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Benzylic hydroxylation of aromatic compounds by P450 BM3

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Cytochrome P450 BM3 monooxygenase from *Bacillus megaterium* and its variants are promising catalysts for organic synthesis. Aiming at the identification of variants for selective hydroxylation of functionalised aromatic compounds, the double mutant F87A L188C showed remarkably improved catalytic activity towards a set of tested toluene derivatives. The apparent catalytic efficiency of this variant towards the model substrate methyl 2-methoxy-3-methylbenzoate was 63.6 s^{-1} M^{-1}, which is 535-fold higher compared to that of wild-type BM3. Furthermore, the double mutant selectively catalysed the benzylic hydroxylation of numerous toluene derivatives, especially in the presence of carbonyl- or carboxyl-functions that are directly attached to the aromatic ring. Preparative scale conversion resulted in efficient production of methyl 3-(hydroxymethyl)-2-methoxybenzoate (73% yield) which proved that F87A L188C is a suitable, efficient and sustainable catalyst for the introduction of benzylic hydroxyl groups in general.

Introduction

The growing interest in C–H bond oxyfunctionalisation methods reflects the importance of these transformations, which enable streamlining of synthetic routes by avoiding the need for pre-functionalised compounds and protection group chemistry.[1] Although tremendous efforts have been reported which successfully examine the use of transition metal catalysts for C–H bond activation, the direct oxyfunctionalisation of saturated C–H bonds remains difficult to achieve *via* conventional organic transformations due to their inertness.[2] Ensuring selectivity is thereby a major challenge, since different types of inactivated C–H bonds often coexist with each other and with other functional groups.[3] Furthermore, chemical C–H bond activation by carbonylation or oxygen addition often requires the use of expensive and hazardous reactants and catalysts.[4] The development of convenient oxidative methods using mild reaction conditions and "green" oxidants is a key issue in modern organic synthesis.[5] Monooxygenases as nature's enzymes for oxyfunctionalisation bear a high synthetic potential. Biocatalytic hydroxylations proceed *via* insertion of an oxygen atom from atmospheric dioxygen into C–H bonds. O_2 – as the ideal oxidant – is cost-effective, sustainable and lacks toxic by-products. Therefore, it is a highly appealing reagent to address key challenges in "green" chemistry.[6,7] However, for traditional synthetic applications molecular oxygen is seldom used. Due to its unreactive nature and poor selectivity of the respective transformations, the synthetic scope and utility of existing aerobic reactions is limited. Enzymes are often valued for their application under mild reaction conditions and their high selectivity in terms of chemo-, regio- and stereoselectivity due to their mutable active site environment for substrate coordination, which allows reaction and selectivity control through steric constraints.

The high number of patents dealing with synthetic access to benzylic alcohol derivatives emphasises the relevance of this class of compounds.[8] The corresponding products are widely used as raw materials for the production of pharmaceuticals, food additives, agrochemicals, plasticising agents, polymers and so on.[9] Common methods for their synthesis are mainly based on the transformation of pre-functionalised compounds, requiring additional efforts towards these starting compounds. Extensively-studied metalloporphyrin catalysts mimic nature with promising results for single-step processes.[10] However, these approaches suffer from high catalyst loads, low conversions and insufficient selectivity.[11] The use of transition metal catalysts in their natural form as prosthetic groups tightly-bound to a protein scaffold provides a promising solution to many of the listed synthetic problems. An early example of enzymatic benzylic hydroxylation was shown by Wubbolts *et al.* in 1994 using a xylene oxygenase.[12] Although the broad substrate spectrum of this enzyme was

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demonstrated, its synthetic applicability was not further investigated. The NADPH-dependent P450 BM3 monooxygenase from *Bacillus megaterium* (CYP102A1) belongs to the cytochrome P450 superfamily of oxidoreductases. It is considered to be a promising biocatalyst for synthetic applications and was selected for our studies towards enzymatic hydroxylation of substituted aromatic compounds. Thereby, the reduced cofactor NADPH supplies the electron pair needed for the reductive cleavage of molecular oxygen and generation of oxygenated products and water. As one of the best-studied P450 enzymes (P for pigment – the enzymes are red due to their heme group; 450 – gives the wavelength of the absorption maximum of the enzyme in its reduced state and complexed with CO), this self-sufficient bacterial catalyst is soluble, exhibits remarkably higher activities than comparable monooxygenases,[13] and can be easily provided in high amounts by recombinant expression in *E. coli*.[14] Furthermore, the substrate spectrum of P450 BM3 was intensively explored and significantly expanded through protein engineering which demonstrates the intrinsic versatility of this monooxygenase. In this manuscript we describe our most recent results on the P450 BM3-catalysed production of benzylic alcohol derivatives.

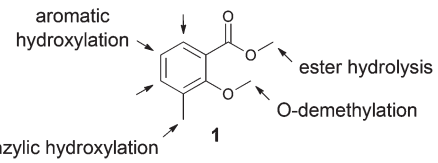


Fig. 1 Possible monooxygenase-catalysed hydroxylations of the model substrate **1** are indicated with arrows.

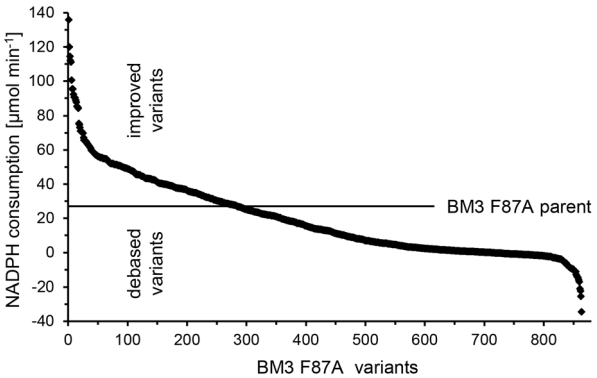


Fig. 2 Activity screen of the generated P450 BM3 F87A pool containing R47SSM, Y51SSM and L188SSM (SSM = site-saturation mutagenesis) sub-libraries towards the model substrate methyl 2-methoxy-3-methylbenzoate (**1**).

Results and discussion

Library generation and activity screening

To investigate the activity and selectivity of P450 BM3 and its variants towards the hydroxylation of functionalised aromatic compounds, a library design based on randomisation of literature-known key residues in the active site was envisaged. Replacement of phenylalanine at position 87 by alanine extended the substrate spectrum towards sterically demanding aromatic compounds by generating additional space above the porphyrin in the active site.[15,16] This F87A variant was subsequently used as the parent for randomisation of three crucial amino acid positions 47, 51 and 188 by site-directed mutagenesis. All three positions have been repeatedly observed to play key roles during substrate binding and in the control of reaction selectivity.[13,17] Corresponding mutants were emphasised in the literature due to their diverse catalytic properties. Mutagenesis of the targeted positions envisaged the generation of a variously featured and highly versatile enzyme library with a broad substrate spectrum and – along with other substrates – methyl 2-methoxy-3-methylbenzoate (**1**) was initially selected as a model substrate. It was synthesised starting from the commercially available 3-methylsalicylic acid by methylation with dimethyl sulphate in 95% yield and simultaneously offers four chemically different types of C–H bonds as well as several aromatic positions for P450 catalysed hydroxylation (Fig. 1), thereby permitting elucidation of the enzymatic preference for specific C–H-bond types and providing knowledge about the tolerance of functional groups. Preliminary investigation of the enzyme library using the NADPH consumption assay indeed proved that compound **1** was a substrate of P450 BM3. Almost one fourth of the investigated variants showed a significant increase in NADPH consumption for **1** when compared to the parent F87A (Fig. 2); BM3 wild-type showed negligible activity.

Overall, substitutions at position 188 proved to be especially beneficial with this sub-library providing the majority of the improved variants. The observed results illustrate the potential and high versatility of the generated mutant library. Sequencing of improved variants revealed that substitution of leucine 188 by alanine, cysteine, isoleucine, and proline increased the ability of P450 to accept substrate **1** up to 7-fold. Therefore, these four variants were selected as promising candidates for further investigation. Noteworthy, according to the MuteinDB database, only one of these amino acid substitutions – the exchange to proline – has been reported previously in P450 BM3 variants related to propane oxidation and coumarin-based screenings.[18]

Product identification

To elucidate the hydroxylation pattern of P450 BM3 regarding the model substrate **1**, the parental mutant F87A was used as the biocatalyst and gave – as suggested by NMR analysis and GC-MS results – the corresponding benzylic alcohol methyl 3-(hydroxymethyl)-2-methoxybenzoate (**2**) as the only product (Fig. 3). This deduction was additionally verified by complete chemical characterisation of the isolated compound. The entire absence of further or side products was a beneficial observation. The resolved hydroxylation pattern is chemically not unexpected due to the activated nature of benzylic C–H bonds, but seldom observed for P450 BM3-catalysed reactions. Benzylic hydroxylation of non-functionalised aromatic

