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Resin Acid Conversion with CYP105A1: An Enzyme with Potential for the Production of Pharmaceutically Relevant Diterpenoids

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Cytochrome P450s are very versatile enzymes with great potential for biotechnological applications because of their ability to oxidize unactivated C–H bonds. CYP105A1 from *Streptomyces griseolus* was first described as a herbicide-inducible sulfonylurea hydroxylase, but it is also able to convert other substrates such as vitamin D_3. To extend the substrate pool of this interesting enzyme further, we screened a small diterpenoid compound library and were able to show the conversion of several resin acids. Binding of abietic acid, dehydroabietic acid, and isopimaric acid to the active site was assayed, and V_{max} and K_m values were calculated. The products were analyzed by NMR spectroscopy and identified as 15-hydroxyabietic acid, 15-hydroxydehydroabietic acid, and 15,16-epoxyisopimaric acid. As the observed products are difficult to obtain by chemical synthesis, CYP105A1 has proved to be a promising candidate for biotechnological applications that combine bioconversion and chemical synthesis to obtain functionalized resin acids.

Introduction

Cytochrome P450 (CYP or P450) monooxygenases form a superfamily of heme-containing enzymes that are widely distributed across all domains of life. Their natural function in most organisms is the degradation and detoxification of drugs and xenobiotics, as well as the biosynthesis of endogenous compounds such as steroids and terpenoids. The key role of these enzymes in important metabolic pathways has led in some cases to a convergent evolution of P450s performing the same reaction in different organisms.[1] It is also common for members of this enzyme family to be involved in the synthesis of a compound in one organism and the degradation of the same compound in another. As a result of this versatility and linked evolution, many P450 enzymes have intriguingly broad substrate ranges, as reflected by the fact that most naturally occurring chemical compounds can serve as substrates for P450-catalyzed conversions. Additionally, P450s are able to catalyze a tremendous variety of reactions (e.g., N-oxidation, sulfoxidation, deamination, dehalogenation, N-, O-, and S-dealkylation); this brings these enzymes into focus for biotechnological applications.[2] In particular, their ability to catalyze the selective insertion of oxygen into activated and nonactivated C–H bonds through hydroxylation and epoxidation makes these proteins very interesting for the production of fine chemicals and pharmaceuticals. As hydroxylation of nonactivated carbon bonds is often difficult to achieve by classical organic synthesis, the use of microorganisms (or their isolated enzymes) represents an environmentally friendly and efficient alternative for this challenge.[3]

Streptomyces are Gram-positive, spore-producing actinobacteria that are predominantly found in soil and decaying vegetation. Approximately two-thirds of clinically useful antibiotics of natural origin are produced by members of this genus.[4] As their secondary metabolome comprises the richest source of biologically active natural compounds, these bacteria are of special interest in the P450 field.[5] The number of P450 genes in distinct species of *Streptomyces* differs, but is typically very high: up to 33 different members of this enzyme family in *Streptomyces avermitilis*, for instance.[6] Because of these characteristics, *Streptomyces* species and their CYPs have high potential for application in the biotechnological production of chemicals. An example of their industrial implementation is the bioconversion of compactin to pravastatin, a therapeutic agent for hypercholesterolemia, by CYP105A3 from *Streptomyces* sp. Y-110.[7,8]

CYP105A1 from *Streptomyces griseolus* was first described as a herbicide-inducible sulfonylurea-converting P450.[9] The crystal structure of this enzyme has been elucidated, and many other compounds like vitamin D_3 and the norisoprenoids α- and β-ionone have been identified as substrates.[10–12] Recently, our group described the hydroxylation of the antidiabetic drugs glibenclamide and glimepiride by CYP105A1.[13] Encouraged by the diversity of these compounds, we decided to fur-

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ther explore the substrate spectrum of this enzyme. As *Streptomyces* species are known to degrade and metabolize complex organic substrates like cellulose and chitin, we concluded that these bacteria also come into contact with secondary metabolites of plant origin. We therefore tested a small compound library consisting of the eight most abundant diterpene resin acids of the abietane and pimarane type and their precursors for substrate conversion by CYP105A1. These compounds are well represented in the resin of conifers,[14,15] and exhibit interesting biological and pharmaceutical properties such as antimicrobial, antiviral, and antifungal, as well as antitumoral and antimutagenic activities.[16–18] Allergenic potential and water toxicity have also been described.[19,20] These compounds might therefore be promising candidates for enzymatically catalyzed functionalization, with possible enhancement of their biological activity.

Results and Discussion

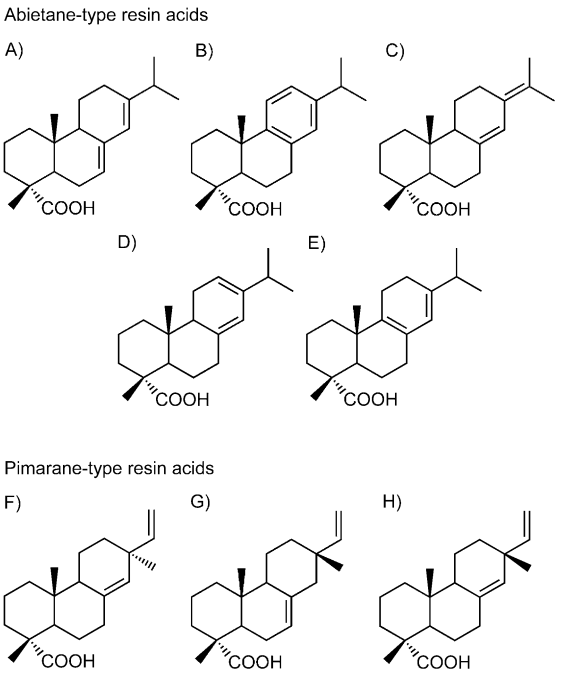
In vitro substrate conversion assay

A characteristic feature of most P450s is their dependence on electron transfer proteins for catalytic function.[21] In this study, the heterologous electron transfer proteins Arh1 (adrenodoxin reductase homologue 1) and Etp1^{fd} (electron transfer protein 1, ferredoxin domain) from *Schizosaccharomyces pombe* were used to support the catalytic function of CYP105A1, as previously described.[13]

