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P450 BM3-catalyzed regio- and stereoselective hydroxylation aiming at the synthesis of phthalides and isocoumarins

Claudia Holec[a], Ute Hartrampf[b], Katharina Neufeld[a], and Jörg Pietruszka[a,b]*

Abstract: Cytochrome P450 BM3 monooxygenases are able to catalyze the regio- and stereoselective oxygenation of a broad range of substrates with promising potential for synthetic applications. To study the ability of P450 BM3 variants for the stereoselective benzylic hydroxylation of 2-alkylated benzoic acid esters, the biotransformation of methyl 2-ethylbenzoate was investigated resulting in both enantiomeric forms of 3-methylphthalide. In case of methyl 2-propylbenzoate as a substrate the regioselectivity of the reaction was shifted towards β-hydroxylation resulting in the synthesis of enantioenriched (*R*)- and (*S*)-configured 3-methylisochroman-1-one. The potential of P450 BM3 variants for the regio- and stereoselective synthesis of phthalides and isocoumarins constitutes a new route to a class of compounds that are valuable synthons for a variety of natural compounds.

Introduction

In the last decades, extensive efforts towards the regio- and stereoselective hydroxylation of organic compounds have been reported aiming at improved accesses to key building blocks and intermediates for the synthesis of natural products and fine chemicals.[1] The selective C-H oxidation of activated or non-activated carbon atoms by classical chemistry has often proven to be difficult due to insufficient regio- and stereoselectivities.[2-4] Enzymes as nature's chiral catalysts offer high potential to overcome these limitations due to their exceptional performance with regard to chemo-, regio- and enantioselectivity. For instance, cytochrome P450 monooxygenases (CYPs, EC 1.14.-.-) - catalyzing the insertion of one oxygen atom from molecular oxygen into C-H bonds while the second is reduced to water - are a prominent example.[5] The P450 BM3 monooxygenase (CYP102A1), a presumed fatty acid hydroxylase from *Bacillus megaterium*, is one of the best characterized and most evolved CYP enzymes.[6] Protein engineering using rational and non-rational approaches has been used to shift the substrate spectrum towards non-natural substrates including aromatic compounds, alkanes, and pharmaceuticals with enhanced regio- and stereoselectivity, improved catalytic rates, and increased total turnovers.[7] Recently, P450 BM3 mutants were established for the regio- and enantioselective α-hydroxylation of ketones yielding chiral acyloins.[8] Moreover, Arnold and co-workers expanded the potential of P450 BM3 by showing non-natural reactivities such as aminations, sulfimidations or carbenoid insertion reactions achieved through protein engineering.[9-11] Our laboratory has engineered P450 BM3 towards the benzylic hydroxylation of a variety of toluene derivatives and the double mutant F87A L188C showed remarkably increased turnovers compared to the wildtype enzyme.[12] In addition, we also emphasized the potential of the P450 BM3 double mutant A74G L188Q for the allylic hydroxylation of ω-alkenoic acids and esters on preparative scale with high to excellent chemo- and enantioselectivities providing (*S*)-configured allylic alcohols.[13] Against this background we investigated the potential of P450 BM3 for the stereoselective hydroxylation of benzoic acid derivatives giving access to optically active phthalides and isocoumarins. Chiral phthalides and isocoumarins often exist as natural products with different biological activities and various methods for an enantioselective synthesis of these chiral lactones have been reported.[14-22] However, a chemical access through stereoselective hydroxylation was not addressed. Nevertheless, Kitayama reported in 1997 a microbial access to (*S*)-3-alkylphthalide derivatives in high optical purity by asymmetric reduction of methyl 2-acetylbenzoate with a *Geotrichum candidum* strain or hydroxylation of 2-alkylbenzoic acids at the benzylic position with a *Pseudomonas putida* or *Aspergillus niger* strain.[23] In recent years, a number of articles reported the application of the P450 BM3 monooxygenase for the synthesis of chiral benzylic alcohols as versatile and pivotal synthons for the pharmaceutical industry, but efficient enantioselective transformations remain challenging.[12,24-26] In this report, we present the synthetic potential of these catalysts for the synthesis of phthalides and isocoumarins, thereby addresses the challenge of enantioselective hydroxylation.

Results and Discussion

Initial investigations towards benzylic hydroxylations with P450 BM3 variant F87A L188C

To investigate the potential of the P450 BM3 enzyme for an enantioselective benzylic hydroxylation, we started our endeavour with the P450 BM3 F87A L188C variant, which was previously described as an efficient catalyst for the benzylic hydroxylation of a set of toluene derivatives by our group.[12] We chose alkyl-substituted methyl benzoate derivatives **1a-c** as test compounds to study the regio- and enantioselective hydroxylation based on previous studies. In former experiments,

