RESEARCH

Production of the forskolin precursor 11β-hydroxy-manoyl oxide in yeast using surrogate enzymatic activities

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Abstract

Background: Several plant diterpenes have important biological properties. Among them, forskolin is a complex labdane-type diterpene whose biological activity stems from its ability to activate adenylyl cyclase and to elevate intracellular cAMP levels. As such, it is used in the control of blood pressure, in the protection from congestive heart failure, and in weight-loss supplements. Chemical synthesis of forskolin is challenging, and production of forskolin in engineered microbes could provide a sustainable source. To this end, we set out to establish a platform for the production of forskolin and related epoxy-labdanes in yeast.

Results: Since the forskolin biosynthetic pathway has only been partially elucidated, and enzymes involved in terpene biosynthesis frequently exhibit relaxed substrate specificity, we explored the possibility of reconstructing missing steps of this pathway employing surrogate enzymes. Using CYP76AH24, a Salvia pomifera cytochrome P450 responsible for the oxidation of C-12 and C-11 of the abietane skeleton en route to carnosic acid, we were able to produce the forskolin precursor 11β-hydroxy-manoyl oxide in yeast. To improve 11β-hydroxy-manoyl oxide production, we undertook a chassis engineering effort involving the combination of three heterozygous yeast gene deletions (mct1/MCT1, whi2/WHI2, gdh1/GDH1) and obtained a 9.5-fold increase in 11β-hydroxy-manoyl oxide titers, reaching 21.2 mg L⁻¹.

Conclusions: In this study, we identify a surrogate enzyme for the specific and efficient hydroxylation of manoyl oxide at position C-11β and establish a platform that will facilitate the synthesis of a broad range of tricyclic (8,13)-epoxy-labdanes in yeast. This platform forms a basis for the heterologous production of forskolin and will facilitate the elucidation of subsequent steps of forskolin biosynthesis. In addition, this study highlights the usefulness of using surrogate enzymes for the production of intermediates of complex biosynthetic pathways. The combination of heterozygous deletions and the improved yeast strain reported here will provide a useful tool for the production of numerous other isoprenoids.

Keywords: Cytochrome P450, Metabolic engineering, Natural products, Terpene, Isoprenoid

Background

Plant natural products have found numerous applications in fragrances, flavors or pharmaceuticals, but the exploitation of many of these compounds is hampered by limited availability or seasonal variation of the raw materials. Reconstruction of natural product biosynthetic pathways in engineered microbes can provide a sustainable alternative and has so far been successful in providing access to important industrial compounds (e.g. artemisinin, resveratrol, vanillin [1–3]). Among the plant natural products with industrial application, terpenes (or isoprenoids) form the largest and more diverse group. Terpenes are synthesized by the successive addition

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of the 5-carbon isoprene building block, giving rise to classes of compounds with increasing skeletal size, such as hemiterpenes (C₅), monoterpenes (C₁₀), sesquiterpenes (C₁₅), and diterpenes (C₂₀) (Fig. 1).

