

Carnosic acid biosynthesis elucidated by a synthetic biology platform

Codruta Ignea^a, Anastasia Athanasakoglou^a, Efstathia Ioannou^b, Panagiota Georgantea^b, Fotini A. Trikkas^c, Sofia Loupassaki^d, Vassilios Roussis^b, Antonios M. Makris^c, and Sotirios C. Kampranis^{a,1,2}

^aDepartment of Biochemistry, School of Medicine, University of Crete, Heraklion 71003, Greece; ^bDepartment of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Athens 15771, Greece; ^cInstitute of Applied Biosciences–Centre for Research and Technology Hellas, Thessaloniki 57001, Greece; and ^dMediterranean Agronomic Institute of Chania, Chania 73100, Greece

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Synthetic biology approaches achieving the reconstruction of specific plant natural product biosynthetic pathways in dedicated microbial “chassis” have provided access to important industrial compounds (e.g., artemisinin, resveratrol, vanillin). However, the potential of such production systems to facilitate elucidation of plant biosynthetic pathways has been underexplored. Here we report on the application of a modular terpene production platform in the characterization of the biosynthetic pathway leading to the potent antioxidant carnosic acid and related diterpenes in *Salvia pomifera* and *Rosmarinus officinalis*. Four cytochrome P450 enzymes are identified (CYP76AH24, CYP71BE52, CYP76AK6, and CYP76AK8), the combined activities of which account for all of the oxidation events leading to the biosynthesis of the major diterpenes produced in these plants. This approach develops yeast as an efficient tool to harness the biotechnological potential of the numerous sequencing datasets that are increasingly becoming available through transcriptomic or genomic studies.

cytochrome P450 | metabolic engineering | yeast | terpene

Chemical synthesis of industrially important plant secondary metabolites is often hampered by their complex stereochemistry and environmental concerns regarding the use of toxic catalysts and solvents. Production of the compounds of interest in heterologous microbial systems can provide a sustainable and environmentally friendly alternative. Several groundbreaking efforts have been reported, including the production of the antimalarial drug artemisinin (1–3), the fragrances sclareol and santalol (4–7), the flavoring agent vanillin (8, 9), and the antioxidant resveratrol (10). Recently, the complete pathway of opioid biosynthesis comprising more than 20 enzymatic activities was reconstructed in yeast, demonstrating the potential of synthetic biology to contribute in the sustainable production of highly complex chemicals (11). In addition to the development of dedicated platform strains (12), such approaches also require detailed knowledge of the biosynthetic pathways and the genes involved. Elucidation of plant secondary biosynthetic pathways can be a demanding process, involving the acquisition of transcriptomic or genomic sequence information and the analysis of a large number of candidate proteins for the identification of the activity responsible for a specific biosynthetic step. Frequently, this approach also requires the time-consuming chemical synthesis of precursors and the structure elucidation of pathway intermediates. Synthetic biology can expedite this process by enabling functional characterization of gene products and synthesis of substrates or intermediates on the same microbial platform. Here, we report on the application of a modular yeast terpene production platform in the elucidation of the biosynthesis of carnosic acid and related diterpenes.

Carnosic acid (1) and carnosol (8) are potent antioxidant (13), antiadipogenic (14), and anticancer agents (15, 16), whereas several carnosic-acid-related diterpenes, such as 12-methoxy-carnosic acid (9) and pisiferic acid (7; Fig. 1A) have strong antiparasitic, antifungal, and antibacterial activities (17–19). These compounds belong to the group of labdane-type diterpenes (20), which also

includes several other members with bioactive properties, such as tanshinones (2–4), which exhibit strong antiinflammatory activity (21) and are effective against various cardiovascular and cerebrovascular disorders (22, 23), and forskolin (5), which activates adenyl cyclase and increases the intracellular levels of cAMP (24–26) (Fig. 1A). Despite their industrial importance, these compounds still remain largely inaccessible by chemical synthesis, calling for the development of biotechnological methods for their production.