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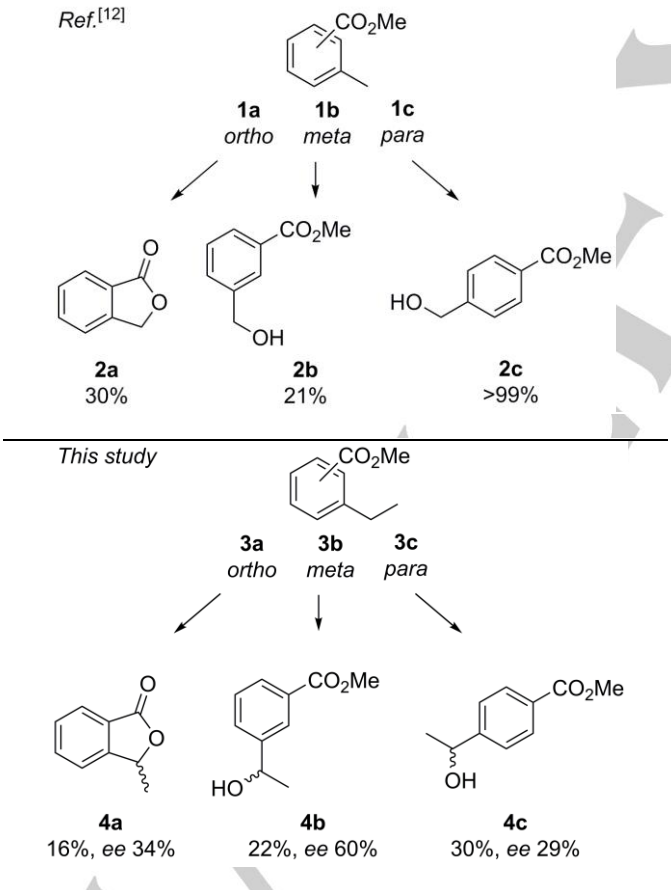
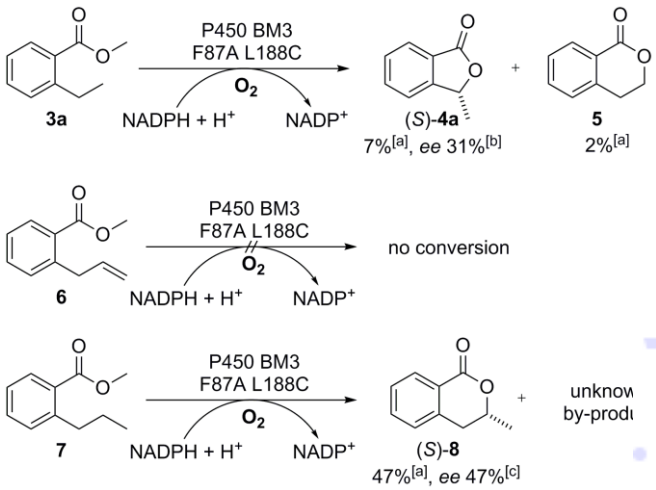
benzylic oxidations were observed with substrates carrying a methyl moiety at the aromatic ring in *ortho*-, *meta*-, or *para*-position to the carbonyl group (Table 1, **2a-c**). Moderate to full conversions were shown and spontaneous lactonization to isobenzofuranone (**2a**) was detected when methyl *o*-methylbenzoate (**1a**) was oxidized in the benzylic position (Table 1). To target the synthesis of chiral benzylic alcohols, we first studied the effect of an ethyl substituent at the aromatic ring in *ortho*-, *meta*-, and *para*-position of methyl benzoates **3a-c** facing a stereoselective hydroxylation in benzylic position. Performing the biotransformations of substrates **3a-c** using the F87A L188C variant on analytical scale indeed confirmed the potential of P450 BM3 for the enantioselective benzylic hydroxylation, since products **4a-c** (Table 1) could be detected in the crude extracts by ^{1}H NMR and gas chromatography utilizing chiral stationary phases. Conversions up to 30% were reached and the enantioselectivity varied between 29-60% (Table 1, **4a**-**c**). In case of methyl 2-ethylbenzoate (**3a**) and methyl 4-ethylbenzoate (**3c**), small amounts of a by-product were detected by ^{1}H NMR analysis in addition to the intended product. In the following, we focused on the biotransformation of

Table 1. Benzylic hydroxylation of benzoic acid derivatives **1** (above, previous study) and **3** (below, this study) by P450 BM3 F87A L188C..



Conversions were determined *via* ^{1}H NMR by comparison with authentic samples and *ee*-values obtained from GC chromatograms.



Scheme 1. Biotransformation of benzoic acid derivatives using the P450 BM3 F87A L188C catalyst. [a] Yield of isolated product; [b] determined by GC and [c] HPLC analysis; absolute configuration was assigned by comparison of the determined rotatory power with a literature reference.[23,27]

methyl 2-ethylbenzoate (**3a**) giving access to optically active 3-methylphthalides (**4a**). In order to identify the by-products of the transformation of compound **3a** a preparative scale biotransformation of methyl 2-ethylbenzoate (**3a**) was performed (Scheme 1). We used crude cell lysate of F87A L188C and reaction conditions based on a previously reported protocol.[12] The reaction was run for 24 h and flash chromatography of the crude product gave lactone (*S*)-**4a** in 7% yield with 31% *ee,* and 51% of substrate **3a** could be re-isolated. Interestingly, isochroman-1-one (**5**) was isolated as a by-product (2%) resulting from a terminal hydroxylation of the non-activated C-H bond of the ethyl group. Despite the poor yield, to the best of our knowledge this is the first time that P450 BM3 is successfully applied for the stereoselective synthesis of phthalides on preparative scale. Next, we investigated the effect of an allyl- and propyl-substituent in *ortho*-position of substrate **3a** (Scheme 1, substrates **6** and **7**). Methyl 2-allylbenzoate (**6**) was considered as a better substrate for the benzylic hydroxylation by P450 BM3 due to an additional activation of the benzylic position by the vinyl group. In addition, the increased size of the substrates **6** and **7** might influence the location of the substrate in the active site of the enzyme resulting in an improved enantioselectivity. Unfortunately, substrate **6** was not a substrate for the CYP enzyme. Surprisingly, the preparative enzyme reaction for substrate **7** resulted in the formation of isocoumarin (*S*)-**8** as the main product with an *ee*-value of 47% (Scheme 1), which is formed by spontaneous lactonization of the alcohol previously produced by hydroxylation of the propyl-chain in β-position. The observed presence of one by-product was beneficial emphasizing the enzyme's selectivity; however, due to insufficient yield the structure of the by-product remained unknown. In summary, an altered regioselectivity favoring the β-hydroxylation was observed when substrate **7** was applied. This

initialized further investigations in the utility of by P450 BM3 for the synthesis of phthalides and isocoumarins.

Library screening for methyl 2-ethylbenzoate (3a)

In the next step of our study, we screened our in-house CYP BM3 mutant library encompassing 121 mutants and the wildtype enzyme to investigate the regio- and enantioselectivity of the hydroxylation of methyl 2-ethylbenzoate (**3a**) and in order to find a more selective BM3 catalyst. The BM3-library is based on the mutated active-site residues R47, Y51, A74, F87 and L188.[6,7b,12,13,28] GC analysis of samples was performed with regard to formation and *ee* of 3-methylisobenzofuran-1(3*H*)-one (**4a**). The results are shown in Figure 1, neglecting the formation of the isocoumarin **5** as a by-product. P450 BM3 variants with Phe in position 87 resulted in wildtype selectivity providing (*R*)-configured product **4a**; 11 of these variants enabled a significant increase in product formation. Notably, opposite enantioselectivities were displayed by variants with a F87A and a F87V mutation; 11 of these mutants gave a lower substrate conversion compared to the respective F87A-parent. Overall, the screened library revealed only moderate *ee*-values with 16-54% (*S*)-*ee* and 54-76% (*R*)-*ee* for the biotransformation of substrate **3a** (detailed screening results see S4.2 and S4.3). Among the variants providing the highest activities and enantioselectivities, four variants for either of the two selectivities were selected for further characterization (Figure 1,

