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Identification of CYP106A2 as a Regioselective Allylic Bacterial Diterpene Hydroxylase

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The cytochrome P450 monooxygenase CYP106A2 from *Bacillus megaterium* ATCC 13368 catalyzes hydroxylations of a variety of 3-oxo-Δ^4-steroids such as progesterone and deoxycorticosterone (DOC), mainly in the 15β-position. We combined a high-throughput screening and a rational approach for identifying new substrates of CYP106A2. The diterpene resin acid abietic acid was found to be a substrate and was docked into the active site of a CYP106A2 homology model to provide further inside into the structural basis of the regioselectivity of hydroxylation. The products of the hydroxylation reaction were analyzed by HPLC and the V_{max} and K_m values were calculated. The corresponding reaction products were analyzed by NMR spectroscopy and identified as 12α- and 12β-hydroxyabietic acid. CYP106A2 was therefore identified as the first reported bacterial cytochrome P450 diterpene hydroxylase. Furthermore, an effective whole-cell catalyst for the selective allylic 12α- and 12β-hydroxylation was applied to produce the hydroxylated products.

Introduction

Cytochromes P450 belong to a superfamily of heme thiolate-containing enzymes that are widespread in nature. They are involved in the degradation of xenobiotics and in several other biological processes such as the biosynthesis of lipids and steroids. Their action of insertion of molecular oxygen into non-activated hydrocarbons is unique. Besides this, they are able to catalyze a variety of different reactions, such as N-oxidation, N-, O-, and S-dealkylation, sulfoxidation, epoxidation, peroxidation, deamination, desulfuration, dehalogenation, N-oxide reduction, and even C–C bond cleavage.[1,2] In classical organic chemistry, the hydroxylation of non-activated carbon bonds is mainly achieved through radical reactions, which are often not selective enough.[3] The use of microorganisms or their isolated enzymes represents an environment-friendly and efficient alternative, especially with regard to their ability to perform reactions in a highly regio- and stereoselective manner.[4] A long-sought and challenging goal in cytochrome P450 research is to capitalize on the selective introduction of molecular oxygen into double bonds, at allylic positions, and also into non-activated C–H bonds for the production of fine chemicals and pharmaceuticals that are difficult to prepare by traditional organic synthesis.[5] Prokaryotic P450s are soluble and therefore easy to purify and to handle, unlike their eukaryotic counterparts, which are, with some exceptions, integral membrane proteins.[3] However, as external monooxygenases, prokaryotic P450s in general need to interact with their soluble electron donor partners to display functional activity.[6,7] The prokaryotic cytochrome P450 CYP106A2 from *Bacillus megaterium* ATCC 13368 is one of only a few cloned bacterial steroid hydroxylases. It hydroxylates 3-oxo-Δ^4-steroids such as testosterone and progesterone mainly in the β orientation at the 15-position.[8,9,10] Additionally, the production of 6β-,[11] 9α-, and 11α-hydroxyprogesterone has been reported.[12] These hydroxylation products or their derivatives are known as pharmaceuticals or useful precursors in the synthesis of anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational, and antitumor compounds. The biological function of CYP106A2 in *Bacillus* is still unknown and its natural electron transfer partners—a NADPH-dependent flavoprotein (megaredoxin reductase) and an iron-sulfur protein (megaredoxin)—have never been cloned. The transfer of electrons can, however, be achieved with different electron donor proteins.[11,13,14] In order to enlarge the substrate spectrum of this unique P450, with its capacity for selective hydroxylation of complex steroidal molecules, to other compounds, we decided to apply a high-throughput substrate screening for this enzyme.

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Results and Discussion

High-throughput substrate screening and binding affinities

For the development of a rational screening method, we utilized the fact that the binding of an organic molecule in the active site of a cytochrome P450 leads to characteristic changes in the cytochrome P450 UV absorption spectrum. In the case of substrate binding, in many cases a so-called type I spectral shift is induced. This is due to the replacement of an axial water molecule from the heme iron coordination sphere by the substrate, which results in a transition of the heme iron from the hexacoordinated low-spin state to the pentacoordinated high-spin state,[15,16] leading to a maximum at ~390 nm and a minimum at ~420 nm. The concentration dependence of the spectral change facilitates the determination of the substrate binding affinity to the enzyme.[17]

We used these enzyme properties to discover substrates of CYP106A2 by means of a high-throughput screening (HTS) of a molecule library consisting of 16671 synthetic organic compounds.[31] These compounds belong to the fragment library of ChemDiv.org, part of the ChemBioNet collection (for detailed structural information about the compounds see also http://www.chembionet.info). Dihydroquinopimaric acid was identified as the only hit (induction of a type I spectrum). It showed an equilibrium dissociation constant (K_D) of 52.93±6.03 μM (Figure 1).

