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Chemo-, Regio-, and Stereoselective Oxidation of the Monocyclic Diterpenoid β-Cembrenediol by P450 BM3

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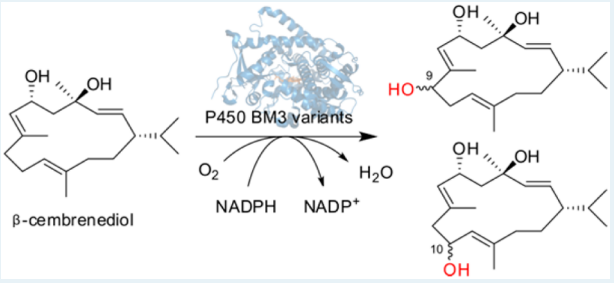
Supporting Information

ABSTRACT: Late-stage oxyfunctionalization of terpenoid scaffolds has been recognized as a powerful tool in the synthesis of complex molecules such as natural products to achieve their efficient diversification. Selective C−H oxidation of such hydrocarbon scaffolds remains challenging for chemical catalysts because of their insufficient regio- and stereoselectivity. To achieve this goal, cytochrome P450 monooxygenases are often used as biocatalysts. Here, we demonstrate the successful P450-catalyzed chemo-, regio-, and stereoselective oxidation of the tobacco cembranoid β-cembrenediol. This 14-membered macrocycle possesses a broad range of biological activities including antitumor-promoting and neuroprotective effects and carries seven potential sites for allylic hydroxylation as well as three epoxidation sites. On the basis of first-sphere active site mutagenesis, we generated in a few rounds a P450 BM3 minimal library and screened for β-cembrenediol oxidation activity. Several P450 BM3 variants were evolved, enabling the regioselective hydroxylation of the neighboring positions C-9 (100% regioselectivity and a diastereomeric ratio of 89:11 in the case of the F87A/I263L mutant) and C-10 (97% regioselectivity and a diastereomeric ratio of 74:26 in the case of the L75A/V78A/F87G mutant) of β-cembrenediol.



KEYWORDS: *enzyme catalysis, allylic hydroxylation, macrocyclic diterpenoids, cytochrome P450, regioselectivity, stereoselectivity*

INTRODUCTION

Terpenoids are a ubiquitous group of natural compounds with a large variety of complex molecular architectures. Terpene biosynthesis begins with the addition of isopentenyl pyrophosphate to dimethylallyl pyrophosphate and proceeds via the head-to-head or head-to-tail coupling of isoprene units to form linear terpenes. The natural diversity of terpenes is introduced in the next steps via cyclizations and rearrangements catalyzed by cyclases.[1] Further chemical modifications are caused by late-stage oxidations of C−H bonds in skeletal structures. Organic chemists have achieved significant progress in the area of polyene cyclization. However, selective oxidation of complex terpene and terpenoid scaffolds remains particularly difficult by using conventional chemical means.[2,3]

Oxygenases provide an enzymatic alternative to C−H bond oxidation. In nature, cytochrome P450 monooxygenases (CYPs) catalyze oxyfunctionalization of a vast variety of organic molecules in the presence of molecular oxygen and the nicotine amide cofactors NADH or NADPH.[4] Numerous native and engineered P450 enzymes have been investigated and explored for their synthetic use. Among the most studied P450 enzymes for protein engineering is P450 BM3 (CYP102A1) from *Bacillus megaterium*.[5−7] P450 BM3 is a cytosolic enzyme that possesses high activity due to its catalytically self-sufficient organization with the electron transferring reductase domain fused to the heme monooxygenase domain.[8] Engineered P450 BM3 variants with substitutions in the substrate binding pocket, introduced by using semirational approaches, were reported to induce high regio- and stereoselectivity for terpenes and terpenoids. For example, a triple P450 BM3 mutant oxidized the monoterpene (*R*)-limonene to form 97% perillyl alcohol,[9] while a double P450 BM3 mutant can oxidize the sesquiterepene (+)-valencene to a mixture of nootkatol and (+)-nootkatone with 95% regioselectivity for C-2 position.[10] Selective P450 BM3 variants have also been reported for more complex molecules. Two mutants were developed for C-7 hydroxylation of the sesquiterpene lactone artemisinin showing 100% regioselectivity: a 16-fold mutant with absolute (*S*)-stereoselectivity and a 17-fold mutant with absolute (*R*)-stereoselectivity for this position.[11] Several P450 BM3 variants have been evolved for highly regioselective hydroxylation of the sesquiterpene lactone parthenolide, either for C-9 (68%) or for C-14 (53%) positions.[12] In the area of steroid hydroxylation, three different P450 BM3 variants with eight mutations were found to oxidize testosterone to form either 16β-hydroxytestosterone (84%), 2β-hydroxytestosterone (100%), or 15β-hydroxytestosterone (57%).[13] Reetz and colleagues engineered P450 BM3 variants

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with high regio- and stereoselectivity for testosterone oxidation using the iterative combinatorial active-site saturation test. Although the parent single mutant produces a mixture of 2β- and 15β-hydroxytestosterone, the best variant with five mutations produced up to 96% of 15β-hydroxytestosterone.[14]

Similar to steroid oxidation, selective oxyfunctionalization of macrocyclic cembranoids is challenging, due to the multitude of potential oxidation sites. Compared to steroids, cembranoids possess several double bonds thus providing several sites for potential allylic hydroxylation as well as for potential epoxidation reactions. Cembranoids constitute a large group of macrocyclic diterpenoids which differ in type, position, and stereochemistry of their oxygen function. Their representatives are mainly found in corals and plants. Physiological functions of individual cembranoids have not been investigated yet in detail, but they have been suggested to be involved in sensing of and interaction with an organism's environment. Many of the isolated cembranoids demonstrate antitumor and cytotoxic activities.[15−17]

β-Cembrenediol ((1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol) (**1**) is a monocyclic diterpenoid that consists of a 14-membered macrocyclic ring. This ring bears an isopropyl group at C-1 and three methyl groups at C-4, C-8, and C-12 and is therefore assigned to the class of cembranoids.[18] Its structure and absolute stereochemistry were determined by X-ray crystallography in 1975.[19] Compound (**1**) was isolated from the plant *Nicotiana tabacum* as a component of the cuticular wax of the leaves and flowers (**1**: 0.005% of dry leaf).[20] Along with its (4*S*)-epimer α-cembrenediol, β-cembrenediol (**1**) belongs to the most abundant cembranoids and key flavor ingredients in tobacco.[17,21]

