Direct Benzene Hydroxylation

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Highly Selective Hydroxylation of Benzene to Phenol by Wild-type Cytochrome P450BM3 Assisted by Decoy Molecules**

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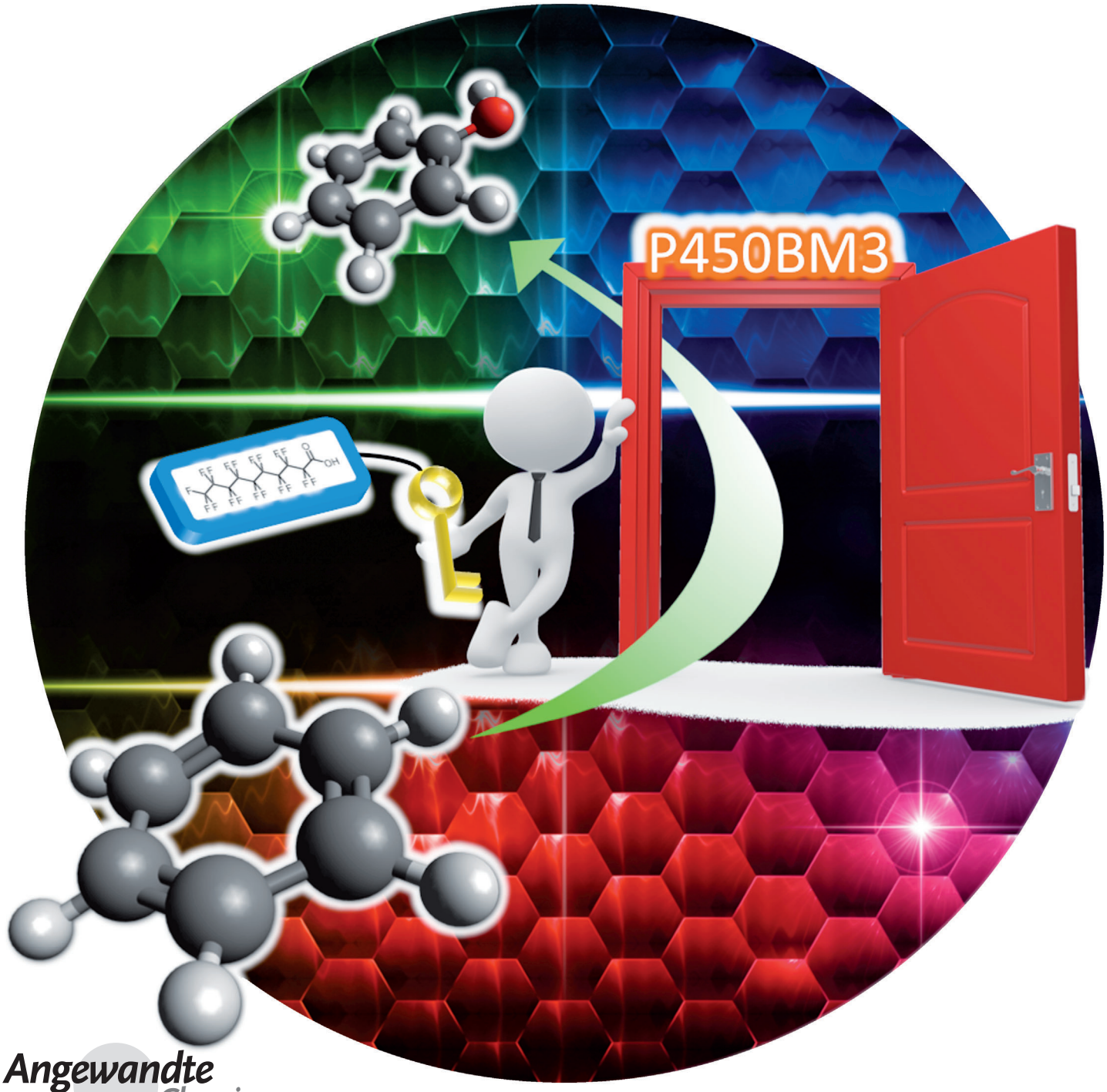
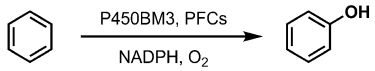


Phenol is a key intermediate in industry for the synthesis of drugs, dyes, and functional polymers. Because phenol is currently produced by the cumene process,[1] which involves high energy consumption and significant formation of side products such as acetone and methylstyrene, direct hydroxylation of benzene has attracted much attention as an alternative method of production. The direct hydroxylation of benzene to phenol using a variety of catalysts,[2] electrochemical oxidation systems,[3] and photochemical systems[4] has thus been extensively investigated. In contrast to many oxidation reactions at high temperature, monooxygenases in nature, such as cytochrome P450, efficiently catalyze the oxidation of inert alkanes and aromatic compounds under mild conditions.[5] Thus, various engineered enzymes have been constructed by site-directed mutagenesis, random mutagenesis, and chemical modification.[6] Biocatalysts for exclusive hydroxylation of the benzene ring using natural enzymes, if they could be developed, would be ideal systems for the production of phenol. Herein we report an efficient and selective hydroxylation of benzene to phenol catalyzed by wild-type cytochrome P450BM3 (P450BM3)[7] with the assistance of decoy molecules.[8]

