*-geranyl acetone **16c** with NMC



16c **9**

[00146] Colorless oil, 1.97 ml, 9.1 mmol, 89% yield. (–)-γ-dihydroionone **9**: : **^{1}H-NMR ($CDCl_3$, 500 MHz):** δ (ppm) 0.87 (s, 3H), 0.92 (s, 3H) 1.10-1.30 (m, 2H), 1.42-1.62 (m, 2H), 1.66-1.70 (m, 1H), 1.76-1.83 (m, 1H), 1.97-2.04 (m, 2H), 2.11 (s, 3H), 2.22-2.45 (m, 2H), 4.50-4.51 (d, *J* = 1.03 Hz, 1H), 4.75-4.77 (m, 1H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** δ (ppm) 20.31 (1C), 22.62 (1C), 23.52 (1C), 26.5 (1C), 28.3 (1C), 30.20 (1C), 32.00 (1C), 34.83 (1C), 42.38 (1C), 53.40 (1C), 109.5 (1C), 149.09 (1C), 209.52 (1C). The data is consistent with the literature.[14]

[00147] *E*/*Z*-geranyl isopropanol **21** with G600N/L607S (SEQ-ID No.17)



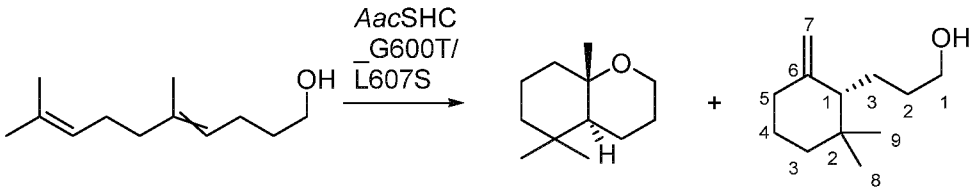
[00148] **21** **24** **25** **18**

[00149] Yellowish oil, 0.020 ml, 0.9 mmol, 10% yield. **^{1}H-NMR ($CDCl_3$, 500 MHz):** δ (ppm) 2*S*,4*S*,8*S*-Tetrahydroedulane **24** : 0,81 (s, 3H), 0,89 (s, 3H), 1,14-1,15 (d, J = 3,1, 3H), 1,23 (s, 3H), 1,28 (s, 1H) 1,33 (s, 1H), 1,42-1,53 (m, 5H), 1,56 (s, 2H), 1,62-1,77 (m, 4H), 3,97-4,04 (m, 1H). 2*R*,4*S*,8*S*-Tetrahydroedulane **25**: 0,74 (s, 3H), 0,87 (s, 3H), 1,09-1,10 (d, J = 3,2,

3H), 1,23 (s, 3H), 1,28 (s, 1H) 1,33 (s, 1H), 1,42-1,53 (m, 5H), 1,56 (s, 2H), 1,62-1,77 (m, 4H), 3,72-3,79 (m, 1H). **^{13}C-NMR ($CDCl_3$, 125 MHz):** 2*R*,4*S*,8*S*-Tetrahydroedulane **25**: δ (ppm) 19.54 (1C), 19.59 (1C), 20.19 (1C), 20.78 (1C), 22.72 (1C), 32.11 (1C), 33.37 (1C), 35.61 (1C), 40.75 (1C), 41.67 (1C), 53.30 (1C), 65.51 (1C), 74.83 (1C). The data is consistent with the literature.[15]

[00150] 4-((R)-2,2-dimethyl-6-methylenecyclohexyl)butan-2-ol **18**: Characteristic methylene signals at **^{1}H-NMR ($CDCl_3$, 500 MHz)**: δ (ppm) 4.53 (d, *J* = 1.25 Hz, 1H) and 4.75 (t, *J* = 1.25 Hz, 1H). From the chiral GC data and the enantiopure monocyclization of **16c** we assume the stereocenter here to be *R.*

[00151] 6,10-dimethylundeca-5,9-dien-2-ol **23** with G600N/L607S (SEQ-ID No.17)



[00152] **23** **27** **20**

[00153] Characteristic C7-methylene signals for **20** at **^{1}H-NMR ($CDCl_3$, 500 MHz)**: 4.55 (*d*, *J* = 1.00 Hz, 1H) and 4.75 (*t*, *J* = 1.30 Hz, 1H). From the chiral GC data and the enantiopure monocyclization of **10c** we assume the stereocenter here to be *R.*

[00154] Table 7 shows the relative conversion rates in % of substrate mixture **16** and isolated **16t** and **16c** with the wild-type enzyme and the variant G600R and the corresponding product selectivities.

