C–H Oxidation

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Enantioselective Allylic Hydroxylation of ω-Alkenoic Acids and Esters by P450 BM3 Monooxygenase**

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Abstract: *Chiral allylic alcohols of ω-alkenoic acids and derivatives thereof are highly important building blocks for the synthesis of biologically active compounds. The direct enantioselective C–H oxidation of linear terminal olefins offers the shortest route toward these compounds, but known synthetic methods are limited and suffer from low selectivities. Described herein is an enzymatic approach using the P450 BM3 monooxygenase mutant A74G/L188Q, which catalyzes allylic hydroxylation with high to excellent chemo- and enantioselectivities providing the desirable secondary alcohols.*

Chiral (ω−2)-hydroxy-ω-alkenoic acids as well as their esters, vinyl lactones, and protected alcohols are pivotal structural elements abundantly present in diverse natural and biologically active compounds including decanolides, oxylipins, antibiotics, and pheromones.[1] Extensive research toward the enantioselective synthesis of these moieties has resulted in multistep approaches comprising prefunctionalization and protection group chemistry as well as functional group interconversions and oxidation state manipulations.[2] A chemoenzymatic route with an alcohol dehydrogenase catalyzed selective reduction as the key step was reported by our group and represents arguably the most efficient access nowadays (2 or 3 steps, 73–77% overall yield, > 99% *ee*, both enantiomers accessible).[3] Significant streamlining could be achieved by the direct asymmetric oxidation of readily available "unactivated" olefins at the allylic position, providing a highly atom-economic single-step process.[4] In this context, significant success in the highly topical field of C–H activation was achieved by Agudo et al. and Li et al. regarding the enzymatic oxidation of cyclic and linear molecules, respectively.[5,6] However, methods for the enantioselective allylic C–H oxidation of simple linear compounds remain a challenge as asymmetry-inducing factors are missing.[7] The enantioselective allylic acetoxylation of terminal olefins using a combination of a Pd^{II}/bis(sulfoxide) system with benzoquinone as the terminal oxidant and Cr^{III}(salen) as a chiral Lewis acid cocatalyst reported by White and co-workers in 2008 was a milestone and *ee* values up to 63% were achieved; the desired products were obtained in 69–89% yield and 50–57% *ee* as roughly 5:1 mixtures with the corresponding linear alcohols.[8] We envisioned that a biocatalytic approach might be preferable to conventional chemistry, since enzymes ensure high selectivity by furnishing completely chiral reaction environments. The well-known and highly promising NADPH-dependent bacterial cytochrome P450 BM3 monooxygenase from *Bacillus megaterium* was the probe system of choice due to its soluble and self-sufficient character, its superior catalytic features compared to other monooxygenases, and the availability of large-scale production and purification protocols.[9,10] The native function of P450 BM3 is supposed to be the subterminal hydroxylation of fatty acids by insertion of one oxygen atom from O_2 into C–H bonds; however, the enzyme's high versatility and evolvability have been proven by protein engineering.[9,11] P450 BM3 mutants were reported to hydroxylate alkanes at the 3-position with enantioselectivity of up to 46% *S-ee* and preferentially catalyze the allylic hydroxylation of terminal alkenes and terminally unsaturated fatty acids over epoxidations; neither the preparative feasibility nor the enantioselectivity of the latter transformations have yet been examined.[12,13] Described herein is an enzymatic access to *S*-configured ω−2-hydroxy-ω-alkenoic acids and esters through P450 BM3 catalyzed allylic hydroxylation. This approach shows the highest enantioselectivity observed to date for the allylic C–H oxidation of terminal linear olefins and simultaneously addresses key issues of modern organic synthesis like the use of mild reaction conditions, aqueous media, sustainable catalysts, and O_2 as a "green" oxidant (Figure 1).[14]

A P450 BM3 library of 65 variants was constructed by mutating active-site residues R47, Y51, A74, F87 and L188, all of which are known determinants of activity and selectivity of this enzyme (for details, see the Supporting Information: S2).[9a,15] This set of mutants served as a versatile catalyst pool for assaying the ability of P450 BM3 to catalyze the allylic hydroxylation of the model substrate ethyl 6-heptenoate (**1a**). Analytical-scale reactions were performed in 96-well plates and analyzed by GC with regard to conversion of starting material and the chemo- and enantioselectivity of product formation. Consistent with previous reports on the P450 BM3 catalyzed oxygenation of terminal olefins,[13] all mutants tested gave the *S*-configured branched allylic alcohol (**2a**)