The 20 carbon atom skeletons of labdane diterpenes, which are characterized by a bicyclic decalin core, are synthesized from geranylgeranyl diphosphate (GGPP; 12) in a modular fashion. Initially, protonation-initiated cyclization by class II diterpene synthases (diTPSs) gives rise to a diphosphate precursor, which is then converted to the basic diterpene skeleton through a divalent-cation-dependent ionization reaction catalyzed by a class I diTPS (reviewed in ref. 20). Subsequent decoration of the various skeletons by modifying enzymes, such as cytochrome P450s (CYPs) (27), gives rise to a plethora of secondary metabolites (28, 29). In *Rosmarinus officinalis* and *Salvia fruticosa*, carnosic acid and carnosol are synthesized from (+)-copalyl diphosphate (CPP; 13) through the intermediates miltiradiene (14), abietatriene (15), and ferruginol (16) (Fig. 1B) (30–32). In *Salvia miltiorrhiza*, the same precursors give rise to the different members of the tanshinone group (33, 34). However, the steps that follow synthesis of 16 and the point where the tanshinone and carnosic acid pathways

Significance

Here we report on the development of a modular terpene production platform as a screening tool for the functional characterization of next-generation sequencing information and on the application of this system in the characterization of the biosynthetic pathway leading to the potent antioxidant carnosic acid in *Lamiaceae*. In addition to revealing an important plant natural product biosynthetic pathway, this work highlights the usefulness of synthetic biology approaches in the elucidation of plant natural product biosynthetic pathways and develops yeast as a tool to exploit the wealth of next-generation sequencing data that are constantly being produced through transcriptomic, genomic, or metagenomic studies. The approach described here will be useful to numerous researchers studying terpene biosynthesis in plants and microorganisms.

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¹To whom correspondence should be addressed. Email: s.kampranis@med.uoc.gr.

²Present address: Department of Plant and Environmental Sciences, University of Copenhagen, 1871 Frederiksberg C, Denmark.

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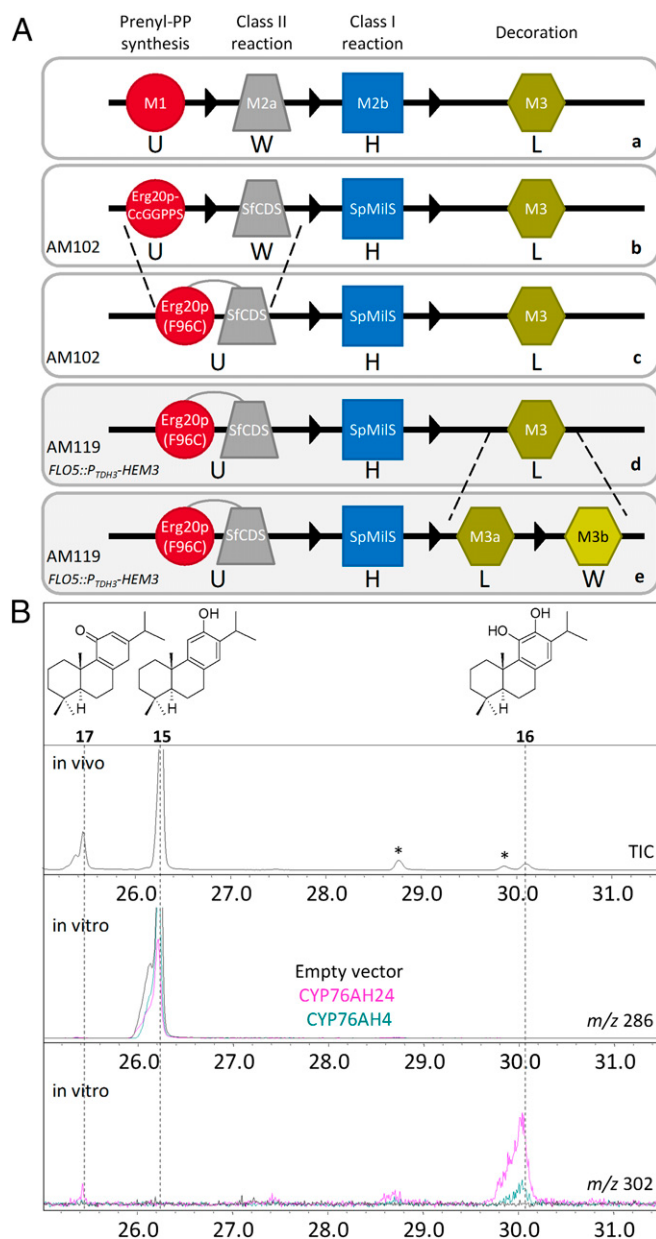