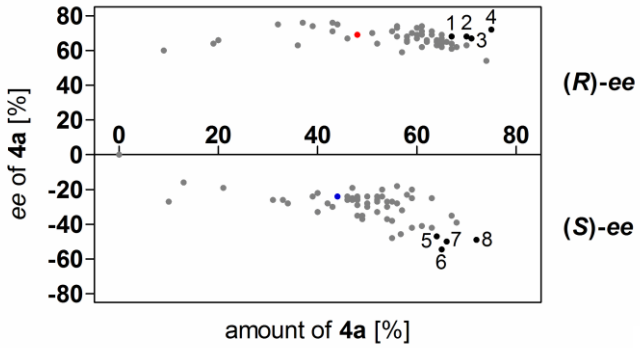


Figure 1. Screening results of the P450 BM3 in-house library for enantioselective benzylic hydroxylation of methyl 2-ethylbenzoate (**3a**). The wildtype (red) gave (*R*)-and the F87A-parent (blue) (*S*)-selectivity; selected BM3 variants are highlighted in black (1: R47N, 2: R47Y, 3: Y51W, 4: Y51V, 5: F87A L188W, 6: F87V L188Q, 7: F87A L188P, 8: A74G F87V); all other variants in grey. All values were determined by GC analysis.

Table 2. Detailed characterization of identified (*R*)- and (*S*)-selective P450 BM3 mutants for the biotransformation of methyl 2-ethylbenzoate (**3a**).

Entry	P450 BM3 variant	Conv. [%]	Product distribution [%]			*CE* [%][a]	*ee* [%][b]
			4a	**5**	by-product		
1	wildtype	70 ± 1	67 ± 2	2 ± 2	1 ± 1	13.5 ± 1.1	59 ± 4 (*R*)
2	R47N	49 ± 2	46 ± 2	3 ± 1	1 ± 0	15.9 ± 0.8	69 ± 3 (*R*)
3	R47Y	85 ± 2	79 ± 1	5 ± 1	1 ± 1	18.5 ± 0.3	74 ± 3 (*R*)
4	Y51V	94 ± 1	70 ± 2	9 ± 0	15 ± 3	12.6 ± 0.5	70 ± 1 (*R*)
5	Y51W	90 ± 1	85 ± 2	3 ± 2	2 ± 0	14.3 ± 1.2	72 ± 1 (*R*)
6	F87A-parent	76 ± 2	57 ± 1	10 ± 1	9 ± 0	18.5 ± 3.3	30 ± 1 (*S*)
7	F87A L188W	98 ± 1	65 ± 1	10 ± 1	23 ± 1	22.9 ± 3.5	57 ± 2 (*S*)
8	F87A L188P	100 ± 0	37 ± 4	13 ± 1	50 ± 3	22.2 ± 1.9	44 ± 6 (*S*)
9	A74G F87V	80 ± 3	64 ± 2	9 ± 1	7 ± 1	14.6 ± 1.8	44 ± 1 (*S*)
10	F87V L188Q	96 ± 0	70 ± 2	8 ± 2	18 ± 1	11.3 ± 0.9	65 ± 3 (*S*)

All values were determined by GC analysis and uncertainty is given as the standard deviation from three measurements. [a] Calculated with previously monitored calibration curves (see S5.1); [b] Absolute configuration was assigned by comparison of the rotatory power of synthesized samples with literature; Conv.: conversion; *CE*: coupling efficiencies.

variants 1-8). Comparison of the identified hits was performed *via* detailed enzyme characterization and enzyme reactions were performed on analytical scale in triplicate; the F87A mutant and the wildtype enzyme served as references. The coupling efficiency (*CE*) was used as an additional parameter for selecting the best biocatalyst and calculated from the amount of formed product(s) as observed by GC compared to the amount of consumed cofactor. No significant differences between the (*R*)-selective mutants were observed (Table 2, entry 1-5). Nevertheless, R47Y was selected as the best catalyst due to higher *ee*-values and better coupling rates (Table 2, entry 3); the latter being a major factor for successful preparative scale biotransformations.[12] In case of (*S*)-selective variants, even though F87V L188Q gave the lowest coupling efficiency, it was favoured for further investigations due to an improved enantioselectivity (65% *ee*, Table 2, entry 10) relative to the F87A-parent (30% *ee*, Table 2, entry 6). Reactions performed with purified enzyme did not lead to any increase in productivity or enantioselectivity (see Figure S4.4). Conclusively, our results show the use of P450 BM3 variants for the synthesis of both configurations of 3-methylisobenzofuran-1(3*H*)-one (**3a**) and amino acid position 87 was identified as a major determinant of P450 BM3 producing enantioenriched 3-methylisobenzofuranone in both enantiomeric forms. Recently, Flitsch and co-workers identified P450cam-RhFRed-variants catalyzing the benzylic hydroxylation of ethylmethylbenzene derivatives by library screening and molecular modelling.[29] The resulting benzylic alcohols were obtained in both configurations with moderate to excellent *ee*-values depending on the amino acid at position 244, which underlines the impact of single amino acid substitutions for inverting the enantioselectivity. However, in most cases protein engineering gives access to only one enantiomer.[30]

Protein engineering: Extension of the P450 BM3 library for the oxidation of methyl 2-ethylbenzoate (3a)

