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An Efficient Route to Selective Bio-oxidation Catalysts: an Iterative Approach Comprising Modeling, Diversification, and Screening, Based on CYP102A1

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Perillyl alcohol is the terminal hydroxylation product of the cheap and readily available terpene, limonene. It has high potential as an anti-tumor substance, but is of limited availability. In principle, cytochrome P450 monooxygenases, such as the self-sufficient CYP102A1, are promising catalysts for the oxidation of limonene or other inert hydrocarbons. The wild-type enzyme converts (4*R*)-limonene to four different oxidation products; however, terminal hydroxylation at the allylic C7 is not observed. Here we describe a generic strategy to engineer this widely used enzyme to hydroxylate exclusively the exposed, but chemically less reactive, primary C7 in the presence of other reactive positions. The approach presented here turns CYP102A1 into a highly selective catalyst with a shifted product spectra by successive rounds of modeling, the design of small focused libraries, and screening. In the first round a minimal CYP102A1 mutant library was rationally designed. It contained variants with improved or strongly shifted regio-, stereo- and chemoselectivity, compared to wild-type. From this library the variant with the highest perillyl alcohol ratio was fine-tuned by two additional rounds of molecular modeling, diversification, and screening. In total only 29 variants needed to be screened to identify the triple mutant A264V/A238V/L437F that converts (4*R*)-limonene to perillyl alcohol with a selectivity of 97%. Focusing mutagenesis on a small number of relevant positions identified by computational approaches is the key for efficient screening for enzyme selectivity.

Animal studies suggest that perillyl alcohol might slow the growth of pancreatic,[1] mammary,[2] colon,[3] and liver tumors.[4] Therefore, perillyl alcohol research is sponsored by the National Cancer Institute (NCI), and the compound is undergoing phase II clinical trials. It can be obtained by the selective 7-hydroxylation of limonene, the cheap and readily available terpene that is the product of the condensation of two molecules of isoprene. The control of site-selectivity among similar C–H bonds is one of the key challenges in developing CH oxidation catalysts. The most difficult application in site-selective C–H bond functionalization is the selective oxidation of a C–H bond that is neither positioned adjacent to a directing group nor is inherently more reactive than alternate sites.[5] The selective 7-hydroxylation of limonene represents such a case, as both C7 and C10 are at terminal allylic positions, and there are five more-reactive positions (three allylic ring positions, and two double bonds for epoxidation). Cytochrome P450 monooxygenases (CYPs) are known to catalyze the oxidation of C–H bonds under environmentally friendly conditions by utilizing molecular oxygen.[6,7] The screening of microbial sources is widely applied to find selective enzyme catalysts. Previously, the extensive screening of 1800 isolates led to the identification of CYP153A11, an enzyme that is capable of the selective conversion of limonene to perillyl alcohol.[8]

Because of the high effort required for screening for selective enzymes from microbial sources, we have been working on a generic approach that turns a well characterized enzyme, the self-sufficient CYP102A1 from *Bacillus megaterium* (also known as P450-BM3), into a selective catalyst for the conversion of limonene to perillyl alcohol. The enzyme is a soluble fusion protein of the monooxygenase and a diflavin reductase,[9] and is relatively stable under process conditions.[10] Therefore, it is one of the most promising CYPs for application in preparative synthesis. The wild-type enzyme is a highly active fatty acid hydroxylase,[11] and has been shown to convert (4*R*)-limonene into a variety of oxidation products. However, perillyl alcohol is not among them.[12] Improving or changing enzyme properties (like selectivity) with the help of random mutant libraries typically also demands a large screening effort, because of the huge size of the combinatorial libraries and the lack of simple high throughput screening or selection assays. Different strategies to enrich mutant libraries and therefore reduce the screening effort have been reported, for example saturation mutagenesis at substrate-interacting residues deduced from the crystal structure of an enzyme–substrate complex.[13] However, the restricted availability of enzyme–substrate complexes clearly limits the applicability of this strategy. Another method is to focus libraries around the complete substrate-binding pocket;[14] this method benefits from relatively small and well defined substrate-binding pockets, as observed in lipases, esterases, and transaminases. However, applying these methods to CYP enzymes with their large, diverse, and flexible substrate binding cavities[15–17] involves again a huge screening effort. Hence, we developed a semi-rational approach that drastically decreases the screening effort. We took