In order to evaluate the conversion of resin acid diterpenoids by CYP105A1, a small compound library containing the eight most abundant resin acid diterpenes and their corresponding alcohols, aldehydes, and olefins (Scheme 1) was tested by using this reconstituted system. It was shown that resin acids of both abietane and pimarane types were converted to one main product. Furthermore, conversion of the alcoholic diterpenoids was observed, but the selectivity and activity of these reactions were not very high, and resulted in unspecific conversion with several products. The aldehydes and olefins could not be converted by CYP105A1. Therefore, we focused on abietic, dehydroabietic, and isopimaric acid for further investigation and characterization of the substrate conversion.

Substrate binding spectra

Binding of a ligand to the active site of cytochrome P450 leads to specific changes in the UV–visible absorbance spectrum. The reason is replacement of a water molecule at the heme iron by the substrate, which results in a transition of the heme from the hexa-coordinated (low-spin) state to the penta-coordinated (high-spin) state.[22,23] This so-called type I spectral shift can be monitored spectrophotometrically and used to determine the binding affinity of a ligand. The three identified substrates abietic acid, dehydroabietic acid, and isopimaric acid induced a type I shift in the spin state of the heme iron. Equilibrium dissociation constants (K_d) for the substrates were determined by substrate titration (Figure 1).



Scheme 1. Structures of the eight resin acid diterpenes. A) abietic acid, B) dehydroabietic acid, C) neoabietic acid, D) levopimaric acid, E) palustric acid, F) pimaric acid, G) isopimaric acid, H) sandaracopimaric acid. The corresponding precursors have an alcohol (CH_2OH), aldehyde (CHO), or methyl group (CH_3) in place of the carboxyl substituent at C4.

The determined K_d value of isopimaric acid is (1.75 ± 0.73) μM. Abietic acid and dehydroabietic acid bind with similar K_d values: (8.75 ± 2.06) μM and (10.66 ± 2.64) μM, respectively. These values in the low micromolar range indicate tight binding of the substrates to the active site, with a higher affinity of this enzyme towards diterpenoid substrates than for CYP106A2, another bacterial P450 with the ability to hydroxylate abietic acid.[24]

Catalytic activity of CYP105A1

The hydroxylation of the three diterpene resin acids was further characterized in terms of their Michaelis–Menten kinetics. HPLC analysis showed that the substrates were converted to single products. The conversion of abietic acid to its product, identified as 15-hydroxyabietic acid (see below), exhibited a V_{max} of (1.18 ± 0.02) nmol product per nmol CYP105A1 per minute, and a K_m of (4.12 ± 0.30) μM. The V_{max} values for the conversion of dehydroabietic acid and isopimaric acid were significantly higher: (8.05 ± 0.19) and (8.55 ± 0.18) nmol of product per nmol CYP105A1 per minute, respectively. The higher reaction velocities of these substrates were accompanied by lower affinity: K_m values of (18.33 ± 1.2) μM for dehydroabietic acid and (13.86 ± 0.84) μM for isopimaric acid (Figure 2). These K_m values are comparable to those described for the hydroxylation of glimepiride and glibenclamide as well as 1α-hydroxyvitamin D_3, and higher compared to those for vitamin D_2 and D_3, for which K_m values in the sub-micromolar range have been described.[13,25,11] However, the overall conver-

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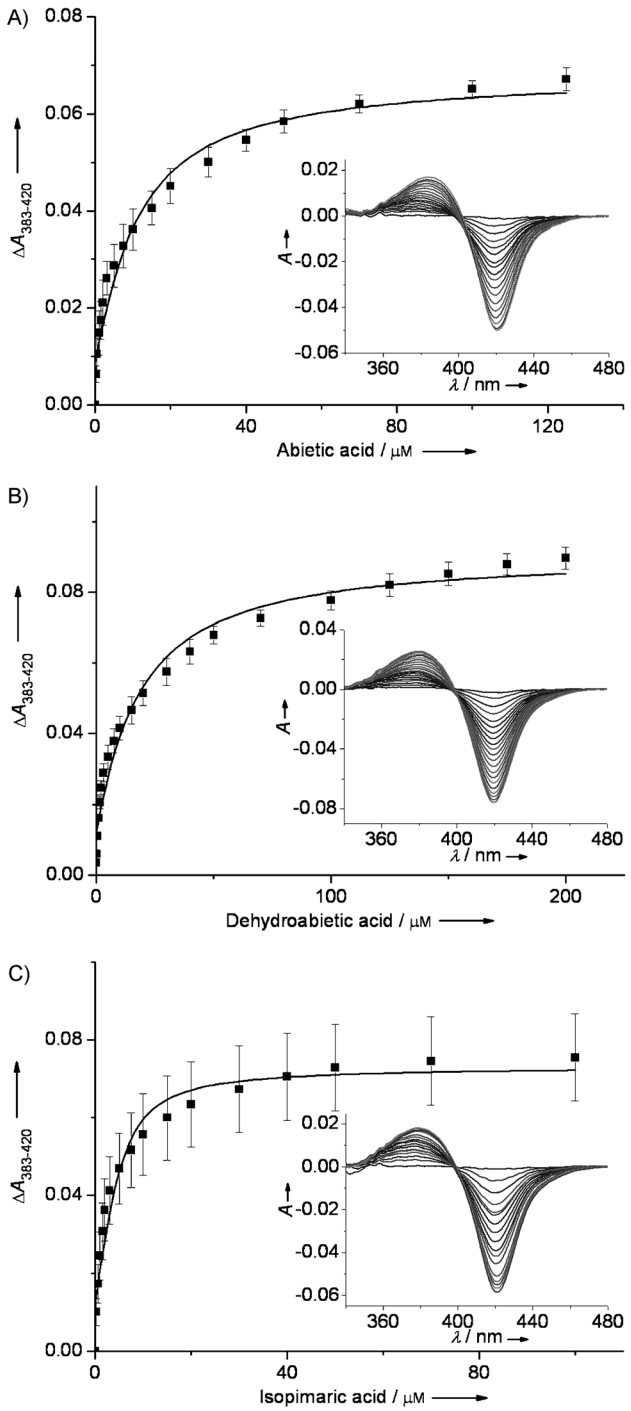