Next, we decided to extend our existing P450 BM3 library by protein engineering to access even better biocatalysts for the benzylic hydroxylation of compound **3a.** Protein engineering to obtain higher selectivity for substrate hydroxylation is challenging, explained by large conformational changes of P450 BM3 during catalysis.[31,32] Against this background, we focussed on P450 BM3 amino acid positions prominent in literature for enhanced regio- and stereoselectivity for different substrates. Amongst others, residues 87 and 328 are known determinants for the regio- and enantioselective hydroxylation with BM3.[7d,33-35] Phenylalanine 87 is directly located in the enzyme's active site and plays a major role in substrate binding and substrate selectivity.[7c,34,36,37] Substitutions of F87 with amino acids carrying smaller side chains result in changes in the active-site volume, thereby enabling the oxidation of unnatural substrates.[7c,37-39] Mutations of residue 328, which sits above the heme cofactor, have been reported to affect substrate binding and substrate specificity of fatty acids as well as linear and cyclic alkanes.[7d,33,40] For example, Arnold and co-workers showed that a A328V substitution shifts the regioselectivity of octane oxidation to 2-octanol with 40% (*S*)-*ee*.[7d] Construction of the F87-library was envisaged by site-saturation-mutagenesis with substitutions to Ala, Gly, Val, Leu, Ile, Ser, Cys, Tyr, His, Asp, Asn, and Arg.[41] P450 BM3 variants F87A and F87V were obtained as described before while the other mutants were generated by QuikChange® PCR (see Supporting Information S2).[12,13] A standard PCR-approach was used for the A328-library generation with primers encoding the amino acids Gly, Val, Leu, Ile, Phe, Tyr, His, Asp, Arg, and Ser. In addition, the generated variants were combined to double variants with the F87A-parent. Screening of the F87-library resulted in overall low conversions and in case of F87D, F87H, F87N, F87R, and F87Y no conversion was detected at all (detailed screening results see S4.3). Interestingly, stressing the strong influence of position 87 on the stereoselectivity, amino acid substitutions for Ile and Leu resulted in wildtype selectivity (Figure 2A), whereas substitutions for Gly, Val and Ser gave the opposite (Figure 2B); *ee*-values remained ≤41% (*S*)-*ee* and ≤21% (*R*)-*ee.* The A328-library screening revealed for the A328-single variants (*R*)-selectivity

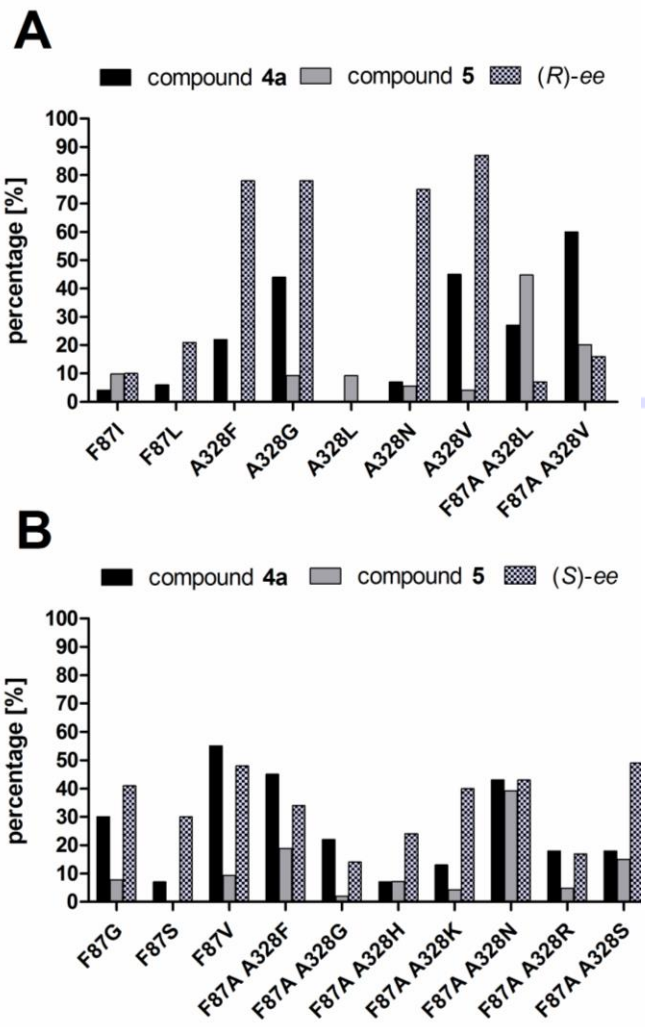


Figure 2. F87/A328-library screening results for the biotransformation of substrate **3a**. P450 BM3 variants resulting in A) (*R*)- and B) (*S*)-*ee* for compound **4a** (dotted grey) considering the amount of product **4a** (black) and by-product **5** (grey). All values were determined by GC analysis.

and A328V, a well-known enzyme variant, was considerably more selective giving an (*R*)-*ee* of 87% (Figure 2A).[42] In summary, the results approved the importance of position 87 and 328 as determinants for improved or inverted selectivity.
In order to enhance the enantioselectivity, a P450 BM3 variant was constructed by combining mutations from the best (*R*)-selective variants: R47Y and A328V. Unfortunately, no cooperative effect in selectivity for the biotransformation of methyl 2-ethylbenzoate (**3a**) was detected. Moreover, it resulted in a slightly decreased selectivity (75% *ee*, 40% conversion) compared to the A328V single mutant (87% *ee*, 48% conversion).

Investigation of P450 BM3-catalyzed C-H activation for the synthesis of isocoumarins

When methyl 2-ethylbenzoate (**3a**) was used as a substrate, the isocoumarin **5** was detected as a by-product because of β-hydroxylation at the ethyl moiety. In addition, we already mentioned the potential of P450 BM3 for the stereoselective synthesis of isocoumarins when methyl 3-propylbenzoate (**7**) was initially studied as a substrate. To further exhibit the oxidative activity of P450 BM3 towards β-hydroxylation of compound **7**, the P450 BM3 libraries were screened regarding regio- and enantioselectivity. As found for substrate **3a**, the presence of an amino acid exchange at position 87 determined the selectivity of the reaction. Screening results of the initial library are shown in Figure 3. As it is the case for substrate **3a**, variants with Phe at position 87 were (*R*)-selective (16-66% *ee*) and mutants with an Ala or Val in the same position showed (*S*)-selectivity (11-69% *ee*, Figure 3A; for detailed screening results see S4.7 and S4.8). We selected four variants of the initial library for the production of each enantiomer of compound **8** (Figure 3A, 1-4 and 5-8) and characterized them in detail regarding conversion, product formation, *ee*-value, and coupling efficiency (Figure 4A and 4B). Reactions with purified enzyme compared to crude cell lysates resulted in overall higher *ee*-values and lower by-product amounts (details see Figure S4.9).