Fig. 2. Characterization of *S. pomifera* CYP76AH24 ortholog as a bifunctional monooxygenase. (A) Diagrammatic representation of the modular design and the platform configuration used. (a) General design of the modular terpene production platform. (b–e) Diagrammatic illustrations of the steps taken to engineer the platform to facilitate the rapid functional characterization of CYP enzymes. (B) Expression of the CYP76AH24 ortholog in optimized multiradiene-producing yeast cells (A, b–d) resulted in the production of **16**, as main compound, and the formation of **17** and **18** minor products. The two peaks indicated by asterisks correspond to degradation products of **17**. In vitro enzymatic assay using a microsomal preparation of yeast cells expressing CPR2 and the *S. pomifera* CYP76AH24 ortholog (pink) or *R. officinalis* ferruginol synthase, CYP76AH4 (teal) confirmed the hydroxylation of **16** at C-11. Microsomal preparations of cells expressing only CPR2 are used as negative control (black).

Examination of the structures of the main *S. pomifera* compounds (Fig. 1A) suggested that **16** is likely their common precursor and that additional CYPs are possibly responsible for the further modification of **16** to the different compounds. To enable analysis of the steps downstream of synthesis of **16**, the yeast modular platform was modified to improve production of **16** and to allow for the simultaneous expression of multiple CYPs.

To improve overall diterpene titers and to make better use of the available auxotrophic selection markers, the Erg20p-CcGGPPS fusion (used as the M1 part) and SfCDS (used as the M2a-specific part) were replaced by a fusion between a mutant form of the yeast FPP synthase [Erg20p(F96C)] and SfCDS. Erg20p(F96C) has been shown to be more efficient in supporting diterpene production than CcGGPPS (5), whereas fusion of a class II synthase N terminally to Erg20p(F96C) was found to facilitate substrate channeling and improve coupling of the subsequent biosynthetic step (5). Coexpression of SfCDS-Erg20p(F96C) (M2a-M1) with SpMiLS (M2b) and CPR/CYP76AH24 (M3) resulted in a threefold increase in the ferruginol titer, from 2.7 to 8.4 mg/L (Fig. 2A, c and SI Appendix, Fig. S2A and B).