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advantage of the fact that enzymes control selectivity by stabilizing the substrate in defined orientations that expose only selected positions of the substrate to the active site, and we focused mutagenesis on residues involved in selectivity control. We previously studied extensively enzyme–substrate interactions by molecular dynamics simulations in order to elucidate the molecular basis of selectivity control in CYP enzymes and mutants.[18,19] The results suggested that structural elements close to the active heme oxygen harbor residues that have a major influence on selectivity. The systematic comparison of this region in 31 CYP crystal structures and over 6300 sequences further showed that common selectivity-determining residues (hotspots) can be identified from the structures and sequences.[20] This study showed that hydrophobic residues are preferentially found at these substrate-interacting hotspot positions, while others (e.g., charged amino acids) are not found at all. Thus we constructed a minimal and highly enriched CYP102A1 mutant library by introducing five hydrophobic amino acids (alanine, valine, phenylalanine, leucine, and isoleucine) at two hotspot positions (87 and 328).[12] The library contained almost exclusively active mutants. Against a selection of four cyclic and acyclic terpenoid substrates including (4*R*)-limonene, eleven variants showed either a strong shift or improved regio- or stereoselectivity during oxidation of at least one substrate, as compared to wild-type CYP102A1. In a further study, the screening of this library with inert cyclic and acyclic alkanes showed variants with high selectivity for the 2-hydroxylation of octane.[21]

The screening of the minimal library with (4*R*)-limonene revealed a total of six different products, and (4*R*)-limonene-8,9-epoxide was identified as the major product.[12] However, the library also contained unselective variants that, unlike the wild-type enzyme, produced perillyl alcohol as a minor product. The work presented here constitutes an extension of the concept of the minimal library, and focuses on those variants that show perillyl alcohol formation. In order to increase selectivity for perillyl alcohol formation, the variant with the highest perillyl alcohol ratio was selected, and the molecular basis of selectivity of this variant was investigated by molecular dynamics simulations. Based on observed binding modes, additional amino acid positions involved in substrate binding in this complex were identified. These positions were addressed by site-directed mutagenesis with the aim of shaping the substrate binding cavity of CYP102A1 to promote selective exposure of C7 of (4*R*)-limonene to the activated heme oxygen, again by allowing substitutions with solely the hydrophobic amino acids alanine, valine, phenylalanine, leucine, and isoleucine. In summary, our approach combines the screening of our previously introduced minimal CYP102A1 mutant library, and subsequent fine tuning of the most successful variant by successive rounds of molecular modeling, mutant design, and screening, while keeping the numbers of generated and screened variants to a minimum.