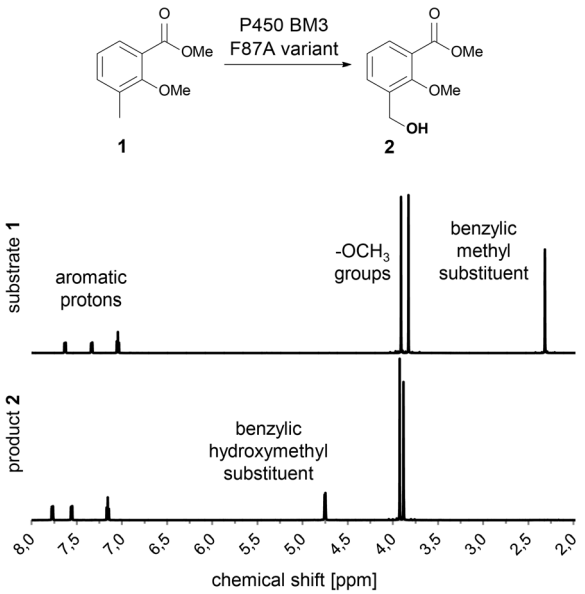


Fig. 3 The F87A variant catalyses selectively the benzylic hydroxylation of substrate **1** providing the corresponding benzylic alcohol product **2**. The shown NMR-spectra of both compounds illustrate the chemical shift of the methyl substituent due to hydroxylation.

compounds often proceeds unselectively and provides product mixtures.[19,20] Aromatic hydroxylations occur often as accompanying reactions.[19,21] To the best of our knowledge, benzylic hydroxylation of functionalised aromatic compounds with P450 BM3 has been described in very few instances. Only for *m*-chlorophenylacetic acid and tolbutamide the corresponding benzylic alcohol products were observed on an analytical scale.[22]

Characterisation of the identified hits

Comparison of the improved variants was performed *via* a detailed enzyme characterisation, which included determination of kinetic constants and coupling efficiencies of the four variants as well as the F87A parent and wild-type reference. K_M and k_{cat} values were determined from Michaelis–Menten graphs of initial NADPH consumption rates at varied substrate concentrations. Coupling efficiency was calculated from the amount of formed product as observed by HPLC compared to the amount of consumed cofactor. Preceding volatility studies ensured that the evaporation step required during sample preparation for the HPLC measurements had no influence on the product to starting material ratio (data not shown). The results in Table 1 show that replacement of phenylalanine at position 87 by alanine significantly improved the K_M value and coupling efficiency of the monooxygenase towards the aromatic model substrate **1**. Furthermore, the high K_M value of the wild-type enzyme explains its low activity within the previous library screening. These improvements – accompanied by an increase of the k_{cat} value – could be even enhanced through additional substitution of Leu188, especially by cysteine or proline. Overall, due to the simultaneous improvement of the catalytic and coupling efficiencies caused by the introduced amino acid substitutions, the apparent catalytic efficiency of P450 BM3 towards the model substrate **1** was improved by two orders of magnitude compared to the wild-type. Determining the "best" double mutant between F87A L188C and F87A L188P based on the available values was difficult. However, against the background of preparative scale application, the cysteine variant was favoured due to higher coupling and this presumption could be experimentally confirmed (see section "Preparative scale application"). Therefore, the F87A L188C double mutant (M2) was selected as a promising catalyst for further investigation on the hydroxylation of substituted aromatic compounds. The obtained k_{cat}, K_M and coupling efficiencies for M2 are comparable to those described in the literature for other non-natural aromatic substrates and are up to several orders of magnitude inferior to those for the natural substrates.[13] However, experimental validation in synthetic applications was required to prove the applicability of M2 as a catalyst.

Investigation of the substrate scope

The high selectivity and productivity of M2 observed for the model substrate **1** suggests that this mutant is a promising catalyst for the synthesis of benzylic alcohol derivatives in general. To analyse its biocatalytic potential for the oxidation of other toluene derivatives – especially functionalised aromatic hydrocarbons – to the corresponding alcohols, the NADPH consumption assay was performed to prove activity towards different potential substrates (Fig. 4a). The screened substrate library included over a dozen compounds, which were selected for their structural and chemical similarity to the model substrate **1**, and included variants, that carried different substituents at the aromatic ring in *ortho*-, *meta*- and

Table 1 Detailed characterisation of the identified P450 BM3 variants and corresponding references

P450 BM3 variant	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]	Coupling efficiency [%]	Substrate oxidation rate [M^{-1} s^{-1}]	Improvement
Wild-type	14.3 ± 3.4	0.17 ± 0.02	12	1 ± 0.0	0.1	1
F87A	3.7 ± 0.3	0.11 ± 0.00	30	11 ± 0.6	3.3	28
F87A L188A	1.2 ± 0.2	0.22 ± 0.01	183	22 ± 0.9	40.3	339
F87A L188C	1.8 ± 0.2	0.44 ± 0.01	244	26 ± 3.1	63.6	535
F87A L188I	4.1 ± 0.4	0.22 ± 0.01	54	18 ± 1.6	9.7	81
F87A L188P	1.2 ± 0.1	0.39 ± 0.01	325	20 ± 1.6	65.0	547

The substrate oxidation rate was calculated by multiplying the catalytic efficiency by the coupling efficiency. The improvement refers to the wild-type.

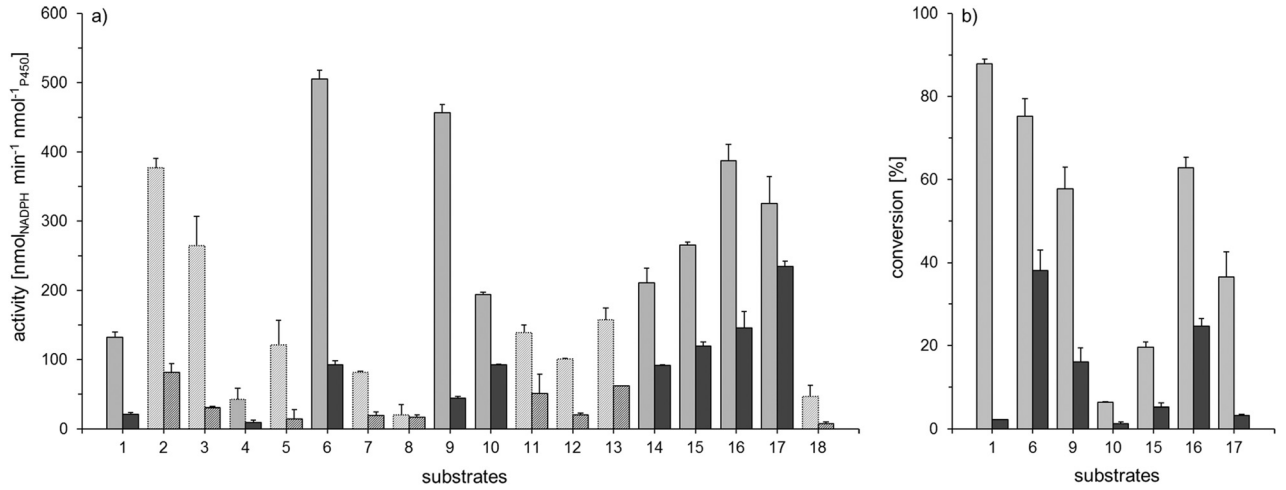
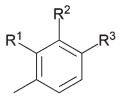


Fig. 4 Substrate screening results for P450 BM3 F87A L188C (grey) and wild-type (black) based on NADPH consumption (a) or conversion measurements (b). Substrates are numbered according to Table 2. Patterned bars indicate no benzylic hydroxylation.

