Aromatic Hydroxylation

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Regioselective *o*-Hydroxylation of Monosubstituted Benzenes by P450 BM3**

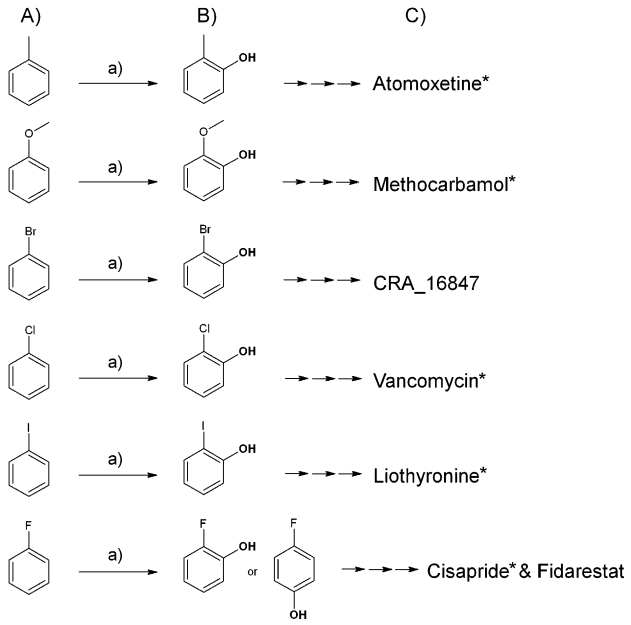
*Alexander Dennig, Nina Lülsdorf, Haifeng Liu, and Ulrich Schwaneberg**

Halogenated phenols are widely used building blocks in the synthesis of vitamins, lipid-lowering agents, and other drugs.[1] Commonly, halogenated phenols are produced in chemosynthetic processes[2] requiring energy-intensive downstream processing steps and hazardous chemicals, for example (Hg/Ti) acetate derivatives for the synthesis of iodophenol.[2b,3] Chloro- and bromophenols are produced by electrophilic halogenation reactions employing Br_2 and Cl_2, yielding mixtures of *o*- and *p*-phenol derivatives as well as additional by-products (e.g. dichlorophenol).[1b,c] Consequently, purification of isomeric products is required for drug synthesis.[1e,4] Guaiacol and its derivatives vanillin and eugenol are intermediates in the perfume and flavor industry and common antioxidants in the treatment of cancer, cardiovascular disorders, and Parkinson's and Alzheimer's diseases.[5] Guaiacol can be isolated from plants or synthesized from catechol by reaction with stoichiometric amounts of NaOH in the presence of other corrosive reagents.[6] The direct chemical hydroxylation of substituted benzenes is unattractive since oxygenation reactions on nonactivated C atoms are unselective[7] and do not occur on the aromatic ring.[8]

Biocatalysis offers alternative and attractive routes to the direct aromatic hydroxylation of benzenes.[4,9] When water can serve as the reaction medium the use of organic solvents is minimized and toxic or corrosive by-products such as HBr and HCl can be avoided.[1a,b,7,9a,10] Especially the potential of regio- and enantioselective catalysis makes direct enzymatic hydroxylations attractive for the production of pharmaceuticals.[4,7,9b] A major limitation for the application of biocatalysts in technical processes is their often insufficient operational stability.[11] Only a few peroxidases are known to halogenate phenols with moderate selectivity.[9a] In this regard, protein engineering offers powerful methods to tailor enzymes to meet specific industrial demands.[12]

P450 BM3, a heme-dependent and industrially important monooxygenase,[13] catalyzes the direct aromatic hydroxylation of benzenes with high selectivity and activity.[12a,d,14] A mechanism in which the hydroxylated substrate forms an epoxide intermediate followed by NIH shift (1,2-hydride shift) to retain the phenol was reported by de Vissier and Shaik[15] and experimentally proven by Whitehouse et al.[14] Recently, the P450 BM3 variant M2 (R47S/Y51W/I401M) was engineered in our group and found to catalyzed the efficient and regioselective aromatic hydroxylation of *p*-xylene.[12a]

Herein we report the aromatic hydroxylation of six monosubstituted benzenes (Scheme 1) with the engineered variant M2 to study the production of *o*-substituted phenols by direct hydroxylation with oxygen at room temperature in water (KPi 50 mM, pH 7.5). In a systematical approach the influence of ring substituents (F, Cl, Br, I, CH_3, and OCH_3) on regioselective hydroxylation was investigated. A list of 25 further compounds investigated including nitrobenzene and aniline can be found in the Supporting Information (Table S1) along with a protocol for the semi-preparative-scale production of guaiacol and *o*-cresol. The highly sensitive, regioselective 4-AAP assay was used to quantify the products[16] and GC-FID and GC-MS were employed with commercial stand-



Scheme 1. A) Aromatic substrates of the P450 BM3 variant M2 (R47S/Y51W/I401M). a) P450 BM3 M2 (0.075 μM), O_2, RT, potassium phosphate buffer (50 mM, pH 7.5), NADPH. B) Products obtained after aromatic hydroxylation with P450 BM3 M2. C) Selected drugs that contain structure motifs in (B) (http://www.drugbank.ca). FDA-approved drugs are marked with an asterisk.