Within the diterpene class, the superfamily of labdane-related diterpenes comprises more than 7000 members and is characterized by a basic decalin core [4]. Their biosynthesis requires the cyclization of geranylgeranyl diphosphate (GGPP), by a class II diterpene synthase (diTPS), to a labdane-type diphosphate (e.g. (+)-copalyl diphosphate ([+]-CPP) or 8-hydroxy-CPP (8OH-CPP)), which is subsequently taken up by a class I diTPS to generate the basic diterpene skeleton (Fig. 1). Through the action of decorating enzymes, such as cytochrome P450s (CYPs), these basic labdane-type skeletons are converted to a diverse array of highly complex molecules [4]. Several labdane-type diterpenes, such as tanshinones, carnosic acid, and forskolin, display potent biological activities. Tanshinones are isolated from the roots of *Salvia miltiorrhiza* and exhibit strong antioxidant and anti-inflammatory activities, and cardiovascular and cerebrovascular therapeutic effects [5–8]. Carnosic acid (14) and carnosol are well known antioxidants produced in rosemary and *Salvia* spp., active as anti-adipogenic [9] and anticancer agents [10, 11]. Forskolin (6) is a complex heterocyclic labdane diterpene produced in the root cork cells of *Coleus forskohlii* plant [12]. By activating adenyl cyclase, it increases the intracellular cAMP levels and acts as a positive inotropic agent and a vaso- and broncho-dilator [13–17]. Forskolin and its derivatives have found application...
in weight-loss supplements [18, 19], in the treatment of open angle glaucoma [20], in the control of blood pressure [21], and in the protection from congestive heart failure [22]. Enantioselective chemical synthesis of forskolin has not yet been successful, calling for the application of biotechnological methods for its production. However, the forskolin biosynthetic pathway has only been partially elucidated. The first steps are believed to involve the formation of (13R)-manoyl oxide (3) from GGPP (1), through 8OH-CPP (2), and the enzymes involved in these reactions have been identified and characterized [12]. The highly oxygenated structure of forskolin requires the oxidation of five different carbon atoms of the manoyl oxide skeleton and the acetylation of the resulting 7β-OH group. One of the early events in this process likely involve oxidation of manoyl oxide at position C-11, since 11-oxo-manoyl oxide (5) (Fig. 2) was isolated from C. forskohlii roots [23, 24], but a catalytic activity responsible for this reaction has not yet been identified.

We recently established a “plug-and-play” platform to facilitate the reconstruction of the diversity of terpenes in yeast [25]. This approach revealed that class I diTPSs and related CYPs frequently exhibit significant substrate promiscuity, a property that can be exploited to provide surrogate enzymatic activities for orphan biosynthetic steps. In the process of biosynthetic pathway elucidation, a Synthetic Biology approach taking advantage of surrogate enzymes for the production of putative precursors of molecules of interest can serve as a useful tool. Production of intermediates or precursors in microbial “chassis”, in titers that enable facile isolation and purification of these compounds, can yield complex substrates for activity screening using in vitro assays or coupled in vivo platforms, greatly speeding up the full process [26]. Here, we apply such an approach in the production of the forskolin precursor 11β-hydroxy-manoyl oxide, taking advantage of two promiscuous enzymes from S. pomifera: one class I diTPS that is normally responsible for the conversion

Fig. 2 Proposed biosynthetic pathway of forskolin and carnosic acid. Both pathways begin from the common diterpene precursor GGPP (1). In C. forskohlii, a class II diTPS converts GGPP to 8OH-CPP (2), which is then taken up by a class I enzyme, to form manoyl oxide (3). This, in turn, becomes oxidized at several positions (C-1, C-6, C-7, C-11), presumably by the action of specific CYPs, and eventually O-acetylated at the C-7 hydroxy to generate forskolin (6). 11β-hydroxy-manoyl oxide (4) and 11-oxo-manoyl oxide (5) are believed to be the first steps in this mechanism, although the enzyme(s) catalyzing these reactions in C. forskohlii have not yet been identified. In S. pomifera, GGPP is converted to CPP (7) and then to miltiradiene (8) by corresponding class II (CPP synthase; CDS) and class I (Miltiradiene synthase; SpMilS) diTPSs. Miltiradiene is non-enzymatically converted to abietatriene (11), the substrate of CYP76AH24. CYP76AH24 catalyzes two successive oxidation events, one on C-12 of abietatriene producing ferruginol (12), and a second one on C-11 of ferruginol producing 11-hydroxy-ferruginol (13). When provided with miltiradiene, in vitro or in yeast cells, CYP76AH24 catalyzes a two step oxidation leading to 11-keto-miltiradiene (10), via 11-hydroxy-miltiradiene (9) [26]. The dashed box encloses the reactions catalyzed by CYP76AH24. CYP76AH46 takes up 11-hydroxy-ferruginol to catalyze a three step oxidation leading to carnosic acid (14) [26]. The promiscuous class I diTPSs, SpMilS, can also accept 8OH-CPP (2) to produce manoyl oxide (3) [25]. To reconstruct the first steps of the forskolin biosynthetic pathway in yeast, CcCLS was used to produce 8OH-CPP, SpMilS was employed to convert 8OH-CPP to manoyl oxide, and CYP76AH24 was exploited to oxidize manoyl oxide (3) to 11β-hydroxy-manoyl oxide (4)
of (+)-CPP to miltiradiene [27], a key intermediate in the biosynthesis of carnosic acid-related diterpenes, and one CYP targeting C-12 and C-11 of the abietatriene skeleton [26]. Combined with a set of heterozygous yeast gene deletions (MCT1, WHI2, GDH1) aiming to improve manoyl oxide availability, we achieved a fourfold increase in manoyl oxide conversion efficiency and a 9.5-fold increase in 11β-hydroxy-manoyl oxide titers, reaching 21.2 mg L⁻¹. This approach sets the basis for the heterologous production of forskolin in yeast and provides a tool for the elucidation of subsequent steps of forskolin biosynthesis, such as hydroxylation at positions C-1, C-6, C-7 or C-9.