A broad range of biological effects have been reported for tobacco cembranoids. In 1985, β-cembrenediol (**1**) and its stereoisomer α-cembrenediol were described as anticancer agents. They inhibit tumor promotion via the early antigen of Epstein−Barr virus in lymphoblastoid Raji cells, which is induced by 12-*O*-tetradecanoylphorbol-13-acetate (**1**: IC_{50} = 22 μM).[22,23] An anti-inflammatory effect higher than that of acetylsalicylic acid was attributed to β-cembrenediol (**1**), which is presumably due to an inhibition of the prostaglandin synthesis (**1**: IC_{50} = 0.39 mM).[24] A behavioral effect could be shown for rats and planarian flatworms after sensitization to nicotine, which opened a potential application in the treatment of nicotine addiction.[25−27] In 2001, β-cembrenediol (**1**) was described as a noncompetitive inhibitor of nicotinic acetylcholine receptors (nAChR). It inhibits an agonist-induced ion current of human $\alpha4\beta2$ neuronal AChRs (**1**: IC_{50} = 19 μM), of human $\alpha3\beta4$ ganglionic AChRs (**1**: IC_{50} = 2 μM) and of embryonic human $\alpha1\beta1\gamma\delta$ muscle AChR.[25] In addition, the potential use of **1** as a neuroprotective drug was shown. Several reports describe a protective effect of **1** against the excitotoxicity induced by *N*-methyl-D-aspartate in hippocampal slices, as well as against the neurotoxicity of organophosphates like paraoxon or diisopropylfluorophosphate.[28−33] A recent report describes a possible application of **1** in the treatment of HIV infection and HIV-associated neurocognitive disorders.[34]

Due to the biological effects of β-cembrenediol (**1**) and its potential as a pharmacologically relevant compound, structure−activity relationship studies have been performed.[23,24,30] In all studies, oxidative analogues were used containing additional oxyfunctionalizations like hydroxy or epoxy groups. The oxidation products of **1** were therefore targets of interest in several studies and were produced by biotransformations. The bacterial cultures of *Bacillus megaterium* NH5,[35,36] *Bacillus megaterium* MO31,[37] cultures of symbiotic marine bacteria like *Bacillus* sp. NC5, NK8, NK7,[38] the fungal culture *Mucor ramannianus* ATCC 9628,[39,40] the plant cell cultures *Tripterygium wilfordii*[41] and *Nicotiana sylvestris*,[42,43] and the cell culture of *Cunninghamella elegans* ATCC 7929[39] and *Cunninghamella* NRRL 5695[40] were used for the production of oxyfunctionalized derivatives of **1**.

The identification of a P450 gene specific for trichome glands in *Nicotiana tabacum* T.I. 1068 was reported in 2001. It could be shown that the function of CYP71D16, the protein encoded by this gene, is the hydroxylation of the cembrenediol precursor cembra-2,7,11-trien-4-ol at position C-6.[44−46] To the best of our knowledge, no P450 enzyme that is capable of further oxidations of cembrenediol has been identified to date.

We herein report P450 BM3 variants that enable regio-, chemo-, and stereoselective oxidation of the model substrate β-cembrenediol (**1**). This resulted in epoxidation as well as in hydroxylation products particularly at allylic positions. Using a straightforward knowledge-based protein-engineering approach, we empirically proved and rationalized the effect of several first-sphere positions on enzyme activity and selectivity toward this substrate. Although the wild-type enzyme led to less than 2% conversion of β-cembrenediol (**1**), the constructed active P450 BM3 variants converted up to 97% of **1**. The evolved P450 BM3 mutants were able to hydroxylate β-cembrenediol (**1**) at either the C-9 or C-10 position, or to catalyze the epoxidation of the C-7/C-8 double bond.

RESULTS AND DISCUSSION

β-Cembrenediol Oxidation by P450 BM3 Variants. We followed a step-by-step optimization of P450 BM3 first for activity toward β-cembrenediol (**1**) and second for chemo- and regioselectivity of the enzyme-catalyzed oxidations. Finally, we analyzed the stereoselectivity of the most regioselective mutants. Because wild-type P450 BM3 converted β-cembrenediol (**1**) only to less than 2%, we suggested that phenylalanine 87 hinders the access of **1** to the heme iron. Due to its location above the heme iron, F87 is the most frequently mutated residue.[7] The replacement of this central position by smaller amino acids generates additional space in the active site (Figure 1A−C) and thus enables the oxidation of sterically demanding substrates.[7] Besides controlling substrate accessibility for oxidation, the amino acid at position 87 has been shown to affect regio- and stereochemistry of P450 BM3-catalyzed reactions.[13,47,48]

Phenylalanine at position 87 was replaced by smaller hydrophobic amino acids. Although the insertion of leucine or valine at this position did not result in any improved conversion compared to the wild-type enzyme (Table S1), variants with smaller amino acid residues (alanine or glycine) led to higher product formation (Table 1). Whereas conversion by variant F87A was only 6% and gave two products, conversion by F87G was 59% and generated more than five products. F87A converted β-cembrenediol (**1**) to the products 9-hydroxy-β-cembrenetriol (**4a**) and 7,8-epoxy-β-cembrenediol (**5**) in a ratio of 78:22, thus addressing C-9 and the neighboring C-7/C-8 double bond, respectively (Scheme 1). Chemo- and regioselectivity was poor in the case of F87G, which produced both epimeric hydroxylation products at C-10 (**2a,b**), the product **5**, and the product **4a** in a ratio of 39:21:13:8. Additionally, 19% of other byproducts were formed by F87G,

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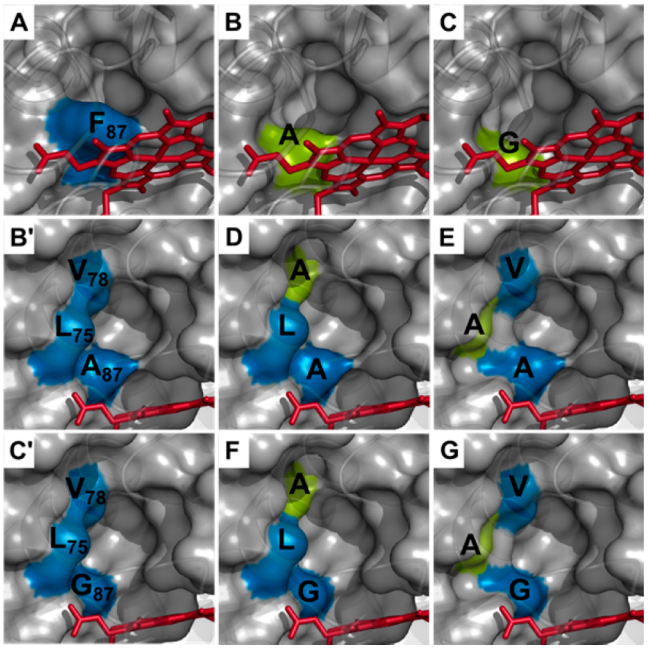

Figure 1. Shape of the substrate binding site in P450 BM3 above the heme (shown in red). The left column shows the respective parental enzyme (amino acids 75, 78, and 87 colored in blue), the middle and right columns illustrate newly introduced mutations (colored in green). Subtle differences in the substrate binding sites are shown for wild-type (A) and single mutants F87A (B) and F87G (C) in the top row, for the double mutants V78A/F87A (D) and L75A/F87A (E) in comparison to the template F87A (B′) in the middle row, and for the double mutants V78A/F87G (F) and L75A/F87G (G) in comparison to the template F87G (C′) in the bottom row. Models are based on P450 BM3 crystal structure (PDB entry: 1ZO9)[52] and were generated using PyMOL 0.99rc6. Panels B and B′ as well as C and C′ present two different views of the F87A and F87G mutant, respectively.

including the enone **3**. The preliminary screening results show that hydroxylation as well as epoxidation of the diterpenoid scaffold **1** is possible with P450 BM3 mutants at 3 out of 16 possible oxidation positions (Scheme 1).