High-level expression of CYPs in yeast may impose stress by heme depletion, and engineering the endogenous heme biosynthetic pathway has been found to improve CYP activity (40). To optimize the platform for CYP expression, one copy of the yeast *HEM3* gene, encoding for the rate-limiting enzyme in the pathway, was introduced in the *FLO5* locus of AM102 yeast cells, under the control of the strong *P_{TDH3}* promoter, to generate strain AM119 (Mat a/α, *P_{GAL1}-HMG2(K6R)::HOx2, ura3, trp1, his3, P_{TDH3}-HMG2(K6R)X2::leu2 ERG9/erg9, UBC7/ubc7, SSM4/ssm4, P_{TDH3}-HEM3::FLO5*) (SI Appendix, Table S2). Using this strain, an additional 2.5-fold increase in the titer of **16** was obtained, reaching 21.2 mg/L (Fig. 2A, d, Fig. 2B, and SI Appendix, Fig. S2A and B). The substrate of ferruginol synthase is **15**, a spontaneous oxidation product of **14** (32). Quantification of **15** and **16** revealed that, whereas in AM102 cells, 32% of **15** was converted to **16**, in AM119 cells, the efficiency of this conversion increased to 62%, suggesting an improvement in the activity of CYP76AH24 in the modified strain (SI Appendix, Fig. S2B). This improvement allowed the identification of additional compounds synthesized by CYP76AH24 (Fig. 2B), which were not detectable using the initial platform configuration (SI Appendix, Fig. S2A). Isolation and structure elucidation revealed two compounds: 11-hydroxy ferruginol (8,11,13-podocarpatriene-11,12-diol) (**17**) (SI Appendix, Figs. S7–S9) and 11-keto multiradiene (**18**) (SI Appendix, Figs. S10–S16 and Table S3). To the best of our knowledge, **18** is reported for the first time in this work. The ability of CYP76AH24 to catalyze additional oxidation events on **16** was confirmed by in vitro studies in which microsomal membranes isolated from yeast cells expressing CYP76AH24 and CPR2 were incubated with **16** in the presence of NADPH (Fig. 2B). Production of **17** was confirmed by GC-MS analysis, using comparison with the isolated structurally characterized compound. In vitro experiments with **14** as substrate also confirmed the formation of **18** by CYP76AH24, albeit with lower efficiency than synthesis of **17** ($k_{\text{cat}} = 1.97 \text{ min}^{-1}$ and $K_M = 72.63 \text{ } \mu\text{M}$ for the reaction with **14** compared with $k_{\text{cat}} = 6.11 \text{ min}^{-1}$ and $K_M = 45.84 \text{ } \mu\text{M}$ for the reaction with **16**; kinetic data provided in SI Appendix, Table S4 and Fig. S25). The significant levels of **18** produced by yeast cells expressing CYP76AH24 are thus possibly due to the high amounts of **14** produced by the platform. In agreement with previous observations (41), **17** was found to be unstable under chromatographic conditions, giving rise to several oxidation products during GC-MS analysis (indicated by asterisks in Fig. 2B and SI Appendix, Fig. S2A). These findings strongly suggest that CYP76AH24 is a bifunctional enzyme that catalyzes successive oxidation events on C-12 and C-11 of the labdane skeleton. All other ferruginol synthase enzymes isolated to date (from *S. miltiorrhiza*, *S. fruticosa*, or *R. officinalis*) have been reported to have single functionality. To investigate whether CYP76AH24 is the only enzyme able to catalyze additional oxidation events on **16** or this activity has previously been unnoticed with the other enzymes, the ferruginol synthase from rosemary, CYP76AH4, was analyzed in vitro. Microsomal preparations of CYP76AH4 were assayed in parallel with CYP76AH24 and both enzymes catalyzed production of **17** (Fig. 2B). Kinetic analysis

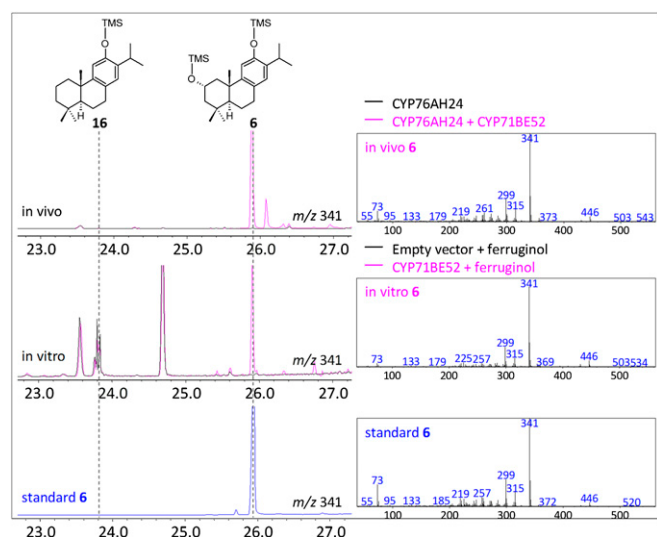


Fig. 3. CYP71BE52 is a salvicol synthase. Expression of *S. pomifera* CYP71BE52 (pink) as an M3b-specific part in the ferruginol-producing yeast platform resulted in the production of **6**, as identified by GC-MS analysis of the TMS-derivatized solvent extract (Top). A microsomal preparation of CYP71BE52 converted **16** to **6** in the presence of NADPH as cofactor (Middle). Formation of **6** was confirmed by comparison with authentic standard (Bottom).

revealed that the two enzymes exhibit similar affinity for **16** ($K_M = 45.8 \pm 9.4 \mu\text{M}$ in the case of CYP76AH24 and $49.5 \pm 11.1 \mu\text{M}$ in the case of CYP76AH4), but also for **14** and **15** as substrates (SI Appendix, Table S4 and Fig. S25).