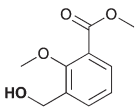
para-position relative to the methyl side chain (Table 2). Activity was detected for every investigated substrate and in all cases M2 showed considerably higher NADPH consumption rates compared to the wild-type BM3. The activity difference towards ethyl 3-methylbenzoate (Table 2, entry 9) was particularly high with the value being 457 $nmol_{NADPH}$ min^{-1} $nmol^{-1}_{P450}$ for the mutant, which is approximately 10-fold higher than the NADPH consumption rate for the wild-type enzyme. Interestingly, half of the investigated compounds showed higher rates compared to the previously examined model substrate **1**. To determine whether the corresponding benzylic alcohol was formed as a product in the course of the reaction, crude extracts of the reaction mixtures were analysed by NMR. Aromatic protons could clearly be assigned besides signals of impurities, which were also extracted from the reaction, and a signal around 4.7 ppm indicated the presence of a hydroxymethyl group. Confirmation of benzylic alcohol formation was performed in most cases *via* comparison of the chemical shifts with authentic standards or published analytical data. The formation of the benzylic alcohol product **5** from ethyl 3-methylbenzoate (Table 2, entry 9) was inferred from the observed NMR signals and subsequently confirmed by isolation and characterisation of this unknown compound. Fortunately, the M2 variant catalysed very selectively the benzylic hydroxylation of half of the examined substrates with an additional product in only one case; most of these substrates provided comparably high NADPH consumption rates in the previous screening. Of note, all toluene derivatives with carbonyl- and carboxyl functions attached to the aromatic ring (Table 2, entries 1, 9, 10, 15, 16 and 17) as well as 3-fluorotoluene (Table 2, entry 4) and 3-trifluoromethyltoluene (Table 2, entry 6) were selectively hydroxylated at the methyl moiety giving the respective benzylic alcohols **2–6** and **9–11**, whereas the oxidation of 4-methoxytoluene (Table 2, entry 14) by the double mutant gave a mixture of *p*-anisyl alcohol (**7**) and the mono-*O*-demethylated product *p*-cresol (**8**). All other substrates were either fully recovered, such as 3-bromotoluene, 3-chlorotoluene, 3-nitrotoluene, 3-methylbenzyl alcohol and toluene (Table 2, entries 2, 3, 5, 8 and 18), or showed products different from benzylic alcohols, such as in the case of 3-hydroxytoluene, 1-(3-methylphenyl)ethanol, 2-methoxytoluene, and 3-methoxytoluene (Table 2, entries 7, 11, 12 and 13). Unsurprisingly, the wild-type enzyme provided significantly lower or no product formation and often deviating product patterns with benzylic hydroxylation in fewer cases. Conclusively, transferability of the previously observed chemoselectivity was successfully proven using a product-based screen. The obtained results suggest an important role of the carbonyl- or carboxyl-moiety within the substrates as their presence strongly influences the substrate acceptance and catalytic selectivity towards the benzylic hydroxylation. However, a correlation between the NADPH consumption rate and the amount of formed product was not found, indicating strong variations of coupling efficiency and monooxygenase stability. A complete absence of product formation despite NADPH consumption is a phenomenon which has already been described in the literature, but still remains to be elucidated.[16] Thus, as the final step, conversion measurements were performed to provide reliable data about the catalytic potential of M2 for benzylic hydroxylation with different substrates (Fig. 4b). This approach allows a combined measurement of catalytic and coupling efficiency with protein stability and performance capacity. Initial studies relying on HPLC analysis failed due to the volatility of the tested substrates. Therefore, conversions were determined by gas chromatographic analysis of reaction extracts for all compounds where benzylic hydroxylation could previously be observed, except for 3-fluorotoluene (Table 2, entry 4; high volatility renders reliable measurements impossible) and 4-methoxytoluene (Table 2, entry 14; formation of product mixtures). M2 gave – as expected – greatly improved conversion rates compared to the wild-type in the order of 2- to 41-fold enhancement and the highest conversion of 88% was observed for model substrate **1**; in four of six cases moderate to good conversions with 75% for 1-methyl-3-(trifluoromethyl)-

Table 2 Tested substrate library and the observed benzylic alcohol derivatives formed during P450 BM3-mediated oxidation

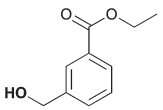


Entry	R^1	R^2	R^3
1	OCH_3	$COOCH_3$	H
2	H	Br	H
3	H	Cl	H
4	H	F	H
5	H	NO_2	H
6	H	CF_3	H
7	H	OH	H
8	H	CH_2OH	H
9	H	$COOCH_2CH_3$	H
10	H	$COCH_3$	H
11	H	$C(OH)CH_3$	H
12	OCH_3	H	H
13	H	OCH_3	H
14	H	H	OCH_3
15	$COOCH_3$	H	H
16	H	$COOCH_3$	H
17	H	H	$COOCH_3$
18	H	H	H

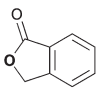
Observed benzylic hydroxylation products



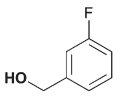
2 (entry 1)



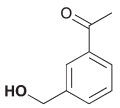
5 (entry 9)



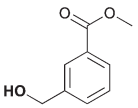
9 (entry 15)[a]



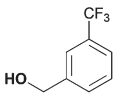
3 (entry 4)



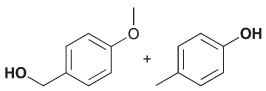
6 (entry 10)



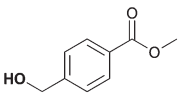
10 (entry 16)



4 (entry 6)



7 + **8** (entry 14)



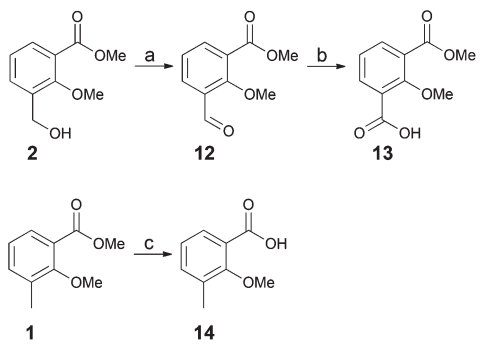
11 (entry 17)

[a] Intramolecular lactonisation as a consequence of hydroxylation.

benzene, 63% for methyl 3-methylbenzoate, 58% for ethyl 3-methylbenzoate and 37% for methyl 4-methylbenzoate were obtained, respectively. Lower conversions were observed for the 3-methylacetophenone and methyl 2-methylbenzoate giving 6% and 20%, respectively. The presented results conclusively demonstrate the suitability of the identified mutant M2 to act as a general catalyst for benzylic hydroxylations. Interestingly, although correlation between NADPH consumption and productivity of P450 BM3 has often been emphasised in the literature, this example contradicts the theory with respect to the comparison of different substrates. This observation stresses the necessity of estimating the productivity of P450 BM3 and its variants for a desired transformation *via* product-based methods.

Preparative scale application

In order to prove the synthetic potential of M2, a preparative scale biotransformation of the initial model substrate **1** was performed. The reaction conditions were based on previously published data related to monooxygenase-catalysed reactions. Crude cell extract containing M2 was applied as the catalyst with a P450 load of 0.03 mol%, whereas Rentmeister *et al.* applied up to 0.1 mol% of purified P450 BM3 and Kühnel *et al.* proved 0.001 mol% to be sufficient for preparative transformations.[23] In addition, a glucose dehydrogenase/glucose system was selected for cofactor regeneration. GDH catalyses the oxidation of β-D-glucose to glucono-1,5-lactone, thereby converting $NADP^+$ to its reduced form and hence providing NADPH for the P450 BM3-catalysed oxidation. No reverse reaction was presumed, since the lactone is unstable under the reaction conditions and spontaneously hydrolyses to β-D-gluconic acid.[24] This regeneration system repeatedly proved itself in combination with P450 BM3 catalysis and alternatives based on glucose-6-phosphate or formate dehydrogenase were either more expensive or commercially unavailable.[25,26] To prevent a decrease of the pH value due to the continuous formation of acid during the reaction process, the controlled addition of base was needed for balancing the pH shift and implemented by using a titrator. At the beginning the reaction mixture was saturated with O_2 to provide sufficient amounts of this component as a reactant, since studies of Schneider *et al.* showed better conversions with higher oxygen concentrations.[27] Furthermore, a catalase from *Micrococcus lysodeikticus* was added to decompose the damaging agent H_2O_2, which is formed by P450 BM3 catalysts as the major uncoupling product upon oxidation of H_2O as a substrate. As published by Maurer *et al.*, addition of this enzyme resulted in higher hydroxylation activity for an extended period of time.[28] Thus, almost full conversion of substrate **1** could be achieved according to HPLC analysis of the crude product and the benzylic alcohol **2** was obtained as the main oxidation product. In addition, a minor signal indicated the presence of a less polar side product. According to GC-MS analysis this side product was supposed to be the corresponding aldehyde **12**. This



Scheme 1 Synthesis of product references. Conditions and reagents: (a) DMP, CH_2Cl_2, 30 min, r.t., quant.; (b) NaH_2PO_4, 2-methyl-2-butene, $NaClO_2$, *t*-BuOH–THF–H_2O, 90 min, r.t., 49%; (c) catalase from bovine liver, KPi buffer (50 mM, pH 7.5), 5d, r.t., quant.

assumption could be proved by comparing the retention times with those of an authentic sample. Therefore, aldehyde **12** was synthesised *via* oxidation of **2** with DMP (Dess–Martin periodinane) in quantitative yield (Scheme 1). An obvious reason why this side product has not been observed previously is the much higher substrate concentration used under the applied reaction conditions as well as the high conversion. Thereby, a much higher concentration of alcohol **2** is reached in the reaction mixture and consequently the product itself can serve as a substrate. From these reflections a higher K_M value of M2 for the alcohol **2** compared to the substrate **1** can be concluded. Even though the obtained HPLC chromatogram did not indicate significant amounts of further products, two additional reference compounds were produced to verify their absence. To examine whether the corresponding acid **13** was also formed during the reaction, the respective reference was produced by Pinnick oxidation of the aldehyde **12** in 49% yield (Scheme 1). Interestingly, no signal was detected indicating its presence. Furthermore, acid **14** was synthesised as a reference compound in quantitative yield for successful exclusion of lipase activity towards substrate **1**, which was assumed to be the most probable side reaction due to the application of crude cell extract (Scheme 1). Therefore, the catalase from bovine liver was used as catalyst for the hydrolysis of substrate **1**, since its hydrolytic activity – most probably attributable to the presence of hydrolytic enzyme impurities in the commercial enzyme preparation – was observed previously in the context of another monooxygenase project (data not shown). Finally, purification of the crude product yielded 73% of the desired alcohol **2** and traces of the aldehyde **12** were isolated (Scheme 2). These results demonstrate that although the apparent catalytic efficiency of M2 towards **1** was rather low (63.6 M^{-1} s^{-1}, which is about six orders of magnitude lower compared to the reported value for P450 BM3 wild-type towards palmitic acid),[29] application of this catalyst for preparative bioconversion was possible and revealed good productivity. The obtained yield is one of the highest ever observed for P450 BM3-catalysed reactions especially with regard to reactions of comparable scale.[23,26] An extended optimisation study would allow further optimisation and the identification of better suited reaction conditions for generating even higher yields. Application of the F87A L188P double mutant under the same conditions gave only 54% conversion and yielded 33% of alcohol **2** as well as traces of the aldehyde **12**. This result illustrates the importance of the coupling efficiency of P450 BM3 catalysts for synthetic applications and confirms the selection procedure applied in the section "Characterisation of the identified hits".