To improve chemo-, regio-, and stereoselectivity further, the single mutants F87G and F87A were chosen as parental enzymes to introduce additional mutations.

P450 BM3 Library Design. In the second round of mutagenesis, we analyzed the effect of further amino acid changes in P450 BM3 variants, which have been reported to convert large non-natural substrates including alkaloids, thioglycosides, polycyclic aromatic hydrocarbons, sesquiterpenoids, or steroids.[11−14,49−51] Among the known mutations, we focused on those amino acids which were most likely to interact directly with the substrate (first-sphere amino acids). All chosen amino acids (A74, L75, V78, I263, A264, and L437) are located in a distance of less than 15 Å to the heme iron. Mutations of these residues are expected to direct the substrate *β*-cembrenediol (**1**) to a certain orientation in the active site and thus influence enzyme regio- and stereoselectivity. A P450 BM3 minimal library was constructed in three rounds, each one introducing a new mutation. For each generated mutant, substrate conversion and product distribution were analyzed. Out of 29 generated variants, 10 double to triple mutants with amino acid changes at positions 75, 78 and in one case at position 263 displayed significant improvement of conversion and selectivity over the parental single mutants F87A or F87G (Table 1 and Table S1). This was not observed for the remaining mutants with amino acid changes at positions 74, 264, and 437 (Table S1). Due to the identification of 10 improved mutants, no further mutagenesis was undertaken.

Screening for Chemo-, Regio-, and Stereoselective P450 BM3 Variants. Screening of the P450 BM3 minimal library enabled the identification of two additional residues which play a key role in the oxidation of *β*-cembrenediol (**1**), namely, V78 and L75 (Figure 1). Both residues lie in the B′-helix above the porphyrine ring, and their exchange by smaller hydrophobic residues was expected to improve the oxidation of **1**.

First, we analyzed activity as well as chemo- and regioselectivity of the designed P450 BM3 variants. The insertion of alanine at position 78 into the single mutant F87A (Figure 1D) increased the conversion of **1** almost 8-fold but decreased the regioselectivity, giving five products (Table 1). The resulting mutant V78A/F87A mainly produced the 9-hydroxy-*β*-cembrenetriol (**4b**) with a ratio of 45% with opposite configuration at C-9 as **4a** (which was produced by the single mutant F87A), whereas the epimer **4a** was obtained with a ratio of only 8%. Next to the products **4a,b**, the V78A/F87A mutant formed 7,8-epoxy-*β*-cembrenediol (**5**, 38%) as well as the products **2a,b** (in total 10%) (Figure 2A). Inserting

Table 1. Conversion and Product Distribution of *β*-Cembrenediol (1) Oxidations by P450 BM3 Mutants[a]

amino acid substitution					product distribution (%)						
					C-10			C-9		C-7/C-8	
L75	V78	F87	I263	conversion (%)	**2a**	**2b**	**3**	**4a**	**4b**	**5**	other products
		A		6 ± 4	-	-	-	78	-	22	-
		A	L	17 ± 1	-	-	-	92	8	-	-
	A	A		47 ± 8	7	3	-	8	45	38	-
A		A		64 ± 8	36	59	4	-	-	-	-
A	A	A		91 ± 2	38	34	4	-	4	3	17
		G		59 ± 2	39	21	2	8	-	13	17
	A	G		67 ± 6	52	27	12	-	-	3	6
A		G		97 ± 4	40	24	27	-	-	-	9
S		G		78 ± 8	42	35	18	-	-	-	6
A	A	G		73 ± 5	59	15	22	-	-	-	3

[a]Experiments were done using cell-free extract of *E. coli* expressing P450 BM3 mutants (2.5 *μ*M final concentration). Mean values and standard deviations are calculated from three separate experiments. Differences from 100% products in total occur from rounding.

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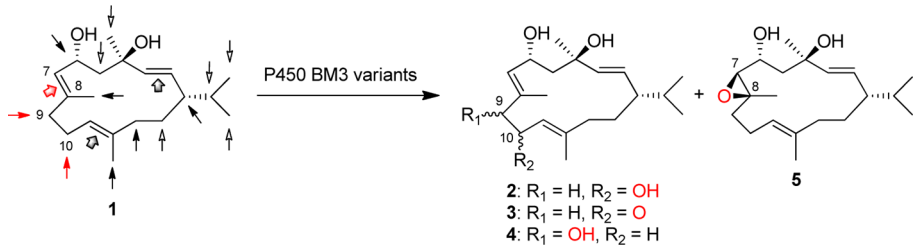
Scheme 1. Potential Oxidation Sites[a] of β-Cembrenediol (1) and Products of P450 BM3 Catalysis



[a]Potential epoxidation sites are marked with broad arrows. Potential allylic (filled arrows) and nonallylic (open arrows) hydroxylation sites are marked with thin arrows.

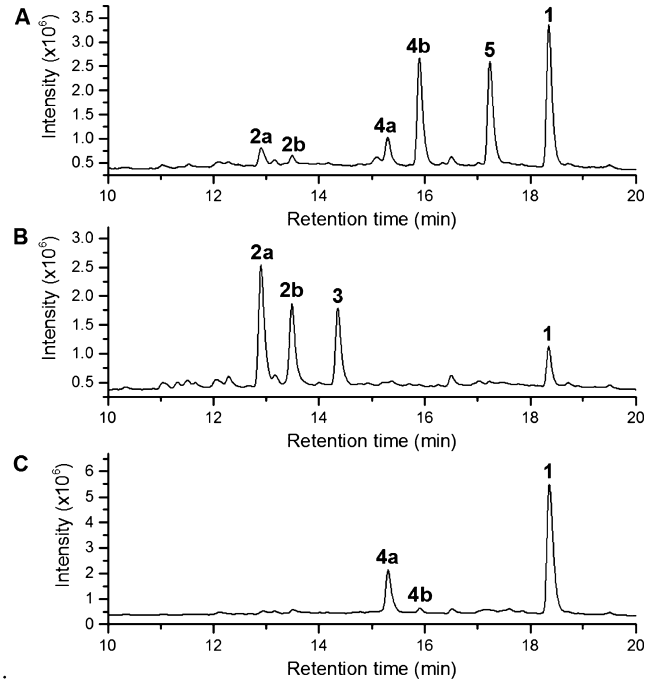


Figure 2. LCMS-chromatograms of β-cembrenediol (**1**) oxidation catalyzed by the P450 BM3 mutants V78A/F87A (A), L75A/F87G (B), and F87A/I263L (C). Oxidation of (**1**) leads to the formation of the epimeric 10-OH-β-cembrenetriols (**2a,b**), the enone (**3**), the epimeric 9-OH-β-cembrenetriols (**4a,b**), and the 7,8-epoxy-β-cembrenediol (**5**). Intensity is shown as the total ion current in the positive ion mode.

alanine at position 75 (instead of 78) into the F87A mutant (Figure 1E) increased the conversion of **1** more than 10-fold. In addition, this double mutant was 100% selective for the oxidation at C-10 (whereas the single mutant F87A and the double mutant V78A/F87A oxidized **1** primarily at the positions C-7, C-8 and C-9). Further addition of the mutation V78A to the double mutant L75A/F87A increased activity even further (91% conversion of **1**); however, the selectivity for position C-10 decreased to 76%. These data illustrate that subtle differences in the shape of the substrate binding site can change the chemo-, regio-, and stereoselectivity dramatically.