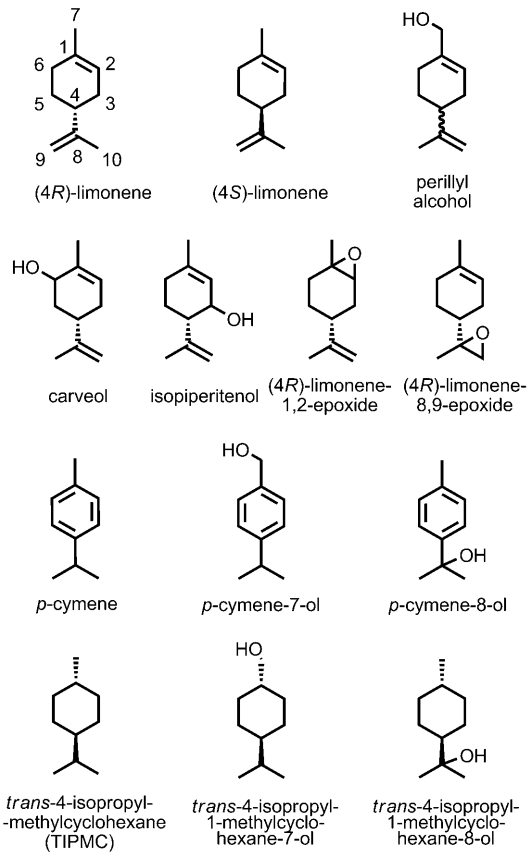
Results

Round 1: Identification of CYP102A1 variants with shifted product spectra

The minimal and highly enriched CYP102A1 mutant library (24 single and double mutants at positions 87 and 328) was screened for enzyme variants that had the ability to convert (4*R*)-limonene to perillyl alcohol. While conversion by the wild-type enzyme did not result in perillyl alcohol, variant A328V formed 27% of the desired product. In order to further improve the selectivity of this variant we studied the impact of the residues at hotspot positions 87 and 328 on substrate orientation. Two variants, F87I/A328I and F87I/A328V, showed similar selectivity; two further variants (F87L/A328L and A328L) resulted in 10–15% perillyl alcohol production, and were not considered for the next round of protein engineering.

Round 2: Improving selectivity of CYP102A1-A328V for perillyl alcohol formation

To reveal the molecular basis of the observed selectivity of variant A328V, the binding of (4*R*)-limonene to its active site was studied by molecular dynamics simulations. As oxidation was observed at both ends of the Y-shaped (4*R*)-limonene (the C8=C9 double bond, and C7; Scheme 1), molecular dynamics (MD)



Scheme 1. Limonene, p-cymene, TIPMC and their derivatives.

simulations were performed by starting with two initial orientations. In orientation I the methyl group harboring C7 was pointed towards the heme center, while in orientation II the isopropene group was pointed towards the heme center. In variant A328V, access to the activated oxygen is restricted by the bulkier amino acids phenylalanine and valine at hotspot positions 87 and 328, respectively. To allow flexibility, five MD simulations for each initial orientation were carried out, and the approach of the substrate to the activated heme oxygen was monitored. During the 80 ns simulation only C6, C7, C9, and C10 of the substrate were at a distance $\leq$4.0 Å from the heme (for 0.08 ns, 0.59 ns, 0.3 ns, and 3.14 ns, respectively; Figure S1 in the Supporting Information). In two simulations that started from orientation I, and one simulation that started from orientation II, the substrate ended up in a stable conformation close to the activated heme oxygen (Figure S2). The two stable conformations after starting from orientation I were very similar, as indicated by an all-atom RMSD of 0.7 Å for limonene after fitting both structures. 81% of the oxidation products observed for variant A328V derived from oxidation at the two ends of the (4*R*)-limonene molecule (Table 1; Scheme 1). These products can be explained by the two productive binding modes observed in the simulations. In both stable complexes the substrate was sandwiched between the two hotspot amino acids (87, 328) and a leucine residue (L437, Figure 1). This result indicated that, in addition to the amino acids in positions 87 and 328, the amino acid at position 437 strongly influences the orientation of the substrate in variant A328V, and therefore the variant's selectivity.

Table 1. Selectivity of (4*R*)-limonene-converting mutants. Errors are $<$10%.