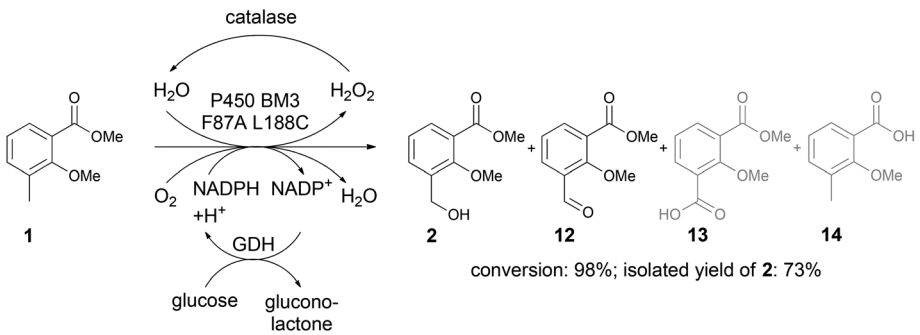
Conclusions

The described biocatalytic approach using the F87A L188C double mutant of P450 BM3 combines direct and selective hydroxylation with an environmentally friendly oxidant under mild reaction conditions and represents a sustainable alternative method for the effective synthesis of benzylic alcohol derivatives. By avoiding highly reactive oxidants and harsh reaction conditions, this approach complements the conventional reduction procedures of pre-functionalised compounds based on active metal or metal hydride reagents, which are often classified as toxic.[30] Furthermore, according to Holland *et al.*, no analogous single-step chemical process can compete with the biocatalytic reaction in terms of selectivity,[3] as chemical oxidation of benzylic C–H bonds suffers from the fact that the alcohol product is often more readily oxidised than the starting material.[31] The reported single-step approach uses cheap and readily accessible toluene derivatives, thereby offering the prospect of a broader substrate scope as well as improved atom-economy.[6] Moreover, application of the described procedure is conceivable for both: biocatalytic steps within a chemical sequence as well as the production of metabolite references.

Experimental section

Chemicals

All chemical reagents were purchased from Sigma-Aldrich, Alfa Aesar, TCI, Applichem or Carl Roth. The $NADP^+$ disodium salt was a gift of Jülich Fine Chemicals. Preparative column chromatography was performed using silica gel 60 (particle size 0.040–0.063 mm, 230–240 mesh). Petroleum ether, EtOAc, diethyl ether and *n*-pentane for column chromatography were



Scheme 2 Preparative scale oxidation of the aromatic model substrate **1** using the identified P450 BM3 M2 catalyst.

distilled prior to use. All other chemicals and solvents were used as purchased without further purification.

Enzymes and spectrophotometric assays

Restriction enzymes with their accompanying buffers were procured from New England Biolabs or Fermentas. *PfuS* DNA polymerase or *Pfu* DNA polymerase were applied in PCR reactions, the latter being a kind gift of Prof. K.-E. Jaeger (IMET, HHU Düsseldorf, Germany). GDH for cofactor recycling was a kind gift of Prof. W. Hummel (IMET, HHU Düsseldorf, Germany) and stored in KPi buffer (20 mM, pH 8.0) at −20 °C upon crude cell extract preparation. Lysozyme from chicken egg white and catalase from bovine liver or from *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich. All spectral experiments were carried out under aerobic conditions at 30 °C. UV/Vis measurements in microtiter plate format were conducted using an Infinite® M1000 or a GENios spectrophotometer plate reader (Tecan) equipped with i-control™ or Magellan™ software, respectively. The reactions were performed in black 96-well flat-bottomed microtiter plates (Grainer Bio-one). Absorbance measurements in cuvette format were conducted using an UV-160 spectrophotometer (Shimadzu) equipped with a CPS controller 2404 temperature control unit. The reactions were performed in disposable cuvettes (1.5 mL volume; 1 cm path length).

Materials and instruments

A thermocycler (Mastercycler® personal, Eppendorf or TProfessional Basic, Biometra) and thin-wall PCR tubes (Thermo Scientific) were used for PCRs. The amount of DNA in cloning experiments was quantified using a spectrophotometer (NanoDrop 1000, peQLab Biotechnology or NanoDrop 2000c, Thermo Fisher Scientific). If required, pH was kept constant at 7.5 during the reaction with aqueous NaOH-solution (1 M) using an 848 Titrino Plus pH stat (Metrohm). Protein purification was performed using an ÄKTApurifier (GE Healthcare) by anion-exchange chromatography on Toyopearl DEAE-650S resin (Tosoh Bioscience). A protein chromatography column was obtained from Kronlab (ECOPLUS glass column, 15 × 125 mm).

Bacterial strains and plasmids

For DNA manipulation and heterologous protein expression, the *E. coli* strains DH5α or BL21 (DE3) were used, respectively. The expression plasmid pET28a(+) containing either the P450 BM3 WT gene from *B. megaterium* or its F87A mutant (cloned *via Nco*I and *Eco*RI restriction sites) was used as template DNA for library generation.

Analytical methods

Analytical TLC was carried out on pre-coated plastic sheets (Polygram® SIL G/UV254, Macherey–Nagel) with detection by ultraviolet irradiation at 245 nm. The Zentralabteilung für Chemische Analysen of the Forschungszentrum Jülich performed elemental analyses. DNA sequencing was performed at MWG-Biotech or GATC Biotech.

^{1}H- and ^{13}C-NMR spectra were recorded on an Advance/DRX 600 nuclear magnetic resonance spectrometer (Bruker) at ambient temperature in $CDCl_3$ at 600 and 151 MHz, respectively. The chemical shifts are given in ppm relative to tetramethylsilane [^{1}H: $\delta(SiMe_4)$ = 0.00 ppm] as an internal standard or relative to the solvent [^{13}C: $\delta(CDCl_3)$ = 77.16 ppm]. NMR signals were assigned by means of H-COSY-, HSQC- and HMBC-experiments.

GC analysis was performed with a CP 9002 gas chromatograph (Chrompack) equipped with a CR 9050 liquid sampler, a flame ionisation detector (FID) and a split injection system, and fitted with a capillary column Optima 5 MS (30 m × 0.25 mm, Macherey and Nagel). The injector and the detector were operated at 250 °C. Hydrogen was used as the carrier gas with a flow rate of 30 mL min^{-1}. The crude products were analysed dissolved in EtOAc according to the following temperature program: 110 °C for 1 min, 5 °C min^{-1} to 150 °C, 150 °C for 4 min. The following retention times were observed (the entries refer to the substrates in Table 2): model substrate **1**: 5.9 min; entry 9: 4.7 min; entry 10: 3.3 min; entry 15: 3.4 min; entry 16: 3.7 min and entry 17: 3.8 min. The retention times for the identified products were: compound **2**: 10.9 min; compound **5**: 9.9 min; compound **6**: 7.7 min; compound **9**: 5.8 min; compound **10**: 8.3 min and compound **11**: 8.5 min. For entry 6 deviating conditions were applied: 40 °C for 5 min, 5 °C min^{-1} to 100 °C, 100 °C for 4 min. The retention times were 4.4 min and 14.3 min for entry 6 and product **4**, respectively.

GC-MS analysis was performed on a HP 6890 series gas chromatograph (Hewlett Packard) equipped with a HP 6890 series injector and a split injection system, fitted with a HP-5 ms column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies) and coupled with a mass selective detector 5973 mass spectrometer. The temperatures of the injector and the detector were fixed at 250 °C and 230 °C, respectively. Helium was used as the carrier gas at 0.57 bar. Mass spectra were collected in the electron impact mode at 70 eV. The column temperature was initially 60 °C for 1 min, then raised to 185 °C at a rate of 15 °C min^{-1}, subsequently raised to 280 °C at a rate of 120 °C min^{-1} and maintained at that temperature for 5 min. Hydroxylated derivatives of substrates were identified by their molecular peak.