[00155]

		(E/Z)-GER 16	(E)-GER 16t	(Z)-GER 16c
WT		23.2	29.0	0.7
G600R		80.4	95.7	68
error WT		4.1	2.5	0.2
error G600R		5.1	0.8	4.3
selectivity WT	bicyclic	100	100	60

selectivity	**bicyclic**	**95**	**100**	**85**
G600R	**monocyclic**	**5**		**15**

[00156] Reaction conditions: E. coli whole cells harboring AacSHC variant resuspended in whole-cell buffer (0.1 M citric acid, 0.1% SDS, pH = 6.0) with an OD_{600} = 20, 20h, 30°C, 4.4 mM substrate (=1 µl in 1ml cell suspension).

[00157] Table 8: Relative conversion rates in % of the substrate Z-geranyl acetone X with all variants at position 600 and the corresponding product selectivities

	Hexahydrochromene	9	14	overall conv.	error
G600R (ID 16)	26.2	1.7	0.7	28.5	0.6
G600M (ID 18)	25.5	0.7	0.6	26.7	1.1
G600T (ID8)	8.8	7.9	3.8	20.4	0.4
G600L (ID 19)	16.1	0.4	0.3	16.8	0.3
G600N (ID 11)	9.9	1.0	1.6	12.4	1.2
G600Q (ID13)	9.6	0.4	0.5	10.4	2.0
G600Y (ID20)	8.1	0.6	0.3	9.0	0.2
G600C (ID15)	6.8	1.5	0.7	9.0	0.4
G600S (ID10)	4.6	2.7	1.2	8.5	0.5
G600K (ID21)	6.6	0.7	0.3	7.5	1.2
G600D (ID12)	5.4	1.2	0.7	7.3	2.5
G600E (ID14)	5.8	1.1	0.3	7.2	1.8
G600V (ID22)	5.4	0.2	0.1	5.7	2.3
G600A (ID9)	2.9	0.9	0.3	4.2	1.0
G600F (ID23)	3.4	0.3	0.1	3.8	2.1
G600I (ID24)	3.4	0.1	0.0	3.6	0.5
G600W (ID25)	2.6	0.4	0.2	3.1	0.3

G600H (ID26)	1.1	0.1	0.1	1.3	0.8
WT (ID1)	0.5	0.1	0.1	0.7	0.4
G600P (ID 27)	0.1	0.0	0.0	0.1	0.0

ID represents the SEQ-ID No. in the sequence listing.

[00158] Reaction conditions: E. coli whole cells harboring AacSHC variant resuspended in whole-cell buffer (0.1 M citric acid, 0.1% SDS, pH = 6.0) with an OD_{600} = 22, 20h, 30°C, 8.48mM substrate (=2 µl in 1ml cell suspension).

[00159] Table 9 Relative conversion rates in % of the substrate Z-geranyl acetone X with the variants at position 607 and the corresponding product selectivities.

	Hexahydrochromene	9	14	overall conv.	error
L607S (ID28)	8.8	6.3	1.8	16.9	1.2
L607M (ID29)	10.5	0.4	0.3	11.2	0.5
L607A (ID30)	5.5	3.3	0.8	9.5	0.6
L607V (ID31)	3.8	1.8	0.5	6.1	0.7
L607G (ID32)	1.2	0.4	0.2	1.7	0.2

ID represents the SEQ-ID No. in the sequence listing.

[00160] Reaction conditions: E. coli whole cells harboring AacSHC variant resuspended in whole-cell buffer (0.1 M citric acid, 0.1% SDS, pH = 6.0) with an OD_{600} = 20, 20h, 30°C, 8.8 mM substrate (=2µl in 1ml cell suspension).