We and Zilly et al. have recently developed a simple and unique system for the hydroxylation of gaseous alkanes, such as propane and butane, catalyzed by wild-type P450BM3.[9] Wild-type P450BM3 exclusively catalyzes the hydroxylation of long-alkyl-chain fatty acids and never hydroxylates small alkanes, because the active site of P450BM3 is optimized for the hydroxylation of fatty acids (Figure 1a, upper)[10] and the first step of the catalytic cycle of P450BM3 starts only when a fatty acid binds to the substrate binding site of P450BM3, which is accompanied by the removal of the water molecule coordinated to the heme iron (Figure 1b, left).[11] However, in the presence of perfluorinated carboxylic acids (PFCs) as inert dummy substrates (decoy molecules), gaseous alkanes can be hydroxylated by wild-type P450BM3. The decoy molecules initiate the activation of molecular oxygen in the same manner as long-alkyl-chain fatty acids and induce the generation of compound **I**[12] (Figure 1b, right). Because the C–F bonds of PFCs are not oxidizable,[13] compound **I** exclusively hydroxylates gaseous alkanes. Moreover, short-alkyl-chain PFCs partially occupy the substrate binding site of P450BM3 to afford a space for small alkanes, which leads to efficient hydroxylation of the small alkanes (Figure 1a, lower). This attractive advantage encouraged us to perform benzene hydroxylation for the selective production of phenol.

The hydroxylation of benzene by P450BM3 was examined by employing a series of PFCs (PFC8–PFC12; Table 1). The catalytic turnover rates of phenol formation were very much dependent on the alkyl chain length of the PFCs. PFC9 and PFC10 afforded the highest turnover rate, 120 min^{-1} per P450.[14] Without PFCs, this reaction did not proceed at all. The coupling efficiency (defined as ([phenol]/[NADPH consumption]) × 100) of PFC9 (24%) was the highest among the PFCs examined, indicating that the active site provided by PFC9 is suitable for the accommodation of benzene. Although hydroxylation of benzene is generally accompanied by further oxidation of phenol,[15] we observed exclusive formation of phenol without any overoxidation products. The selectivity towards phenol production was more than 99%, according to high-performance liquid chromatography (HPLC) analysis (see the Supporting Information). Phenol is expected to escape rapidly from the active site of P450BM3 because the heme cavity consists of hydrophobic amino acid residues (see the Supporting Information) and the hydrophobic nature of PFC. In fact, phenol was less reactive than benzene under the same conditions.[16] Furthermore, only small amounts of hydroquinone and catechol were detected when phenol (10 mM) was added as a substrate (see the Supporting Information). The turnover rate and coupling efficiency of the benzene hydroxylation are higher than those of a P450BM3 mutant (28 min^{-1} per P450, 14%) developed by random mutagenesis.[17] Although there are a few reports on the catalytic turnover rate for benzene hydroxylation catalyzed by isolated P450s,[18] to the best of our knowledge, this is the first example demonstrating the exclusive formation of phenol by the hydroxylation of benzene with P450. The [D_6]benzene hydroxylation had a higher turnover rate of

Table 1: Turnover rate, NADPH consumption, and coupling efficiency of benzene hydroxylation,[a] and the dissociation constant of decoy molecules.[b]



Decoy[c]	Rate[d]	NADPH consumption[d]	Coupling efficiency [%][e]	K_d [mM][f]
PFC8	38 ± 6	230 ± 41	17	1900
PFC9	120 ± 9	500 ± 13	24	980
PFC10	120 ± 5	600 ± 39	19	290
PFC11	83 ± 6	560 ± 17	15	91
PFC12	71 ± 5	590 ± 26	12	30
none	n.d.	–	–	–

[a] Reaction conditions: P450BM3 (0.5 μM), decoy molecule (100 μM), benzene (10 mM), NADPH (5 mM). [b] from Ref. [9a]. [c] Decoy compounds are all linear perfluorinated carboxylic acids; the number shown indicates the chain length. [d] Rates and NADPH consumption are given in units of [min^{-1} per P450]. Uncertainty is given as the standard deviation from at least three measurements. [e] ([Phenol]/[NADPH consumption]) × 100. [f] K_d = dissociation constant. n.d. = not detected.