HPLC separations were performed with a Jasco System consisting of a PU-2080 plus pump, a LG-2080-08S gradient unit, a DG-2080-53 degasser and an AS-2057 plus sampler equipped with a MD-2010 plus multiwavelength detector and using a HyperClone™ ODS (C18) column (125 × 4 mm, 5 μm, 120 Å, Phenomenex). For all measurements the column temperature was 25 °C, the flow rate was 0.5 mL min^{-1} and 220 nm was used as the detection wavelength. Mixtures of ACN and H_2O were used as solvents and the elution programs are mentioned hereafter.

Synthesis of the model substrate and reference compounds

Methyl 2-methoxy-3-methylbenzoate (**1**) was synthesised according to a published procedure.[32] Under an atmosphere of dry

nitrogen, anhydrous K_2CO_3 (197.10 mmol), anhydrous $MgSO_4$ (32.85 mmol) and dimethyl sulphate (131.40 mmol) were added to a solution of 3-methylsalicylic acid (65.72 mmol) in dry acetone (200 mL). The solution was stirred for 10 h and then refluxed for 24 h. In the case of incomplete conversion, further portions of dimethyl sulphate were added. After cooling to r.t., methanol (100 mL) was added and the mixture was stirred vigorously for 1 h. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. 200 mL EtOAc were added to the crude product and the organic phase was successively washed with water, aqueous HCl-solution (2 M), water and brine (100 mL each), subsequently dried with $MgSO_4$ and the solvent was again removed under reduced pressure. Chromatography on silica gel with petroleum ether–EtOAc (19 : 1) provided compound **1** (11.197 g, 95%) as a colourless oil. The analytical data were consistent with those previously reported.[33–35] R_f = 0.67 (petroleum ether–EtOAc, 7 : 3); IR (film): 3073, 2992, 2951, 2861, 2841, 2820, 1726, 1592, 1468, 1434, 1417, 1380, 1293, 1260, 1230, 1192, 1173, 1135, 1089, 1008, 977, 876, 819, 774, 730 cm^{-1}; ^1H NMR: δ = 2.32 (s, 3 H, CH_3), 3.83 (s, 3 H, OCH_3), 3.91 (s, 3 H, $COOCH_3$), 7.05 (dd, J = 7.8, 7.5 Hz, 1 H, 5-H), 7.34 (dd, J = 7.5, 1.3 Hz, 1 H, 4-H), 7.63 (dd, J = 7.8, 1.3 Hz, 1 H, 6-H) ppm; ^{13}C NMR: δ = 16.1 (CH_3), 52.5 ($COO\underline{C}H_3$), 61.6 (OCH_3), 123.6 (C-5), 124.7 (C-1), 129.2 (C-6), 132.8 (C-3), 135.2 (C-4), 158.5 (C-2), 167.0 ($\underline{C}OOCH_3$) ppm; MS: m/z (%): 180 (57), 165 (9), 162 (10), 151 (29), 149 (100), 147 (62), 133 (32), 119 (47), 105 (24), 91 (58), 77 (21), 65 (12), 51 (11).

Methyl 3-formyl-2-methoxybenzoate (**12**) was synthesised according to a published procedure.[35] Dess–Martin periodinane (2.55 mmol) was added at ambient temperature to a solution of methyl 3-(hydroxymethyl)-2-methoxybenzoate (**2**) (2.04 mmol) in CH_2Cl_2 (15 mL) and the mixture was stirred for 30 min. Excess oxidant was quenched by the addition of saturated $Na_2S_2O_3$- and saturated $NaHCO_3$-solution (6 mL each) and vigorous stirring for 45 min. Subsequently, the mixture was extracted with CH_2Cl_2 (3 × 15 mL), the combined extracts were dried with $MgSO_4$ and the solvent was removed under reduced pressure to provide the desired aldehyde **12** (397 mg, quant.) as a yellowish oil. The analytical data were consistent with those previously reported.[36] R_f = 0.50 (petroleum ether–EtOAc, 7 : 3); mp close to r.t.; IR (film): 3076, 3001, 2953, 2868, 2749, 1728, 1687, 1584, 1469, 1460, 1435, 1423, 1386, 1294, 1270, 1245, 1192, 1173, 1130, 1080, 996, 895, 879, 817, 789, 769, 736, 695, 665 cm^{-1}; ^1H NMR: δ = 3.97 (s, 3 H, $COOCH_3$), 4.02 (s, 3 H, OCH_3), 7.29 (ddd, J = 7.7, 7.7, 0.7 Hz, 8.03 (dd, J = 7.7, 1.9 Hz, 1 H, 4-H), 8.09 (dd, J = 7.7, 1.9 Hz, 1 H, 6-H), 10.45 (d, J = 0.7 Hz, 1 H, COH) ppm; ^{13}C NMR: δ = 52.7 ($COO\underline{C}H_3$), 65.1 (OCH_3), 124.2 (C-5), 125.6 (C-1), 130.5 (C-3), 132.6 (C-4), 137.8 (C-6), 163.4 (C-2), 165.6 ($\underline{C}OOCH_3$), 189.4 (COH) ppm; MS: m/z (%): 194 (32), 163 (68), 149 (59), 147 (32), 135 (53), 134 (41), 133 (92), 120 (38), 119 (58), 105 (100), 92 (32), 91 (43), 77 (96), 76 (55), 75 (33), 65 (19), 63 (60), 51 (30), 50 (36).

2-Methoxy-3-(methoxycarbonyl)benzoic acid (**13**) was synthesised according to a published procedure.[37] NaH_2PO_4 (1.63 mmol) and 2-methyl-2-butene (7.32 mmol) were added at ambient temperature to a solution of methyl 3-formyl-2-methoxybenzoate (**12**) (1.63 mmol) in a *tert*-butanol–THF–water-mixture (15 mL/5 mL/4 mL). Sodium chlorite (5.70 mmol) was slowly added and the reaction mixture was stirred for 90 min. The mixture was quenched with aqueous HCl-solution (3 mL, 1 M) and extracted with EtOAc (4 × 20 mL). The combined extracts were washed with brine (30 mL), dried with $MgSO_4$ and the solvent was removed under reduced pressure. Chromatography on silica gel with petroleum ether–EtOAc + AcOH (9 : 1 + 1% (v/v)) provided acid **13** (168 mg, 49%) as a white solid. The analytical data were consistent with those previously reported.[38] R_f = 0.20 (petroleum ether–EtOAc + AcOH, 8 : 2 + 1% (v/v)); mp 101 °C; calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80; found: C, 57.20 ± 0.10; H, 4.86 ± 0.02; IR (film): 2956, 2585, 1728, 1679, 1589, 1467, 1420, 1403, 1311, 1292, 1245, 1205, 1181, 1162, 1124, 1090, 997, 982, 911, 865, 839, 823, 777, 767, 715, 690 cm^{-1}; ^1H NMR: δ = 3.97 (s, 3 H, $COOCH_3$), 4.05 (s, 3 H, OCH_3), 7.33 (dd, J = 7.8, 7.7 Hz, 1 H, 5-H), 8.07 (dd, J = 7.7, 1.8 Hz, 1 H, 4-H), 8.24 (dd, J = 7.8, 1.8 Hz, 1 H, 6-H) ppm; ^{13}C NMR: δ = 52.8 ($COO\underline{C}H_3$), 64.5 (OCH_3), 123.9 (C-3), 124.7 (C-5), 125.4 (C-1), 136.9 (C-4), 137.0 (C-6), 159.7 (C-2), 165.5 ($\underline{C}OOCH_3$), 166.6 (COOH) ppm; MS: m/z (%): 210 (8), 209 (11), 192 (17), 179 (53), 177 (60), 149 (100), 147 (64), 133 (15), 119 (37), 105 (27), 91 (20), 77 (21), 65 (26), 51 (10).

2-Methoxy-3-methylbenzoic acid (**14**) was synthesised by adding catalase from bovine liver (50 mg, 148.5 kU) to a solution of methyl 2-methoxy-3-methylbenzoate (**1**) (1.22 mmol) in KPi buffer (50 mL, 50 mM, pH 7.5) and the mixture was stirred for 5 d at ambient temperature. Subsequently, the mixture was acidified to pH ≈ 1 with an aqueous HCl-solution (30 mL, 1 M) and filtered over celite. The aqueous phase was extracted with CH_2Cl_2 (4 × 30 mL), the combined extracts were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Chromatography on silica gel with petroleum ether–EtOAc + AcOH (9 : 1 + 1% (v/v)) provided acid **14** (207 mg, quant.) as a white solid. The analytical data were consistent with those previously reported.[33,34] R_f = 0.32 (petroleum ether–EtOAc + AcOH, 9 : 1 + 1% (v/v)); mp 83 °C; IR (film): 3064, 3001, 2953, 2924, 2859, 2827, 2657, 2566, 1751, 1690, 1592, 1467, 1423, 1405, 1376, 1308, 1229, 1281, 1192, 1163, 1090, 1001, 977, 916, 822, 811, 765, 742, 725 cm^{-1}; ^1H NMR: δ = 2.37 (s, 3 H, CH_3), 3.92 (s, 3 H, OCH_3), 7.17 (dd, J = 7.8, 7.5 Hz, 1 H, 5-H), 7.43 (dd, J = 7.5, 1.3 Hz, 1 H, 4-H), 7.95 (dd, J = 7.8, 1.3 Hz, 1 H, 6-H) ppm; ^{13}C NMR: δ = 16.1 (CH_3), 62.2 (OCH_3), 122.2 (C-1), 125.0 (C-5), 130.8 (C-6), 131.9 (C-3), 137.1 (C-4), 158.1 (C-2), 167.2 (COOH) ppm; MS: m/z (%): 166 (47), 149 (18), 148 (37), 137 (30), 133 (53), 119 (100), 105 (37), 93 (29), 91 (76), 78 (27), 77 (64), 65 (17), 51 (31).