[00161] Table 10: Relative conversion rates in %, corresponding selectivities and total turnover numbers (TTN) of the wild-type enzyme and the engineered enzymes.

[00162]

Enzymeconc. in g/l	1.54	1.32	1.5	1.54	1.64	1.38

Enzymeconc. in mol/l	2.15569E-05	1.848E-05	2.1E-05	2.156E-05	2.296E-05	1.932E-05
MW = 71439 g/mol	**WT**	**G600R**	**G600T**	**N1 (+L607A)**	**N2 (+Y420F)**	**NMC (+306V)**
chromene	0.5	21.9	6.4	4.4	1.8	1.0
9	0.1	1.5	7.4	22.2	63.8	95.2
14	0.1	0.7	1.1	1.4	2.2	2.1
overall conversion	0.7	24.1	14.9	28.0	67.8	98.3
error conversion	0.2	3.0	0.5	3.2	0.9	2.4
TTN	2.9	114.8	62.4	114.4	260.0	447.8
error TTN	0.8	14.3	2.1	13.1	3.4	10.9

[00163] 10 mg lyophilized E. coli whole cells harboring AacSHC variant (18-22 µM) resuspended in 1 mL whole-cell buffer (0.1 M citric acid, 0.1% SDS, 10mM 2-Hydroxypropyl)-β-cyclodextrin, pH = 6.0), 24h, 30°C, 8.8 mM substrate.

[00164] Table 11: Relative conversion rates in % of the substrate Z-geranyl acetone X with the variants N2, Y420F/L607A, Y420F/G600T/L607A/Y609F and Y609F and the corresponding product selectivities

[00165]

	chromene	9	14	overall conv.	error
N2 (Y420F/G600T/L607A) (ID5)	1.4	58	1.8	60.2	5.4
Y420F/L607A (ID33)	2.6	8.2	0.5	12	1.1
Y420F/G600T/L607A/Y609F (ID34)	1	1	0.2	2.2	0.1
Y609F (ID35)	0	0	0	0	0

ID represents the SEQ-ID No. in the sequence listing.

[00166] Reaction conditions: E. coli whole cells harboring AacSHC variant resuspended in whole-cell buffer (0.1 M citric acid, 0.1% SDS, pH = 6.0)

with an OD_{600} = 22, 20h, 30°C, 8.8 mM substrate (=2µl in 1ml cell suspension).

[00167] Biotransformation of 16 using other cyclases

[00168] In the following experiment the capability of other cyclases to perform the monocyclization reaction is shown. Therefore, the thermophilic squalene-hopene cyclase from *Thermesynechococcus elongatus* (*Tel*SHC) which naturally harbors a phenylalanine at position 429 (corresponding position in AacSHC Y420) was chosen. The results show 2% conversion of the substrate 16 towards monocyclic product 9, therefore, confirm the findings of the present invention and show the general capability of squalene-hopene cyclases to perform this reaction (Figure).

[00169] Literature

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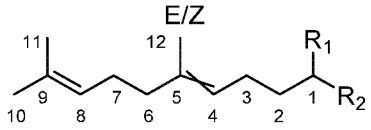
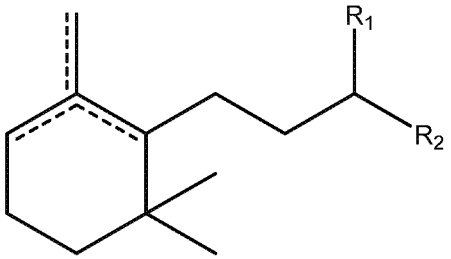
Claims

1. Enzyme mutant with Squalene-hopene cyclase activity, selected from mutants of a wild-type enzyme comprising an amino acid sequence selected from SEQ-ID Nos. 1 to 3 or an amino acid sequence derived therefrom with a degree of sequence identity in the range of from 60 to 99,9 % of SEQ-ID No. 1 to 3, wherein the mutant catalyzes at least the one-step monocyclization of a substrate of general formula (I)



to a monocyclic compound of formula (II)