CYP102A1 variant	(4*R*)-limonene-1,2-epoxide [%]	(4*R*)-limonene-8,9-epoxide [%]	isopiperitenol [%]	carveol [%]	perillyl alcohol [%]
A328V	5	54	5	3	27
A328VL/437A	10	78	0	1	10
A328VL/437I	7	57	3	4	25
A328VL/437V	10	55	2	3	24
A328VL/437F	3	27	0	0	60
A328VL/437F/A264V	2	1	0	0	97

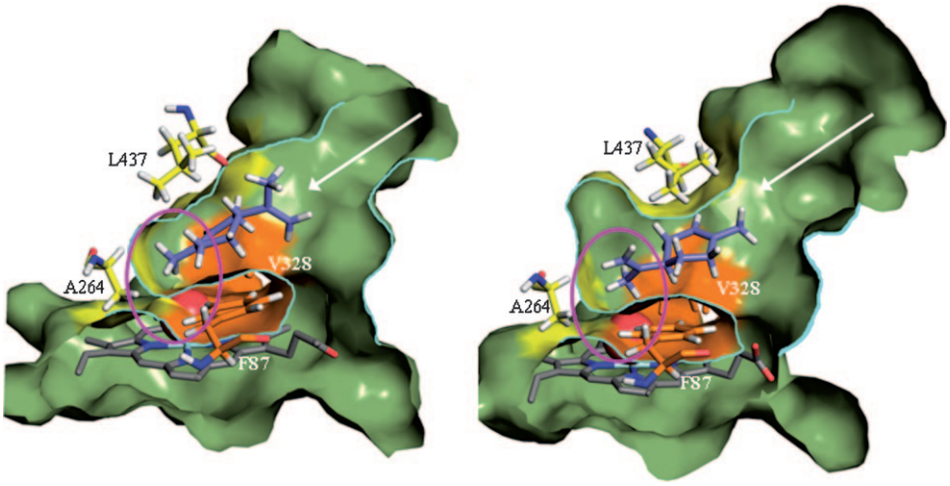


Figure 1. Snapshots of stable binding orientations of (4*R*)-limonene (blue/white) after 10 ns of simulation in variant A328V starting from A) orientation I and B) orientation II. The side chains of amino acids in hot spot positions 87 and 328 (orange) strongly restrict access to the activated heme oxygen (red sphere). In both simulations the substrate is sandwiched between F87, V328 and L437. The size of the amino acid in position 264 controls the available space in the immediate vicinity of the activated heme oxygen (magenta ellipse). White arrows indicate the entrance route of the substrate to the active site.

As it is rather difficult to predict the impact on substrate orientation of a particular amino acid substitution at position 437, a second minimal library was designed to identify the optimal amino acid at position 437. As substrate-interacting amino acids in cytochrome P450 monooxygenases are preferentially hydrophobic,[20] isoleucine, phenylalanine, alanine, and valine were chosen as substitutes for leucine at position 437, and these were introduced into variant A328V. The small double-mutant library was screened for perillyl alcohol formation. While alanine, valine, and isoleucine did not improve the selectivity of the enzyme for perillyl alcohol formation, phenylalanine at position 437 increased the selectivity to 60% (Table 1). In addition, double mutant A328V/L437F showed, almost exclusively, oxidation at the two ends of the Y-shaped (4*R*)-limonene molecule.

Round 3: Improving CYP102A1-A328V/L437F selectivity for perillyl alcohol formation

The double mutant in complex with (4*R*)-limonene was modeled by starting from orientations similar to those for A328V. The MD simulations revealed binding orientations of the substrate very similar to those observed for variant A328V (data not shown). The observed stable conformations identified an additional position with the potential to affect selectivity for perillyl alcohol formation. Position 264 confined the substrate binding cavity in the I-helix region, and restricted the available space in the immediate vicinity of the activated heme oxygen (Figure 1). As the two ends of the (4*R*)-limonene molecule clearly differ in their bulkiness, the size of the amino acid side chain in position 264 determines the size of the substrate moiety of the Y-shaped (4*R*)-limonene that can reach to the activated heme oxygen. The methyl group harboring C7 occu-