Figure 1. Type I spectral changes caused by binding of A) abietic acid, B) dehydroabietic acid, and C) isopimaric acid to CYP105A1. The binding assay was conducted in tandem cuvettes containing 5 μM CYP105A1 in 50 mM potassium phosphate buffer (pH 7.4). Substrates were dissolved in DMSO and added in different concentrations, followed by recording of difference spectra.

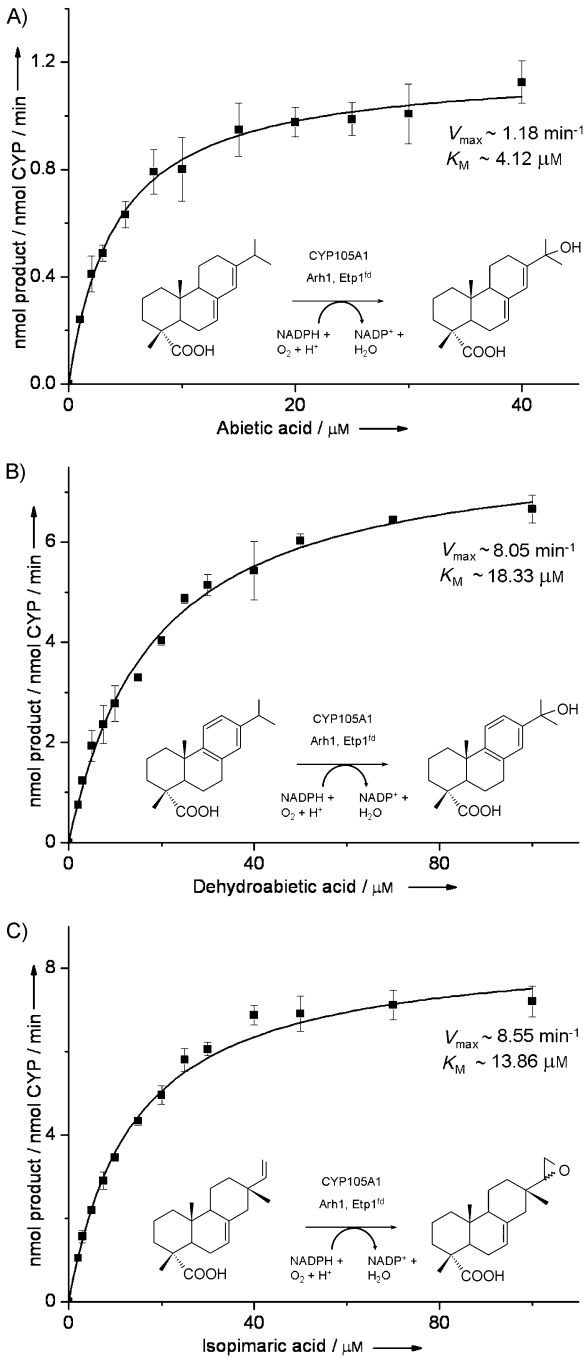

Figure 2. Initial rates of A) abietic acid, B) dehydroabietic acid, and C) isopimaric acid conversion by CYP105A1. Reactions were carried out in a final volume of 250 μL in 50 mM HEPES (pH 7.4) with 0.05% Tween 20 at 30 °C in the presence of a NADPH regenerating system.

sion rates were considerably higher because of the higher V_{max} values with the diterpenoid substrates. Taken together, this catalytic efficiency towards resin acids is the highest described so far for CYP105A1, thus making this enzyme an interesting candidate for diterpenoid functionalization.

Substrate docking

In order to gain deeper insights into substrate binding of the resin acids to the active site of CYP105A1, docking experiments with abietic, dehydroabietic, and isopimaric acids and abietadienol were performed by using Autodock 4.0.[26,27] The best docking scores for abietic and dehydroabietic acids were obtained with orientation of the isopropyl group of the resin acid substrates directly above the heme prosthetic group (Figure 3).

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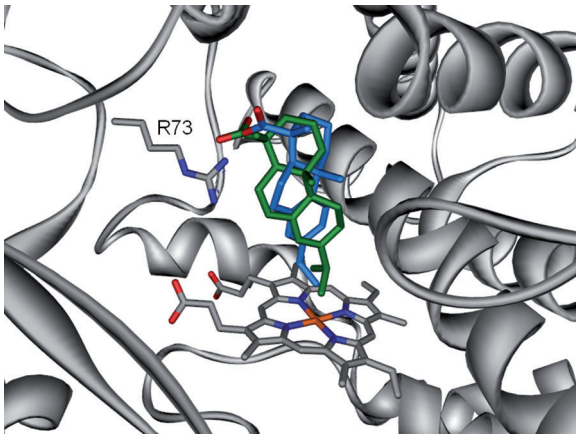

Figure 3. Docking results show the carboxylate groups of abietic acid (green) and dehydroabietic acid (blue) forming salt bridges with the side chain of R73, whereas the isopropyl groups are at a short distance from the iron of the prosthetic heme group.