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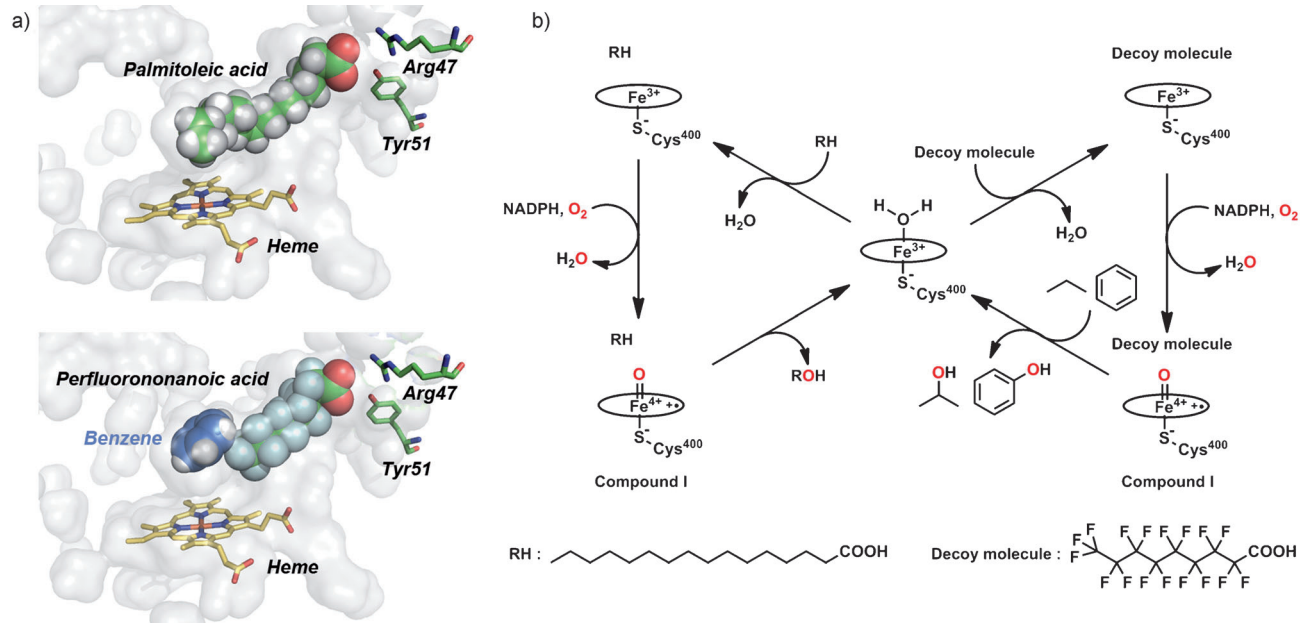
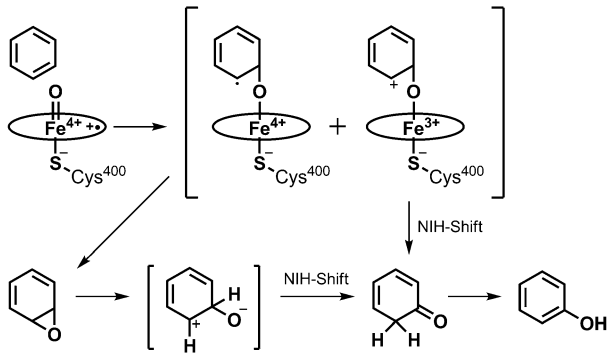


Figure 1. a) The active site of P450BM3 containing palmitoleic acid (upper; PDB code: 1FAG) and the proposed active-site structure of P450BM3 containing perfluorononanoic acid (PFC9) and benzene (lower). b) General reaction mechanism of P450BM3 (left) and the reaction mechanism of propane and benzene hydroxylation catalyzed by P450BM3 with PFC9 as a decoy molecule (right).

135 min^{-1} per P450 (NADPH consumption of 435 min^{-1} per P450 and coupling efficiency of 31%; see the Supporting Information), which indicates that the rate-determining step does not include C–H bond cleavage, but is rather well explained by assuming epoxide formation followed by rearrangement reactions, including a hydride shift, known as an NIH shift (Scheme 1).[19] The observed deuterium kinetic isotope effect (<1) is attributed to the change in carbon atoms from sp^2 to sp^3 for the formation of epoxide, which generally affords an inverse isotope effect.[20]

Toluene was also hydroxylated, and PFC9 gave the highest turnover rate (220 min^{-1} per P450) for the selective *ortho* hydroxylation (Table 2). The coupling efficiency reached 56%.