pies considerably less space than the branched isopropene moiety that contains the double-bond forming carbons (C8 and C9). This feature of the substrate molecule, and the binding orientation observed for variants A328V and A328V/L437F reveal a strategy for how to prevent 8,9-epoxide formation and to increase the selectivity for 7-hydroxylation: the size of the pocket in the direct vicinity of the heme centre can be reduced by substituting A264 with the slightly more bulky valine. While a smaller pocket would be expected to hinder the approach of the more bulky isopropene moiety to the activated heme oxygen, the smaller methyl group would be expected to remain able to reach the activated heme oxygen and therefore to be selectively oxidized. As the size of the side-chain at position 264 is limited (because of the proximity to the heme center), larger hydrophobic residues were not tested at this position. As expected, the substitution of A264 with valine in variant A328V/L437F strongly increased selectivity for the 7-hydroxylation of (4*R*)-limonene to 97% (Figure S3). The product formation rate for perillyl alcohol with purified variant A264V/A328V/L437F within the first minute was $72\pm8\,\mu mol\,min^{-1}\,\mu mol^{-1}$ P450, and $37\pm6\%$ of the substrate was converted to perillyl alcohol after two hours.

Selectivity towards molecules of similar shape

To determine the impact of the three mutations on regioselectivity towards molecules of similar shape but with different intrinsic reactivity, (4*S*)-limonene, *p*-cymene, and *trans*-4-isopropyl-1-methylcyclohexane (Scheme 1) were converted by variant A264V/A328V/L437F.

The (*S*)-enantiomer of limonene was preferentially hydroxylated at C7 by the triple mutant, though with a slightly lower selectivity (87%), as compared to (4*R*)-limonene.

In contrast to (4*R*)-limonene, *p*-cymene contains a benzyl ring and is therefore entirely planar. The reactivity of C7 in *p*-cymene was comparable to that of C7 in (4*R*)-limonene. In contrast to the wild-type enzyme, which forms preferentially *p*-cymene-8-ol (75%) and only small amounts of the 7-hydroxy product (<10%), mutant A264V/A328V/L437F converted *p*-cymene-like (4*R*)-limonene with high regioselectivity for C7 (99%) to *p*-cymene-7-ol.

In contrast to (4*R*)-limonene and *p*-cymene, *trans*-4-isopropyl-1-methylcyclohexane (TIPMC) is not planar and contains no double bonds. Therefore the molecule is more bulky and less reactive, especially at C7. The highly unselective wild type enzyme hydroxylates TIPMC preferentially at ring positions (and a minor product derived from hydroxylation at C8), however, formation of the 7-hydroxy product was not observed. In variant A328V a strong elevation of the 8-hydroxy product at the expense of the ring-hydroxylation was observed, and this variant also showed hydroxylation at the non-activated position C7 to form TIPMC-7-ol (7%). The substitution of leucine by phenylalanine at position 437 increased the ratio for the 7-hydroxy product to 27%. However, conversion of this substrate by the triple mutant A264V/A328V/L437F was not observed, as access to the heme oxygen was too narrow. This clearly shows that even non-activated terminal carbons are oxidized by our mutants, and that the positions that are controlling selectivity for (4*R*)-limonene are also driving selectivity towards TIPMC. The lower selectivity for the terminal hydroxylation for TIPMC in comparison to (4*R*)-limonene and *p*-cymene is probably attributable to absence of the planar character of the hexane ring.

Discussion

Cytochrome P450 monooxygenases catalyze oxidation reactions at physiological conditions by using molecular oxygen. In contrast to conventional oxidation chemistry, this constitutes an environmentally friendly alternative. In addition, there is an increased demand for biologically produced flavorings and aromatic compounds. The latter are often selective oxidation products of terpenes and terpenoids—both common CYP102A1 substrates. The fatty acid hydroxylase CYP102A1 is one of the most promising monooxygenases for application in preparative synthesis.[22] Various mutations (often in the substrate entrance region) have been described that widen the substrate scope toward more bulky molecules[23,24] However, for many of the substrates the wild-type enzyme (or variants with extended substrate spectra) show low selectivity, or the desired product is not obtained. Thus, an efficient strategy to design an enzyme with desired selectivity is needed.