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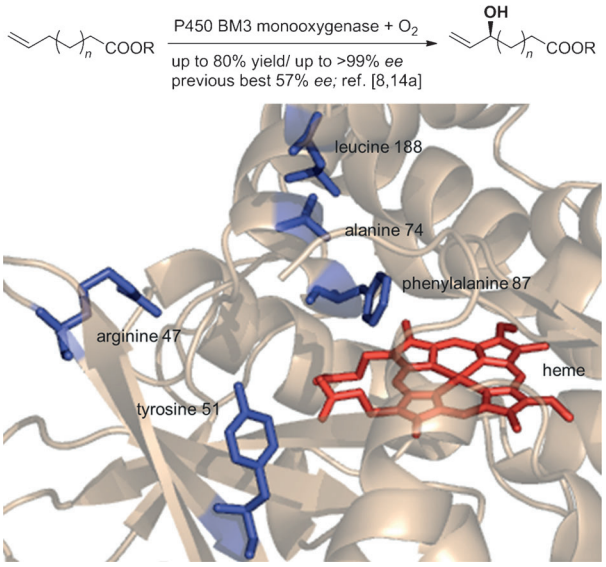
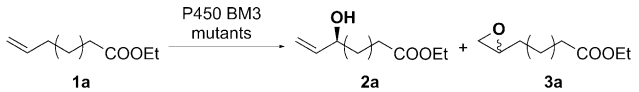


Figure 1. Active site of P450 BM3 (PDB: 1BVY). The highlighted amino acids mark the location of mutagenesis aimed at the generation of a versatile catalyst pool. These biocatalysts faciliated the allylic C–H oxidation of linear terminal olefins giving the highest enantioselectivity observed to date.

as the major product accompanied by the corresponding epoxide (**3a**) as the minor product. Conversions up to 59% were reached, the alcohol selectivity varied between 65–97% and the enantioselectivity amounted to 87–98% *ee* (Table 1, entry 1; for details, see the Supporting Information: S3). Moreover, 6-heptenoic acid (**4a**) was detected as a constituent of the crude product and its formation could be traced back to hydrolase activity in the crude cell lysates (see the Supporting Information: S4). The mutants A74G, L188Q, and A74G/L188Q (GQ) showed the most advantageous combinations of all parameters tested and were subjected to a comparative characterization. The preceding experiment was repeated using defined amounts of purified biocatalysts and coupling efficiencies (amount of NADPH used for substrate oxidation) were examined, providing a further measure for the quality of the monooxygenases (Table 1, entries 2–4). The P450 BM3 double mutant GQ appeared to be most suitable for the selective allylic hydroxylation of substrate **1a**, giving the best results for three of four tested variables with a conversion of (49 ± 1)%, an alcohol selectivity of (90 ± 0)%, an *ee* of (95 ± 1)%, and a coupling efficiency of (52 ± 1)%. Its general suitability for the asymmetric production of allylic alcohols from ω-alkenoic acid related compounds was additionally proven by assaying the P450 BM3 library using ethyl 8-nonenoate (**1b**); a detailed comparison of the identified hits confirmed GQ to be the best mutant (for details, see the Supporting Information: S3) and suggested that rescreening of the library with structurally related substrates would be redundant. Interestingly, Reetz and co-workers[5] drew a similar conclusion while studying cyclic olefins, which increases the synthetic potential of the P450 BM3 monooxygenase.

The substrate scope of mutant GQ was systematically examined by testing its ability to catalyze the stereoselective allylic hydroxylation of ω-alkenoic acids and respective ethyl esters with chain lengths of C_6 to C_{11}. Semi-preparative-scale reactions were analyzed by ^{1}H NMR spectroscopy; all tested esters **1a–f** and longer-chain acids **4e,f** formed the alcohols, but the shorter-chain esters and longer-chain acids gave higher conversions (Table 2, products **2a–f** and **5e,f**). Observed epoxide signals were below the integration limit and hence, neglected, but spontaneous lactonization of products **2a** and **2c** occurred yielding the γ- and δ-ring

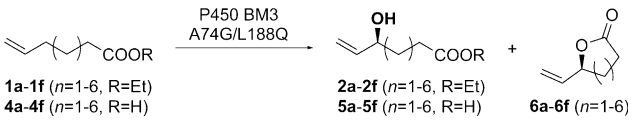
Table 1: Screening and characterization of P450 BM3 monooxygenase mutants for the stereoselective allylic hydroxylation of ethyl 6-heptenoate (**1a**).