Site-saturation library generation and mutant validation

Three sites around the substrate binding channel (Arg47, Tyr51, Leu188) were randomised by site-directed saturation mutagenesis using a QuikChange protocol to create the desired mutant library. Plasmid pET28a(+) P450 BM3 F87A was used as the template and the primers for each saturation library contained all possible codons (NNN, N = A, T, G, C) at

the position for the particular residue. The sequences of the forward primers used were: for R47: 5′-GGAGAAATCTTT-AAATTCGAGGCGCCTGGTNNNGTAACGCGC-3′; for Y51: 5′-GC-CTGGTCGTGTAACGCGCNNNTTATCAAGTCAGCGTCTA-3′; for L188: 5′-GATGAAGCAATGAACAAGNNNCAGCGAGCAAATCCAG-ACG-3′. The reverse primers complement their corresponding forward primer. The PCR reaction was performed *via* a 2-step-protocol. In the first step, forward and reverse primers were applied separately to PCR mixtures. For randomisation of position 51 and 188 both PCR mixtures contained *PfuS* DNA polymerase (0.25 μL, 5 U μL^{-1}), buffer (2.5 μL, 10×), dNTPs (0.5 μL, 10 mM each), either forward or reverse primer (2 μL, 5 μM), template DNA (≈ 25 ng) and water (added to a total volume of 25 μl). The PCR conditions were 30 s at 98 °C, followed by 4 cycles of 10 s at 98 °C, 30 s at 60 °C and 3 min at 72 °C. Both PCR mixtures were combined after cooling to 12 °C and thermocycled for 13 further cycles to perform the second round of PCR. Finally, an extension step at 72 °C for 5 min was applied. For randomisation of position 47 the *Pfu* DNA-polymerase (0.25 μL, 5 U μL^{-1}) was employed and DMSO (1.25 μL, 5% (v/v)) was added to the reaction mixtures. The PCR conditions were adjusted to 30 s at 95 °C, followed by cycles of 30 s at 95 °C, 60 s at 55 °C and 24 min at 68 °C. The final extension step was conducted at 68 °C for 40 min. The PCR product solution was purified using a NucleoSpin® Extract II Kit (Macherey–Nagel) and subsequently treated with *Dpn*I to remove template DNA (50 U, 15 h at 37 °C). Agarose gel electrophoresis confirmed the presence of a product band. After another purification step initial cloning was done in *E. coli* strain DH5α, which gives high transformation efficiency and good plasmid yield, followed by heterologous expression in BL21 (DE3) cells. Standard heat shock procedures and chemically-competent cells were employed. The DH5α transformants were selected by overnight incubation at 37 °C in LB medium supplemented with kanamycin sulphate (50 μg mL^{-1}) and the initial quantity of transformants in these cultures (>600 per randomised position) was simultaneously controlled by plating spot checks on LB_{Kan} agar plates. Plasmid DNA isolation from *E. coli* cultures was performed using the QIAprep Spin Miniprep Kit (Qiagen). Application to the above mentioned cultures gave the desired plasmid DNA libraries for each targeted position. Accordingly, cultures of improved mutants gave the desired plasmid samples containing the gene of interest. To ensure the absence of any spurious mutations within the identified improved variants, the genes encoding each mutant protein were fully sequenced using the following oligonucleotides: Primer 1 forward (T7_promotor): 5′-TAATACGACTCACTATAG-GG-3′; Primer 2 forward: 5′-GCCGCTTGATGACGAGAAC-3′; Primer 3 forward: 5′-CAGGCTGCAACGCTTGATTC-3′; Primer 4 reverse (T7_terminator): 5′-CTAGTTATTGCTCAGCGG-3′.

Cultivation and expression of native and mutant CYP102A1

P450 BM3 WT and its mutants were heterologously produced in *E. coli* strain BL21 (DE3) using the pET28a(+) expression vector according to published procedures.[39]

Enzyme library expression in microtiter plates. Cloning of the generated plasmid DNA libraries was done in *E. coli* strain BL21 (DE3) and the transformants were cultivated on LB_{Kan} agar plates at 37 °C. After 14 h of growth, single clones were transferred, by using toothpicks, into flat-bottomed 96-well microtiter plates (Nunc) containing LB_{Kan} medium (150 μL per well). To ensure almost complete coverage of all possible substitutions within the library, 270 clones per targeted position were chosen. WT and F87A parent mutant samples as well as empty vector controls were added in duplicate onto each plate as references. After being shaken at 70% humidity, 900 rpm and 37 °C for 15 h to grow the precultures to saturation, an aliquot (10 μL per well) was used to inoculate expression cultures and the plates were stored with addition of glycerol (50% (v/v) in water, 100 μL per well) as master plates at −80 °C. Replicates were generated *via* inoculation of microtiter plates containing LB_{Kan} media (150 μL per well) with a 96-pin replicator starting from defrosted master plates. Expression was performed in deep-well plates (96 × 2 mL, Eppendorf), each well containing TB medium (500 μL) supplemented with kanamycin sulphate (50 μg mL^{-1}), isopropyl-β-D-thiogalactoside (IPTG) (0.5 mM), δ-aminolevulinic acid hydrochloride (0.5 mM), thiamine hydrochloride (100 μg mL^{-1}) and trace element solution (0.5 μL containing 3.4 mM $CaCl_2$, 0.6 mM $ZnSO_4$, 0.6 mM $MnSO_4$, 54.0 mM Na_2–EDTA, 61.8 mM $FeCl_3$, 0.6 mM $CuSO_4$ and 0.8 mM $CoCl_2$ in water). Clones were cultivated at 70% humidity, 900 rpm and 30 °C for 24 h and the cells were harvested by centrifugation (4 °C, 4000 rpm, 15 min). After discarding the supernatants, the cells were stored at −20 °C until further use.

Preparation of enzyme variants used for characterisation and hydroxylation experiments. LB_{Kan} agar plates were streaked with *E. coli* BL21 (DE3) transformants harboring the pET28a(+) vector containing the desired P450 BM3 variant gene. Single clones were used to inoculate LB_{Kan} cultures (10 mL), which were grown at 37 °C for 14 h. Baffled shaking flasks (3 L) containing enriched TB media (1 L, supplemented as described previously except for the IPTG concentration, which was 0.1 mM in this case) were inoculated with these overnight cultures to an OD_{600} value of 0.04. Growth and expression was performed at 120 rpm and 25 °C for 36 h. The cells were harvested by centrifugation (4 °C, 4000 rpm, 15 min) and stored at −20 °C until further use.

Preparation of crude cell extracts

To obtain crude cell extracts for bioconversion experiments, the collected *E. coli* cells were thoroughly resuspended in KPi buffer (10–30% (w/v), 50 mM, pH 7.5) and disrupted using a French press (Thermo Electron) equipped with a pressure cell (Glen Mills) at 1000–1500 psi for 1–2 cycles. The cell extract was recovered by centrifugation (4 °C, 20 000 rpm, 10 min) and used for further experiments.

NADPH consumption assay

Library screening. To obtain crude cell extracts for the screening procedure, the harvested *E. coli* pellets in the

deep-well plates were thoroughly resuspended in KPi buffer containing lysozyme (240 μL per well, 5 mg mL^{-1} lysozyme, 50 mM, pH 7.5). After incubation for 60 min at 900 rpm and 37 °C, the plates were centrifuged (4000 ppm, 15 min, 4 °C) and the supernatants were used in the activity assay. The catalytic activity of P450 BM3 variants towards the substrate of interest was measured spectrophotometrically by monitoring the rate of NADPH consumption *via* its fluorescence (λ_{ex} = 340 nm; λ_{em} = 460 nm) according to published procedures.[40] The assay was conducted in 96-well microtiter plates. The assay solutions containing crude cell extract (100 μL; if necessary diluted with 50 mM KPi buffer pH 7.5 to obtain quantifiable activities), a buffer mixture (60 μL, 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0) and substrate (20 μL, 20 mM in DMSO) were preincubated for 5 min at 900 rpm and r.t. Subsequently, the reaction was started by the addition of NADPH (20 μL, 3 mM in KPi buffer 50 mM, pH 7.5) and the decrease in NADPH concentration was monitored for 5 min with an average 13 s interval between each reading cycle. The observed initial NADPH consumption rates were corrected for the background NADPH oxidation in the absence of substrate. NADPH turnover was calculated using a calibration curve, which was obtained by measuring the fluorescence of defined NADPH concentrations under the same conditions. Improved clones that showed higher NADPH consumption rates than the parent mutant (10 clones per plate) were rescreened in triplicate following the same procedure, but this time the previously generated master plates were employed to inoculate overnight culture plates with the potentially interesting variants. Mutants exhibiting high activities during the rescreening were sequenced. Overall, mutants of the 188 sub-library were especially beneficial and exchanges of Leu by Ala, Cys, Glu and Pro (with codon changes from CTG to GCG, TGC, GAA and CCG, respectively) were identified.