Directed evolution is a powerful method to change and improve enzyme properties such as activity, stability and selectivity.[25] However, this approach relies on effective screening assays, because a large number of variants have to be generated and screened. This is straightforward with assays for activity or stability. However, if directed evolution is applied to improve regio-, chemo-, or stereoselectivity, the reaction products have to be quantitatively analyzed. Even though high-throughput screening methods for selectivity on the basis of GC analysis have been developed,[26] increasing efforts have been undertaken to limit the size of mutant libraries by focusing mutagenesis on potential substrate-interacting residues, selected by examination of the crystal structure information.[13,14] However, because of the large and diverse substrate binding cavity of CYP enzymes,[15–17] the number of potential substrate-interacting residues is of the order of ten; thus a combinatorial library would be too large to be screened. However, by an integrated approach of sequence analysis, molecular modeling, and screening of focused libraries, we drastically reduced the size of mutant library to be screened. After only three rounds of modeling, design, and screening we were able to extend the product spectra of CYP102A1 to include perillyl alcohol (round 1: screening of the previously designed minimal CYP102A1 library). Subsequently, we identified two additional mutations at position 437 (round 2) and position 264 (round 3), where amino acid substitutions increased the selectivity for the new product up to 97%. This is among the highest regioselectivities for terminal hydroxylation ever reported for the comprehensively investigated CYP102A1 enzyme.[27] With our approach, the screening of only 29 variants was sufficient to turn a generally unselective enzyme (which does not show terminal hydroxylation activity) into a catalyst that

equals CYP153A11, a specialized enzyme for terminal hydroxylation.[28]

The initial activity measured for perillyl alcohol formation by the most selective variant is in the same range as that observed for CYP153A11. Nevertheless, there is room for improvement of activity. As the substrate entrance to the active site cavity has a strong influence on activity, we expect that mutations in this region (far away from the heme) could improve activity drastically without interfering with selectivity. As an alternative, directed evolution could be applied to improve overall stability, solubility, and activity, as was shown previously for CYP102A1 to improve its activity toward limonene and other compounds.[29]

The conversion of molecules of similar shape but with different intrinsic reactivity confirmed our observations from modeling: the orientation of the substrate close to the activated heme oxygen is a major determinant of regio- and chemoselectivity. Because of this shape selectivity, nonactivated carbon atoms are oxidized even in the presence of other reactive atoms. This is in agreement with previous reports that showed that CYP102A1 can be engineered for hydroxylation at the nonactivated terminal positions of alkanes.[30]

As mutants of CYP102A1 have been shown to accept a wide range of substrates,[31] and the minimal library was highly enriched for selective catalysts towards cyclic and acyclic alkanes,[21] we are confident that our approach is generic and can be successfully applied to develop highly selective oxidation catalysts for a wide range of substrates.

Experimental Section

Yeast extract and tryptone/peptone from caseine were purchased from Roth (Karlsruhe, Germany). NADPH tetrasodium salt was obtained from Codexis (Jülich, Germany). *Trans*-4-isopropyl-1-methylcyclohexane was purchased from Chemos (Regenstauf, Germany). All other chemicals used in this work were purchased from Fluka or Sigma and were of analytical grade or higher.

Mutant expression: Wild type CYP102A1 and its mutants were heterologously expressed from pET22b and pET28a+ vectors in *E. coli* as reported previously.[32] For the introduction of site directed mutations, the QuikChange site-directed mutagenesis kit from Stratagene was used.

Enzyme purification: Purification was done by metal-affinity chromatography by using Ni-NTA matrices from Qiagen. Protein lysates, filtered through a 1–2 μm filter, were applied to a 5 mL bed volume column that was pre-equilibrated with purification buffer [potassium phosphate (50 mM, pH 7.5), KCl (300 mM), imidazole (20 mM), and PMSF (0.1 mM)]. Nonspecifically bound proteins were washed from the column with four column volumes of purification buffer containing 80 mM imidazole. The bound protein was eluted with purification buffer containing imidazole (200 mM). Purified sample was dialyzed overnight against potassium phosphate buffer [2 L, 50 mM, pH 7.5, containing PMSF (0.1 mM)], and frozen at −30 °C until use.