The insertion of the V78A mutation into the single mutant F87G (Figure 1F) increased the conversion of **1** to a minor degree (67% versus 59% conversion) and changed the regioselectivity considerably toward C-10. The epimers **2a** and **2b** and the enone **3** accounted for 91% in the case of V87A/F87G, compared with 62% in the case of the single mutant F87G. Adding alanine at position 75 (instead of 78) into F87G (Figure 1G) led to the highest conversion of **1** (97%), whereas the selectivity of oxidation at position C-10 remained equally high (91%) (Figure 2B). Comparable results were achieved with the double mutant L75S/F87G and the triple mutant L75A/V78A/F87G, albeit with decreased conversions of 78% and 73%, respectively. This demonstrates that the generation of minor additional space in the binding pocket due to insertions of smaller amino acids at position 78 and 75 in the F87G mutant increases the regioselectivity toward position C-10.

The single mutant F87A was the only catalyst producing 9-hydroxy-β-cembrenetriol **4a** as the main product, although with very low activity (6% conversion of **1**). By insertion of an amino acid change in the I-helix, namely, at position 263 from isoleucine to leucine, conversion was slightly improved (17%). In addition, the chemo- as well as the regioselectivity increased, giving solely the hydroxylation products at C-9 (**4a,b**) (whereas F87A additionally produced the epoxide **5** (22%)).

In the next step, we analyzed the stereoselectivity of the most regioselective P450 BM3 mutants in more detail using purified enzymes. This allowed us to exclude the possibility of further oxidation of alcohols to ketones by native *E. coli* proteins. The diastereomeric ratios of the epimers 9-OH-β-cembrenetriol (**4a,b**) and 10-OH-β-cembrenetriol (**2a,b**) were determined (Table 2 and Table S2). Whereas the double mutant F87A/I263L preferably formed 9-OH-β-cembrenetriol **4a** (89:11 dr), the mutant V78A/F87A mostly produced the corresponding epimer **4b** (79:21 dr) (Table 2). In the case of the 10-OH-β-cembrenetriol production, the triple mutant L75A/V78A/F87G showed a preference for the production of stereoisomer **2a** (65:35 dr after 0.5 h). Due to further oxidation of alcohol **2b** to the corresponding enone **3** (catalyzed by the corresponding P450 BM3 mutant), the diastereomeric ratio increased in favor of stereoisomer **2a** (74:26 dr after 24 h). The same

Table 2. Diastereomeric Ratios of the Epimeric 9-OH-(4a,b) and 10-OH-β-Cembrenetriols (2a,b)[a] after 0.5 and 24 h Reaction Time

amino acid substitution				dr		
L75	V78	F87	I263	0.5 h	24 h	diastereomers
		A	L	89:11	n.d.	**4a:4b**
	A	A		21:79	n.d.	**4a:4b**
A		G		51:49	61:39	**2a:2b**
S		G		46:54	49:51	**2a:2b**
A	A	G		65:35	74:26	**2a:2b**

[a]Experiments were done in duplicate using purified enzymes (1 μM). n.d. = not determined.

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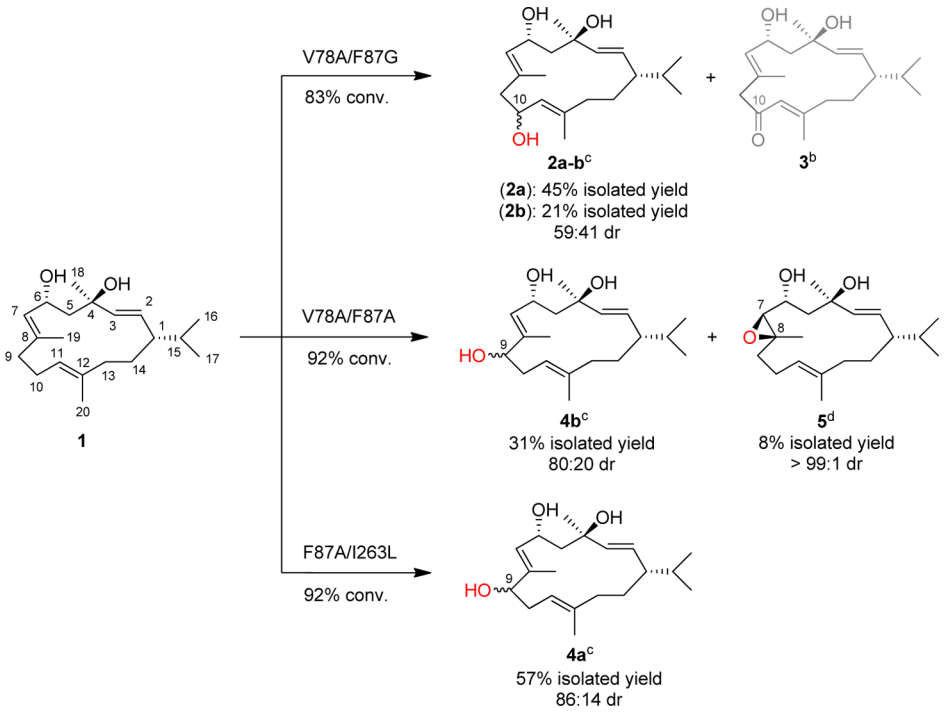
Scheme 2. Preparative Scale Oxidation of β-Cembrenediol (1) by P450 BM3 Mutants[a]



[a]Conversion (conv.), diastereomeric ratios (dr), and isolated yields refer to the preparative scale experiments. The crude product was analyzed by LCMS. The calculation of conversion values is based on the ratio of all product peaks to the sum of product and substrate peaks. Products with an abundance of >13% are shown. [b]The structure shown in gray is assumed with the help of MS-measurements. All other structures were determined by 2D-NMR experiments. [c]The exact configuration of the newly formed stereocenters in the epimers **2a,b** as well as **4a,b** was not determined. [d]The (7*R*,8*R*)-stereoisomer of **5** was not detectable.

stereospecific alcohol oxidation was observed for the double mutant L75A/F87G (51:49 dr after 0.5 h and 61:39 dr after 24 h) (Figure S1), whereas the L75S/F87G mutant did not display any stereoselectivity (Table 2).