These results suggest that synthesis of **17** is likely the event that follows formation of **16** en route to **1** in *S. pomifera* and *R. officinalis*. To elucidate the subsequent steps in this pathway, the ability of additional CYPs to act on the products of CYP76AH24 was investigated. To this end, the remaining 14 CYPs were cloned in the yeast vector pWTDH3myc and introduced under tryptophan selection as a second M3 module, M3b, alongside CYP76AH24 (M3a) (Fig. 24, e). Using this reconfigured platform, two additional CYPs acting on CYP76AH24 products were identified. The first one, CYP71BE52, was found to catalyze oxidation of **16** at position 2α , giving rise to **6**, one of the major diterpenes of *S. pomifera*. In yeast culture extracts, **6** was identified by comparison of retention time and mass spectrum with authentic standard (Fig. 3, Top and Bottom and SI Appendix, Figs. S17 and S18), whereas microsomal preparations of CYP71BE52 and CPR2-producing cells were used in enzymatic assays to confirm conversion of **16** to **6** in vitro (Fig. 3, Middle; kinetic parameters for the in vitro reaction of CYP71BE52 with **16** are provided in SI Appendix, Table S4 and Fig. S25). CYP71BE52 was the third most abundant transcript among the identified CYP family genes, suggesting that it likely encodes for the main enzyme responsible for the production of **6** in *S. pomifera* glandular trichomes.

In addition to CYP71BE52, the screen also revealed CYP76AK6, the second most abundant transcript after CYP76AH24, as an enzyme able to oxidize several compounds produced by CYP76AH24 in *S. cerevisiae*. Three compound groups were detected by GC-MS analysis of trimethylsilyl (TMS) derivatized ethyl acetate extracts of culture media (SI Appendix, Supplementary Methods 1.4). The first group of products displayed characteristic fragment ions with 335 and 431 m/z (Fig. 4, Top). Comparison of retention time and mass spectrum with authentic standard confirmed one of these products as **1** (Fig. 4, Bottom), likely synthesized by successive oxidation events at C-20 of **17**. Structure elucidation of the additional compounds in this group was not possible due to the low amounts produced in yeast. However, based on the similarity of their mass spectra with that of **1**, and the fact that oxidized C-20 of the labdane

skeleton is labile and frequently undergoes elimination during GC-MS analysis (42), it is likely that these are the corresponding C-20 aldehyde and alcohol intermediates. The second group of compounds included two peaks exhibiting strong 348 and 430 m/z fragment ions, which are characteristic of **8** (SI Appendix, Fig. S3A, a), a potent antioxidant resulting from the oxidation of **1**. One of these compounds was confirmed to be **8** by comparison with authentic standard (SI Appendix, Figs. S3A, b, S19, and S20). The remaining compound in this group could result from oxidative degradation and rearrangement of carnosic acid to a molecule bearing a catechol, quinone, or semiquinone feature at C-12–C-11 (13, 43, 44), but insufficient amounts precluded characterization at this stage. The last group of compounds displayed 343 m/z as the most abundant fragment ion (SI Appendix, Fig. S3B, a). One of these compounds matched the retention time and mass spectrum of TMS-derivatized **7** standard (SI Appendix, Figs. S3B, a and d, S21, and S22). Purification of the yeast products followed by structure elucidation also revealed production of pisiferol (**19**) (SI Appendix, Figs. S3B, a and c, S23, and S24), the product of the first oxidation event on C-20 of ferruginol en route to **7**. Identification of the remaining compound in this group (indicated by an asterisk, SI Appendix, Fig. S3B, a) was not possible at this stage, but this peak likely corresponds to pisiferol, the intermediate between **19** and **7**. Taken together, these results suggest that CYP76AK6 is a multifunctional enzyme that catalyzes successive oxidation events at C-20 of the labdane skeleton. An enzymatic assay using a combination of microsomal preparations of CYP76AH24 and CYP76AK6 in the presence of **16** as substrate confirmed the ability of CYP76AK6 to produce in vitro a similar blend of molecules to those obtained from yeast cells (Fig. 4 and SI Appendix, Fig. S3B, b). However, production of **8** was not detected in the in vitro reaction, suggesting that enzymatic or nonenzymatic