Enzyme activity measurements: CO-difference spectra measurements with an extinction coefficient of 91 $mM^{-1} cm^{-1}$ were used to determine CYP concentrations as described elsewhere.[33] CYP activity was determined by measuring product formation over time. The final reaction mixture (1 mL) contained Tris-HCl (50 mM, pH 7.5), < 2% (*v/v*) DMSO, substrate (0.5 mM), CYP enzyme (0.5 μM), a cofactor (NADPH) recycling system (5 U glucose-6-phosphate dehydrogenase) and β-D-glucose-6-phosphate Na salt (100 mM). The reaction was started by adding NADPH (100 μL, 2 mM). The mixture was left for 1, 5, 10, 20, 30, 60, and 120 min at 30 °C with gentle mixing, and the reaction was terminated by adding HCl (20 μL, 37%) prior to extraction.

GC-MS analysis: Diethyl ether was used to extract the aqueous reaction mixture twice. The organic phase was dried over magnesium sulfate. Analysis of the reaction products was performed on a Shimadzu QP2010 GC/MS with EI-ionization; the GC was equipped with a FS-supreme-5 capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 μm). For the analysis of (4*R*)-limonene, (4*S*)-limonene, *p*-cymene, *trans*-4-isopropyl-1-methylcyclohexane, and their oxidation products, the GC was programmed as follows: 80 °C, 2 min. iso; 5 °C min^{-1} to 180 °C; 30 °C min^{-1} to 250 °C; injector temperature 250 °C.

The oxidation products, carveol, (4*R*)-limonene-8,9-epoxide, and perillyl alcohol were identified by comparison to authentic samples. (4*R*)-limonene-1,2-epoxide, *p*-cymene-7-ol, *p*-cymene-8-ol, *trans*-4-isopropyl-1-methylcyclohexane-7-ol ,and *trans*-4-isopropyl-1-methylcyclohexane-8-ol were identified by comparison of their characteristic mass fragmentation patterns in the NIST mass spectrometry database.[34] Isopiperitenol was identified by comparison of the mass spectra to literature data.[35] Regioselectivity was determined from gas chromatograms by integrating and comparing the product peaks.

For quantitative GC analysis the FID response was calibrated for perillyl alcohol. Mixtures of potassium phosphate buffer [50 mM, pH 7.5, containing perillyl alcohol final concentration: 30–500 μM)] and nerol (100 μM) as an internal standard were extracted with diethyl ether. The ratio of the area corresponding to the perillyl alcohol to that of the internal standard was plotted against perillyl alcohol concentration to give a straight line.

Molecular modeling

Molecular dynamics simulations: Molecular dynamics (MD) simulations were carried out to study how (4*R*)-limonene is accommodated in the active site cavity of CYP102A1 variants A328V and A328V/L437F. The mutations A328V and L437F were introduced into the structure of CYP102A1 (PDB ID: 1BU7, chain A) with the Pymol 0.99 program.[36] The (4*R*)-limonene molecule was manually placed in the active site cavity. The complexes were simulated in explicit water by using the Amber 9 molecular dynamics simulation program.[37] The system was coupled to an external pressure and temperature bath.[38] The Amber force field ff03 was used, and the partial charges of (4*R*)-limonene and the heme group including the activated oxygen were assigned by ab initio calculations and restrained electrostatic potential (RESP) fitting as described previously.[19] During the first 600 ps the protein was restrained by a harmonic potential, while the substrate remained free. The restraints were gradiently decreased, and the system was finally simulated unrestrained for 10 ns. The systems were equilibrated after 1.4 ns, and the last 8 ns of each simulation was used for analysis.

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