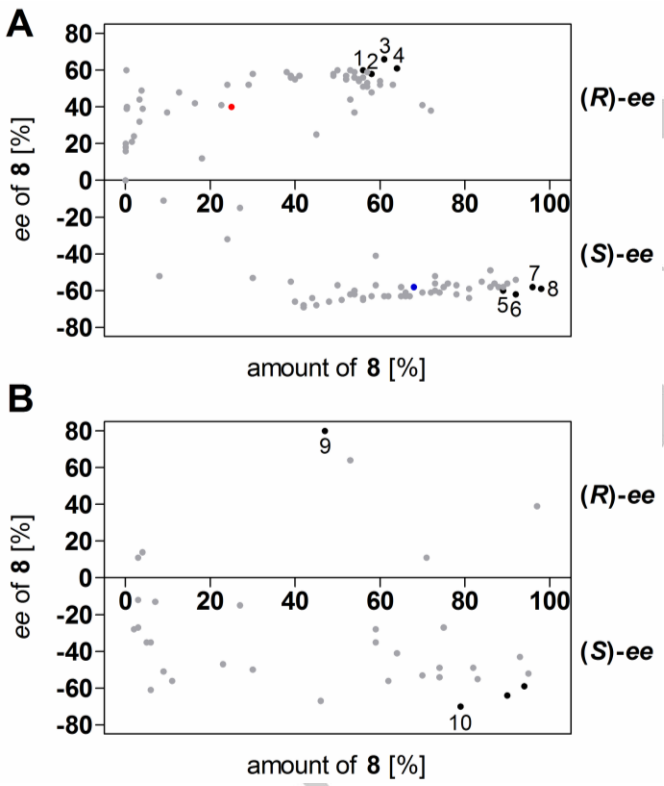


Figure 3. Screening results of the P450 BM3 library for enantioselective hydroxylation of methyl 2-propylbenzoate (**7**). All values were determined by HPLC analysis. A) Screening results of the original library: the wildtype (red) gave (*R*)- and the F87A (blue) (*S*)-selectivity; selected BM3 variants for detailed characterization are highlighted in black (1: R47Y, 2: R47H, 3: R47W, 4: R47V, 5: F87A R47T, 6: F87A Y51P, 7: F87A L188Y, 8: F87A L188V), all other variants are shown in grey. B) Screening results of the F87-/A328-library; selected BM3 variants for detailed characterization are highlighted in black (9: A328G, 10: F87A A328S), all other variants are shown in grey.

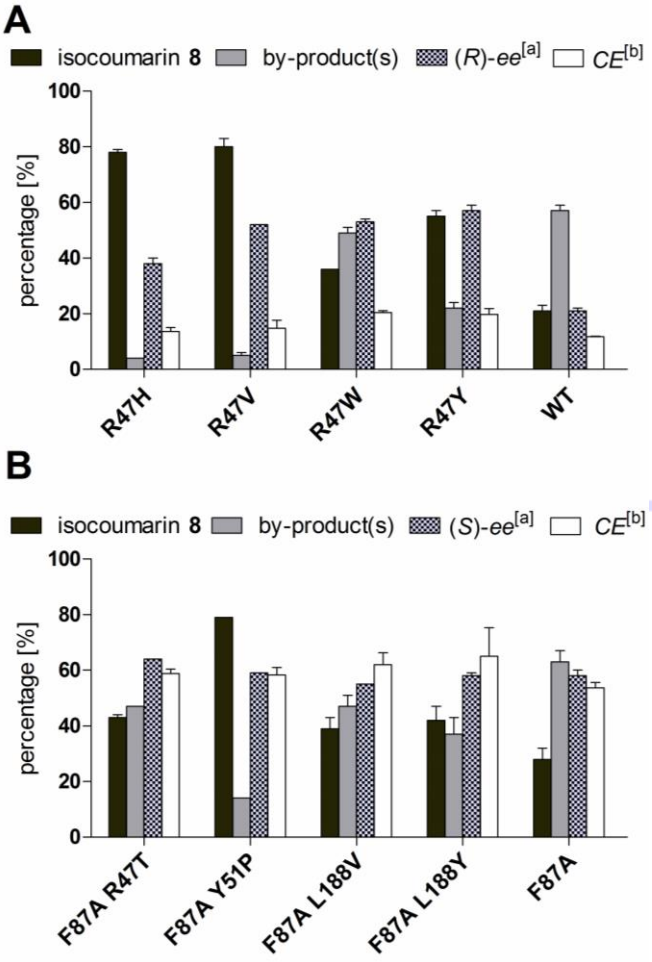


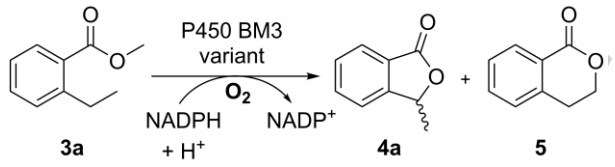
Figure 4. Detailed characterization of A) (*R*)-selective and B) (*S*)-selective P450 BM3 variants for the biotransformation of methyl 2-propylbenzoate (**7**). The wildtype and the F87A mutant were included as references. Uncertainty is given as the standard deviation from three measurements. [a] Determined by HPLC analysis on chiral stationary phase; absolute configuration was assigned by comparison of the rotatory power of synthesized samples with literature; [b] Calculated by GC analysis with previously monitored calibration curves (see S5.1).

The enzyme variant R47V gave the highest amount of (*R*)-configured isocoumarin **8** (80%) with low by-product content (5%). Mutant R47H showed also high amounts of product **8** (78%), but a 2.5-fold *ee*-increase in case of R47V (1.8-fold increase for R47H) relative to the wildtype with an (*R*)-*ee* of 21% favoured the valin-containing variant for further investigations. With regard to the (*S*)-product, mutant F87A Y51P was chosen due to high formation of compound **8** (79%) and low by-product content relative to the F87A mutant as well as other variants (Figure 4B). The *ee* was comparable to the F87A-parent (59% *ee* for F87A Y51P, 58% *ee* for F87A-parent). Screening of the extended F87/A328-library revealed mutant A328G with an improved *ee*-value of 80% with wildtype selectivity (Figure 3B, variant 9) and variant F87A A328S convinced through an *ee*-value of 70% giving the F87A-parent selectivity (Figure 3B, variant 10). Double and triple mutants were constructed by combining A328G with R47V and A328S with the F87A Y51P mutant. The resulting variants were analyzed in detail regarding conversion, product formation and *ee*-value, but unfortunately, as observed for substrate **3a** no additive boost in selectivity could be achieved (S4.10). However, none of the F87-single mutants reached enantioselectivities ≥54% (*S*)-*ee* and ≥64% (*R*)-*ee* (for details see S4.8).