Screening of further toluene derivatives. To elucidate the activity of P450 BM3 M2 regarding a set of toluene derivatives, a NADPH consumption assay was performed. The activity assay was conducted following the procedure described above, but this time the cell disruption was performed as described in the section "Preparation of crude cell extracts" and defined monooxygenase concentrations of the P450 BM3 M2 mutant and the WT enzyme were applied. All measurements were performed in duplicate. The assay solutions contained crude cell extract (100 μL, 0.3 μM for the mutant and 0.7 μM for the WT enzyme, respectively; dilution of the initial extract with 50 mM KPi buffer pH 7.5), a buffer mixture (60 μL, 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0), substrate (20 μL, 100 mM in DMSO) and NADPH (20 μL, 1 mM in KPi buffer 50 mM, pH 7.5). The obtained activity values were further normalised by the applied monooxygenase concentration.

Purification of CYP102A1

P450 BM3 variants were purified following a published procedure.[41] To obtain crude cell extracts for protein purification, lysis was performed in Tris–HCl buffer (10–30% (w/v), 10 mM, pH 7.8). The extract was recovered by centrifugation (4 °C, 20 000 rpm, 60 min) and further cleared by filtration through a low protein-binding filter (0.45 μm). P450 BM3 was purified using Tris–HCl buffer (buffer A: 100 mM, pH 7.8; buffer B: 100 mM, pH 7.8 containing 2 M NaCl). The elution profile used was as follows (CV = column volume): washing: 3 CV with 0% buffer B and 3 CV with 3% buffer B steps; elution: 3.5 CV with 3–9.3% buffer B gradient.

Quantification of CYP102A1

P450 BM3 concentrations in crude extract or purified samples were determined in triplicate from CO-binding difference spectra of the reduced heme iron using an extinction coefficient of ε = 91 mM^{-1} cm^{-1} according to Omura and Sato.[42] The protein sample (100–1000 μL) was filled up to 2 mL with KPi buffer (50 mM, pH 7.5) in a 2 mL reaction tube and a methyl viologen solution (7 μL, 1% (w/v) in water) as well as a few grains of sodium dithionite were added. Mixing by inverting the reaction tube several times yielded a blue solution, half of which was transferred to another reaction tube and saturated with CO by aeration for 1 min. Subsequently, the absorption of both samples at 450 and 500 nm was measured to determine the monooxygenase concentration.

Determination of k_{cat} and K_M values for CYP102A1

The kinetic constants of P450 BM3 variants towards substrate **1** were determined based on the NADPH consumption assay. The measurements were done in triplicate and performed as described in the section "NADPH consumption assay". Besides the four identified mutants, the parent mutant F87A and the WT enzyme were tested as references. The final monooxygenase concentration in the reaction mixture was chosen individually for each mutant to allow detection of quantifiable activities. Substrate concentrations were varied from 1.0–22.5 mM in the reaction mixture and background activity was measured in the absence of substrate. The reaction mixtures containing purified enzyme (160 μL, 0.6–3.3 μM; purified samples were diluted with Tris–HCl buffer 100 mM, pH 7.8 supplemented with 180 mM NaCl to obtain quantifiable results) and gradient concentrations of substrate (20 μL, 10–225 mM dissolved in DMSO) were preincubated for 5 min at 900 rpm and r.t. Subsequently, the reactions were initiated by the addition of NADPH (20 μL, 3 mM in KPi buffer 50 mM pH 7.5) and the decrease in NADPH concentration was monitored for 2 min. Kinetic constants were estimated by plotting initial NADPH consumption rates *versus* applied substrate concentrations and fitting the values to a one-site binding hyperbola equation (Michaelis–Menten equation) by non-linear regression using Origin 7G software. In view of the stoichiometric incorporation of heme in P450 BM3, all catalytic rates were normalised to the heme content measured using CO-difference spectroscopy.

Coupling efficiency of CYP102A1

To measure the coupling efficiency of P450 BM3 variants towards substrate **1**, purified monooxygenase (580 μl, 2.6 μM; purified samples were diluted with Tris–HCl buffer 100 mM,

pH 7.8 supplemented with 180 mM NaCl) was diluted with a buffer mixture (300 μL, 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0) and mixed with the substrate (20 μL, 20 mM in DMSO) in a 2 mL reaction tube. After preincubation for 5 min at 300 rpm and 30 °C, NADPH (100 μl, 2 mM in KPi buffer 50 mM, pH 7.5) was added and the sample was incubated for 2 h at 30 °C and 300 rpm to allow complete cofactor consumption. Subsequently, the reaction mixture was acidified with aqueous HCl-solution (50 μL, 1 M) and extracted with MTBE (500 μL). The organic layer was partially (300 μL) transferred into the well of a polypropylene microtiter plate and evaporated within 90 min at r.t. The crude product was solved in ACN (200 μL) and the sample subjected to HPLC analysis. The system was run with ACN–H_2O (40 : 60) and under these conditions the alcohol **2** eluted at 3.2 min and the substrate **1** at 8.4 min. The amount of product formed was calculated based on the observed signals and using a calibration curve, which was obtained by measuring defined substrate-to-product mixtures. The coupling efficiency was calculated as the proportion of the amount of product formed to the amount of NADPH consumed. All measurements were performed in triplicate and control experiments were carried out in the absence of substrate and enzyme, respectively.

Product analysis

Bioconversion of methyl 2-methoxy-3-methylbenzoate (1). The bioconversion was performed on a 7 mL scale in a closed solvent-resistant 50 mL reaction vessel at 300 rpm and 30 °C in KPi buffer (50 mM, pH 7.5). The reaction mixture contained P450 BM3 F87A (11.6 μM; crude cell extract), a buffer mixture (30% (v/v), 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0), substrate (40 mM, 283 μmol), DMSO (1% (v/v)) and $NADP^+$ (100 μM, 3 mM in KPi buffer 50 mM, pH 7.5) as well as GDH (10.5 U) and glucose (400 mM, 3.3 M in KPi buffer 50 mM, pH 7.5, sterile filtered) for cofactor regeneration. After incubation for 4 h the reaction mixture was acidified with aqueous HCl-solution (350 μL, 1 M) and extracted with EtOAc (3 × 10 mL) using a centrifugation step (r.t., 10 000 rpm, 10 min) to facilitate phase separation. The extracts were combined, dried with $MgSO_4$ and concentrated by evaporation under reduced pressure. The crude product was analysed by TLC, GC-MS and NMR, whereby all methods indicated the presence of a single hydroxylation product. Flash chromatography on silica gel with petroleum ether–EtOAc (9 : 1 → 1 : 1) provided methyl 3-(hydroxymethyl)-2-methoxybenzoate (**2**) (3.5 mg, 18 μmol, 6%, brsm 9%) as an orange oil.

Bioconversion of further toluene derivatives. To elucidate the hydroxylation pattern of P450 BM3 M2 regarding a set of toluene derivatives, a NMR based screening with the focus on the formation of benzylic alcohol products was conducted. Furthermore, the WT enzyme was also tested as a reference to allow the evaluation of the double mutant as a highly improved biocatalyst. The bioconversion was performed on a 10 mL scale in a closed solvent-resistant 50 mL reaction vessel at 300 rpm and 30 °C in KPi buffer (50 mM, pH 7.5). The reaction mixture contained P450 BM3 WT or M2 (2.5 μM; crude cell extract), a buffer mixture (30% (v/v), 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0), DMSO (1% (v/v)) and $NADP^+$ (100 μM, 50 mM in KPi buffer 50 mM, pH 7.5) as well as GDH (5.0 U) and glucose (400 mM, 3.3 M in KPi buffer 50 mM, pH 7.5, sterile filtered) for cofactor regeneration and catalase (6 kU) for detoxification. Potential substrates were added to a final concentration of 10 mM (100 μmol). The solution was gas-flushed with molecular oxygen for 2 min and the resulting reaction mixture was incubated for 24 h. After addition of aqueous HCl-solution (500 μL, 1 M), the reaction was extracted with $CDCl_3$ (1.2 mL) using a centrifugation step (r.t., 10 000 rpm, 10 min) to facilitate phase separation. The extract was dried with $MgSO_4$ and subjected to NMR and GC-MS analysis. Control experiments were carried out in the absence of substrate and with empty vector crude cell extracts, respectively, to exclude catalysis by host-derived factors. Commercially available authentic standards were used to confirm the formation of isobenzofuran-1(3*H*)-one **9**, *p*-cresol **8** and (4-methoxyphenyl)methanol **7**. All other products, except for compound **5**, were identified by comparison with analytical data obtained from the literature. In the case of enzyme product **5** formation of this benzylic alcohol was deduced in a first case from the aromatic signals and the presence of a benzylic methylene group signal around 4.7 ppm.