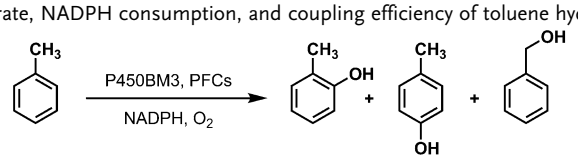
Because the *ortho* selectivity in toluene hydroxylation was not much affected by the length of the PFCs, we assume that the regioselectivity is mainly controlled by amino acid residues at the active site of P450BM3. Phe87, which is located on the distal side of the heme (see the Supporting Information), could interact with toluene to induce the *ortho* position selective hydroxylation. In fact, the *ortho* selectivity was reduced in the case of the F87A mutant of P450BM3.[21] The *ortho*-selective hydroxylation was also observed in the hydroxylation of anisole and chlorobenzene (Table 3; see also the Supporting Information). We did not observe any color change in the reaction of anisole hydroxylation even though *o*-hydroxyanisole (guaiacol) formed. Guaiacol is a substrate commonly used to examine the oxidation activity of heme enzymes such as peroxidase by affording its dimer along with a color change.[22] Moreover, even though the *ortho* demethylation of anisole is a common reaction for human and rat hepatic P450s,[23] the *ortho* demethylation of anisole to give phenol and formaldehyde was not observed. Furthermore, the hydroxylation of the electron-poor benzene derivatives nitrobenzene and acetophenone gave the *o*-hydroxylated product, despite their *meta*-preference in many reactions. These results clearly show that the *ortho* position of monosubstituted benzenes was selectively hydroxylated irrespective of substituents. Although co-crystal structure analysis of P450BM3 with PFC9 would be required to clarify the active-site structure of a P450BM3–PFC9 complex and determine the possible locations and orientations of benzene and its derivatives in the heme pocket, we have not yet succeeded in the co-crystallization.



Scheme 1. Proposed reaction mechanism for benzene hydroxylation by P450BM3.[19e]

In conclusion, we have demonstrated selective hydroxylation of benzene to phenol catalyzed by wild-type P450BM3 through the simple addition of PFCs as decoy molecules. Although we did not replace any amino acid residues, the catalytic turnover rate and the coupling efficiency are higher than those of the engineered P450BM3 mutants prepared by

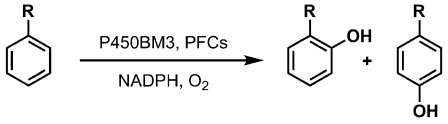
Table 2: Turnover rate, NADPH consumption, and coupling efficiency of toluene hydroxylation.[a]



Decoy[b]	Rate[c]				NADPH consumption[c]	Coupling efficiency [%][d]
	o-cresol	*m*-cresol	*p*-cresol	benzyl alcohol		
PFC8	120 ± 2	n.d.	3.2 ± 0.1	7.7 ± 0.5	260 ± 12	51
PFC9	220 ± 7	n.d.	5.3 ± 0.2	16 ± 0.4	430 ± 34	56
PFC10	210 ± 23	n.d.	5.0 ± 0.7	18 ± 2	520 ± 41	45
PFC11	150 ± 10	n.d.	3.5 ± 0.3	10 ± 1	410 ± 30	39
PFC12	140 ± 15	n.d.	3.3 ± 0.4	7.2 ± 0.9	570 ± 7	27

[a] Reaction conditions: P450BM3 (0.5 μM), decoy molecule (100 μM), toluene (10 mM), NADPH (5 mM). [b] Decoy compounds are all linear perfluorinated carboxylic acids; the number shown indicates the chain length. [c] Rates and NADPH consumption are given in units of [min^{-1} per P450]. Uncertainty is given as the standard deviation from at least three measurements. [d] ([Products]/[NADPH consumption]) × 100. n.d. = not detected.

Table 3: Turnover rates for the hydroxylation of benzene derivatives.[a]



Substrate R =	Rate[b] *ortho*	*meta*	*para*
OCH_3	260 ± 4	n.d.	38 ± 0.3
Cl	120 ± 4	n.d.	14 ± 0.6
NO_2	0.9 ± 0.05	n.d.	n.d.
$COCH_3$	2.9 ± 0.1	n.d.	n.d.

[a] Reaction conditions: P450BM3 (0.5 μM), PFc9 (100 μM), substrate (10 mM), NADPH (5 mM). [b] Rates are given in units of [min^{-1} per P450]. Uncertainty is given as the standard deviation from at least three measurements. n.d. = not detected.

directed evolution.[17] The benzene hydroxylation reported here is an example of how a P450BM3–decoy molecule system can be used to develop biocatalysts as an alternative to repeated mutagenesis. We believe that the catalytic turnover rate and coupling efficiency for benzene hydroxylation would be further improved by optimizing the structure of decoy molecules to increase their binding constants toward that of P450BM3 (Table 1).[9a] Moreover, it would be possible to control the regioselectivity of the hydroxylation of benzene derivatives by changing the structure of decoy molecules.

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