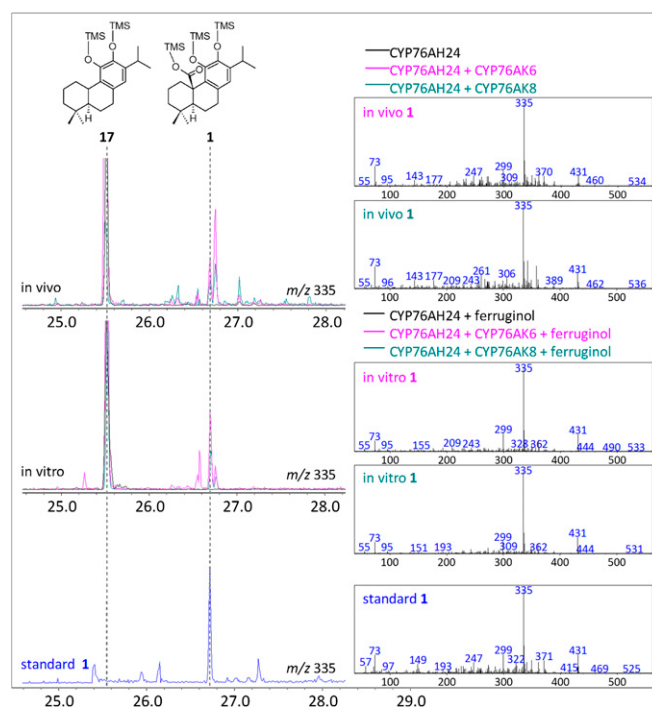


Fig. 4. CYP76AK6 and its homolog, CYP76AK8, catalyze successive oxidation events at C-20. Formation of **1** by CYP76AK6 (pink) and CYP76AK8 (teal) in yeast cells coexpressing CYP76AH24 and SpMILS. TMS-derivatized extracts of yeast cultures analyzed by GC-MS revealed the formation of **1** (Top), identified by comparison with authentic standard (blue) (Bottom). Enzymatic assays containing microsomal preparations of CYP76AH24 ortholog and CYP76AK6 (pink) or CYP76AK8 (teal), **16** and NADPH revealed the formation of **1** in vitro (Middle).

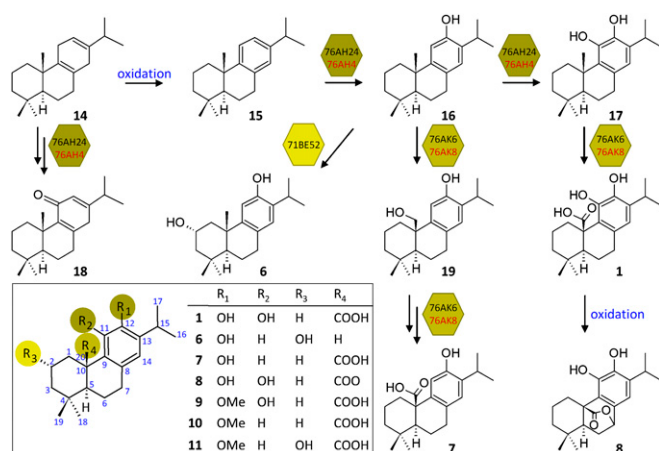


Fig. 5. Proposed mechanism for the biosynthesis of carnosic-acid-related diterpenes in *S. pomifera* and *R. officinalis*. The biosynthesis of labdane-type diterpenes in *S. pomifera* (black) and *R. officinalis* (red) is initiated by the action of bifunctional enzymes, CYP76AH24 or CYP76AH4, respectively, which are responsible for the hydroxylation of **15** initially at position C-12 to produce **16** and subsequently at position C-11 to yield **17**. The same bifunctional enzymes can also catalyze two successive oxidation events on **14** to yield **18**. A *S. pomifera* enzyme, CYP71BE52, catalyzes oxidation of **16** at position 2 α to synthesize **6**. CYP76AK6 or CYP76AK8 catalyze successive oxygenations at position C-20 of **16** and **17** to yield **7** via **19**, and **1**, respectively. In the yeast platform, **1** is further oxidized to **8** by a yet undefined mechanism. The combined activities of CYP76AH24, CYP71BE52, and CYP76AK6 on the abietatriene skeleton (Inset) are sufficient to explain the biosynthesis of the main diterpenes (**1**, **6**–**11**) isolated from *S. pomifera* and *R. officinalis*.

oxidation events occurring in the process of yeast cultivation are likely responsible for the conversion of **1** to **8** and other oxidative degradation products.