Entry	P450 BM3 mutant	Conv. [%]	**2a**:**3a**	*ee* [%]	CE [%]
1	enzyme library	0–59	65:35–97:3	87–98	–
2	A74G	47 ± 3	89(±0):11	96 ± 1	36 ± 1
3	L188Q	37 ± 2	85(±0):15	93 ± 1	51 ± 1
4	A74G/L188Q	49 ± 1	90(±0):10	95 ± 1	52 ± 1

Reaction conditions: Σ500 μL: 200 μL P450 BM3 crude cell lysate, 2 mM substrate, 2 vol% DMSO, 300 U catalase, 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), GDH-glucose-NADPH-recycling (entry 1); as previous except for using 3 μM purified P450 BM3 and 10 mM substrate without DMSO (entries 2–4); Σ1 mL: 1.5 μM purified P450 BM3, 400 μM substrate, 2 vol% DMSO, 30 vol% activity buffer, 200 μM NADPH (CE column). All values were determined by GC analysis and uncertainty is given as the standard deviation from three measurements; absolute configuration was assigned by comparison with an authentic sample. conv.: conversion; CE: coupling efficiency.

Table 2: Investigation of the substrate scope and selectivity of the P450 BM3 A74G/L188Q catalyzed allylic hydroxylation of ω-alkenoic acids and esters.



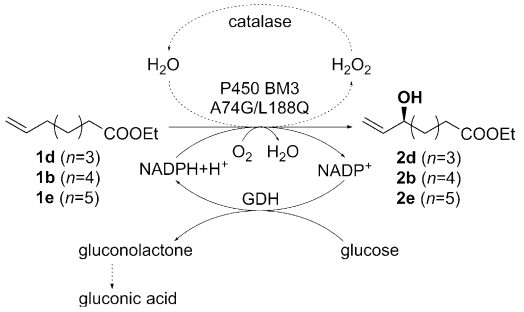
Entry	Substrate			Product(s)	Conv. [%][a]	*ee* [%][b]
1	**1c**	*n* = 1	R = Et	**2c** (**6a**)	49[c]	96
2	**1a**	*n* = 2	R = Et	**2a** (**6b**)	42[d]	93
3	**1d**	*n* = 3	R = Et	**2d**	70	97
4	**1b**	*n* = 4	R = Et	**2b**	49	> 99
5	**1e**	*n* = 5	R = Et	**2e**	34	n.d.
6	**1f**	*n* = 6	R = Et	**2f**	18	n.d.
7	**4a**	*n* = 1	R = H	**5a**	0	–
8	**4b**	*n* = 2	R = H	**5b**	0	–
9	**4c**	*n* = 3	R = H	**5c**	0	–
10	**4d**	*n* = 4	R = H	**5d**	< 5	–
11	**4e**	*n* = 5	R = H	**5e**	6	n.d.
12	**4f**	*n* = 6	R = H	**5f**	28	n.d.

Reaction conditions: Σ3 mL: 3 μM purified P450 BM3, 10 mM substrate, 2 vol% DMSO, 1.8 kU catalase, 30 vol% activity buffer, GDH-glucose-NADPH-recycling. [a] Determined by ^{1}H NMR analysis. [b] Determined by GC analysis; absolute configurations were assigned by comparison with authentic samples. [c] **2c**:**6a** ≈ 6:4. [d] **2a**:**6b** ≈ 9:1. Conv.: conversion; n.d. = not determined.

compounds **6a** and **6b**.[16] GC analyses of the formed products on chiral stationary phases revealed the high enantioselectivity of the GQ-catalyzed oxidations leading to *S*-alcohols in all cases; the highest value was > 99 % *ee* for product **2b** and the lowest was 93 % *ee* for ester **2a**. This feature further supported the general applicability of the GQ mutant for the asymmetric production of allylic alcohols starting from substrates exhibiting an ω-alkenoic acid core; this is highly advantageous as the synthetic usability of biocatalysts is often limited by high substrate specificity. The demonstrated substrate spectrum was broadened even further by testing different esters of 6-heptenoic acid with the aim of at enhanced enantioselectivity; allylic alcohols were formed in all cases with *ee* values mostly close to the previously mentioned 93 % (for details, see the Supporting Information: S3; decrease of stereocontrol was observed only when bulky or polar moieties were present directly adjacent to the ester oxygen).