[*] A. Dennig, N. Lülsdorf, Dr. H. Liu, Prof. Dr. U. Schwaneberg
Institute of Biotechnology, RWTH Aachen University
Worringerweg 1, 52074 Aachen (Germany)
E-mail: u.schwaneberg@biotec.rwth-aachen.de
Homepage: http://www.biotec.rwth-aachen.de/

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Supporting information for this article (including the protocol for semi-preparative-scale production of *o*-phenol compounds, GC-FID chromatograms, and GC-MS data for all obtained products)is available on the WWW under http://dx.doi.org/10.1002/anie.201303986.

Table 1: Catalytic performance of P450 BM3 wild-type and variant M2 for the aromatic hydroxylation of six substrates.

Variant	Substrate	iTOF[a] [s^{-1}]	TOF[b] [300 s^{-1}]	U/mg_{P450}[c]	*C* [%][d]	Phenol [%] *o*-	*m*-/*p*-
WT	anisole	n.d.	362.3 ± 91.9	n.d.	11 ± 4	> 90	n.d./< 10
M2		38.6 ± 1.8	2427.4 ± 172.7	19.5	44 ± 4	> 95	n.d./< 5
WT	toluene	n.d.	83.5 ± 8.4	n.d.	10 ± 3	> 95	< 1[e]
M2		14.6 ± 0.6	1062.9 ± 107.6	7.4	48 ± 4	> 99	n.d.
WT	fluorobenzene	n.d.	n.d.	n.d.	0	n.d.	n.d.
M2		n.d.	67.2 ± 30.7[g]	0.02	6 ± 3	49	n.d./51
WT	chlorobenzene	n.d.	237.5 ± 38.6	n.d.	6 ± 3	> 96	< 4
M2		12.3 ± 1.3	1923.4 ± 116.5	6.2	28 ± 4	> 99	< 1
WT	bromobenzene	n.d.	169.8 ± 12.3	n.d.	5 ± 3	> 97	< 3
M2		7.7 ± 1.3	1993.7 ± 88.3	3.8	29 ± 6	> 99	n.d./< 1
WT	iodobenzene	n.d.	n.d.	n.d.	0	n.d.	n.d.
M2		2 ± 0.7	205.4 ± 20.8	0.97	23 ± 8	> 99	n.d.[f]

[a] Initial turnover frequency (iTOF) [$\mu mol_{product}\,\mu mol_{P450}^{-1}\,s^{-1}$]. [b] TOF [$\mu mol_{product}\,\mu mol_{P450}^{-1}\,300\,s^{-1}$]. [c] $\mu mol_{phenol}\,min^{-1}\,mg_{P450}^{-1}$. [d] Coupling efficiency. [e] 3% yield of benzyl alcohol. [f] 51.1% yield of phenol (GC area; see also Figure S5 in the Supporting Information); values determined after 300 s are partially limited by NADPH. [g] Determined after 30 min reaction time. n.d. = not detected.

ards to determine the regioselectivity of the reaction (Supporting Information). Notably, aromatic hydroxylations were performed most efficiently in aqueous solution without additional cosolvents that could interfere in the purification of the products, despite the poor solubility of the substrates in water (< 2 $g\,L^{-1}$).[17]

Table 1 and Figure S1 in the Supporting Information show that wild-type P450 BM3 catalyzes product formation with moderate activities which do not exceed 0.6 U/mg_{P450} (guaiacol). The wild-type enzyme does not convert fluoro- and iodobenzene at a detectable level. Variant M2 could efficiently hydroxylate all six substrates including fluoro- and iodobenzene. Based on initial turnover frequency (iTOF) values, the aromatic hydroxylation of anisole was performed with particular efficiency with 19.5 U/mg_{P450}. An iTOF of 38.6 s^{-1} and specific activity of 19.5 U/mg_{P450} is, to the best of our knowledge, the highest reported rate of product formation for a monooxygenase-catalyzed direct aromatic hydroxylation of a benzene compound.[13] Interestingly, the productivity for the formation of guaiacol is about three times higher than for *o*-cresol (7.4 U/mg_{P450}), likely due to differences in steric demands (Table 1, Scheme 1).[12a,13] Toluene and bromo- and chlorobenzene are also converted much faster by P450 BM3 M2 than by the wild-type enzyme (12.7-fold, 8-fold, and 11.7-fold, respectively) even under NADPH-limited conditions (TOF values in Table 1 and Figure S1).