**Results and discussion**

Establishing manoyl oxide production in yeast using a surrogate terpene synthase

Due to the so far limited knowledge of the final steps of its biosynthetic pathway, we set out to reconstruct forskolin biosynthesis in yeast using surrogate dTPS and CYP activities. In our previous work, we discovered that several class I dTPSs examined were able to accept alternative substrates and to yield different products with similar efficiency [25]. One such example was *S. pomifera* miltiradiene synthase (SpMilS), an enzyme believed to accept (+)-CPP as its physiological substrate to produce miltiradiene. Miltiradiene is a common precursor of several class I diTPSs examined were able to accept additional oxidation steps. In yeast, overexpression of multiple CYP oxidation events, strain AM119 was developed by chromosomal integration of the *HEM3* gene under the control of the strong *PTDH3* promoter in the 3′-UTR of the *FLO5* locus of strain AM102 [26]. In order to establish manoyl oxide production in AM119, in addition to SpMilS, the fusion between the *Cistus creticus* 8OH-CPP synthase (*CcGCLS*) and a GGPP-producing yeast *Erg20p* mutant (*Erg20p(F96C)) was also expressed to provide high levels of the 8OH-CPP precursor (Fig. 3a). Under these conditions, the manoyl oxide titer at the end of shake-flask batch cultivation reached 41 mg L⁻¹ of yeast culture. The reported titer for manoyl oxide production using the *C. forskolii* manoyl oxide synthase is 10 mg L⁻¹ [33].

Oxidation of manoyl oxide by CYP76AH24

Recently, we discovered CYP76AH24 from *S. pomifera* as an enzyme responsible for the oxidation of C-12 of abietatriene to produce ferruginol and the subsequent oxidation of C-11 of ferruginol to yield 11-hydroxy-ferruginol (Fig. 2), two important intermediates in the carnosic acid biosynthetic pathway. When provided with miltiradiene, CYP76AH24 catalyzed two successive oxidation events on C-11, giving rise to 11-keto-miltiradiene (Fig. 2; [26]). Thus, CYP76AH24 appears to exhibit relatively relaxed substrate selectivity, being able to accept several structurally related molecules and to catalyze their oxidation at C-12 or C-11. Assuming that additional compounds with similar structure may also be oxidized by CYP76AH24, we decided to investigate its ability to catalyze conversion of manoyl oxide to related oxidized structures. Manoyl oxide-producing AM119 yeast cells were engineered to express CYP76AH24 and popular CPR [33] from the bidirectional vector pESC-Leu under the *Pgal10 and Pgal1* promoters, respectively.