To explore whether the same steps are also responsible for carnosic acid biosynthesis in *R. officinalis*, a transcriptome database available online (medicinalplantgenomics.msu.edu) was used to identify the closest rosemary homolog of CYP76AK6 through blastx analysis. CYP76AK8, which is encoded by the ORF of *R. officinalis* locus 4766 isotig 5 and exhibits 75% similarity at the amino acid sequence level with CYP76AK6, was amplified from *R. officinalis* cDNA and tested for the production of **1** in the 11-hydroxy-ferruginol-producing yeast cells. The product profile of *S. cerevisiae* cells expressing CYP76AK8 was similar to that of CYP76AK6, including **1**, **8**, **7**, and **19** (Fig. 4 and SI Appendix, Fig. S3 A and B, a). Moreover, in vitro reaction combining individual microsomal preparations of CYP76AH24 and CYP76AK8 with **16** as substrate and NADPH cofactor resulted in the production of the same compounds, except for **8** (Fig. 4 and SI Appendix, Fig. S3 A and B, b), whereas kinetic parameters for the in vitro reactions of CYP76AK6 and CYP76AK8 with **16** and **17** were found to be comparable (SI Appendix, Table S4 and Fig. S25), suggesting that CYP76AK8 is the rosemary ortholog of CYP76AK6. Investigation of the order of biosynthetic events using in vitro assays revealed that although CYP76AK6 and CYP76AK8 can oxidize C-20 of both **16** and **17**, CYP76AH24 or CYP76AH4 are unable to modify **7**. The titer of **1** obtained in shake-flask cultivation using the described configuration was in the range of 1 mg/L of culture. Considering the number of biosynthetic steps introduced downstream of the mevalonate pathway in this platform, further optimization through state-of-the-art metabolic engineering approaches is expected to achieve significant improvement in product yields.

Discussion

The combined activities of the CYPs identified here, targeting four positions of the abietane skeleton, C-2, C-11, C-12, and C-20,

are sufficient to explain all of the oxidation events involved in the biosynthesis of the major diterpenes identified in *S. pomifera* (Fig. 5), and to clarify the pathway leading to **1** and **8** in both *S. pomifera* and *R. officinalis*. In the first step of this mechanism, **15** is converted to **16** by CYP76AH24 or CYP76AH4, which is then taken up by CYP76AK6 or CYP76AK8 to produce **7**. Further oxidation of **16** by CYP76AH24 or CYP76AH4 produces **17**, which is converted to **1** by CYP76AK6 or CYP76AK8. In yeast cultures, **1** is converted to **8**, either by nonenzymatic oxidation or through a yet unidentified yeast enzymatic system. In *S. pomifera*, CYP71BE52 oxidizes **16** at position 2 α to produce salviol. *O*-methylation at position 12 of **1**, **7**, or 2 α -hydroxy-pisiferic acid produces the corresponding methoxy derivatives. Further studies will extend this approach to the identification of the methyltransferase(s) responsible for this step, using the already available transcriptomic information.

Although the example presented in this report only goes as far as the characterization of CYP activities responsible for the oxidation of mitradadiene-derived structures, the developed approach has broader application. The modular structure of the platform allows its facile reconfiguration for the production of other terpene skeletons, so that, for example, when a library of M3 parts is available, this can be tested in parallel on different mono-, sesqui-, di-, or triterpenoid substrates. This is particularly useful in the analysis of libraries derived from transcriptomic studies, which encode biosynthetic activities responsible for the production of a diverse group of terpene compounds. The design is expandable, allowing the incorporation of additional module-specific parts to study events beyond oxidation (e.g., acetylation, methylation, etc.).