Preparative scale applications for the synthesis of phthalides and isocoumarins

To test the potential of the best mutants for synthetic applications, preparative scale reactions were performed. Reactions were run for 24 h and conversions were calculated by GC or HPLC analysis. For the synthesis of phthalide **4a**, variant F87V L188Q resulted in 9% of the isocoumarin **5**, whereas the other reactions gave the isobenzofuranone **4a** as the only product. The absence of by-products was confirmed by ^{1}H NMR analysis of the crude product. No correlation could be achieved between conversion and yield, as even at almost full conversion a huge loss in yield was noticed. For mutant R47Y only 32% of product **4a** was isolated whereas 98% conversion of substrate **3a** to the desired product **4a** was measured (Table 3, entry 2). In addition, lower conversions and TTNs were obtained for variants A328V and R47Y A328V, the latter resulting in a decreased *ee*-value compared to the analytical scale results (Table 3, entry 4 in comparison to 75% (*R*)-*ee*, 40% conversion). The formation of non-isolated by-products was assumed, which we investigated by time course experiments of substrate conversion and product formation. A preparative enzyme reaction with R47Y was run over 12 h, samples were extracted at different time points and analyzed by GC. No linear correlation between conversion of substrate **3a** and product formation was observed, which might be a consequence of non-homogenous dispersion of the substances in the aqueous reaction mixture (Figure S4.5). Therefore, identical reactions were performed on analytical scale (substrate loading: 10 mM) enabling the extraction of the whole reaction at different time points and the results illustrate a non-linear correlation between substrate conversion and product formation (Figure S4.5). To quantify this observation, 4.4 µmol of substrate **3a** were consumed at a time point of 12 h, but only 1.1 µmol product **4a** were formed. We surmise a polymerization of compounds during the enzyme reaction, which is not detectable by GC or NMR and results in a minor productivity of the applied reaction. Aside from these results, we demonstrated the potential of P450 BM3 for the enantioenriched synthesis of both enantiomers of 3-methyl isobenzofuranones **4a** on preparative scale. The results set the stage for further optimization of the biotransformation.

Table 3. Preparative scale hydroxylation of substrate **3a** using crude cell lysates of the most selective P450 BM3 variants.

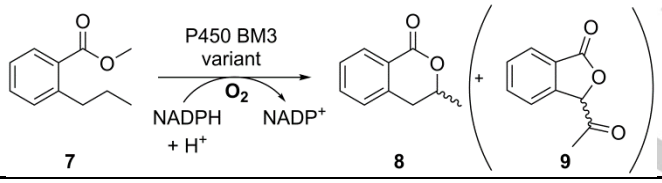


Entry	P450 BM3 variant	Conv. (**4a**:**5**) [%][a]	Yield [%][b]	TTN	*ee* [%][c]
1	F87V L188Q	62 (53:9)	27	825	60 (*S*)
2	R47Y	98 (98:0)	32	978	66 (*R*)
3	A328V	7 (7:0)	n.d.	-	79 (*R*)
4	R47Y A328V	17 (17:0)	12	367	79 (*R*)

[a] Calculated from signal intensities in GC chromatograms; [b] Yield of isolated product **4a**; [c] determined by GC analysis on chiral stationary phase; absolute configuration was assigned by comparison of the rotatory power with literature. Conv.: conversion.

When performing the biotransformation of substrate **7** with mutants listed in Table 4 on preparative scale, poor yields were detected even when moderate amounts of the desired product were formed (Table 4, entry 4 and 5). Similar to the previous case, a possible explanation might be the formation of non-detectable by-products formed during the biotransformation. Overall, product **8** was observed as the main product, but additional signals in the HPLC-chromatograms confirmed the formation of further, less polar by-products, which could not be identified by GC/MS analysis. Flash chromatography of the crude extract yielded compound **8** as the only product and in some cases small amounts of compound **9** were isolated as a by-product, addressing the influence of over-oxidation on preparative scale applications. As found for mutants R47V and A328G, in the preparative scale application minor conversions were obtained compared to analytical scale reactions (Table S4.10, entry 1 and 2: R47V: 87% and A328G: 70% conv. in comparison to Table 4, entry 1 and 2), which might be a consequence of the 5-fold increase in substrate concentration on preparative scale resulting in substrate or product inhibition of the enzyme. Therefore, an extended study to optimize the reaction conditions is envisaged to achieve better productivity of the engineered P450 BM3 variants. When focussing on the

Table 4. Preparative scale hydroxylation of substrate **7** by the most selective P450 BM3 variants using purified enzyme.



Entry	P450 BM3 variant	Conv. (**8**) [%][a]	Yield [%][b]	TTN	*ee* [%][c]	*CE* [%][d]
1	R47V	45 (34)	11	183	68 (*R*)	14.9 ± 2.8
2	A328G	34 (26)	10	167	85 (*R*)	14.3 ± 1.1
3	R47V A328G	26 (9)	n.d.	-	17 (*R*)	6.7 ± 0.1
4	F87A Y51P	>99 (57)	21	350	57 (*S*)	58.3 ± 2.8
5	F87A A328S	98 (74)	27	450	69 (*S*)	25.2 ± 3.4
6	F87A Y51P A328S	86 (70)	52	867	66 (*S*)	33.8 ± 7.9

[a] Calculated from relative signal intensities according to HPLC chromatograms; [b] Yield of isolated product **8**; [c] determined by HPLC analysis on chiral stationary phase; absolute configuration was assigned by comparison of the rotatory power with literature; [d] determined by GC and calculated with previously monitored calibration curves (see S4.1); Conv.: conversion; *CE*: coupling efficiencies.

selectivity, application of double mutant R47V A328G revealed only 17% (*R*)-*ee* with 26% conversion; on analytical scale 68% (*R*)-*ee* and 98% conversion were obtained (Table S4.10, entry 3). The poor coupling efficiency of 7% for substrate **7** emphasizes a loose fixation of the substrate in the active site of the enzyme leading to low selectivities. Noteworthy, the importance of the coupling efficiency for synthetic applications is illustrated by high conversions of substrate **7** observed for the (*S*)-selective mutants (Table 4, entry 4-6), and former preparative scale applications with P450 BM3 reported by our group approved this assumption.[12] Compared to the (*R*)-selective mutants, overall higher coupling efficiencies were obtained (25-58%, Table 4, entry 4-6) and no decrease in selectivity was observed. However, our results demonstrated the first P450 BM3-catalyzed biotransformation on preparative scale towards the synthesis of enantioenriched isocoumarins as prominent building blocks for a variety of natural compounds.[18-22]