### Table 1 List of *S. cerevisiae* strains used

| Strain | Genotype | Source |
|--------|----------|--------|
| AM119  | Mat a/α, *Pgal1*-M2G2(K6R):HOX2, ura3, trp1, his3, *PTDH3*-M2G2(K6R):X2::::eu2 ERG9:erg9, ubc7UBC7, ssm4/SSM4, *PTDH3*-HEM3-FLOS | Ref. [26] |
| AM119-1| Mat a/α, *Pgal1*-M2G2(K6R):HOX2, ura3, trp1, his3, *PTDH3*-M2G2(K6R):X2::::eu2 ERG9:erg9, ubc7UBC7, ssm4/SSM4, *PTDH3*-HEM3-FLOS, *PTDH3*-CGGPPS1-FLOS | This study |
| AM119-2| Mat a/α, *Pgal1*-M2G2(K6R):HOX2, ura3, trp1, his3, *PTDH3*-M2G2(K6R):X2::::eu2 ERG9:erg9, ubc7UBC7, ssm4/SSM4, mct1/MCT1, *PTDH3*-HEM3-FLOS, *PTDH3*-CGGPPS1-FLOS | This study |
| AM119-3| Mat a/α, *Pgal1*-M2G2(K6R):HOX2, ura3, trp1, his3, *PTDH3*-M2G2(K6R):X2::::eu2 ERG9:erg9, ubc7UBC7, ssm4/SSM4, mct1/MCT1, *wh2/WHI2, gdh1/GDH1, *PTDH3*-HEM3-FLOS, *PTDH3*-CGGPPS1-FLOS | This study |
| AM119-4| Mat a/α, *Pgal1*-M2G2(K6R):HOX2, ura3, trp1, his3, *PTDH3*-M2G2(K6R):X2::::eu2 ERG9:erg9, ubc7UBC7, ssm4/SSM4, mct1/MCT1, *wh2/WHI2, gdh1/GDH1, *PTDH3*-HEM3-FLOS, *PTDH3*-CGGPPS1-FLOS | This study |
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(Fig. 3b). GC–MS analysis of the dodecane extracts of the corresponding cultures revealed one new peak compared to the empty vector control, likely corresponding to a manoyl oxide oxidation product (Fig. 4a). To examine whether additional manoyl oxide oxidation products are formed, which are either retained by yeast cells or inefficiently volatilized during GC–MS analysis, yeast cultures were extracted with pentane and the extracts were derivatized with Sylon-HTP (Sigma-Aldrich) prior to analysis. No additional peak was detected, suggesting that CYP76AH24 likely catalyzes the synthesis of one main oxidized form of manoyl oxide.

In order to identify the structure of the new compound, a large scale yeast culture (1 L) producing the unknown hydroxy-manoyl oxide derivative was established and the dodecane extract was fractionated and analyzed as described in the “Methods” section. Analysis of its NMR spectra revealed the identity of the new product as 11β-hydroxy-manoyl oxide (Additional file 1: Figs. S1 and S2, Table S2). To confirm that 11β-hydroxy-manoyl oxide is indeed the product of the CYP76AH24-catalyzed reaction and not the result of an in vivo bioconversion event, the enzymatic activity of CYP76AH24 was analyzed in vitro using purified yeast microsomal fractions in the presence of manoyl oxide and NADPH co-factor. A single product with retention time and mass spectrum matching that of purified 11β-hydroxy-manoyl oxide was detected (Fig. 4b–d). Kinetic analysis using the yeast microsomal preparation of CYP76AH24 revealed that this reaction occurs with high efficiency compared to the oxidation of C-11 of miltiradiene or ferruginol by CYP76AH24 (kcat/KM = 315 × 10^3 M^{-1} min^{-1} for manoyl oxide vs. 133 × 10^3 M^{-1} min^{-1} for ferruginol and 29.3 × 10^3 M^{-1} min^{-1} for miltiradiene; Fig. 5; Table 2).
Chassis engineering to improve manoyl oxide and 11β-hydroxy-manoyl oxide production