Oxidation of β-Cembrenediol on a Preparative Scale. P450 BM3-catalyzed oxidation of β-cembrenediol (**1**) was carried out on a preparative scale with three different P450 BM3 mutants. In comparison to the screening experiments without shaking, high conversions (>83%) were achieved when reaction mixtures were gently agitated and purified enzymes were used. The V78A/F87G mutant converted **1** to give **2a** (45% isolated yield) and **2b** (21% isolated yield). Conversion of **1** by the F87A/I263L mutant yielded **4a** (57% isolated yield), whereas the V78A/F87A mutant produced **4b** (31% isolated yield) besides epoxide **5** (8% isolated yield) (Scheme 2).

Structure Elucidation of the Oxidation Products. In order to identify the structures of the oxidation products, compounds **2a,b**, **4a,b**, and **5** were isolated by semipreparative HPLC and elucidated by NMR spectroscopy and mass spectrometry. Full assignment of the ^{1}H and ^{13}C chemical shifts were achieved by evaluating their $^{1}H,^{1}H$−COSY, $^{1}H,^{13}C$-HSQC, and $^{1}H,^{13}C$-HMBC spectra (Figure S9−S33).

Products **2a,b** and **4a,b** showed six proton signals between 4 and 6 ppm, corresponding to the olefinic protons (H-2, H-3, H-7 and H-11) and the protons next to an alcohol group (H-6 and the newly hydroxylated position). Taking into account the number of signals between 4 and 6 ppm, an epoxidation reaction (and therefore the loss of olefinic protons) could be excluded. In the case of a hydroxylated product, correlation signals in the COSY spectra of protons between 4 and 6 ppm were determined by the position at which **1** is oxidized. A newly introduced hydroxy group at C-10 (**2a,b**) gave three correlation signals of two protons each (H-2/H-3, H-6/H-7, H-10/H-11) (Figure S2). Thus, the proton bound to the newly hydroxylated carbon atom must be located in direct neighborhood of H-11. Having a hydroxy group at C-9 (**4a,b**), both protons H-9 and H-11 showed COSY correlation signals with the same methylene protons at 2.2−2.4 ppm, that can be assigned unambiguously to H-$10_{a,b}$ (Figure S3). Compounds **2a** and **2b** were thus confirmed to be the epimers (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6,10-triols with opposite absolute configuration at C-10,[30,53] whereas compounds **4a** and **4b** were confirmed to be the epimers (1*S*,2*E*,4,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6,9-triols with opposite absolute configuration at C-9.[39]

In contrast to products **2** and **4**, product **5** showed only four proton signals between 4 and 6 ppm (one signal less than substrate **1**). In close accordance to literature data, product **5** was identified as (1*S*,2*E*,4*R*,6*R*,7*S*,8*S*,11*E*)-7,8-epoxy-2,11-cembradiene-4,6-diol.[54] The methine signal at δ = 2.83 (d) was assigned to the newly epoxidized C-7-atom. It showed a vicinal coupling to H-6 (J = 8.0 Hz), which correlates with a (7*S*,8*S*)-configuration of the newly formed epoxide.

Product **3** was analyzed by HRMS (ESI) and showed a molecular ion peak of 343.2243 (calculated for $C_{20}H_{32}NaO_3$ $[M + Na]^+$ m/z: 343.2244). Product **3** is therefore assumed to be (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol-10-one.

Characterization of the P450 Catalysts. The mutants with the highest selectivity and activity for each of the products were analyzed with regard to their substrate oxidation rate and coupling efficiency (Table 3). Uncoupling between NAD(P)H

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Table 3. Catalytic Properties of β-Cembrenediol-Hydroxylating P450 BM3 Variants

amino acid substitution				coupling efficiency[a] (%)	substrate oxidation rate[b] (min^{-1})
L75	V78	F87	I263		
		A	L	23 ± 1	1.2 ± 0.0
	A	A		27 ± 2	4.4 ± 0.2
A		G		41 ± 2	22.7 ± 1.4
S		G		37 ± 4	5.7 ± 0.7
A	A	G		56 ± 2	13.0 ± 0.4

[a]Ratio between moles of consumed substrate and moles of consumed NADPH. [b]Moles of consumed substrate per mole of P450 per minute.

consumption and product formation can occur within the catalytic cycle of P450s, especially when non-natural substrates are used.[49] Consequently, electrons abstracted from NAD(P)H are not completely used for substrate oxidation but partially utilized for the production of reactive oxygen species and/or water in the so-called "P450 shunt-pathways". The ratio of consumed substrate to consumed NADPH is referred to as "coupling efficiency" In general, coupling efficiencies and substrate oxidation rates were higher for all tested F87G-based mutants in comparison to F87A-based mutants. The L75A/F87G mutant demonstrated the highest substrate oxidation rate for 10-OH-β-cembrenetriols (**2a** and **2b**) (23 min^{-1}, and a coupling efficiency of 41%). A higher coupling efficiency (56%) but lower substrate oxidation rate (13 min^{-1}) were observed with the L75A/V78A/F87G mutant. The 9-OH-β-cembrenetriol (**4a**)-producing double mutant F87A/I263L showed a substrate oxidation rate of 1.2 min^{-1} and a coupling efficiency of 23%.

CONCLUSION

In summary, we constructed cytochrome P450 biocatalysts that selectively oxidize the monocyclic diterpenoid β-cembrenediol (**1**). These monooxygenases selectively introduced one atom of molecular oxygen into a scaffold carrying 13 positions as potential hydroxylation sites with seven allylic positions among them and additionally three double bonds as potential epoxidation sites. Among the potential hydroxylation reactions, only allylic hydroxylation products were verified. Out of the seven possible allylic positions, hydroxylations were detected exclusively at the two neighboring positions C-9 and C-10 (97% regioselectivity for C-10 in case of the L75A/V78A/F87G mutant and 100% regioselectivity for C-9 in case of the F87A/I263L mutant), illustrating the remarkable regioselectivity of the constructed monooxygenases. Among the hydroxylation reactions, we achieved stereoselectivities with diastereomeric ratios of 74:26 for C-10 (with the L75A/V78A/F87G mutant) and 89:11 for C-9 (with the F87A/I263L mutant) at best. Out of three potential epoxidation sites, an epoxidation reaction was determined solely at one double bond (C-7/C-8), though in this case, hydroxylated products were also observed. Next to the high regioselectivity, this epoxidation reaction occurs stereoselectively with an excellent diastereomeric ratio of >99:1 (with the V78A/F87A mutant). Although 9- and 10-OH-β-cembrenetriols had previously been produced by biotranformations with *Bacillus* sp. NC5 and NK8,[39] as well as *Bacillus megaterium* NH5,[36] our approach resulted in up to 10-fold higher yields.