Transcription of genes involved in secondary metabolite biosynthesis is frequently influenced by environmental factors or developmental phase. Such variations could influence the efficiency of pathway elucidation approaches based on transcriptional analysis. The effectiveness of the overall effort can be greatly improved if transcriptomic analysis is carried out in parallel to metabolomic studies, and, if possible, on the same sample or under very similar conditions. In the case study presented here, metabolomic and transcriptomic analysis was combined in the same plants (37). Following next-generation sequencing, genes were prioritized for analysis on the basis of their transcription levels and by increasing the representation of the CYP71 and CYP76 subfamilies, members of which have previously been found to be involved in terpene oxidation (29). Rational short listing of candidate genes can help minimize time and reduce cost. In the current effort, the three CYPs involved in carnosic-acid-related diterpenes in *S. pomifera* corresponded to the top three expressed CYP transcripts in the glandular trichome library. Because these CYPs also belong to the targeted CYP subfamilies, it is unclear at this stage whether a selection of candidates based on transcript abundance would, in general, be advantageous over selection, based on sequence relatedness. For major metabolites, prioritization based on gene expression levels is likely to be more successful, whereas sequence-based selection may work better for minor compounds or for transcriptional datasets obtained under different conditions than those of the metabolic profile.

Taken together, these results highlight the effectiveness of using engineered yeast as a tool for the elucidation of biosynthetic pathways. The yeast system can expedite structure elucidation of products or pathway intermediates by readily providing sufficient amounts of compounds through the high titers achieved using the engineered yeast strains. This also makes adequate quantities of substrates and standards available for subsequent confirmation and characterization of the pathway steps through in vitro studies or for the evaluation of the biological activity of isolated compounds. Yeast is a commonly used microorganism that does not require dedicated culture systems or specialized equipment, whereas scaling up can be achieved using established infrastructure. Due to its relatively short growth cycle, yeast allows for fast

turnaround times, enabling analysis of a large number of samples in a short time. Thus, in combination with DNA synthesis and rapid cloning techniques, this system can serve as a tool to exploit the wealth of next-generation sequencing data that is constantly being produced through transcriptomic, genomic, or metagenomic studies. Additionally, because many of these compounds have industrial importance, coupling pathway identification with production in the same organism minimizes transfer to an industrial setting. As yeast is a favorable host for industrial fermentation, once a specific pathway is analyzed, the system is already set up for further optimization of heterologous production of specific compounds using metabolic engineering approaches, such as flux analysis, pathway balancing, or metabolic channeling.

Materials and Methods

Yeast Strains Construction. Yeast strain AM119 was generated using AM102 (38) as follows. The *HEM3* gene was cloned into EcoRI-XhoI restriction sites of the plasmid construct COD7 (*P_{TDH3}-HEM3-CYC1t*, *LoxP-HIS5-LoxP*) (38) using primers 5-*HEM3*-EcoRI and 3-*HEM3*-XhoI (*SI Appendix, Table S5*). The COD7/*HEM3* construct was PCR amplified with primers 5-*FLO5*-COD7 and 3-*FLO5*-COD7 (*SI Appendix, Table S5*), which incorporate flanking sequences with complementarity to the 3'UTR of *FLO5* gene. Selection marker excision gave rise to strain AM119.

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In Vitro Enzymatic Assay and Kinetic Analysis. The kinetic parameters of the different CYPs were determined as previously described (36) using varying concentrations (1–75 μ M) of each substrate (*SI Appendix, Table S4*). The enzymatic reactions were incubated with mild shaking at 30 °C for 3 h and terminated by extraction with 100 μ L of decane or ethyl acetate containing 10 μ g/mL sclareol as internal standard. A total of 2 μ L of extracts was analyzed by GC-MS using the conditions described in *SI Appendix*. To evaluate the combined enzymatic activity of CYPs in vitro, a 0.5-mL reaction containing 90 mM Tris-HCl (pH 7.5), 1 mM NADPH, 80 pmol of CYP76AH24 or CYP76AH4 microsomal protein, and 100 μ M ferruginol was incubated with shaking at 30 °C for 18 h. Subsequently, 80 pmol of CYP76AK6 or CYP76AK8 was added and the reaction was incubated for an additional 18 h and terminated by extraction with 200 μ L pentane or ethyl acetate. The extract was gently evaporated and derivatized with Sylon HTP. A total of 2 μ L of derivatized extract was analyzed by GC-MS. All assays were carried out in duplicates.

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