Titers of 11β-hydroxy-manoyl oxide produced by AM119 cells reached 2.3 mg L⁻¹, indicating a 5.3 % efficiency of manoyl oxide conversion (as estimated by the ratio of 11β-hydroxy-manoyl oxide titer to the sum of manoyl oxide and 11β-hydroxy-manoyl oxide titres). One factor limiting the efficiency of manoyl oxide oxidation may be a relatively low intracellular concentration of manoyl oxide. The estimated Michaelis–Menten constant \((K_M = 11.6 \mu M)\) for the oxidation of manoyl oxide by CYP76AH24 suggests that if the intracellular concentration of manoyl oxide is in the low micromolar range, the enzyme will not function at maximum efficiency. Thus, increasing the yield of manoyl oxide synthesis may help improving the efficiency of conversion. To this end, a metabolic engineering effort aiming to improve strain AM119 as a chassis for diterpene production was undertaken.

Incorporation of a plant GGPP synthase—Initially, we aimed to improve the endogenous GGPP pool by constitutive expression of the \(C.\ creticus\) GGPP synthase (CcGGPPS) from a chromosomally integrated copy. The mature form of CcGGPPS was incorporated in the \(3'\)-UTR \(FLO8\) locus of AM119 strain, under the control of the \(P_{TDH3}\) constitutive promoter, generating strain AM119-1. No significant difference in either manoyl oxide or 11β-hydroxy-manoyl oxide titers was observed, suggesting that the Erg20p mutant used for GGPP production is providing sufficient levels of GGPP for 8OH-CPP synthesis and that this step is not limiting the pathway.

Heterozygous deletion of \(MCT1\)—Isoprenoid biosynthesis in yeast proceeds mainly through the mevalonic acid pathway, which greatly relies on the availability of acetyl-CoA precursor. The acetyl-CoA pool is drained by competing pathways, such as ethanol production or membrane lipid and fatty acid biosynthesis, which divert acetyl-CoA from isoprenoid biosynthesis. One such pathway that competes for the availability of acetyl-CoA is the mitochondrial fatty acid biosynthesis pathway (or octanoyl-ACP pathway), which is responsible for the synthesis of lipoic acid and other mitochondrial membrane lipids [34]. This pathway is highly conserved and completely independent of the yeast cytosolic fatty acid synthase apparatus. In yeast, the second enzyme in this pathway is the malonyl-CoA:ACP transferase Mct1p (Fig. 1). Downregulation of \(MCT1\) is expected to redirect the acetyl-CoA substrate from fatty acid biosynthesis towards the mevalonate pathway and subsequently terpene biosynthesis. However, deletion of \(MCT1\) has been reported to result in a respiratory-deficient phenotype and small rudimentary mitochondria [35]. Deletion of one of the two alleles of a certain gene in a yeast diploid strain results in a decrease in the levels of the corresponding proteins by approximately 50 % [36]. To alleviate potential negative effects on cellular growth and viability caused by complete deletions, we previously reported the combination of monoallelic gene deletions as an efficient alternative which allowed the development of improved yeast strains for the production of the sesquiterpene \(β\)-caryophyllene [37]. Heterozygous deletion mutant yeast strains are stable and maintain their improved characteristic over time [37]. To downregulate the mitochondrial fatty acid synthase pathway without

**Table 2 CYP76AH24 kinetic parameters with different substrates**

| Substrate       | \(K_M\) (\(\mu M\)) | \(k_{cat}\) (min⁻¹) | \(k_{cat}/K_M\) \((\times 10^3 \text{min}^{-1} \text{M}^{-1})\) | Source      |
|-----------------|---------------------|---------------------|-------------------------------------------------|-------------|
| Manoyl oxide    | 11.6 ± 1.06         | 3.65 ± 0.12         | 315                                             | This study  |
| Ferruginol      | 45.84 ± 9.37        | 6.11 ± 0.45         | 133                                             | Ref. [26]   |
| Mitiradiene     | 72.63 ± 43.19       | 1.97 ± 0.72         | 27.1                                            | Ref. [26]   |
the deleterious effects of complete MCT1 elimination, only one of the two MCT1 alleles was deleted in strain AM119-1 to give rise to strain AM119-2 (Table 1). Heterozygous MCT1 deletion improved 11β-hydroxy-manoyl oxide production by over twofold, reaching titers of 5.8 mg L⁻¹ and boosted manoyl oxide conversion rate to 10.2 % (Fig. 6).