We demonstrate how first-sphere active site mutagenesis based on a small number of amino acids effectively improves activity and regioselectivity of a P450 BM3 biocatalyst within only two to three generations. Insertion of smaller amino acids at positions 75 and 78 resulted in a shift of the chemo-, regio-, and stereoselectivity, illustrating that subtle differences in the shape of the substrate binding site force the macrocycle **1** in distinguishable orientations. Co-crystallization of the most selective P450 BM3 mutants with β-cembrenediol (**1**) in future work could provide a more fundamental understanding of substrate-directing amino acids in the enzyme's active site.

To the best of our knowledge, β-cembrenediol (**1**) is the first cembranoidic substrate that is described to be oxidized by a bacterial P450 monooxygenase. Taking β-cembrenediol (**1**) as an example for monocyclic diterpenoids, this study highlights the potential for late-stage C−H oxyfunctionalizations. Because no comparable methods for chemical synthesis are available to address this synthetic goal, this work shows the usefulness of P450 BM3 as a biocatalyst in chemoenzymatic synthesis of large complex molecules.

EXPERIMENTAL SECTION

Cloning. The *cyp102a1* gene from *Bacillus megaterium* (GenBank J04832) encoding P450 BM3 was cloned into the expression vector pET-28a (Novagen) as described elsewhere.[55] A mutant library of P450 BM3 variants was prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol.

Expression and Purification. Wild-type P450 BM3 and its mutants were heterologously expressed in *E. coli* BL21(DE3) cells. For precultures, 5 mL of Lysogeny Broth medium supplemented with 30 μg/mL kanamycin were inoculated with a single colony and incubated at 37 °C and 180 rpm overnight. For the initial mutant screening, a 0.5 l flask with 50 mL of Teriffic Broth (TB) medium was supplemented with kanamycin (30 μg/mL), inoculated with 1% (v/v) of an overnight culture and incubated at 37 °C and 180 rpm until $O.D._{600}$ of 0.6−0.8. At this point isopropyl-β-D-thiogalactopyranoside (100 μM), $FeSO_4$ (100 μM), and δ-aminolevulinic acid (500 μM) were added. Cell cultures were stirred at 30 °C and 140 rpm for 18−20 h. Cells were harvested by centrifugation (11,325 rcf, 20 min, 4 °C), resuspended in potassium phosphate buffer (50 mM, pH 7.5) containing phenylmethylsulfonyl fluoride (100 μM) and disrupted by sonication on ice. The cell debris was removed by centrifugation (58 545 rcf, 30 min, 4 °C).

For production of purified proteins, P450 BM3 mutants were expressed in a scale of 400 mL of TB-medium in a 2 l flask as described above. Purification was done by metal (nickel) affinity chromatography on two consecutive His-Trap FF crude 5 mL columns (GE Healthcare). Proteins were eluted from the column with a step gradient from 20 to 200 mM of imidazole. Pooled fractions were desalted by size exclusion chromatography on a HiPrep 26/10 Desalting column (GE Healthcare).

The gene of glucose dehydrogenase (GDH) from *Bacillus megaterium* (*gdhIV*, GenBank D10626)[56] was amplified from the template pETDUET-glcdh (kindly donated by J. Schrader, DECHEMA Forschungsinstitut, Frankfurt am Main) and subcloned into the plasmid pET-22b (Novagen) between the restriction sites for endonucleases *Nde*I and *Xho*I. Expression was done in *E. coli* BL21(DE3) cells as described above with the exception of media supplementation with ampicillin (100 μg/mL) and cell incubation after induction with isopropyl-β-D-thiogalactopyranoside (100 μM) at 25 °C.

Enzyme Assays. P450 BM3 concentrations were determined from CO-difference spectra as described previously and

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calculated using the following extinction coefficients: $\varepsilon_{450-490}$ = 91 mM^{-1} cm^{-1} or $\varepsilon_{420-490}$ = 111 mM^{-1} cm^{-1}.[57] Upon reduction with sodium dithionite (50 mM) and bubbling with CO, absorption spectra (400−500 nm) were recorded five times consecutively.

$NADP^+$ reduction activity of GDH (which was used for cofactor regeneration) was measured spectrophotometrically at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). Reaction solutions contained 100 mM glucose, 0.1 mM $NADP^+$, and 100 μL of cell-free extract in a final volume of 1 mL of potassium phosphate buffer (50 mM, pH 7.5).

Analytical Scale Conversions. Screening of P450 BM3 mutants was performed with *E. coli* cell-free extracts in 0.5 mL scale. In a standard reaction 100 μM β-cembrenediol (**1**) dissolved in ethanol (1% (v/v) ethanol), 2.5 μM P450 BM3 and 100 μM NADPH were used. Cofactor regeneration was done with 20 mM glucose and 3−5 U/ml GDH. In order to remove hydrogen peroxide, which can be formed due to uncoupling reactions, 600 U/ml catalase from bovine liver (Sigma-Aldrich) was added. Reactions were performed in potassium phosphate buffer (50 mM, pH 7.5) at 25 °C without shaking. After 16.5 h reaction mixtures were extracted twice with 300 μL ethyl acetate. The combined organic phases were concentrated under reduced pressure and the residues were resolved in methanol for LCMS-analysis. For the initial screening, all products were identified by their m/z values. Calculations of conversion values are based on the consumed substrate in respect of a control reaction with cell-free extract obtained from *E. coli* cells transformed with an "empty" vector. Product distributions are based on the observed peak areas. All experiments were performed in triplicate.

For the determination of the stereoselectivity, conversions were performed with purified enzymes for 24 h similar to the screening experiments. In variation from this, concentrations of 200 μM β-cembrenediol (**1**), 200 μM NADPH, and 1 μM purified P450 BM3 in a reaction volume of 0.2 mL were used. Prior to the extraction with ethyl acetate, 50 μM of the internal standard cyclotetradec-4-ene-1,1-diylbis(methylene)diacetate was added. Conversion values were based on the absolute β-cembrenediol (**1**) concentration, which was determined by calibration with an internal standard. All experiments were performed in duplicate.

Preparative Scale Conversions and Product Purification. Conversions for product purification were performed similar to the analytical scale conversions. Several reactions were done using 1−2.5 μM purified P450 BM3 in a final volume of 8 mL for 6 h (V78A/F87G mutant) or 19 h (V78A/F87A and F87A/I263L mutants) under luffing shaking. Reactions were extracted with ethyl acetate (3 × 5 mL), and the organic layers of all reactions were combined. Product separation was performed by semipreparative HPLC with a H_2O/methanol gradient. Conversion of 19 mg of β-cembrenediol (**1**) by V78A/F87G afforded **2a** as colorless solid (9 mg; 45%) and **2b** as colorless oil (4 mg; 21%). Oxidation of 19 mg of β-cembrenediol (**1**) by V78A/F87A produced **5** as colorless oil (1.6 mg; 8%) and **4b** as colorless oil (6 mg; 31%). Conversion by BM3 V78A/F87A was repeated to yield **5** (3 mg in total). Conversion of 17 mg of β-cembrenediol (**1**) by F87A/I263L resulted in **4a** as colorless oil (10 mg; 57%).