The performance of biocatalysts in preparative-scale reactions is a highly important property not necessarily inferable from screening results because of different reaction conditions and enzyme behavior. To this end, the usability of the identified GQ mutant was tested using ethyl 7-octenoate (**1d**), ethyl 8-nonenoate (**1b**), ethyl 9-decenoate (**1e**), and 10-undecenoic acid (**4f**), as these substrates gave better results than the alternatives of corresponding chain lengths in the preceding assay. Substrates **1c** and **1a** were not investigated due to the high volatility of these compounds and lactone formation of the respective alcohols **2c** and **2a**, both factors complicating product isolation. Reaction conditions were set following previous studies and included addition of O_2 as a reactant and catalase for the decomposition of H_2O_2, a toxic P450 BM3 decoupling product, to H_2O.[17] A GDH/glucose system effected NADPH regeneration and titrator-mediated pH adjustment ensured a value of 7.5 throughout the reaction (GDH: glucose dehydrogenase). Minor optimization was performed by testing the impact of varying DMSO amounts (a P450 BM3 mutant specific parameter depending on the accessibility of the active site for cosolvent molecules),[18] different O_2 addition options (a substrate-specific parameter depending on the compound's volatility), and several catalyst-loading protocols (an enzyme-/substrate-specific parameter depending on the stability of the former in presence of the latter) on the reaction's output using the example of ester **1d** as the substrate (Table 3, entries 1–10). Purified P450 BM3 and GDH were used for the oxidation of esters to exclude hydrolase activity, which would otherwise reduce the amount of P450 BM3 substrate present. The highest yield was achieved with 2 vol % DMSO, an initial saturation of the reaction mixture with O_2, and continuous addition of GQ affording 45 % of alcohol **2d** (Table 3, entry 10). Strikingly, even though Reetz and co-workers stressed the importance of maintaining the substrate-to-enzyme ratio in order to obtain optimal enantioselectivities, in this study a constant *ee* value of 97 % was observed independent of the applied reaction conditions. Substrates **1b** and **1e** were oxidized under the established conditions affording products **2b** and **2e** in 24 % and 20 % yield as well as > 99 % and 97 % *ee*, respectively (Table 3, entries 11 and 12).

Table 3: Preparative-scale oxidation of ethyl ω-alkenoates using purified P450 BM3 A74G/L188Q.



Entry	Substrate		Product	P450 BM3 [10^{-2} mol %]	DMSO [vol %]	Yield [%][a]	*ee* [%][b]
1	**1d**	$n=3$	**2d**	3.0	0.5	23	97
2	**1d**	$n=3$	**2d**	3.0	1.0	27	97
3	**1d**	$n=3$	**2d**	3.0	2.0	32	97
4	**1d**	$n=3$	**2d**	3.0	3.0	21	97
5[c]	**1d**	$n=3$	**2d**	3.0	2.0	21	97
6[c]	**1d**	$n=3$	**2d**	6.0	2.0	26	97
7	**1d**	$n=3$	**2d**	6.0	2.0	33	97
8	**1d**	$n=3$	**2d**	9.0	2.0	27	97
9	**1d**	$n=3$	**2d**	6.0[d]	2.0	37	97
10	**1d**	$n=3$	**2d**	4.8[e]	2.0	45	97
11	**1b**	$n=4$	**2b**	4.8[e]	2.0	24	> 99
12	**1e**	$n=5$	**2e**	4.8[e]	2.0	20	97[g]

Reaction conditions: Σ60 mL: purified P450 BM3, 0.6 mmol substrate, 36 kU catalase, 30 vol % activity buffer, GDH-glucose-NADPH-recycling. [a] Yield of isolated product. [b] Determined by GC analysis; absolute configurations were assigned by comparison with authentic samples. [c] Continuous O_2 supply. [d] Initially $3\times\cdot10^{-2}$ mol % catalyst plus an additional portion of 3×10^{-2} mol % after 3 h. [e] Initially 3×10^{-2} mol % catalyst plus three additional portions of 6×10^{-3} mol % after 2, 4, and 6 h. [g] Determined by HPLC analysis after acetylation with 3,5-dinitrobenzoyl chloride; absolute configuration was assigned by comparison with an authentic sample.