The wild-type and M2 enzymes have excellent regioselectivity (> 90% *o*-hydroxylation) and M2 reaches almost quantitative values for the *o*-hydroxylations of toluene and halogenated substrates. The only exception is fluorobenzene (Table 1) which is converted with a ratio of 49:51 (ratio of *o*- to *p*-hydroxylation); the *m*-hydroxylation that was reported for eukaryotic P450s is not observed.[18] The fluoro substituent is highly electronegative[19] and can potentially reduce π–π interactions with phenylalanine 87 (F87). This decrease in the π–π interactions could result in the loss of precise steric control and yield a mixture of *ortho* and *para* products.[20] The importance of amino acid residue F87 for the aromatic hydroxylation of *p*-xylene was reported previously by us and attributed to strong π–π interactions(Figures 1 and 2).[12a,21] The regioselective hydroxylation of substituted benzenes by wild-type BM3 and the M2 variant is controlled through space by the binding and orientation of benzene substrates in a defined manner towards T268, a key residue in dioxygen activation and substrate recognition (Figure 1).[13,22] Small compounds such as benzenes have to be positioned correctly in the active site for regiospecific hydroxylation since the large substrate-binding pocket of P450 BM3 can harbor, for instance, polycyclic aromatic hydrocarbons.[23] P450 BM3 controls reactions through space, and the intrinsic reactivity of substrates does not play a dominant role during catalysis in

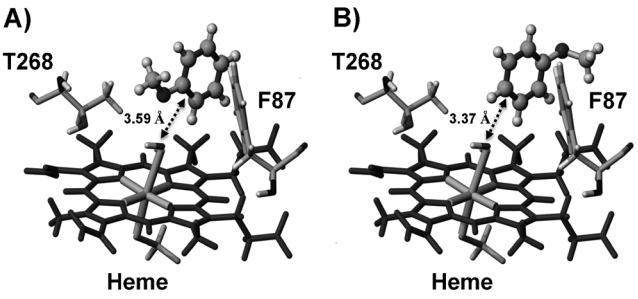

Figure 1. Active site of P450 BM3 (PDB: 1BU7) with the docked anisole substrate (ball-and-stick representation) in T-shape orientation and highest interaction energies (A: −4.98 kcal mol^{-1}, B: −5.46 kcal mol^{-1}).[29] Conformation A suggests hydroxylation at the *ortho* position; conformation B suggests hydroxylation preferentially at the *para* position. The heme center and the residues T268 (dioxygen activation)[22] and F87 (indispensable for aromatic hydroxylation)[12a] are depicted in stick representation. The distance (in Å) between the C atoms of anisole closest to the water ligand bound to the heme iron are indicated with arrows.

contrast to most chemical catalysts.[24] Figure 2 shows the substrate docked into an active site cavity located between the key catalytic residues F87 and T268 and the heme iron. The substrate fits excellently in a T-shape orientation with strong binding energies of −4.98 and −5.46 kcal mol^{-1}.

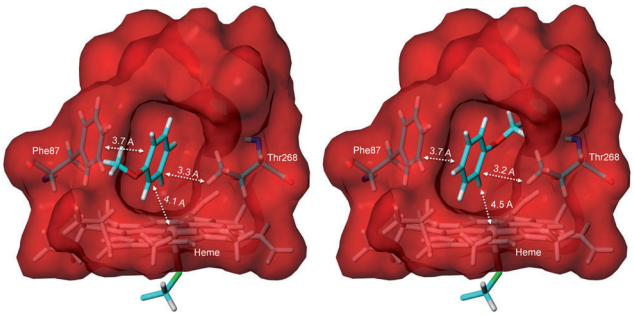

Figure 2. Active-site cavity P450 BM3 (PDB: 1BU7) with anisole docked in two T-shaped binding orientations. The red surface indicates the space filling of residues in the P450 BM3 active site at a distance of less than 4 Å to anisole. Distances are given in Å between anisole and the key residues F87 and T268 as well as heme iron. Both orientations would lead preferentially to the formation of *o*-phenols. Docking was achieved using the VINA docking plug-in[32] for YASARA.[29]