Structure Elucidation. *(1S,2E,4R,6R,7E,11E)-2,7,11-Cembratriene-4,6-diol (1).* ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.80 (d, J = 6.8 Hz, 3H, 17-H), 0.82 (d, J = 6.8 Hz, 3H, 16-H), 1.25−1.32 (m, 1H, 14-H_a), 1.39 (s, 3H, 18-H), 1.45−1.49 (m, 1H, 15-H), 1.50 (s, 3H, 20-H), 1.52−1.60 (m, 2H, 1-H, 14-H_b), 1.70 (d, J = 1.2 Hz, 3H, 19-H), 1.86 (dd, J = 14.2, 8.9 Hz, 1H, 5-H_a), 1.88−1.94 (m, 1H, 13-H_a), 1.99−2.06 (m, 2H, 5-H_b, 13-H_b), 2.07−2.25 (m, 4H, 9-H, 10-H), 4.77−4.81 (m, 1H, 6-H), 4.97−5.02 (m, 1H, 11-H), 5.21 (dd, J = 15.6, 9.3 Hz, 1H, 2-H), 5.26 (dq, J = 9.6, 1.2 Hz, 1H, 7-H), 5.39 (d, J = 15.6 Hz, 1H, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 15.0 (C-20), 15.9 (C-19), 19.4 (C-17), 20.6 (C-16), 23.1 (C-10), 27.7 (C-14), 28.7 (C-18), 32.9 (C-15), 36.5 (C-13), 38.8 (C-9), 46.3 (C-1), 52.5 (C-5), 64.5 (C-6), 71.5 (C-4), 124.4 (C-11), 130.4 (C-2), 131.4 (C-7), 133.0 (C-12), 136.1 (C-3), 136.5 (C-8).

(1S,2E,4R,6R,7E,11E)-2,7,11-Cembratriene-4,6,10-triol (2a). HRMS (ESI) m/z: [M + Na]$^+$ calcd for $C_{20}H_{34}NaO_3$, 345.2400; found, 345.2397. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.80 (d, J = 6.8 Hz, 3H, 17-H), 0.82 (d, J = 6.8 Hz, 3H, 16-H), 1.27−1.36 (m, 1H, 14-H_a), 1.39 (s, 3H, 18-H), 1.42−1.48 (m, 1H, 1-H), 1.48−1.53 (m, 1H, 15-H), 1.60−1.67 (m, 1H, 14-H_b), 1.68 (s, 6H, 19-H, 20-H), 1.85 (dd, J = 14.4, 8.3 Hz, 1H, 5-H_a), 1.92 (td, J = 13.2, 4.0 Hz, 1H, 13-H_a), 2.02 (dd, J = 14.4, 1.3 Hz, 1H, 5-H_b), 2.05−2.15 (m, 2H, 9-H_a, 13-H_b), 2.57 (dd, J = 12.2, 5.2 Hz, 1H, 9-H_b), 4.46−4.53 (m, 1H, 10-H), 4.76 (ddd, J = 9.7, 8.3, 1.3 Hz, 1H, 6-H), 5.16−5.19 (m, 1H, 11-H), 5.21 (dd, J = 15.4, 9.1 Hz, 1H, 2-H), 5.31−5.33 (m, 1H, 7-H), 5.34 (d, J = 15.4 Hz, 1H, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 14.6 (C-20), 16.3 (C-19), 19.4 (C-17), 20.4 (C-16), 27.1 (C-14), 28.5 (C-18), 33.0 (C-15), 36.2 (C-13), 46.4 (C-1), 48.8 (C-9), 52.2 (C-5), 64.1 (C-6), 64.6 (C-10), 71.4 (C-4), 128.0 (C-11), 130.3 (C-2), 133.1 (C-8), 133.4 (C-7), 136.2 (C-3), 140.3 (C-12).

(1S,2E,4R,6R,7E,11E)-2,7,11-Cembratriene-4,6,10-triol (2b). $[\alpha]_D^{20}$ +73 (c 0.1, $CHCl_3$). HRMS (ESI) m/z: [M + Na]$^+$ calcd for $C_{20}H_{34}NaO_3$, 345.2400; found, 345.2398. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.79 (d, J = 6.8 Hz, 3H, 17-H), 0.83 (d, J = 6.8 Hz, 3H, 16-H), 1.22 (brs, 1H, 4-OH), 1.25 (brs, 1H, 10-OH), 1.30 (brs, 1H, 6-OH), 1.37 (s, 3H, 18-H), 1.35−1.41 (m, 1H, 14-H_a), 1.47−1.53 (m, 1H, 15-H), 1.57−1.62 (m, 1H, 14-H_b), 1.69 (s, 3H, 20-H), 1.69−1.75 (m, 2H, 1-H, 5-H_a), 1.85 (s, 3H, 19-H), 1.95−2.02 (m, 1H, 13-H_a), 2.02−2.09 (m, 2H, 5-H_b, 13-H_b), 2.25 (dd, J = 15.4, 8.1 Hz, 1H, 9-H_a), 2.37−2.43 (m, 1H, 9-H_b), 4.63−4.68 (m, 1H, 10-H), 4.83 (ddd, J = 9.9, 9.1, 1.8 Hz, 1H, 6-H), 5.03−5.07 (m, 1H, 7-H), 5.22 (dd, J = 15.7, 9.4 Hz, 1H, 2-H), 5.30 (dq, J = 8.4, 1.2 Hz, 1H, 11-H), 5.40 (d, J = 15.7 Hz, 1H, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 16.9 (C-20), 19.40 (C-19), 19.41 (C-17), 20.3 (C-16), 28.0 (C-14), 29.3 (C-18), 33.4 (C-15), 38.2 (C-13), 45.6 (C-9), 47.0 (C-1), 52.2 (C-5), 64.4 (C-6), 66.6 (C-10), 71.4 (C-4), 127.4 (C-11), 130.3 (C-2), 131.0 (C-7), 135.5 (C-8), 136.1 (C-3), 138.5 (C-12).