Oxidation of acid **4f** was performed according to previous insights with crude cell lysates of P450 BM3 and GDH instead of the purified enzymes, as hydrolase activity was irrelevant in this case. Different catalyst amounts were tested due to varying behavior of P450 BM3 in purified and crude preparations (observed in previous studies, data not shown) and the crude product was methylated using CH_2N_2 to facilitate isolation of the desired alcohol by chromatography (Table 4). The highest yield was achieved with 9×10^{-2} mol % biocatalyst; alcohol **7** was obtained over two steps in 80 % yield (Table 4, entry 3). Again, the *ee* appeared to be independent of the enzyme-to-substrate ratio, and was in the range of 87–92 %. Besides testifying to the preparative-scale applicability of the GQ mutant, these results indicate that conversion values obtained in screening experiments, even though perfectly suited for a relative comparison of different P450 BM3 mutants, are not meaningful regarding catalytic productivity in preparative-scale synthesis. Furthermore, it seems that P450 BM3 exhibits higher activity and stability in crude cell lysates than in purified samples in general and this effect is even more distinct when high enzyme concentrations are applied.

Table 4: Preparative-scale oxidation of 10-undecenoic acid (**4f**) using P450 BM3 A74G/L188Q crude cell lysate.

4f **7**

Entry	P450 BM3 [10^{-2} mol%]	Yield [%][a]	*ee* [%][b]
1	3.0	30	>89
2	6.0	68	>87
3	9.0	80	92
4	15.0	69	>90

Reaction conditions: Σ60 mL: P450 BM3 crude cell lysate, 0.6 mmol substrate, 36 kU catalase, 30 vol% activity buffer, GDH-glucose-NADPH-recycling. [a] Yield of isolated product. [b] Determined by HPLC analysis after acetylation with 3,5-dinitrobenzoyl chloride; absolute configuration was assigned by comparison with an authentic sample and its optical rotation.

In summary, we have identified the P450 BM3 double mutant A74G/L188Q as a highly active and selective biocatalyst for the asymmetric allylic hydroxylation of readily available ω-alkenoic acids and their esters providing *S*-configured allylic alcohols and exhibiting a broad substrate range. The fact that high chemo- and enantioselectivity was observed with substrates of varying chain length and almost independent of the carbonyl moiety present, indicates that selectivity is not attributable to specific interactions with defined amino acid residues in the active site, but represents a rather general orientation of ω-alkenoic acid related compounds within the substrate channel. The relatively rigid carbonyl moiety is a conceivable cause of fixed compound orientation, as minor levels of enantioselectivity were previously observed for comparable, more flexible alkanes.[12] The potential of the GQ mutant for asymmetric synthesis in preparative-scale reactions was further demonstrated by producing desired building blocky for the synthesis of biologically active compounds with up to 80% yield and unprecedented enantioselectivities of up to >99% *ee*, thereby providing pivotal knowledge for the application of this highly promising monooxygenase for synthetic purposes in general.

Experimental Section

Enzymatic oxidation of ω-alkenoic acids and esters (preparative scale): A sterile, three-necked flask equipped with a cross-shaped magnetic stir bar was charged with P450 BM3 mutant A74G/L188Q (9 μM, crude cell lysate for oxidation of acids; 3 μM, purified enzyme for oxidation of esters), 30 vol% activity buffer (50 mM KPi, 50 mM Tris-HCl, 250 mM KCl, pH 8.0), $NADP^+$ (200 μM for oxidation with crude cell lysate, 100 μM for oxidation with purified catalyst), 2 vol% DMSO, 15 U GDH, 400 mM glucose (sterile 3.3 M stock solution in 50 mM KPi buffer, pH 7.5), 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 0.6 mmol substrate. 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 aqueous 1M NaOH solution, and the oxidation proceeded at 30°C within 16 h; in the case of purified enzyme, additional portions were added in the course of the oxidation (3× 0.6 μM after 2, 4, and 6 h). Progress was followed by monitoring the amount of base consumed over time and the reaction was stopped by saturating the solution with NaCl once the titration plot indicated saturation. Protein was denatured at 4°C overnight; the reaction was acidified with aqueous 1M HCl to pH ≈ 2 and extracted with EtOAc (40 mL). The first extraction step was performed using centrifugation to achieve phase separation. The pellet formed at the interface was transferred to the organic phase and conventional extraction steps followed (4 × 40 mL). The extracts were combined, dried with $MgSO_4$, and concentrated in vacuo; acids were additionally methylated with CH_2N_2. Column chromatography on silica gel using *n*-pentane/Et_2O (8:2→6:4) gave the desired products.

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