An important parameter for describing the performance of P450 monooxygenases is the coupling efficiency, a measure of the efficient use of NADPH.[13] High coupling efficiencies require that substrates are positioned in specific orientations so that an efficient transfer of the activated oxygen can be achieved from BM3 to the targeted C atom.[25] The BM3 wild-type showed coupling efficiencies ranging from 5 to 11% (Table 1). No NADPH consumption was observed for iodobenzene, suggesting that it is not a substrate for BM3 wild-type. One reason could be, as reported for DMSO, an interaction of the substrate with amino acid residue R47 which restricts the entrance to the active site.[26] In the case of fluorobenzene, NADPH was consumed without being hydroxylated, indicating that the substrate is bound within the active site inducing electron transfer to the heme iron (low spin to high spin)[27] which enables oxygen binding.[28] Oxygen is finally reduced to hydrogen peroxide or another reactive oxygen species when the bound substrate is "loosely fitting"[13] and no hydrogen atom is positioned for abstraction.[25a] Compared to BM3 wild-type, variant M2 displays significantly improved coupling efficiencies ranging from 23 to 48% for all investigated substrates (Table 1). The coupling efficiencies in the reactions with toluene and anisole very high for a P450 monooxygenase (48%).[13] Coupling efficiencies of M2 for bromo- and chlorobenzene were improved by six- and fivefold, making P450 BM3M2 a very efficient catalyst for converting substituted benzenes. A semi-preparative-scale conversion of anisole and toluene with 10^{-4} mol% P450 BM3 M2 yielded the respective phenolic products at levels of 0.67 g L^{-1} (6195 TTN) 0.31 g L^{-1} (2870 TTN).

Table 1 reveals that product formation rates and coupling efficiencies differ significantly in contrast to the regioselectivity values. The main differences between the substituents (OCH_3, CH_3, Cl, Br, I, F) are steric demand and electronegativity.[19] The π–π interactions between residue F87 and monosubstituted benzenes can occur in four orientations: a) π–π stacked, b) dimer, c) T-shaped, and d) inverse T-shaped.[21] Depending on the binding state the distance between two aromatic rings can vary from 3.5 (b) to 6.0 Å (d) leading to interaction energies ranging from −5.38 (a) to −0.88 kcal mol^{-1} (d) for benzene and hexafluorobenzene dimers.[21] The high regioselectivity of BM3 and variant M2 is a first indication that the selected benzene substrates (Table 1) have a strongly preferred orientation for *o*-hydroxylation.

The docking of anisole into the P450 BM3 active site yielded only the T-shape orientation relative to F87 (A and B in Figure 1) with binding energies of −4.98 (A) and −5.46 kcal mol^{-1} (B). The two orientations (inversion of the OCH_3 group) with the highest binding energies are shown in Figure 2, suggesting an interaction with F87 in a T-shape orientation. Conformation A would direct the *ortho* position directly towards the reactive oxygen species which is located between T268 and the heme center.[14,15] Conformation B would preferentially yield *p*- and *m*-hydroxylated phenols, which were produced in minor amounts (Table 1). The latter results indicate that conformation A (Figure 1) is the preferred orientation mode for BM3 wild-type and M2. Despite the good agreement between the docking studies and the experimental data, the docking studies neglect the dynamics within the binding pocket and heme during the hydroxylation reactions.[13] The following correlation between atom size, electronegativity, and reactivity of the halogenated substituents was experimentally observed: M2 activity: Cl > Br > I (Table 1).[14] Fast chlorobenzene hydroxylation could be attributed to an electron-withdrawing effect which could promote faster formation of the epoxide intermediate.[15] Electron-rich substituents such as iodine tend to stabilize the epoxide intermediate, therefore re-aromatization could be slower according to the proposed mechanism.[30] In addition, hydroxylation by P450 BM3 M2 leads to dehalogenation of iodobenzene to phenol (51.1%, GC).[31]

In summary, we have reported the first direct hydroxylation of halogenated benzenes with nearly perfect regioselectivity for chloro-, bromo-, and iodobenzene by an engineered P450 BM3 variant (M2) and the first P450-catalyzed hydroxylation of iodobenzene. All phenols were produced at room temperature in water without co-solvent and with molecular oxygen. The reported phenols are important synthons and direct hydroxylation offers novel options for the synthesis of hydroxylated halobenzenes. Notably, the engineered M2 variant has excellent selectivity (> 99%) and for a P450 enzyme an excellent activity (e.g. anisole hydroxylation: 19.5 U/mg$_{P450}$; 0.67 g L^{-1} product; TTN of 6195).

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