(1S,2E,4R,6R,7E,11E)-2,7,11-Cembratriene-4,6,9-triol (4a). $[\alpha]_D^{20}$ +103 (c 0.1, $CHCl_3$). HRMS (ESI) m/z: [M + Na]$^+$ calcd for $C_{20}H_{34}NaO_3$, 345.2400; found, 345.2397. ^{1}H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.79 (d, J = 6.8 Hz, 3H, 17-H), 0.82 (d, J = 6.8 Hz, 3H, 16-H), 1.24−1.32 (m, 1H, 14-H_a), 1.39 (s, 3H, 18-H), 1.50 (s, 3H, 20-H), 1.45−1.59 (m, 3H, 1-H, 14-H_b, 15-H), 1.71 (s, 3H, 19-H), 1.89−1.96 (m, 3H, 5-H_a, 13-H_a, OH), 1.99−2.04 (m, 1H, 13-H_b), 2.05−2.10 (m, 1H, 5-H_b), 2.33−2.44 (m, 2H, 10-H), 4.17−4.23 (m, 1H, 9-H), 4.83−4.88 (m, 1H, 6-H), 4.97−5.03 (m, 1H, 11-H), 5.20 (dd, J = 15.6, 9.2 Hz, 1H, 2-H), 5.34 (d, J = 15.6 Hz, 1H, 3-H), 5.59−5.64 (m, 1H, 7-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 15.1 (C-19), 15.2 (C-20), 19.4 (C-17), 20.6 (C-16), 27.6 (C-14), 28.7

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(C-18), 30.8 (C-10), 32.9 (C-15), 36.6 (C-13), 46.2 (C-1), 52.7 (C-5), 64.0 (C-6), 71.5 (C-4), 73.1 (C-9), 119.3 (C-11), 128.7 (C-7), 130.6 (C-2), 134.9 (C-12), 136.0 (C-3), 137.9 (C-8) ppm.

*(1S,2E,4R,6R,7E,11E)-2,7,11-Cembratriene-4,6,9-triol (**4b**).* $[\alpha]_D^{20}$ +149 (c 0.1, $CHCl_3$). HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{20}H_{34}NaO_3$, 345.2400; found, 345.2399. ^1H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.79 (d, *J* = 6.8 Hz, 3H, 17-H), 0.83 (d, *J* = 6.8 Hz, 3H, 16-H), 1.23−1.31 (m, 1H, 14-H_a), 1.38 (s, 3H, 18-H), 1.51 (s, 3H, 20-H), 1.45−1.60 (m, 3H, 1-H, 14-H_b, 15-H), 1.70 (d, *J* = 1.4 Hz, 3H, 19-H), 1.76−1.91 (m, 3H, 5-H_a, 13-H_a, OH), 1.99−2.07 (m, 2H, 5-H_b, 13-H_b), 2.21−2.29 (m, 1H, 10-H_a), 2.31−2.38 (m, 1H, 10-H_b), 2.53 (brs, 2H, 2 x OH), 4.13 (dd, *J* = 11.3, 5.5 Hz, 1H, 9-H), 4.64−4.71 (m, 1H, 11-H), 4.81 (ddd, *J* = 10.0, 8.3, 1.2 Hz, 1H, 6-H), 5.22 (dd, *J* = 15.7, 9.1 Hz, 1H, 2-H), 5.34 (d, *J* = 15.7 Hz, 1H, 3-H), 5.39 (dq, *J* = 9.7, 1.4 Hz, 1H, 7-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 9.9 (C-19), 15.3 (C-20), 19.4 (C-17), 20.5 (C-16), 27.5 (C-14), 28.5 (C-18), 31.5 (C-10), 32.8 (C-15), 36.2 (C-13), 46.2 (C-1), 52.2 (C-5), 63.9 (C-6), 71.9 (C-4), 78.5 (C-9), 120.7 (C-11), 130.8 (C-2), 132.9 (C-7), 134.4 (C-12), 135.8 (C-3), 137.5 (C-8).

*(1S,2E,4R,6R,7S,8S,11E)-7,8-Epoxy-2,11-cembradiene-4,6-diol (**5**).* The analytical data were in close accordance to those of literature.[54] $[\alpha]_D^{20}$ +71 (c 0.1, $CHCl_3$). HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{20}H_{34}NaO_3$, 345.2400; found, 345.2400. ^1H NMR (600 MHz, $CDCl_3$): δ [ppm] = 0.82 (d, *J* = 6.8 Hz, 3H, 17-H), 0.86 (d, *J* = 6.8 Hz, 3H, 16-H), 1.35 (s, 3H, 19-H), 1.45 (s, 3H, 18-H), 1.36−1.47 (m, 2H, 9-H_a, 14-H_a), 1.53 (s, 3H, 20-H), 1.47−1.54 (m, 1H, 15-H), 1.56−1.65 (m, 2H, 1-H, 14-H_b), 1.88 (dd, *J* = 14.9, 4.9 Hz, 1H, 5-H_a), 1.90−1.95 (m, 1H, 13-H_a), 1.99−2.05 (m, 1H, 9-H_b), 2.05−2.08 (m, 1H, 13-H_b), 2.08−2.12 (m, 2H, 5-H_b, 10-H_a), 2.16−2.25 (m, 1H, 10-H_b), 2.83 (d, *J* = 8.0 Hz, 1H, 7-H), 3.99 (ddd, *J* = 8.0, 4.9, 3.7 Hz, 1H, 6-H), 4.99−5.06 (m, 1H, 11-H), 5.26 (dd, *J* = 15.7, 9.4 Hz, 1H, 2-H), 5.49 (d, *J* = 15.7 Hz, 1H, 3-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ [ppm] = 14.6 (C-20), 17.4 (C-19), 19.9 (C-17), 20.4 (C-16), 22.9 (C-10), 28.3 (C-14), 28.8 (C-18), 32.2 (C-15), 36.0 (C-13), 36.7 (C-9), 46.6 (C-1), 50.0 (C-5), 63.4 (C-8), 64.9 (C-6), 68.9 (C-7), 71.5 (C-4), 123.5 (C-11), 131.3 (C-2), 135.2 (C-12), 136.9 (C-3).

Coupling Efficiencies and Substrate Oxidation Rates. Coupling efficiencies were determined in 200 μL reactions containing 93−116 μM β-cembrenediol (**1**), 0.13−1.75 μM purified P450 BM3, and 86−171 μM NADPH in potassium phosphate buffer (50 mM, pH 7.5) at 25 °C. NADPH consumption was followed at 340 nm in a spectrophotometer (TECAN Infinite M200Pro). The exact NADPH concentration consumed in each reaction was determined from the absorbance difference between start and end of the reaction using ε = 6.22 mM^{-1} cm^{-1}. After complete NADPH consumption, 50 μM of the internal standard cyclotetradec-4-ene-1,1-diylbis(methylene)diacetate was added to the reaction solution which was subsequently extracted twice with ethyl acetate. The combined organic layers were concentrated under reduced pressure and resolved in methanol for LCMS-analysis. The exact substrate concentration applied in each experiment was determined in a control reaction without NADPH. The absolute concentrations of β-cembrenediol (**1**) in each reaction were assigned by using an internal standard calibration. The coupling efficiency was calculated from the ratio between moles of consumed substrate and moles of consumed NADPH. Substrate oxidation rates are based on the moles of consumed substrate in relation to the applied moles of P450 and the time required for complete NADPH consumption. Reported mean and standard deviation values are based on experiments performed in triplicate.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/cs5020404.

Experimental procedures, screening results of all tested BM3 mutants, additional data corresponding to the time-dependent oxidation of **1** using purified enzyme and 1D- and 2D-NMR spectra of **1**−**5** (PDF)

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Notes

The authors declare no competing financial interest.

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