Heterozygous deletion of WHI2—Whi2p is a cytoplasmic scaffold protein required, together with its partner Psr1, for the proper activation of the general stress response. WHI2 was identified as a positive genetic interactor of HMG2 [38] and the heterozygous deletion whi2/WHI2 was previously found to strongly synergize with ubc7/UBC7 (already present in the AM119 background) in enhancing sesquiterpene production [37]. Heterozygous deletion of WHI2 (whi2/WHI2) in the AM119-2 background generated strain AM119-3. In this chassis, 11β-hydroxy-manoyl oxide production increased by 83 % to 10.6 mg L⁻¹, while manoyl oxide conversion reached 14.2 % (Fig. 6).

GDH1 deletion—NADP(+) -dependent glutamate dehydrogenase 1 (Gdh1p) synthesizes glutamate from ammonia and α-ketoglutarate (Fig. 1). A computational approach identified GDH1 as a gene the deletion of which could improve carbon flux through the mevalonate pathway, by increasing the pool of NADPH available for Hmg1p and Hmg2p [39]. Deletion of GDH1 in a haploid strain increased production of the sesquiterpene cubebol by 85 %. However, GDH1 deletion also caused a significant decrease in the growth rate [39]. To avoid possible growth inhibition, a heterozygous GDH1 deletion strain, AM119-4, was derived from AM119-3 (Table 1). When tested for 11β-hydroxy-manoyl oxide production, AM119-4 was 2 times more efficient than AM119-3, yielding 21.9 mg L⁻¹ (Fig. 6), without any detectable growth retardation. The combination of the three heterozygous deletions (mct1/MCT1, whi2/WHI2, gdh1/GDH1) lead to an overall 9.5-fold increase of 11β-hydroxy-manoyl oxide titer over the base strain (AM119), and a fourfold improvement of manoyl oxide conversion (Fig. 6).

Conclusions

Aiming to identify surrogate enzymes for the heterologous biosynthesis of forskolin, we identified CYP76AH24 as an enzyme that specifically catalyzes the introduction of one oxygen atom at the C-11β position of manoyl oxide to form the corresponding alcohol. An enzymatic activity capable of catalyzing this step is reported here for the first time. Further oxidation of 11β-hydroxy-manoyl oxide at the same position will generate the 11-oxo functionality present in forskolin. The efficiency (kcat/KM) of manoyl oxide oxidation by CYP76AH24 is high and comparable to the oxidation of C-11 of ferruginol, one of the presumed in planta substrates of CYP76AH24 (Table 2). The use of surrogate enzymes has also proved to be an efficient method for the production of manoyl oxide. The overall performance of the platform using SpMilS as the manoyl oxide synthase compares favorably to the published titers of manoyl oxide achieved using the corresponding C. forskohlii enzyme in Escherichia coli and in Synechocystis sp. PCC 6803 (40–100 vs. 10 mg L⁻¹ in [33] and 0.46 mg L⁻¹ in [40, 41], respectively).

Examination of the efficiency of the oxidation of manoyl oxide in yeast reveals that as the manoyl oxide titer increases, so does the efficiency of conversion of manoyl oxide to 11β-hydroxy-manoyl oxide (Fig. 6b). This suggests that at conditions where manoyl oxide production rates are low, most manoyl oxide does not accumulate
long enough inside the cell (or in the ER) so as to be efficiently oxidized. This observation may provide important insights for future development of CYP-mediated oxidation of terpenes in yeast, as it indicates that efficient channeling of substrates between the terpene synthase step and the CYP-mediate oxidation is required.