Conclusions

In summary, we applied the P450 BM3 monooxygenase for the first time for the regio- and stereoselective synthesis of phthalides and isocoumarins starting from simple 2-alkylated benzoic acid esters on preparative scale. Screening of a set of P450 BM3 variants for the hydroxylation of methyl 2-ethylbenzoate (**3a**) resulted not only in mutants, which gave (*S*)-3-methylisobenzofurane [(*S*)-**4a**] with 30–66% *ee* through α-hydroxylation, but also biocatalysts with opposite enantioselectivity providing the (*R*)-enantiomer with 30–44% *ee* were identified. In case of methyl 3-propylbenzoate (**7**) as a substrate, a shift in the regioselectivity towards β-hydroxylation was observed, yielding 3-methylisocoumarin (**8**) after a spontaneous lactonization of the obtained hydroxylation product. Again, both enantiomers were accessible with up to 69% (*S*)- and 85% (*R*)-*ee* by using different mutants. The synthetic potential of the P450 BM3 catalysts was tested on preparative scale: The observed yields emphasize that synthetic and screening data are difficult to correlate directly. However, our results illustrate the potential of P450 BM3 catalysts giving access to optically active phthalides and isocoumarins as valuable building blocks for a variety of natural compounds. The lack of chemical alternatives towards enantioselective hydroxylation aiming at the synthesis of phthalides and isocoumarins highlights the P450 BM3 monooxygenase as a promising catalyst for future synthetic applications.

Experimental Section

P450 BM3 library generation

For DNA manipulation *E. coli* strain Xl1 Blue was used. The expression plasmid pET28a(+) containing the P450 BM3 wildtype (WT) gene from *Bacillus megaterium* with an additional *Bsu*36I and *Bam*HI restriction site was used as a template for library generation (details see S2).[13] The P450 BM3 F87-library consisting of 12 variants was constructed by exchanging the amino acid codon for F87 within the WT gene for 10 different amino acids codons (C, D, G, H, I, L, N, R, S, and Y) by QuikChange® PCR. The P450 BM3 variants F87V and F87A were obtained as described previously.[12,13] The P450 BM3 A328 library consisting of 24 variants was constructed by exchanging the amino acid codon for A328 within the WT gene for 12 different amino acid codons (D, F, G, H, I, K, L, N, R, S, V, and Y) by PCR-based generation of 12 insert DNA fragments containing the desired mutations and subsequent ligation into the WT DNA-template. For detailed PCR protocols see Supporting Information S2.

Enzyme production, purification and activity measurements

For heterologous protein production, *E. coli* strain BL21 (DE3) was used. Flask- and microtiter plate-expression, purification and quantification of P450 contents were performed as previously described.[12-13] Glucose dehydrogenase (GDH) was generously provided by Prof. W. Hummel (IMET, HHU Düsseldorf) and its activity was determined as described elsewhere.[12]

P450 BM3-catalyzed biotransformations for substrate studies

Reactions were performed in 15 mL reaction vessels using a final volume of 3 mL containing 3 µM P450 BM3 crude cell lysate, 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), 10 mM substrate, 0.3 U GDH, 400 mM glucose, 100 µM $NADP^+$, and 1.8 kU catalase from *Micrococcus lysodeikticus* in 50 mM KPi buffer (pH 7.5). In detail, an O_2-saturated stock solution (introduction of O_2 over 5 min under stirring) containing all components except for substrate was prepared and added to the substrates of interest to initiate the reactions. Oxidations proceeded in closed reaction vessels under an atmosphere of O_2 at 30 °C and 300 rpm for 22 h. Samples were extracted with 1.5 mL chloroform-*d*1 upon acidification with 300 µL of 1 M aq. HCl *via* vortexing for 30 sec and centrifugation (2 min, r.t., 12.000 rpm) to assure phase separation. The organic phase was transferred over a glass pipette filled with $MgSO_4$ into a NMR vial to provide the desired crude extracts for ^1H NMR and GC analysis.

Library screening of substrates 3a and 7

The crude cell extracts for the screening procedure were obtained as described previously.[12] Reactions were performed in 96/2000 µL-deep-well-plates (conical bottom, solvent resistant) using a final volume of 500 µL containing 200 µL P450 BM3 crude cell lysate, 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), 2 mM substrate, 2 vol% DMSO, 0.1 U GDH, 400 mM glucose, 100 µM $NADP^+$, and 300 U catalase from *Micrococcus lysodeikticus* in 50 mM KPi buffer (pH 7.5). In detail, an O_2-saturated stock solution (introduction of O_2 over 5 min under stirring) comprising GDH, glucose, $NADP^+$, catalase, activity and KPi buffer was added to mixtures of the different crude cell lysates with substrate and DMSO to initiate the reactions. The plates were sealed with aluminium foil. Oxidations proceeded at 30 °C and 900 rpm for 22 h and were stopped by addition of 20 µL of 1 M aq. HCl. Samples were extracted by re-sealing the plates upon addition of 400 µL ethyl acetate and incubation at r.t. and 500 rpm for 4 h. Centrifugation (10 min, r.t., 4600 rpm) assured phase separation and allowed transfer of 200-400 µL of the organic phases into new 96/500 µL-deep-well-plates (conical bottom, solvent resistant) containing $MgSO_4$. Participation of the solid by centrifugation (10 min, r.t., 4600 rpm) and transfer of 100-300 µL of the organic phase to 1.5 mL vials provided the desired crude extracts appropriate for GC analysis. For the analysis of the library screening with methyl 2-propyl-benzoate (**7**), 100-300 µL of the organic phase were transferred into a 96/250 µL-deep-well-plate (solvent resistant) and the solvent was evaporated. The remaining residue was dissolved in 300 µL of a mixture of *n*-heptane/2-propanol (90:10) and analyzed by HPLC.

P450 BM3-catalyzed biotransformations for examination of conversion and enantioselectivity

Reactions were performed in 2 mL reaction vessels using a final volume of 1 mL containing 3 µM P450 BM3 (purified sample or crude lysate), 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), 10 mM substrate, 0.1 U GDH, 400 mM glucose, 100 µM $NADP^+$, and 600 U catalase from *Micrococcus lysodeikticus* in 50 mM KPi buffer (pH 7.5) in triplicate. In detail, an O_2-saturated stock solution (introduction of O_2 over 5 min under stirring) comprising the P450 BM3 sample, GDH, glucose, $NADP^+$, catalase, activity and KPi buffer was added to the substrate to initiate the reactions. Oxidations proceeded in closed reaction vessels at 30 °C and 300 rpm for 22 h. Samples were extracted with 500 µL ethyl acetate upon acidification with 20 µL of 1 M aq. HCl *via* vortexing for 30 sec and centrifugation (2 min, r.t., 14.000 rpm) to assure phase separation. Transfer of 200 µL of organic phases into 1.5 mL vials provided the desired crude extracts for analysis. In case of substrate **3a**, GC analyses regarding substrate conversion, product mixture composition and enantioselectivity of benzylic hydroxylation were performed in analogy to the library screening experiment. In case of substrate **7**, conversion measurement and determination of enantioselectivity by HPLC took place as mentioned for the library screening procedure.