In the case of isopimaric acid, the optimum orientation had the vinyl group above the heme. The substrates were stabilized by salt bridges between their carboxyl group and the side chain of an arginine residue (R73). This amino acid plays an important role in CYP105A1 as it is one of three arginine residues that describe the distal pocket wall of the active site. The function of these amino acids in the hydroxylation of vitamin D_3 has been extensively studied by site-directed mutagenesis.[10] However, R73 was found to have a rather unfavorable role in the hydroxylation of 1α-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3 due to close contact to the hydrophobic CD ring of the substrates.[10] In contrast, R73 seems to play a major role during hydroxylation of the resin acids, through stabilization of the substrate in the active site. This is supported by the fact that the corresponding aldehydes and olefins, which lack the carboxyl group, are not (or only very weakly) converted by CYP105A1. The docking experiments also provided an explanation for the rather unspecific product formation of the alcohol substrates. Docking of abietadienol revealed two possible orientations of the substrate in the active site. The first was similar to those observed for the resin acid substrates, with an orientation of the isopropyl group above the heme, thereby bringing C16 and C17 close enough to the iron for hydroxylation. In the second orientation, the substrate molecule was turned about 180° around the longitudinal axis, which led to orientation of the methyl group and the alcohol function of C4 in close proximity to the heme.

Whole-cell conversion

To obtain higher yields from the CYP105A1-mediated resin acid conversions, a recombinant whole-cell system with *Bacillus megaterium* was used. This has been successfully applied for the conversion of glimepiride and glibenclamide.[13] Compared to the application of purified enzymes, whole-cell systems in the biocatalysis with P450 enzymes have the advantages of higher stability of P450 in a living organism and the regeneration of expensive cofactors by the host cell.[28]

In order to prevent side reactions and unspecific conversions of constituent parts of the complex medium, the whole-cell conversions were performed in 50 mM potassium phosphate buffer (pH 7.4). With this system, 30.2 mg of abietic acid in a culture volume of 500 mL could be completely converted within 24 h. The isolated products were subjected to NMR spectroscopy.

Conclusions

The aim of this study was to broaden the substrate spectrum of CYP105A1 from *S. griseolus*. The natural function of this P450 is not known, but a diverse variety of substrates has been shown to be converted by CYP105A1. Among these are compounds such as herbicides, antidiabetic drugs, vitamin D_3, and norisoprenoids. *Streptomyces* are well known for their high potential in synthesizing antibiotics. Although the involvement of P450 enzymes in such pathways is common, analysis of the genetic context of the CYP105A1 gene did not reveal its location in a biosynthetic cluster, and therefore gives no hint to its involvement in a particular biosynthetic pathway. Taking the diversity of the reported substrates into account, we concluded that the physiological role of CYP105A1 might be degradation of various xenobiotics. This assumption is supported by the finding that other members of the CYP105 family also have the ability to accommodate a broad range of structurally diverse compounds.[29] As a soil-dwelling organism that also grows on decaying vegetation (e.g., waste wood), *S. griseolus* naturally comes into contact with a lot of biomaterial containing plant secondary metabolites like terpenoids. For this reason we chose a small compound library of diterpenoids to be tested for conversion with CYP105A1, and eight resin acid diterpenes of the abietane and pimarane type were identified as new substrates. The high affinity of the resin acids, as demonstrated by their low K_d values, as well as their efficient conversion by CYP105A1, provide an indication of the important role of this P450 in the detoxification of compounds such as plant secondary metabolites. Although conversion of the norisoprenoids α- and β-ionone has been reported, the product formation of these substrates was very low and unselective.[12] In light of the fact that ionones are obtained from the degradation of carotenoids, this work is the first description of terpenoids built of isoprene subunits as substrates for CYP105A1. The binding and conversion of the most interesting substrates, namely abietic acid, dehydroabietic acid, and isopimaric acid, were investigated in more detail. All reactions on these resin acids were shown to be regioselective, and resulted in only one product. The different products were analyzed by NMR spectroscopy. In the case of abietic and dehydroabietic acid, they were identified as 15-hydroxyabietic acid and 15-hydroxydehydroabietic acid. The pimarane-type isopimaric acid, which lacks the isopropyl function in favor of a methyl and vinyl group at C13, was converted to 15,16-epoxyisopimaric acid. Interestingly, the corresponding aldehydes and olefins did not show any product formation in a reconstituted substrate-con-

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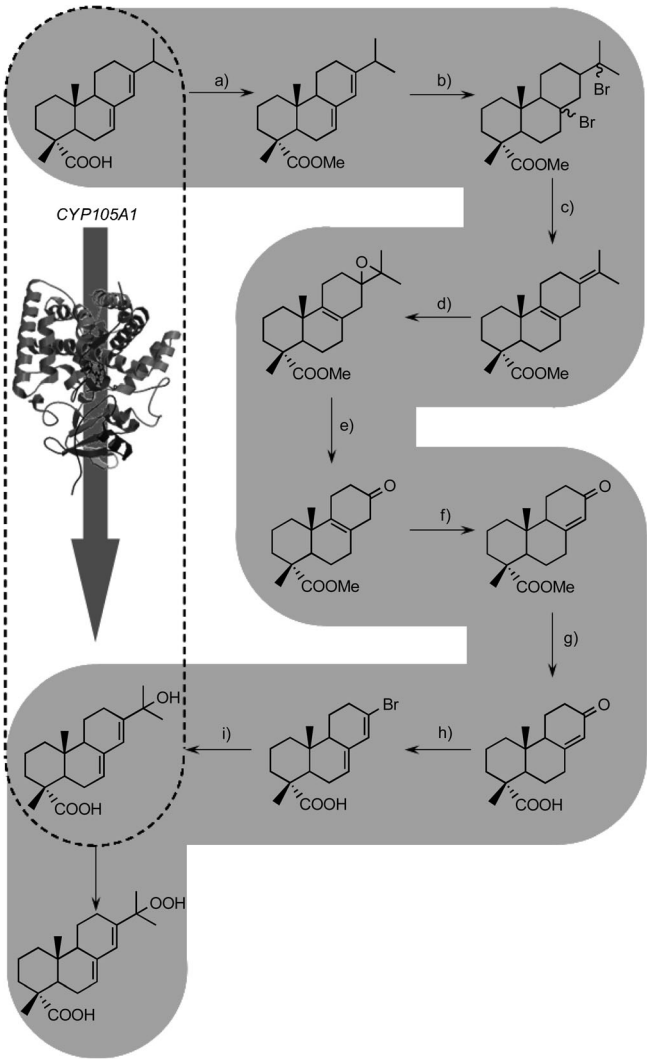
version assay, whereas the alcohols were converted in an unselective manner. The structural basis for these differences in substrate acceptance was elucidated by docking studies; these showed a clear preference for those orientations of the ligands that led to the experimentally observed hydroxylation patterns.