By combining heterozygous deletions in three yeast genes we achieved significant yield improvements. This further supports the notion that monoallelic deletions can provide a useful genetic engineering tool in cases where complete gene deletion may have adverse effects in host cell physiology, as is the case here with MCT1 and GDH1. Further fine-tuning of gene dosage could be achieved by complementary approaches, such as the integration of weaker promoters or destabilizing sequences in the mRNA [42].

The yeast platform described in this report opens the way for the elucidation of the biosynthesis of the unusual series of tricyclic (8,13)-epoxy-labdanes, providing either the starting material for in vitro reactions aiming to identify downstream steps of the pathway or a platform for the in vivo screening of candidate biosynthetic genes, as previously described for the elucidation of the carnosic acid pathway [26]. In addition, 11β-hydroxy-manoyl oxide produced by this platform can be further derivatized by chemical synthesis to yield analogues with potentially interesting properties. As already described for other terpenes [25], combinatorial biosynthesis coupled with protein engineering of labdane skeleton-acting CYPs can be applied to further derivatize the 11β-hydroxy-manoyl oxide molecule towards a broad range of potentially bioactive compounds.

**Methods**

**Chemicals and enzymes**

Standard compounds were obtained from: geranylinalool (Aldrich, 48809), geranylgeraniol (Sigma, G3278), sclareol (VIORYL SA, Athens, Greece). Manoyl oxide standard was from our in-house collection, isolated from natural sources and characterized by NMR spectroscopy. PCR amplifications were performed using Phusion High-Fidelity DNA Polymerase (New England BioLabs, M0530) and MyTaq DNA polymerase (BIO-21105, Bioline). Restriction enzymes from New England BioLabs were used for cloning purposes. NucleoSpin Plasmid Kit (740588, Macherey–Nagel) was used for plasmid DNA purification, while QIAquick Gel Extraction Kit (#28704, Qiagen) was used for gel extraction and DNA purification.

**Yeast media, expression vectors, and strains**

Yeast cells were cultivated in Complete Minimal (CM) medium, composed of 0.13 % (w/v) dropout powder (all essential amino acids), 0.67 % (w/v) Yeast Nitrogen Base w/o AA (Y2025, US Biological) and 2 % d-(+)-glucose monohydrate (16301, Sigma). For galactose-based medium, glucose was substituted with 2 % d-(+)-galactose (G0625, Sigma) and 1 % Raffinose pentahydrate (R1030, US Biological). Constructs pYES2myc/CcCLS-ERG20(F96), pHTDH3myc/SpMilS and pESC-Leu:CRP2-CYP76AH24 were previously described [25, 26, 43].

The generation of yeast strain AM119 (Mat a/α, P_Gal1-HMG2(K6R)::HOX2, ura3, trp1, his3, P_TDHS-HMG2(K6R)X2::leu2 ERG9/erg9, ubc7/UBC7, ssn4/SSM4, P_TDHS-HEM3-FLO5) has previously been described [26]. AM119 was used as the starting strain to generate strain AM119-1. Plasmid construct COD7/CcGGDPS (PTDH3-CcGGDPS-CYC1t, LoxP-HIS5-LoxP) [37], which harbors the mature form of C. creticus GGPPS, was PCR amplified using primers 5-FLO8-COD7 and 3-FLO8-COD7 (Additional file 1: Table S1) incorporating flanking sequences complementary to the 3′UTR of FLO8 gene. Following transformation, selection and excision of the selection marker, strain AM119-1 was generated.

The pUG27 cassette [44] was PCR amplified with primers MCT1-pUGF and MCT1-pUGR and used to transform AM119-1 cells to inactive one allele of MCT1. Proper integration of the cassette was verified by PCR on genomic DNA of selected colonies using primers MCT1prom and MCT1pUGR. Upon excision of the selection marker, strain AM119-2 was generated.

To generate strain AM119-3, the pUG27 cassette was PCR amplified with primers WHI2-640-pUGF and WHI2-2790-pUGR and was used to transform AM119-2 cells to inactive one allele of WHI2. Proper integration of the cassette was verified by PCR on genomic DNA of selected colonies using primers WHI2prom and WHI2-2790-pUGR, followed by selection marker excision.