Determination of coupling efficiencies

Reactions were performed in 2 mL reaction vessels using a final volume of 1 mL. Purified P450 BM3 samples (3 µM P450 concentration) were mixed with activity buffer (300 µL; 50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0; final concentration 30 vol%) and 400 µM of the substrate (in DMSO) in 50 mM KPi buffer (pH 7.5) in triplicate. In detail, an O_2-saturated stock solution (introduction of O_2 over 1 min) comprising the P450 BM3 sample, activity and KPi buffer was added to the substrate and pre-incubated for 5 min at 300 rpm and 30 °C before reactions were initiated by addition of NADPH (final concentration 400 µM). Oxidations proceeded in closed reaction vessels at 30 °C and 300 rpm for 2 h. Samples were extracted with 500 µL MTBE upon acidification with 50 µL of 1 M aq. HCl *via* vortexing for 30 sec and centrifugation (2 min, r.t., 14.000 rpm) to assure phase separation. In case of both substrates **3a** and **7**, respectively, 250 µL of the organic phase where used for GC analyses. Quantification of substrate conversion was performed with a product-based calibration curve (see Figure S5.1). Different product/substrate-ratios were mixed and the molar ratios determined by ^1H NMR analysis (100 mM solutions of **4a** and **8** in chloroform-*d*1 mixed in different ratios, ≥50% of substrate **3a** or **7**). The product/substrate mixtures were analyzed by GC on a chiral stationary phase and their ratios determined from the obtained chromatograms by integration of starting material and product signals.

Preparative scale biotransformations

*Benzylic hydroxylation of methyl 2-ethyl benzoate (**3a**)*: A sterile, three-necked flask equipped with a cross-shaped magnetic stir bar was charged with the desired P450 BM3 mutant (3 µM crude cell lysate), 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), 100 µM $NADP^+$, 15 U GDH, 400 mM glucose, 36 kU catalase from *Micrococcus lysodeikticus*, and 50 mM KPi buffer (pH 7.5) to a final volume of 210 mL. The solution was saturated with O_2 by introducing the gas for 5 min while stirring and the reaction was initiated by addition of 2.20 mmol substrate **3a**. The flask was immediately attached to a Metrohm 848 Titrino Plus pH stat, which continuously adjusted the reaction pH to 7.5 by addition of 1 M aq. NaOH solution, and the oxidation proceeded at 30 °C within 24 h at 500 rpm. After 6 h, further catalyst (3 µM crude cell lysate), GDH (15 U), and $NADP^+$ (100 µM) were added. Progress was followed *via* the amount of base consumed over time and the reaction was stopped by acidifying with 1 M aq. HCl to pH 4 once the curve slope indicated saturation. The solution was saturated with ammonium sulfate and the proteins were denaturated at 4 °C overnight. The reaction was extracted with MTBE (5×60 mL) and the combined organic phases were dried with $MgSO_4$ and concentrated *in vacuo*. Chromatography on silica gel with *n*-pentane/Et_2O (99:1→80:20) gave the desired product **4a** as a colourless oil. For detailed compound characterization see S7.

*β-Hydroxylation of methyl 2-proyl benzoate (**7**)*: A sterile, three-necked flask equipped with a cross-shaped magnetic stir bar was charged with the desired P450 BM3 mutant (3 µM purified enzyme), 1.35 g bovine serum albumin, 30 vol% activity buffer (50 mM KPi, 50mM Tris-HCl, 250 mM KCl, pH 8.0), 100 µM $NADP^+$, 15 U GDH, 400 mM glucose, 36 kU catalase from *Micrococcus lysodeikticus*, and 50 mM KPi buffer (pH 7.5) to a final volume of 60 mL. The solution was saturated with O_2 by introducing the gas for 5 min while stirring and the reaction was

initiated by addition of 0.60 mmol substrate **7**. The flask was immediately attached to a Metrohm 848 Titrino Plus pH stat, which continuously adjusted the reaction pH to 7.5 by addition of 1 M aq. NaOH solution, and the oxidation proceeded at 30 °C within 24 h at 500 rpm. After 6 h, further catalyst (3 µM purified enzyme), GDH (15 U), and $NADP^+$ (100 µM) were added. Progress was followed *via* the amount of base consumed over time and the reaction was stopped by acidifying with 1 M aq. HCl to pH 4 once the curve slope indicated saturation. The solution was saturated with ammonium sulfate and the proteins were denaturated at 4 °C overnight. The reaction was extracted with MTBE (5×60 mL) and the combined organic phases were dried with $MgSO_4$ and concentrated *in vacuo*. Chromatography on silica gel with *n*-pentane/Et_2O (90:10→80:20) gave the desired product **8** as a colourless oil. For detailed compound characterization see S7.

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Keywords: benzylic hydroxylation • biotransformations • C-H activation • P450 enzymes • protein engineering

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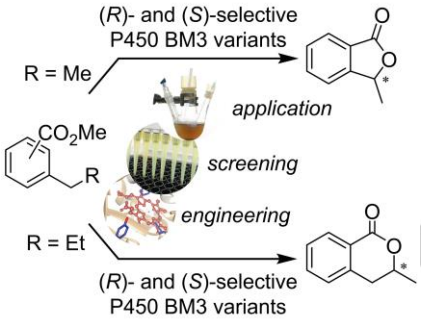
Entry for the Table of Contents

FULL PAPER

Finding the best catalyst: A set of P450 BM3 variants was tested for stereoselective hydroxylation of benzoic acid derivatives aiming at the synthesis of phthalides and isocoumarins. We obtained variants giving access to (*R*)- and (*S*)-configured products constituting a new route to a class of compounds which are valuable synthons for a variety of natural products.



*Claudia Holec, Ute Hartrampf, Katharina Neufeld, Jörg Pietruszka**

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P450 BM3-catalyzed regio- and stereoselective hydroxylation aiming at the synthesis of phthalides and isocoumarins