Conversion measurements

Conversion measurements were carried out on a 1 mL scale in closed solvent-resistant 2 mL reaction vessels at 300 rpm and 30 °C in KPi buffer (50 mM, pH 7.5). The reaction mixture contained P450 BM3 WT or M2 (3.0 μM in KPi buffer 50 mM, pH 7.5, crude cell extract), a buffer mixture (30% (v/v), 25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0), substrate (400 μM), DMSO (2% (v/v)) and $NADP^+$ (100 μM) as well as GDH (0.3 U) and glucose (400 mM; sterile filtered stock solution) for cofactor regeneration and catalase (600 U) for detoxification. The resulting solution was incubated for 24 h to ensure completion of the enzymatic reaction. Subsequently, the reaction mixture was acidified with aqueous HCl-solution (50 μL, 1 M) and extracted with EtOAc (400 μL). The organic phase was dried with anhydrous $MgSO_4$ and the sample was subjected to GC analysis. Conversion values were determined based on the observed signal intensities for product and starting material. All measurements were performed in triplicate and control experiments were carried out in the absence of substrate and enzyme, respectively.

Determination of GDH activity

The catalytic activity of glucose dehydrogenase was measured spectrophotometrically by monitoring the rate of NADPH formation in the course of the reaction through its absorption at 340 nm in cuvettes. The assay solutions containing crude cell extract (779 μL; dilution of the initial extract with 50 mM KPi buffer pH 7.5 to obtain quantifiable activities) and glucose (121 μL, 3.3 M in KPi buffer 50 mM, pH 7.5) were preincubated for 5 min at r.t. Subsequently, the reaction was started by the addition of $NADP^+$ (100 μL, 3 mM in KPi buffer 50 mM,

pH 7.5) and the increase in NADPH concentration was monitored for 1 min with an average 5 s interval between each reading cycle. Turnover values were calculated using an extinction coefficient for NADPH of $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$.

Biotransformations

P450 BM3-catalysed oxidations were performed at 30 °C in KPi buffer (50 mM, pH 7.5) and utilisation of a titrator allowed us to maintain the pH at 7.5 during the reaction. 100 mL three-necked flasks were filled with a total reaction volume of 60 mL and cross-shaped magnetic stir bars ensured extensive mixing at 500 rpm. The catalyst was added as crude cell extract (3 μM, 0.03 mol%) and the reaction mixtures contained 30% (v/v) of a buffer mixture (25 mM Tris–HCl, 25 mM KPi and 250 mM KCl, pH 8.0), a catalase from *Micrococcus lysodeikticus* (600 U mL^{-1}) and availability of NADPH was ensured through addition of 200 μM $NADP^+$, 400 mM glucose (3.3 M in KPi buffer 50 mM, pH 7.5, sterile filtered) and 15 U GDH. Initially, the reaction mixtures were saturated with O_2 by bubbling O_2 through the solution for 5 min. Subsequently, 600 μmol of substrate **1** were added and the reaction mixtures were incubated overnight for 15–24 h. For an efficient work-up, NaCl was added to allow saturation of the reaction mixture overnight at 4 °C. Afterwards the reaction mixture was acidified with aqueous HCl (2 M) to a pH around 3.0 and extracted with EtOAc (40 mL). To facilitate phase separation the sample was centrifuged (4000 rpm, 20 min, r.t.). The organic phases and the protein pellet, which formed the interphase, were transferred into a new flask and the aqueous phase was extracted further (6 × 20 mL). The extracts were combined, dried with anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. For the HPLC analysis of the crude products, the ACN concentration was raised during the first 40 min from 5% to 40% and then maintained at 40% for 10 min. Under these conditions acid **13** eluted at 8.7 min, acid **14** at 12.1 min, alcohol **2** at 19.0 min, aldehyde **12** at 30.2 min and substrate **1** at 37.0 min, respectively. The conversion values were determined based on the observed signal areas. Flash column chromatography on silica gel with *n*-pentane–diethyl ether (6 : 4 → 1 : 1) allowed purification of the benzylic alcohol product **2**. Applying the P450 BM3 double mutant M2 as the enzyme catalyst gave 73% of alcohol **2** (86 mg) as an orange oil and 3% of the aldehyde **12** (4 mg). Utilisation of the F87A L188P double mutant gave 33% of **2** (39 mg, brsm 60%) and 2% of **12** (2 mg), respectively.[43]

Characterisation of 2

Methyl 3-(hydroxymethyl)-2-methoxybenzoate. R_f = 0.17 (petroleum ether–EtOAc, 7 : 3); anal. calcd for $C_{10}H_{12}O_4$: C, 61.22; H, 6.16; found: C, 60.79 ± 0.05; H, 6.18 ± 0.01; IR (film): 3432, 3076, 2999, 2951, 2872, 2835, 1712, 1593, 1465, 1433, 1424, 1365, 1298, 1267, 1230, 1193, 1134, 1085, 1061, 1000, 887, 870, 812, 778, 764, 731 cm^{-1}; ^1H NMR: δ = 2.32 (brs, 1 H, OH), 3.88 (s, 3 H, OCH_3), 3.91 (s, 3 H, $COOCH_3$), 4.75 (d, J = 5.1 Hz, 2 H, CH_2), 7.16 (dd, J = 7.8, 7.5 Hz, 1 H, 5-H), 7.56 (dd, J = 7.5, 1.5 Hz, 1 H, 4-H), 7.77 (dd, J = 7.8, 1.5 Hz, 1 H, 6-H); ^{13}C NMR: δ = 52.4 ($COO\underline{C}H_3$), 61.0 (CH_2), 62.7 (OCH_3), 124.0 (C-5), 124.3 (C-1), 131.3 (C-6), 133.1 (C-4), 135.6 (C-3), 158.3 (C-2), 166.6 ($\underline{C}OOCH_3$) ppm; MS: *m*/*z* (%): 196 (16), 181 (23), 165 (34), 149 (100), 135 (75), 119 (27), 105 (23), 91 (16), 77 (36), 65 (17), 51 (10).

Ethyl 3-(hydroxymethyl)benzoate (**5**) was provided by isolation from combined reaction mixtures and purification *via* flash column chromatography on silica gel with *n*-pentane–diethyl ether (7 : 3). R_f = 0.30 (petroleum ether–EtOAc, 7 : 3); anal. calcd for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71; found: C, 66.58 ± 0.11; H, 6.87 ± 0.01; IR (film): 3424, 2982, 2960, 2933, 2906, 2872, 1716, 1699, 1610, 1590, 1465, 1445, 1393, 1368, 1279, 1190, 1104, 1083, 1018, 930, 902, 864, 818, 746, 697, 671 cm^{-1}; ^1H NMR: δ = 1.39 (t, J = 7.1 Hz, 3 H, CH_3), 2.17 (brs, 1 H, OH), 4.37 (q, J = 7.1 Hz, 2 H, CH_2), 4.74 (s, 2 H, CH_3), 7.43 (dd, J = 7.8, 7.6 Hz, 1 H, 5-H), 7.56 (ddd, J = 7.6, 1.3, 0.6 Hz, 1 H, 4-H), 7.96 (ddd, J = 7.8, 1.3, 1.2 Hz, 1 H, 6-H), 8.03 (dd, J = 1.2, 0.6 Hz, 1 H, 2-H) ppm; ^{13}C NMR: δ = 14.4 (CH_3), 61.2 (CH_2), 64.9 (CH_2OH), 128.2 (C-2), 124.7 (C-6), 128.9 (C-5), 131.4 (C-4), 130.8 (C-1), 141.3 (C-3), 166.7 (COO) ppm; MS: *m*/*z* (%): 180 (17), 151 (26), 152 (17), 135 (100), 133 (26), 123 (18), 107 (41), 105 (29), 89 (46), 79 (36), 77 (62), 51 (22).

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43 Additional experiments showed that up-scaling of the M2-catalysed biotransformation is possible and thorough mixing is the limiting factor. In this manner, applying 12 mmol of substrate **1** (20-fold up-scaling) gave 64% conversion and yielded 51% of alcohol **2** (brsm 82%).

Furthermore, application of purified protein under similar conditions gave slightly lower conversions and yields, but comparable product compositions with alcohol **2** to aldehyde **12** ratios around 10 : 1. In this manner, applying 3 mmol of substrate **1** (5-fold up-scaling with continuous O_2-supply and higher catalyst load) gave 75% conversion and yielded 64% of alcohol **2**. Therefore, it was concluded that purified M2 – even though stabilised *via* addition of BSA – shows lower stability under the reaction conditions compared to M2 in crude cell extract. Furthermore, this result proved that aldehyde **12** was indeed an "overoxidation product" of M2 catalysis.