The hydroxylation of abietic acid at C15 is extremely interesting, because the easy aromatization of ring C is a major pitfall in chemical synthesis of hydroxylated abietic acid derivatives. Furthermore, 15-hydroperoxyabietic acid (15-HPA) has been identified as a major contact allergen, responsible for the contact dermatitis of colophonium, one of the ten most commonly encountered allergic reactions.[19,30] The chemical hemisynthesis of this compound from abietic acid is very time- and labor-intensive (Scheme 2). A combined approach that made use of the biotransformation of abietic acid into 15-hydroxyabietic acid together with a subsequent chemical synthesis to obtain the hydroperoxide would be beneficial environmentally and economically, and could help to produce workable amounts of 15-HPA, which are needed to study the reaction of 15-HPA with skin proteins.

A synthetic route for the epoxidation of the vinyl group in pimaric acid has been described, but this requires several steps.[31] The chemical synthesis and NMR analysis of 15,16-epoxyisopimaric acid have not yet been published. However, derivatives of isopimaric acid might be interesting, as this compound is described as a potent opener of large-conductance calcium-activated K^+ (BK) channels.[32]

In summary, we found CYP105A1 to be a resin acid diterpene hydroxylase capable of converting different abietane- and pimarane-type diterpenoids. It must be highlighted that the catalytic efficiency and regioselectivity of this wild-type enzyme make it an ideal candidate for biotechnological applications.



Scheme 2. Schematic overview of the chemical hemisynthesis of 15-HPA from abietic acid as described in ref. [38] and ref. [40]. a) LiOH·H_2O, DMF, RT, 4 h, DMS, 0 °C, 15 min, 98%; b) HBr, AcOH, RT, 6 h, 47%; c) LiOH·H_2O, DMF, 80 °C, 4 h, 71%; d) MCPBA, CH_2Cl_2, 0 °C, 2 h, 90%; e) H_5IO_6, THF, −30 to −10 °C, 1 h, 87%; f) HCl, MeOH, 0 °C, 4 h, 96%; g) *t*BuOK, DMSO, RT, 45 min, 80%; h) $(COBr)_2$, DMF, CH_2Cl_2, −78 °C to RT, 5 h, 74%; i) *t*BuLi, Et_2O, Aceton anhydr., −78 °C to RT, 2 h, 70%. CYP105A1-mediated hydroxylation of abietic acid at C15 bypasses reaction steps (a) to (i).

Experimental Section

Reagents and chemicals: Bulk amounts of abietic acid, dehydroabietic acid, and isopimaric acid were purchased from Orchid Cellmark (New Westminster, BC, Canada). All other chemicals were obtained from standard sources and were of the highest purity available.

Protein expression and purification: Recombinant expression of CYP105A1 in *Escherichia coli* JM109 was performed as previously described[10] with minor modifications. Cells were grown in 2 L baffled flasks containing TB medium (1 L; 24 g yeast extract, 12 g peptone, 4 mL glycerine, 2.31 g KH_2PO_4 12.54 g K_2HPO_4, and 1000 mL distilled H_2O) at 37 °C. When the cell density reached $OD_{600} = 0.8$, induction was started by addition of IPTG (1 mM final concentration). At the same time, the heme precursor δ-Ala was added (final concentration 0.5 mM), and the temperature was reduced to 25 °C. Cells were harvested after 42 h and stored at −20 °C. For the purification of his-tagged CYP105A1, the cytosolic fraction of the cells was applied to a Ni-NTA agarose column (Macherey–Nagel) equilibrated with potassium phosphate buffer (50 mM, pH 7.4) containing NaCl (300 mM). After washing with five column volumes of the same buffer, the protein was eluted with a linear gradient of histidine (0–40 mM) in potassium phosphate buffer (50 mM, pH 7.4) containing NaCl (300 mM). The protein fractions were pooled in potassium phosphate buffer (50 mM, pH 7.4), concentrated by using centrifugal filter devices (exclusion size 50 kDa, 4500 *g*; Merck–Millipore) and applied to a Superdex 75 gel filtration column (GE Healthcare, Solingen, Germany) in potassium phosphate buffer (50 mM, pH 7.4). Determination of the P450 concentration was conducted by measuring reduced CO difference spectra in potassium phosphate buffer (1 M, pH 7.4) in a double-beam spectrophotometer (Shimadzu) with an extinction coefficient of 91 $mM^{-1}cm^{-1}$ as described by Omura and Sato.[33] The heterologous electron transfer proteins Arh1 and $Etp1^{fd}$ from *S. pombe* were expressed and purified as previously described.[34,35]

In vitro substrate conversion assay: A reconstituted in vitro system containing CYP105A1 (1 μM), $Etp1^{fd}$ (20 μM), Arh1 (3 μM), and a cofactor-regenerating system was used to screen for substrate conversion, with glucose-6-phosphate (5 mM) and glucose-6-

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phosphate dehydrogenase (2 U mL^{-1}; Roche) and $MgCl_2$ (1 mM) in HEPES (50 mM, pH 7.4) and Tween 20 (0.05% *v/v*). The potential substrates were dissolved in ethanol and added (final concentration 200 μM). The final ethanol concentration in the reaction mixture was 1% (*v/v*). Reactions were performed in a total volume of 250 μL at 30 °C in a Thermomixer (1000 rpm; Eppendorf). The reactions were started by addition of NADPH (1 mM) and stopped after 30 min by addition of ethyl acetate (250 μL). After a second extraction with ethyl acetate (250 μL), the organic phases were combined and dried.

HPLC and TLC analysis: HPLC analysis was performed on a system consisting of a PU-2080 HPLC pump, AS-2059-SF autosampler, and MD-2010 multiwavelength detector (Jasco). A Nucleodur 100–3 C18 column (125×4 mm, 1 mL min^{-1}; Macherey–Nagel) was used at 40 °C under isocratic conditions (mobile phase: methanol/water 80:20, with acetic acid (0.05%)). Compounds were detected at 240 and 210 nm. The chromatograms were monitored for 20 min. Thin layer chromatography (TLC) was used to monitor conversion of substrates lacking a conjugated pi-electron system. The samples were spotted onto silica TLC plates (4×8 cm; Sigma–Aldrich), and chromatography was performed in a solvent tank containing hexane and ethyl acetate (70:30) with acetic acid (1% *v/v*) as a mobile phase. Compounds were visualized with an anisaldehyde stain (4-methoxybenzaldehyde (2% *v/v*) in acetic acid/sulfuric acid/ethanol 1.5:5:93.5) and subsequent heating with a hot air gun.

Substrate binding analysis: Substrate binding was assayed by following the substrate-induced spin-shift of CYP105A1. Spin-shift assays were conducted at room temperature under aerobic conditions in tandem quartz cuvettes (Hellma, Mülheim, Germany) in a UV-2101PC double beam spectrophotometer (Shimadzu). One chamber of each cuvette contained CYP105A1 (5 μM) in potassium phosphate buffer (50 mM, pH 7.4), and the other chamber contained potassium phosphate buffer (50 mM) without protein. Substrates dissolved in DMSO were added in equal amounts to the chamber containing the enzyme (sample cuvette) and the chamber containing only buffer (reference cuvette). Final ligand concentrations were in the range 0–200 μM, and spectra were recorded from 480 to 340 nm. All measurements were performed a minimum of three times, and mean values were calculated for each data set. The peak-to-trough difference (ΔA) was plotted against ligand concentration, and fitted to a nonlinear tight-binding equation.[36] K_d was determined by using Origin 8.5 (OriginLab, Northampton, MA).

Kinetic analysis: Determination of enzyme activity was performed by using the reconstituted in vitro system described above. Reactions were performed with Etp1^{fd} (10 μM), Arh1 (1.5 μM), and CYP105A1 (0.5 μM) in a total reaction volume of 500 μL, and substrate concentrations were varied (0–40 μM for abietic acid, 0–100 μM for dehydroabietic acid and isopimaric acid). The reactions were stopped after 1 min (abietic acid) or 3 min (dehydroabietic acid and isopimaric acid). After extraction and dissolving of the dried residuum, samples were subjected to HPLC analysis. The amount of each product was determined from the relative peak area, and the respective activity was calculated. Each reaction was performed at least three times, and mean values were calculated. K_m and V_{max} were determined by plotting the product-formation rate against substrate concentration and fitting with a hyperbolic equation in Origin 8.5 software.

Whole-cell conversion in *B. megaterium*: *B. megaterium* MS941 cells were transformed with a vector containing CYP105A1 cDNA, and cultivated in complex medium (500 mL) as previously described.[13] Following a 24 h expression period, the cells were harvested by centrifugation (4000 *g*) and resuspended in potassium phosphate buffer (500 mL, 50 mM, pH 7.4). The substrate dissolved in ethanol was added (final concentration 0.2 mM). After 24 h, the culture was extracted twice with ethyl acetate. The combined organic phases were dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was dissolved in methanol/water (85:15, with acetic acid (0.1%)) and purified by HPLC with a Nucleodur 100–5 C18 column (250×8 mm, 3 mL min^{-1}; Macherey–Nagel) at 40 °C. The combined fractions containing the product were evaporated to dryness and analyzed by NMR spectroscopy.

Computational methods: Substrate docking experiments were performed on the basis of the crystal structure of CYP105A1 from *S. griseolus* (PDB ID: 2ZBX); the four missing residues (not resolved in the X-ray data) were modeled by using the SWISS-MODEL server as previously described.[13] Docking was performed with AUTODOCK (version 4.00)[26,27] after preparation of the ligands by using AutoDockTools (Windows version 1.5.2).[37] A total of 100 docking runs were carried out for each ligand. Default parameters were applied except for the mutation rate (increased to 0.05).

NMR measurements: NMR spectra were recorded in $CDCl_3$ with a DRX 500 NMR spectrometer (Bruker). All chemical shifts are relative to $CHCl_3$ at $\delta = 7.24$ (^{1}H NMR) or $CDCl_3$ at $\delta = 77.00$ (^{13}C NMR). The 2D NMR spectra were recorded as gs-HH-COSY, gs-NOESY, gs-HSQC, and gs-HMBC.

Conversion of abietic acid: The conversion of abietic acid with CYP105A1 led to the known 15-hydroxyabietic acid, which could easily be verified by comparison of its ^{1}H and ^{13}C NMR data (see below) with those from literature.[38] The detailed NMR data for 15-hydroxyabietic acid are: ^{1}H NMR (500 MHz, $CDCl_3$, 300 K): $\delta = 6.04$ (d, $J = 2.5$ Hz, H-14), 5.46 (br s, H-7), 2.30 (m, H-12a), 2.07 (m, H-5), 2.05 (m, H-12b), 1.94 (m, H-9), 1.90 (m, H-6a), 1.87 (m, H-6b), 1.87 (m, H-1a), 1.83 (m, H-11a), 1.78 (m, H-3a), 1.67 (m, H-3b), 1.58 (m, 2H; 2×H-2), 1.32 (s, 3H; 3×H-16), 1.30 (s, 3H; 3×H-17), 1.24 (s, 3H; 3×H-19), 1.18 (m, H-11b), 0.81 ppm (s, 3H; 3×H-20); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K): $\delta = 183.78$ (C18) 144.55 (C13), 135.04 (C8), 122.99 (C7), 122.54 (C14), 72.54 (C15), 50.65 (C9), 46.25 (C4), 44.83 (C5), 38.25 (C1), 37.19 (C3), 34.43 (C10), 28.72 (C16), 28.55 (C17), 25.75 (C6), 25.63 (C12), 22.56 (C11), 18.03 (C2), 16.75 (C19), 13.99 ppm (C20).

Conversion of dehydroabietic acid: As for abietic acid, the treatment of dehydroabietic acid with CYP105A1 led to the hydroxylation at C15 of the abietane skeleton. NMR data for the resulting 15-hydroxydehydroabietic acid could not be found in literature, but the data of its methyl ester were in good accordance with our data:[39] ^{1}H NMR (500 MHz, $CDCl_3$, 300 K): $\delta = 7.22$ (dd, $J = 8.5$, 2.0 Hz, H-12), 7.19 (d, $J = 8.5$ Hz, H-11), 7.14 (d, $J = 2.0$ Hz, H-14), 2.94 (m, H-7a), 2.87 (m, H-7b), 2.30 (m, H-1a), 2.22 (dd, $J = 12.5$, 2.0 Hz, H-5), 1.85 (m, H-6a), 1.80 (m, H-3a), 1.77 (m, H-2a), 1.73 (m, H-2b), 1.70 (m, H-3b), 1.54 (m, H-6b), 1.54 (s, 6H; 3×H-16 and 3×H-17), 1.50 (m, H-1b), 1.27 (s, 3H; 3×H-19), 1.20 ppm (s, 3H; 3×H-20); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K): $\delta = 183.95$ (C18), 147.82 (C13), 146.04 (C9), 134.74 (C8), 124.93 (C14), 124.14 (C11), 121.91 (C12), 72.34 (C15), 47.36 (C4), 44.59 (C5), 37.89 (C1), 36.70 (C3), 36.92 (C10), 31.63 (C16), 31.61 (C17), 30.11 (C7), 25.06 (C20), 21.71 (C6), 18.50 (C2), 16.26 ppm (C19).

Conversion of isopimaric acid: The conversion of isopimaric acid with CYP105A1 did not lead to the formation of an alcohol but an epoxide function. In contrast to the substrate the NMR data of the conversion product missed the signals for olefinic C15 and C16. Therefore C15 ($\delta = 60.74$ ppm) and C16 ($\delta = 43.50$ ppm) as well as H-15 ($\delta = 2.69$ ppm, dd, $J = 4.5$ and 3.0 Hz) and H-16 (2.65 and

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2.61 ppm, both dd, both $J=4.5$ and 3.0 Hz) revealed resonances of a mono-substituted epoxide. The resulting 15,16-epoxyisopimaric acid is not mentioned in literature. 2D NMR HHCOSY, HSQC, HMBS, and NOESY supported the structure. NMR data: ^{1}H NMR (500 MHz, $CDCl_3$, 300 K): $\delta=5.32$ (m, H-7), 2.69 (dd, $J=4.5$, 3.0 Hz, H-15), 2.65 (dd, $J=4.5$, 3.0 Hz, H-16a), 2.61 (dd, $J=4.5$, 3.0 Hz, H-16b), 1.99 (m, H-14a), 1.98 (m, H-6a), 1.91 (m, H-5), 1.85 (m, H-14b), 1.82 (m, H-1a), 1.76 (m, H-3a), 1.73 (m, H-9), 1.68 (m, H-6b), 1.65 (m, H-3b), 1.55 (m, H-11a), 1.53 (m, 2H; 2×H-2), 1.41 (m, H-12a), 1.34 (m, H-11b), 1.29 (m, H-12b), 1.25 (s, 3H; 3×H-19), 1.10 (m, H-1b), 0.87 (s, 3H; 3×H-20), 0.75 ppm (s, 3H; 3×H-17); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K): $\delta=183.95$ (C18), 134.79 (C8), 121.33 (C7), 60.74 (C15), 52.07 (C9), 46.22 (C4), 44.96 (C5), 43.50 (C16), 42.97 (C14) 38.80 (C1), 36.99 (C3), 35.03 (C10), 33.94 (C13), 31.98 (C12), 25.18 (C6), 19.45 (C11), 18.53 (C17), 17.91 (C2), 17.17 (C19), 15.28 (C20).

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Keywords: 15-hydroxyabietic acid · biocatalysis · cytochromes P450 · hydroxylation · terpenoids

[1] N. B. Jensen, M. Zagrobelny, K. Hjernø, C. E. Olsen, J. Houghton-Larsen, J. Borch, B. L. Møller, S. Bak, *Nat. Commun.* **2011**, *2*, 273.
[2] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128 – 145.
[3] K. S. Rabe, V. J. Gandubert, M. Spengler, M. Erkelenz, C. M. Niemeyer, *Anal. Bioanal. Chem.* **2008**, *392*, 1059 – 1073.
[4] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, 2nd ed., John Innes Foundation, Norwich, **2000**.
[5] S. D. Bentley, K. F. Chater, A.-M. Cerdeño-Tárraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby et al., *Nature* **2002**, *417*, 141 – 147.
[6] D. C. Lamb, H. Ikeda, D. R. Nelson, J. Ishikawa, T. Skaug, C. Jackson, S. Omura, M. R. Waterman, S. L. Kelly, *Biochem. Biophys. Res. Commun.* **2003**, *307*, 610 – 619.
[7] M. K. Julsing, S. Cornelissen, B. Bühler, A. Schmid, *Curr. Opin. Chem. Biol.* **2008**, *12*, 177 – 186.
[8] J.-W. Park, J.-K. Lee, T.-J. Kwon, D.-H. Yi, Y.-J. Kim, S.-H. Moon, H.-H. Suh, S.-M. Kang, Y.-I. Park, *Biotechnol. Lett.* **2003**, *25*, 1827 – 1831.
[9] J. A. Romesser, D. P. O'Keefe, *Biochem. Biophys. Res. Commun.* **1986**, *140*, 650 – 659.
[10] H. Sugimoto, R. Shinkyo, K. Hayashi, S. Yoneda, M. Yamada, M. Kamakura, S. Ikushiro, Y. Shiro, T. Sakaki, *Biochemistry* **2008**, *47*, 4017 – 4027.
[11] N. Sawada, T. Sakaki, S. Yoneda, T. Kusudo, R. Shinkyo, M. Ohta, K. Inouye, *Biochem. Biophys. Res. Commun.* **2004**, *320*, 156 – 164.
[12] A. Çelik, S. L. Flitsch, N. J. Turner, *Org. Biomol. Chem.* **2005**, *3*, 2930 – 2934.
[13] M. Kleser, F. Hannemann, M. Hutter, J. Zapp, R. Bernhardt, *J. Biotechnol.* **2012**, *157*, 405 – 412.
[14] B. Hamberger, T. Ohnishi, B. Hamberger, A. Séguin, J. Bohlmann, *Plant Physiol.* **2011**, *157*, 1677 – 1695.
[15] C. I. Keeling, J. Bohlmann, *Phytochemistry* **2006**, *67*, 2415 – 2423.
[16] H. Ohtsu, R. Tanak, Y. In, S. Matsunaga, H. Tokuda, H. Nishino, *Planta Med.* **2001**, *67*, 55 – 60.
[17] S. S. Feio, S. Franca, A. M. Silva, B. Gigante, J. C. Roseiro, M. J. M. Curto, *J. Appl. Microbiol.* **2002**, *93*, 765 – 771.
[18] B. Gigante, C. Santos, A. M. Silva, M. J. M. Curto, M. S. J. Nascimento, E. Pinto, M. Pedro, F. Cerqueira, M. M. Pinto, M. P. Duarte, A. Laires, J. Rueff, J. Gonçalves, M. I. Pegado, M. L. Valdeira, *Bioorg. Med. Chem.* **2003**, *11*, 1631 – 1638.
[19] A. T. Karlberg, K. Bohlinder, A. Boman, U. Hacksell, J. Hermansson, S. Jacobsson, J. L. Nilsson, *J. Pharm. Pharmacol.* **1988**, *40*, 42 – 47.
[20] J. M. Leach, A. N. Thakore, *Prog. Water Technol.* **1977**, *9*, 787 – 798.
[21] F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim. Biophys. Acta Gen. Subj.* **2007**, *1770*, 330 – 344.
[22] C. R. Jefcoate, *Methods Enzymol.* **1978**, *52*, 258 – 279.
[23] J. B. Schenkman, I. Jansson in *Methods in Molecular Biology, Vol. 107* (Eds: I. R. Phillips, E. A. Shephard), Humana Press, Totowa, **1998**, pp. 25 – 33.
[24] S. Bleif, F. Hannemann, M. Lisurek, J. P. von Kries, J. Zapp, M. Dietzen, I. Antes, R. Bernhardt, *ChemBioChem* **2011**, *12*, 576 – 582.
[25] K. Hayashi, H. Sugimoto, R. Shinkyo, M. Yamada, S. Ikeda, S. Ikushiro, M. Kamakura, Y. Shiro, T. Sakaki, *Biochemistry* **2008**, *47*, 11964 – 11972.
[26] R. Huey, G. M. Morris, A. J. Olson, D. S. Goodsell, *J. Comput. Chem.* **2007**, *28*, 1145 – 1152.
[27] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639 – 1662.
[28] V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2012**, *30*, 26 – 36.
[29] Y. Yasutake, N. Imoto, Y. Fujii, T. Fujii, A. Arisawa, T. Tamura, *Biochem. Biophys. Res. Commun.* **2007**, *361*, 876 – 882.
[30] A. M. Downs, J. E. Sansom, *Contact Dermatitis* **1999**, *41*, 305 – 310.
[31] B. Arreguy-San Miguel, M. Taran, P. Picard, B. Delmond, *Synth. Commun.* **1989**, *19*, 979 – 986.
[32] Y, Imaizumi, K. Sakamoto, A. Yamada, A. Hotta, S. Ohya, K. Muraki, M. Uchiyama, T. Ohwada, *Mol. Pharmacol.* **2002**, *62*, 836 – 846.
[33] T. Omura, R. Sato, *J. Biol. Chem.* **1964**, *239*, 2370 – 2378.
[34] K. M. Ewen, B. Schiffler, H. Uhlmann-Schiffler, R. Bernhardt, F. Hannemann, *FEMS Yeast Res.* **2008**, *8*, 432 – 441.
[35] B. Schiffler, M. Bureik, W. Reinle, E.-C. Müller, F. Hannemann, R. Bernhardt, *J. Inorg. Biochem.* **2004**, *98*, 1229 – 1237.
[36] J. W. Williams, J. F. Morrison, *Methods Enzymol.* **1979**, *63*, 437 – 467.
[37] M. F. Sanner, *J. Mol. Graphics Modell.* **1999**, *17*, 57 – 61.
[38] L. Haberkorn, V. Mutterer, E. Gimenez Arnau, J.-P. Lepoittevin, *Synlett* **2001**, *2001*, 1723 – 1726.
[39] K. Krohn, E. Budianto, U. Floerke, B. M. Hausen, *Liebigs Ann. Chem.* **1992**, 911 – 919.
[40] A. Abad, M. Arno, L. R. Domingo, R. J. Zaragoza, *Tetrahedron* **1985**, *41*, 4937 – 4940.

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