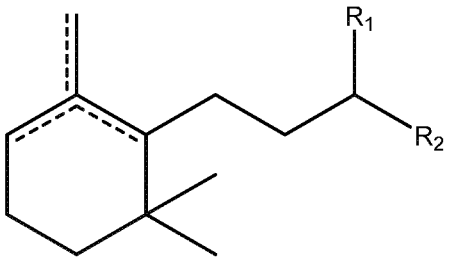
wherein at least one of substituents R^1 und R^2 is selected from the group consisting of =O, -OH, thiol, amino, ester, halogen, nitro or nitrile groups and wherein at least one of substituents R^1 und R^2 is selected from hydrogen, alkyl or alkylene groups.

2. Enzyme mutant in accordance with claim 1, comprising
   a) a mutation in position G600 of SEQ-ID No. 1 or
   b) a mutation in an amino acid sequence selected from amino acid sequences SEQ-ID Nos. 2 to 3 wherein the position of the mutation corresponds to position G600 of SEQ-ID No. 1.
3. Enzyme mutant in accordance with any of the preceding claims in which up to 10 % of the amino acid groups are modified compared to SEQ-ID No. 1 or SEQ-ID No. 2 to 3 by deletion, insertion, substitution, addition or a combination thereof.
4. Enzyme mutant in accordance with any of the preceding claims in which the mutation in position G600 of SEQ-ID No. 1 or in a position corresponding to

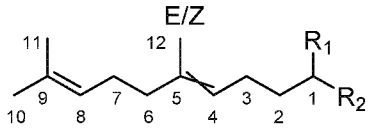
position G600 in SEQ-ID No. 1 in one of the amino acid sequences SEQ-ID No. 2 to SEQ-ID No. 3 is a substitution selected from the group consisting of G600A, G600S, G600C, G600T, G600N, G600D, G600Q and G600E.

5. Enzyme mutant in accordance with any of the preceding claims comprising in addition at least one mutation in one of positions Y420 or L607 of SEQ-ID No. 1 or at least one mutation in an amino acid sequence selected from amino acid sequences SEQ-ID No 2 to SEQ-ID No. 3 wherein the position of the mutation corresponds to position Y420 respectively L607of SEQ-ID No. 1.
6. Enzyme mutant in accordance with any of the preceding claims comprising three mutations in positions Y420, G600 und L 607 of Sequence-ID No. 1 or three mutations in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position of the mutation corresponds to position Y420, G600 and L607 of SEQ-ID No. 1.
7. Enzyme mutant in accordance with any of the preceding claims comprising four mutations in positions A306, Y420, G600 und L 607 of Sequence-ID. No. 1 or four mutations in an amino acid sequence selected from amino acid sequences SEQ-ID Nos 2 to 3 wherein the position of the mutations correspond to positions A306, Y420, G600 and L607 of SEQ-ID No. 1.
8. Enzyme mutant corresponding to any of claims 1 to 4 comprising an amino acid sequence selected from SEQ-ID No. 4 to 8 and 34.
9. Nucleic acid sequence, encoding for an enzyme mutant in accordance with any of the preceding claims.
10. Expression cassette, comprising a nucleic acid sequence in accordance with claim 9.
11. Recombinant vector, comprising under the control of at least one regulative element, at least one nucleic acid sequence in accordance with claim 9 or at least one expression cassette in accordance with claim 10.
12. Recombinant microorganism comprising at least one nucleic acid sequence in accordance with claim 9 or at least one expression cassette in accordance with claim 10 or at least one recombinant vector in accordance with claim 11.