Strain AM119-3 was subsequently transformed with a pUG27 PCR amplified cassette with primers GDH1-F-646-pUG and GDH1-R-2653 and to inactive one allele of GDH. Proper integration of the cassette was verified by PCR on genomic DNA of selected colonies using primers WHI2prom and WHI2-2790-pUG, and the selection marker was excised to give rise to strain AM119-4.

**Yeast strain cultivation, terpene quantification and extraction from yeast cells**

Cultivation of yeast cells for the production of terpene compounds was performed as previously described [25]. For the expression of genes placed under the galactose-inducible promoters P_Gal1 and P_Gal10 (the CcCLS-Erg20p(F96C) fusion, poplar CPR2 and CYP76AH24), yeast cultures were grown until OD600 = 0.7–1 and subsequently switched to galactose-rafínose based selective
growth medium (10–25 mL). Terpene extraction was performed by dodecane overlay (10 %) or liquid–liquid extraction using aliquots of 1 ml cultures and pentane as extracting solvent. Where appropriate, pentane extracts were derivatized using Sylon HTP (hexamethyltrisilazane:trimethylchlorosilane:pyridine, 3:1:9) (Supelco, Bellefonte, PA) as previously described [26]. GC–FID was employed for quantification and identification of terpene products as described in [45]. Identification of produced compounds by GC–MS analysis was carried out by comparison with commercial or in-house standards.

**Microsomal protein preparation and cytochrome P450 quantification**

Yeast cultures (250 mL) were used to isolate microsomes from cells engineered to express CYP76AH24 with the method in [46], with an additional final ultracentrifugation step at 100,000 g for 60 min. The concentration of CYP76AH24 was determined by the spectroscopic difference at 450 nm of CYP enzymes due to CO binding [47], using the extinction coefficient 91 mM$^{-1}$ cm$^{-1}$. Background correction of the endogenous CYPs was deduced using microsomes purified from cells carrying an empty vector.

**In vitro enzymatic assay and kinetic analysis**

The enzymatic activities of CYP76AH24 was evaluated as previously described [25] using varying concentrations (1–75 μM) of manoyl oxide as substrate. The enzymatic reactions were incubated with shaking at 30 °C for 30 min and terminated by extraction with 100 μL of decane containing 10 μg/mL sclareol as internal standard. 2 μL of extracts were analyzed by GC–MS using the conditions previously described [45]. All assays were carried out in duplicates.

**Additional file**

Additional file 1: Table S1. List of primers. Table S2. Experimental and bibliographic 1H and 13C NMR data (in CDCl3) of 11β-hydroxy-manoyl oxide (4). Figure S1. 1H NMR spectrum of 11β-hydroxy-manoyl oxide (4). Figure S2. 13C NMR spectrum of 11β-hydroxy-manoyl oxide (4).

**Abbreviations**

CYP: cytochrome P450; GGPP: geranylgeranyl diphosphate; dTPS: diterpene synthase; CPP: copalyl diphosphate; 8OH-CPP: 8-hydroxy-(+)-copalyl diphosphate; CcCGPPS. *Cistus creticus* geranylgeranyl diphosphate synthase; SpWiS. *Salvia pomifera* mitradiene synthase; CcCLS. *Cistus creticus* 8-hydroxy-copalyl diphosphate synthase; CPR2: *Populus trichocarpa* × *Populus deltoides* cytochrome P450 reductase 2; NMR: nuclear magnetic resonance; CM medium: complete minimal medium; ACP. acyl carrier protein.

**Authors’ contributions**

Cl conducted experimental work and participated in the drafting of the paper. FAT, AA, SL, PG conducted some experimental work. EI and VR assisted in the data analysis and review of the manuscript. AMM assisted in data analysis, experimental design and review of the manuscript. SCK is responsible for the design, data analysis, drafting and reviewing the paper. All authors read and approved the final manuscript.
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Competing interests
The authors declare that they have no competing interests.

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