Binding of a substrate is a necessary but not sufficient prerequisite for substrate conversion, so in the next step the catalytic activity of CYP106A2 towards dihydroquinopimaric acid had to be studied. For this, CYP106A2 was reconstituted with heterologous electron transfer partners—bovine adrenodoxin reductase (AdR) and bovine adrenodoxin, together with NADPH—and the substrate conversion was monitored. The reaction product was analyzed by reversed-phase HPLC. It could be demonstrated clearly that dihydroquinopimaric acid was converted by the CYP106A2-dependent system into one product (see the Supporting Information). Dihydroquinopimaric acid thus represents the first substrate of CYP106A2 to lack a 3-oxo-Δ^4 moiety,[18] prompting the assumption that this structure motif is not essential for substrate binding and conversion.

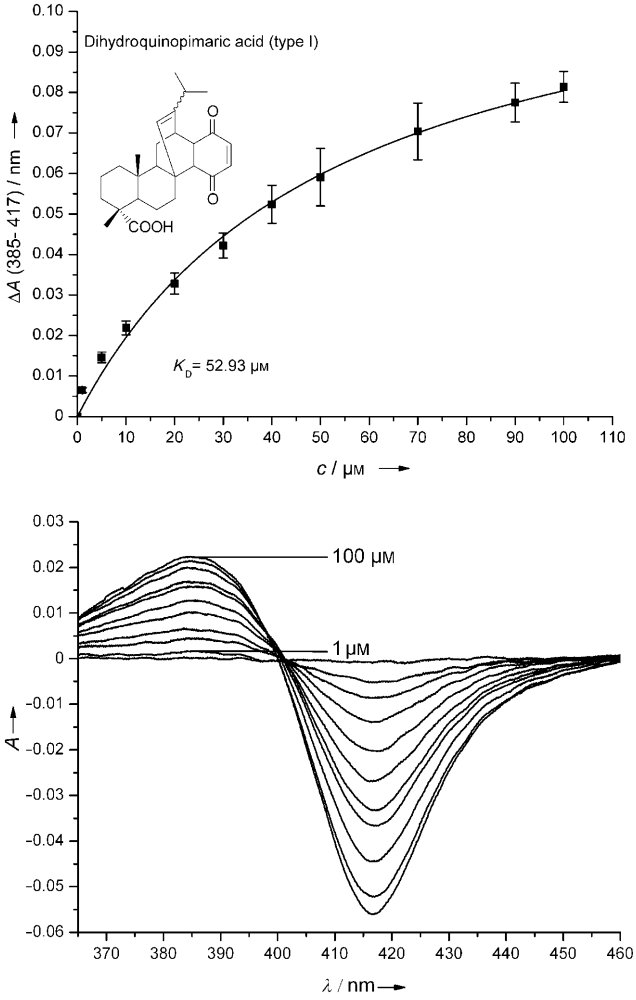


Figure 1. Type I spectral changes caused by binding of dihydroquinopimaric acid to CYP106A2. The concentration dependence of the spectral changes allows the binding affinities of the ligand to be estimated. The manual binding assay was conducted in tandem cuvettes containing CYP106A2 (10 μM) in potassium phosphate buffer (pH 7.4, 50 mM). Dihydroquinopimaric acid, dissolved in DMSO at a stock concentration of 5 mM, was added to give final concentrations ranging from 0–100 μM and monitored by recording of difference spectra.

Substrate prediction

To verify this assumption, abietic acid, a plant-derived diterpene resin acid obtained from colophony, which shares some structural similarities with dihydroquinopimaric acid, was selected as a potential further substrate. Abietic acid belongs to the chemically diverse group of plant terpenoids. This high diversity is probably a reflection of their versatile biological activities in nature, which have made them a widely used resource for traditional and modern human exploitation as, for example, pharmaceuticals, flavors, fragrances, food supplements in the form of vitamins, sweeteners, or pesticides.[19] Diterpene resin acids are important defense compounds of conifers against herbivores and pathogens. The biological activities of natural abietane acids have been reviewed in detail.[20] Abietic acid has shown antiallergic, anti-inflammatory, phytoalexin-like, and anticonvulsant activities and is therefore an optimal target for structural modifications with regard to possible enhancement of its biological activity.

Interestingly, abietic acid—like the steroidal substrates of CYP106A2[21]—did not induce a type I spectrum during binding. This property had been observed with CYP106A2 and the substrate deoxycorticosterone and afterwards was also noticed for the binding of substrates to other P450s.[36] Nevertheless, we demonstrated conversion of abietic acid in the presence of CYP106A2, indicating that our prediction of this diterpene as a potential substrate for CYP106A2 due to structural similarities between abietic acid and dihydroquinopimaric acid had been successful. The corresponding reaction products were analyzed by NMR spectroscopy and were identified as 12α- and 12β-hydroxyabietic acid (Figure 2, Scheme 1). The synthesis and NMR

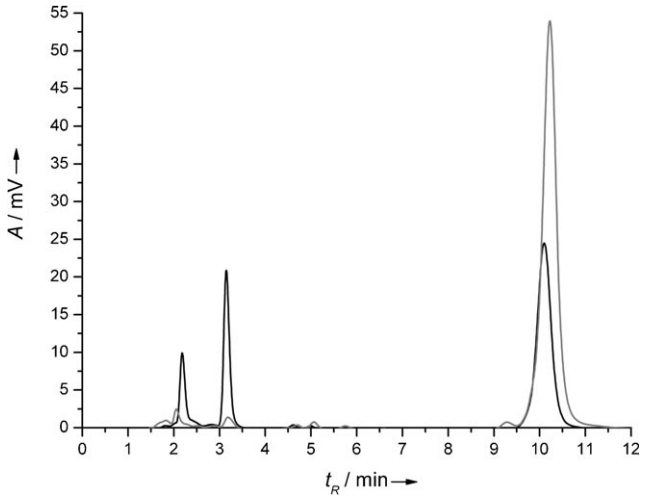


Figure 2. HPLC chromatogram of the in vitro conversion of abietic acid by CYP106A2. The reaction was carried out in a final volume of 500 μL in HEPES (pH 7.4, 50 mM) at 30 °C for 30 min in the presence of a NADPH regenerating system. The concentrations used were: CYP106A2 (0.5 μM), AdR (1 μM), and Adx_{4-108} (10 μM). 12β-Hydroxyabietic acid (t_R = 2.183 min), 12α-hydroxyabietic acid (t_R = 3.150 min), and abietic acid (t_R = 10.100 min) are shown.

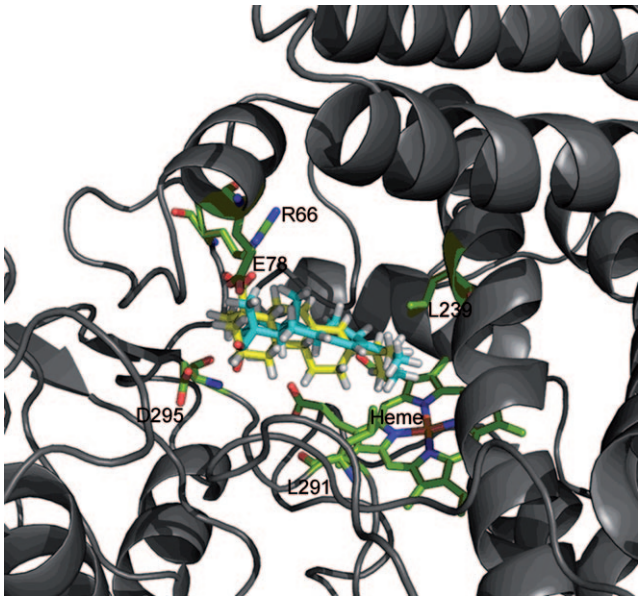
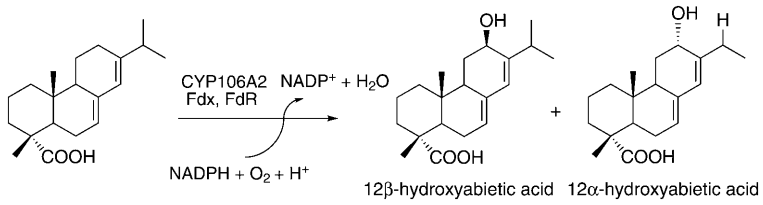


Figure 3. The active site of CYP106A2 containing abietic acid as substrate in the most favorable orientations for α- (yellow) and β- (cyan) hydroxylation of C12. The prosthetic heme group, the substrate, and the residues R66, E78, L239, L291, and D295 are drawn in stick representation.

analysis of the latter had not previously been published. The diastereomers were formed in the in vitro reaction in a ratio of 2:1 (α/β).



Scheme 1. CYP106A2-catalyzed hydroxylation of abietic acid at the 12-position.

Substrate docking

To gain further insights into the structural basis of the regioselectivity of hydroxylation, we used a homology model recently created in our group[9] to dock abietic acid into the active site with the aid of the program FlexX.[22,23] The best docking orientations for hydroxylation in the α (yellow) and β (cyan) positions (Figure 3) revealed possible hydrogen bonds between the acid group of the substrate and the side chains of the residues D295, E78, and R66. Additionally, the isopropyl group of abietic acid was stabilized through hydrophobic pincers, consisting of the leucine residues L291 and L239 (Figure 3). This binding mode position of the substrate, with C-12 in close proximity to the prosthetic heme group of the protein, is thus in very good accordance with the observed hydroxylation pattern. Furthermore, all of these residues (R66, E78, L239, L291, and D295) are located within the putative substrate recognition sites 1, 4, and 5,[24] which represent the most variable regions essential for substrate orientation and catalysis in cytochrome P450 structures. On comparing the orientations positioned most favorably for α- and β-hydroxylation, it can be observed that for hydroxylation in the β-position the ligand is rotated by approximately 100° relative to the ligand orientation for α-hydroxylation (Figure 3; see also the Supporting Information). The resulting orientation shows a different interaction pattern of its acid group (interaction with the residues E78 and D295) in relation to the orientation favorable for α-hydroxylation (interaction with the residues R66 and E78) and is energetically less favorable than the "α"-orientation (docking scores: −9.604 for α and −4.962 for β). Furthermore, the distance between C-12 and the heme group is slightly increased. This is in good agreement with the experimentally determined lower hydroxylation rate for 12β-hydroxyabietic acid in the in vitro reaction.

Although molecular docking experiments provide reliable results for ligand placement and allow the structural interpretation of experimental results, for a detailed, quantitative study of the differences in the energetics of substrate binding and conversion, simulations based on biophysical molecular dynamics or even quantum mechanical investigations would be necessary. However, the detailed analysis and discussion of such studies would increase the size of this publication considerably and go beyond the scope of the computational studies performed in this context: namely to provide a structural interpretation of the experimental data. However, the corresponding investigations would be worth performing as a study on their own.

Catalytic activity

The regioselective hydroxylation of the diterpene abietic acid by CYP106A2 was further analyzed by Michaelis–Menten kinet-

ics. The enzyme performed the conversion to 12α-hydroxyabietic acid with a V_{max} value of about 13.5 nmol product per min per nmol CYP106A2 and a K_M of about 107 μM, whereas the 12β-hydroxylation activity reached only 57% of the 12α-hydroxylation activity. The V_{max} value in that case was about 8.0 nmol 12β-hydroxyabietic acid per min per nmol CYP106A2 and the K_M value was 123 μM (Figure 4). The achieved K_M values are better than or comparable to the K_M values described for progesterone hydroxylation by this enzyme.[12] The presented reaction is extremely interesting, because the enzyme is capable of performing a regioselective, allylic hydroxylation in one step, which is difficult to accomplish by traditional organic synthesis.[25]

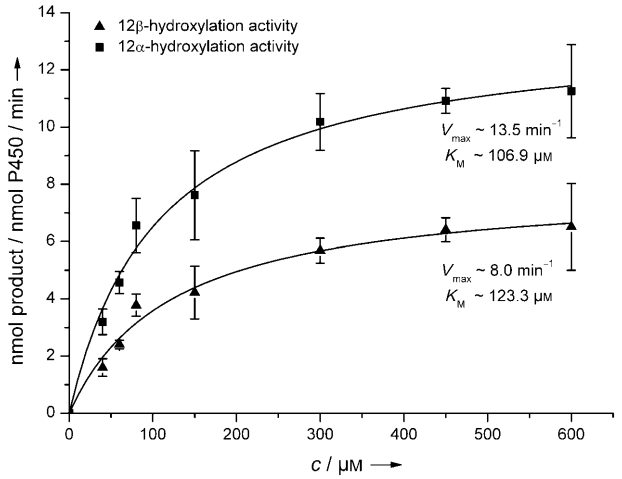


Figure 4. Initial rates [(nmol product) (nmol P450)$^{-1}$ min^{-1}] of abietic acid hydroxylation by CYP106A2. Reactions were carried out in a final volume of 250 μL in Hepes (pH 7.4, 50 mM) at 30 °C over 10 min in the presence of a NADPH-regenerating system. For the hydroxylation activity, CYP106A2 was reconstituted through the use of CYP106A2 (0.5 μM), AdR (1 μM), and Adx_{4-108} (10 μM) with differing concentrations of abietic acid (40–600 μM).

Whole-cell conversion

The general challenge for the application of P450s in organic synthesis and industrial processes is the regeneration of the cofactor NAD(P)H and the provision of redox partners.[26] To address this task we applied an *E. coli* whole-cell biocatalyst containing Adx and AdR as redox partners, which had successfully been utilized for the conversion of 11-deoxycorticosterone into 15β-hydroxy-11-deoxycorticosterone.[18] Surprisingly, the Gram-negative bacteria did not show any abietic acid conversion, which is probably due to inability of the hydrophobic acid to cross the outer membrane of the *E. coli* cells.

In a second attempt we therefore focused on the Gram-positive bacterium *Bacillus megaterium* ATCC 13368, carrying the endogenous CYP106A2 and its natural electron transfer partners. A knockout strain of *Bacillus megaterium* ATCC 13368 lacking the capacity to synthesize CYP106A2 served as a negative control.[13]

No conversion occurred with the knockout strain, indicating that the reaction was catalyzed in the whole-cell approach by CYP106A2 (Figure 5). Interestingly, 12β-hydroxyabietic acid was built up to a higher content than in the in vitro reaction. The in vivo reaction resulted in a 2:1 mixture of 12β- and 12α-hydroxyabietic acid. *Bacillus megaterium* ATCC 13368 was able to convert 0.2 mM abietic acid within 48 h in a culture volume of 1000 mL, yielding 38.2 mg of 12β-hydroxyabietic acid and 25.8 mg of 12α-hydroxyabietic acid. The HPLC elution profile shows two additional peaks with retention times lower than that of 12β-hydroxyabietic acid. These peaks were also present after chloroform extraction of the complex medium containing the *Bacillus megaterium* ATCC 13368 strain without addition of abietic acid and so are not due to a CYP106A2-dependent conversion of abietic acid. The two additional peaks can therefore be assigned to ingredients of the medium.

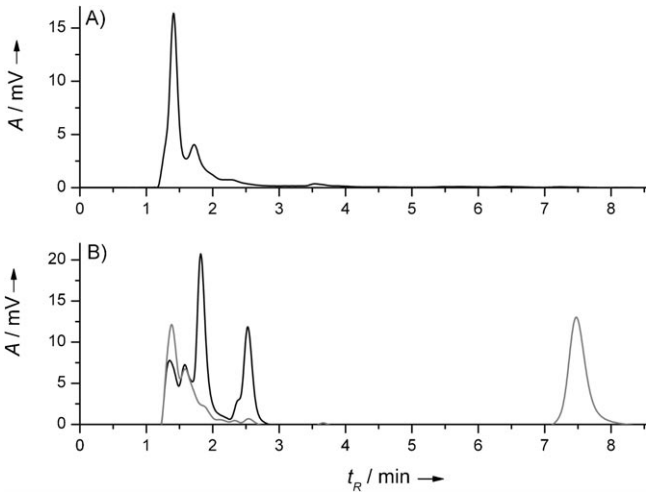


Figure 5. A) HPLC chromatogram (after chloroform extraction) of the complex medium containing the *Bacillus megaterium* ATCC 13368 strain without addition of abietic acid. Ingredients of the medium (t_R = 1.408, 1.717 min). B) HPLC chromatogram of the in vivo conversion of abietic acid by *Bacillus megaterium* ATCC 13368 (black) and the knockout strain (gray). Reactions were carried out in 1000 mL complex medium at 30 °C by vigorous shaking for 48 h. 12β-Hydroxyabietic acid (t_R = 1.817 min), 12α-hydroxyabietic acid (t_R = 2.525 min), and abietic acid (t_R = 7.475 min) are shown.

Conclusions

Our aim was to characterize new substrates for CYP106A2. We successfully identified dihydroquinopimaric acid as a hit (induction of a type I spectrum) with an equilibrium dissociation constant (K_D) of 52.93 ± 6.03 μM (Figure 1) by screening a molecule library consisting of 16671 synthetic organic compounds. This is the first substrate of CYP106A2 to lack a 3-oxo-Δ^4 moiety.[18] By subsequently choosing the structurally similar diterpene abietic acid for further experiments, we demonstrated that the 3-oxo-Δ^4- structure motif is not essential for binding and conversion and that CYP106A2 is able to hydroxylate a diterpene of the abietane type. Surprisingly, abietic acid did not induce a type I spectrum during binding, but an in vitro conversion of

abietic acid with CYP106A2 led to two products. These were analyzed by NMR and identified as 12α- and 12β-hydroxyabietic acid (Figure 2). The two products were formed in a ratio of 2:1.

To predict which residues might affect substrate binding, abietic acid was docked into a CYP106A2 homology model. The docked substrate displays different orientations in the binding pocket of CYP106A2 for α- and β-hydroxylation in C12. The most favorable orientation for α-hydroxylation shows an interaction of the acid group of abietic acid with the amino acids D295 and E78 of CYP106A2, whereas for β-hydroxylation the substrate is rotated by approximately 100° relative to the α-hydroxylation orientation and therefore shows an interaction with residues E78 and D295 of the protein. The β-orientation is energetically less favorable than the α-orientation, which is in good accordance with the observed in vitro hydroxylation pattern. Furthermore, the isopropyl group of the substrate seems to be stabilized through two leucine residues, L291 and L239 (Figure 3), with the C-12 of abietic acid being in an optimal hydroxylation position over the prosthetic heme group of the protein. For a quantitative study of the differences in the energetics of substrate binding and conversion further bioinformatic studies would be necessary, which might be interesting to investigate in the future.

In order to evaluate the catalytic activity of the regioselective hydroxylation, the K_M and V_{max} values of the abietic acid hydroxylation were determined. The 12β-hydroxylation activity reached only 57% of the 12α-hydroxylation activity (Figure 4), but the achieved K_m values were in the same range as described for progesterone hydroxylation by CYP106A2.

A long-sought and challenging goal for the application of P450s in the synthesis of chemicals and pharmaceuticals is the regeneration of the cofactor NAD(P)H and the need for interactions with redox partner(s). We therefore attempted to create a whole-cell biocatalyst for the regioselective hydroxylation of abietic acid. Interestingly, an *E. coli* whole-cell biocatalyst expressing CYP106A2 and its heterologous redox partners AdR and Adx, which had been successfully used for the conversion of 11-deoxycorticosterone, did not show any product formation after incubation with abietic acid. Probably the acid can not cross the outer membrane of the Gram-negative bacteria.

We therefore focused our further experiments on the Gram-positive bacterium *Bacillus megaterium* ATCC 13368, carrying the endogenous CYP106A2 and its natural electron transfer partners. This strain converted 0.2 mM of abietic acid into 38.2 mg of 12β-hydroxyabietic acid and 25.8 mg of 12α-hydroxyabietic acid. Surprisingly, the product distribution in the in vivo reaction was shifted towards the 12β-hydroxylated product (relative to the in vitro reaction, in which the 12α-hydroxylated product had dominated). The in vivo reaction resulted in a 2:1 mixture of 12β- and 12α-hydroxyabietic acids, whereas in the in vitro reaction a ratio of 1:2 for the 12β-and 12α-hydroxylated products occurred.

In summary, we have combined a high-throughput method and a rational approach for identifying new potential substrates of CYP106A2. Additionally, a whole-cell catalyst for the selective 12α- and 12β-hydroxylation of abietic acid was applied to produce the selectively hydroxylated products. CYP106A2 was identified as the first reported bacterial cytochrome P450 diterpene hydroxylase capable of carrying out a one-step regioselective allylic hydroxylation of a diterpene, abietic acid.

Experimental Section

Reagents and chemicals: Abietic acid was purchased from ACROS (Geel, Belgium). All other chemicals were from standard sources and were of highest purity available.

Microorganism and media: *Bacillus megaterium* ATCC 13368 and a knockout strain of *Bacillus megaterium* ATCC 13368 lacking the capacity to synthesize CYP106A2 were a kind gift from Dr. R. Rauschenbach (Bayer–Schering AG).[13] All experiments were carried out in a complex medium consisting of yeast extract (Difco, Detroit, MI, 24 g), glycerine (4 mL), soytone (Difco, 12 g), KH_2PO_4 (2.31 g), K_2HPO_4 (12.5 g), and distilled H_2O (1000 mL). *B. megaterium* was stored on nutrient agar (Difco) plates at 4 °C, which in the case of the knockout strain contained tetracycline (12.5 mg L^{-1}).

Enzyme expression and purification: Recombinant bovine adrenodoxin reductase and a truncated form of bovine adrenodoxin (adrenodoxin 4–108) were expressed and purified as described elsewhere.[27,28] The cDNA of CYP106A2 was a kind gift from Dr. R. Rauschenbach. Expression and purification of CYP106A2 were performed as described in the literature.[12,21] The protein concentration was calculated with use of $E_{414} = 9.8\ mM^{-1}\,cm^{-1}$ for adrenodoxin ,[29] $E_{450} = 11.3\ mM^{-1}\,cm^{-1}$ for adrenodoxin reductase,[30] and $E_{417} = 91\ mM^{-1}\,cm^{-1}$ for CYP106A2.[12]

Automated library screening: A library consisting of 16671 small molecules was screened.[31] This was done with the aid of a highly automated liquid handling workstation (Sciclone 3000, Caliper Lifesciences) and a microplate reader for absorbance scanning (Safire, Tecan) in 384-well clear flat-bottomed polystyrene microplates (3702, Corning) in a final volume of 50 μL. The CYP106A2 concentration was 10 μM in Tris·HCl (pH 7.5, 50 mM), NaCl (250 mM), glycerol (20%), Tween 20 (0.05%), and dithiothreitol (DTT; 1 mM). Ligand concentrations were 100 μM. Cholest-4-en-3-one (100 μM) was used as reference compound for the type I binding mode. The compound-dependent changes in the characteristic region of the absorption spectra were recorded (350–450 nm) and the corresponding difference spectra were calculated. Compounds exhibiting difference spectra that resembled the type I control were regarded as primary hits and were retested for concentration dependence in a validation screen.

Manual spectroscopic binding assay: The manual binding assay was conducted in tandem cuvettes containing CYP106A2 (10 μM) in potassium phosphate buffer (pH 7.4, 50 mM). Compounds dissolved in DMSO at a stock concentration of 5 mM were added to give final concentrations ranging from 0–100 μM, as described elsewhere,[32,33] followed by recording of difference spectra. Each measurement was performed a minimum of three times. The titration data were analyzed by plotting ΔA against c_0, where ΔA is the change in absorbance and c_0 the total concentration of the ligand, and the data were finally fitted by hyperbolic regression with the aid of Origin (OriginLab Corporation, Massachusetts, USA), for the determination of the equilibrium dissociation constant (K_D).

Modeling and docking: The modeling was performed as described elsewhere.[9] Molecular docking calculations were performed for

the substrate abietic acid. For the calculations, the FlexX docking program was used.[22,23]

In vitro conversion and enzyme activity: The in vitro conversion of potential substrates was carried out with a reconstituted system in a final volume of 500 μL at 30 °C for 30 min in HEPES buffer (pH 7.4, 50 mM). The reconstituted system contained adrenodoxin reductase (1 μM), adrenodoxin 4–108 (10 μM), CYP106A2 (0.5 μM), a NADPH-regenerating system [$MgCl_2$ (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 U), and NADPH (0.1 mM)], and the potential substrate (150 μM). The reaction was stopped and the compounds were extracted with chloroform (500 μL). After a second extraction with chloroform (500 μL), the chloroform phases, containing compounds/products were combined prior to solvent evaporation. The residue was dissolved in methanol (200 μL) and subjected to HPLC analysis. Enzyme activity with the abietic acid substrate was measured in a reconstituted system in a final volume of 250 μL at 30 °C for 10 min in HEPES buffer (pH 7.4, 50 mM). The reconstituted system contained adrenodoxin reductase (1 μM), adrenodoxin 4–108 (10 μM), CYP106A2 (0.5 μM), a NADPH-regenerating system [$MgCl_2$ (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 U), and NADPH (0.1 mM)], and abietic acid (40–600 μM). The reaction was stopped and the compounds were extracted with chloroform (250 μL). After a second extraction with chloroform (250 μL) the compound-containing chloroform phases were combined prior to solvent evaporation. The residue was dissolved in methanol (200 μL) and subjected to HPLC analysis. 11-Ketoboswellic acid (KBA, 5 mM stock, 3 μL) was used as internal standard. The relative amounts of the products were determined through the relative peak area of the internal standard and the corresponding activities were calculated. Each reaction was performed a minimum of three times. Data were fitted by hyperbolic regression with the aid of Origin (OriginLab Corporation, Massachusetts, USA).

HPLC analysis: HPLC experiments were carried out with a Jasco system consisting of a Pu-980 HPLC pump, an AS-950 sampler, a UV-975 UV/Vis detector, and an LG-980–02 gradient unit (Jasco, Gross-Umstadt, Germany). The mobile phases [methanol/water (0.1% TFA) 85:15 or methanol/water (0.1% TFA) 75:25] were degassed before use. A reversed-phase Waters Nova Pak Nukleosil C_{18} (4 μm, 3.9×150 mm) column (Waters, Milford, MA, USA) or a reversed-phase ec MN Nucleodor C18 (5 μm, 4.0×125 mm) column (Macherey–Nagel) was used for these studies. The flow rate was 1.0 mL min^{-1}. The compound solution (5 μL) was injected each time. The column temperature was maintained at 40 °C by use of a BFO-04 column thermostat (W.O. Electronics, Langenzersdorf, Austria). The absorbance was measured at 254 nm.

In vivo substrate conversion: The complex medium (1000 mL), supplemented if necessary with the appropriate amount of tetracycline, was inoculated with a single colony either of *Bacillus megaterium* ATCC 13368 or of the knockout strain of *Bacillus megaterium* ATCC 13368 and incubated at 250 rpm at 30 °C. After 72 h of incubation, abietic acid (0.2 mM) was added to the culture. After a 48 h incubation period, the cultures were extracted with chloroform. The organic phase was dried over anhydrous $MgSO_4$ and concentrated to dryness. The yellowish residue was chromatographed on a silica gel column with hexane/ethyl acetate (7:3) as an eluent, which resulted in homogeneous fractions. The fractions were evaporated to dryness and analyzed by NMR spectroscopy.

NMR measurements: All NMR experiments were performed with a Bruker DRX 500 NMR spectrometer. NMR spectra were recorded in $CDCl_3$. Chemical shifts are given in parts per million (ppm) on the δ scale relative to the solvent peaks $CHCl_3$ at $\delta_H = 7.27$ ppm and $CDCl_3$ at $\delta_C = 77.0$ ppm. ^{13}C multiplicities were determined by use of the DEPT-135 pulse sequence. All assignments of 12α- and 12β-hydroxyabietic acid were confirmed by 2D NMR ^1H,^1H COSY, HSQC, HMBC and NOESY spectra.

12α-Hydroxyabietic acid: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 0.83$ (s, 3H; H-20), 1.08 (d, $J = 7$ Hz, 3H; H-16), 1.11 (d, $J = 7$ Hz, 3H; H-17), 1.21 (m; H-1α), 1.27 (s, 3H; H-19), 1.34 (dd, $J = 13$, 3 Hz; H-11β), 1.62 (m, 2H; H-2α and H-2β), 1.69 (m; H-3β), 1.83 (m; H-3α), 1.86 (m; H-1β), 1.89 (ddd, $J = 13$, 3, 3 Hz; H-11α), 1.95 (m; H-6α), 2.12 (m; H-6β), 2.17 (dd, $J = 12$, 3.5 Hz; H-5α), 2.33 (ddd, $J = 13.5$, 3, 3 Hz; H-9α), 2.43 (sep, $J = 7$ Hz; H-15), 4.29 (dd, $J = 3$, 3 Hz; H-12β), 5.54 (ddd; $J = 5$, 2.5, 2.5 Hz; H-7), 5.85 ppm (brs; H-14); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 14.38$ (C-20), 16.67 (C-19), 17.98 (C-2), 21.72 (C-17), 22.33 (C-16), 25.83 (C-6), 30.55 (C-11), 32.57 (C-15), 34.00 (C-10), 37.20 (C-3), 37.97 (C-1), 43.53 (C-9), 44.91 (C-5), 46.21 (C-4), 66.43 (C-12), 124.31 (C-7), 125.72 (C-14), 134.36 (C-8), 143.94 (C-13), 183.60 ppm (C-18). 12α-Hydroxyabietic acid is a known oxidation product when levopimaric acid is dissolved in dilute base and treated with hypochlorous acid.[34] The above data are in good accordance with those from the literature.[34,35]

12β-Hydroxyabietic acid: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 0.85$ (s, 3H; H-20), 1.06 (d, $J = 7$ Hz, 3H; H-16), 1.08 (d, $J = 7$, 3H; H-17), 1.15 (m; H-1α), 1.27 (s, 3H; H-19), 1.27 (m; H-11β), 1.60 (m, 2H; H-2α and H-2β), 1.69 (m; H-3β), 1.79 (m; H-3α), 1.89 (m; H-1β), 1.90 (m;H-6α), 2.02 (m; H-5α), 2.05 (m; H-9α), 2.06 (m; H-6β), 2.13 (ddd, $J = 12$, 4.5, 3.5; H-11α), 2.72 (sep, $J = 7$ Hz; H-15), 4.44 (dd, $J = 10$, 4.5 Hz; H-12α), 5.50 (ddd; $J = 5$, 2.5, 2.5 Hz; H-7), 5.79 ppm (brs; H-14); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 14.10$ (C-20), 16.71 (C-19), 17.98 (C-2), 20.66 (C-17), 22.71 (C-16), 25.62 (C-6), 28.03 (C-15), 33.20 (C-11), 34.41(C-10), 37.17 (C-3), 38.15 (C-1), 44.22 (C-5), 46.16 (C-4), 48.45 (C-9), 69.52 (C-12), 122.29 (C-7), 123.52 (C-14), 134.79 (C-8), 147.23 (C-13), 184.19 ppm (C-18).

Abbreviations: AdR: adrenodoxin reductase. Adx: adrenodoxin. CYP: cytochrome P450. HEPES: [*N*-(2-hydroxyethyl)-piperazine]-*N*′-ethanesulfonic acid.

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