13. A process for the manufacture of compounds of formula (II)



wherein at least one of substituents R^1 and R^2 is selected from the group consisting of =O, -OH, thiol, amino, ester, halogen, nitro or nitrile groups and wherein at least one of substituents R^1 und R^2 is selected from hydrogen, alkyl or alkylene groups wherein compounds of formula (I)

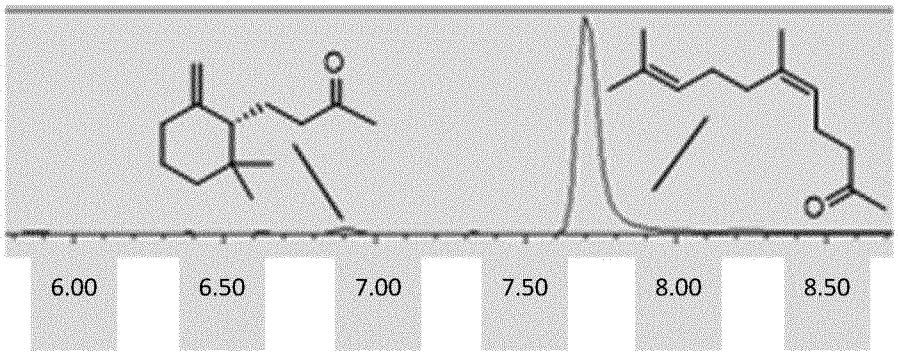


wherein R^1 and R^2 are as defined above,
are cyclized with an enzyme mutant in accordance with any of claims 1 to 9 or in the presence of a microorganism expressing an enzyme mutant in accordance with any of claims 1 to 9.

14. Use of an enzyme mutant in accordance with any of claims 1 to 8, a nucleic acid sequence in accordance with claim 9, an expression cassette in accordance with claim 10, a recombinant vector in accordance with claim 11 or a recombinant microorganism in accordance with claim 12 for the cyclization of compounds of formula (I) as defined in claim 1 to compounds of formula (II) as defined in claim 1.

15. Use in accordance with claim 14 for the manufacture of dihydroionone derivatives, in particular (+)-γ-dihydroionone, starting from geranylacetone, nerylacetone, calmusal, calmusol or mixtures thereof.

Figure:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/082104

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/88 C12P17/02 C12P17/04 C11B9/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P A23L C11B

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	DATABASE Geneseq [Online] 25 June 2009 (2009-06-25), "A. acidocaldarius ATCC27009 Shc polypeptide sequence, SEQ ID NO:83.", XP002805799, retrieved from EBI accession no. GSP:AWV29295 Database accession no. AWV29295	1-7,9-12
A	abstract & US 2009/117557 A1 (WANG HUA [US] ET AL) 7 May 2009 (2009-05-07) sequence 83 paragraphs [0017], [0142] ----- -/--	8,13-15

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

* Special categories of cited documents :

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

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

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

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

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

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

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

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

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Date of the actual completion of the international search	Date of mailing of the international search report
3 May 2022	11/05/2022

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	Tudor, Mark

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SERRA STEFANO: "An expedient preparation of enantio-enriched ambergris odorants starting from commercial ionone alpha : Synthesis of enantio-enriched ambergris odorants", FLAVOUR AND FRAGRANCE JOURNAL., vol. 28, no. 1, 21 November 2012 (2012-11-21), pages 46-52, XP055893418, GB ISSN: 0882-5734, DOI: 10.1002/ffj.3126 cited in the application abstract page 46 - page 52	1-15
A	SEITZ M ET AL: "Synthesis of heterocyclic terpenoids by promiscuous squalene-hopene cyclases", CHEMBIOCHEM,, vol. 14, no. 4, 4 March 2013 (2013-03-04), pages 436-439, XP002760197, ISSN: 1439-4227, DOI: 10.1002/CBIC.201300018 [retrieved on 2013-02-18] cited in the application page 438, right-hand column, paragraph 2	1-15
A	CHRISTINE FULL ET AL: "Conserved Tyr residues determine functions of Alicyclobacillus acidocaldarius squalene-hopene cyclase", FEMS MICROBIOLOGY LETTERS, vol. 183, no. 2, 1 February 2000 (2000-02-01), pages 221-224, XP055020877, ISSN: 0378-1097, DOI: 10.1111/j.1574-6968.2000.tb08961.x abstract page 221, right-hand column, lines 1-6 page 224, left-hand column, paragraph 7 - right-hand column, paragraph 1	1-15

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/082104

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: