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# Improved oxidation of aromatic and aliphatic hydrocarbons using rate enhancing variants of P450Bm3 in combination with decoy molecules

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Samuel D. Munday,[a] Osami Shoji,[b] Yoshihito Watanabe,[b] Luet-Lok Wong[c] and Stephen G. Bell[a]*

**Enzyme performance can be improved using decoy molecules or engineered variants to accelerate the activity without affecting selectivity. Here we combine a generic rate accelerator variant of cytochrome P450Bm3 with decoy molecules to enhance the oxidation activity of a range of small organic molecules. This combined approach offers superior biocatalytic efficiency without modifying the product distribution.**

Cytochrome P450s (CYPs) catalyse the oxidation of a broad array of organic substrates, allowing them to perform physiological roles as varied as steroid hormone and antibiotic synthesis and the breakdown of xenobiotics.[1-3] The signature reaction of CYP enzymes is the insertion of a single oxygen atom from molecular dioxygen into a carbon–hydrogen bond to give the corresponding alcohol, the second oxygen atom being reduced to water.[4] The direct hydroxylation of benzenes and alkanes by chemical methods involves high energy consumption and the formation of side products. CYP-mediated enzymatic oxidation reactions have the potential to provide an efficient biocatalytic route to generate alcohols from alkanes, or phenols from benzenes, which are not feasible using contemporary organic synthetic methods.[5-8]

CYP102A1 (P450Bm3) from *Bacillus megaterium* is a soluble 119.5 kDa enzyme in which the P450 heme domain is fused to a reductase domain.[6] It requires only NADPH and oxygen to function, and hydroxylates fatty acids at sub-terminal positions close to the ω-terminus.[9] Self-sufficient CYP102 family systems exhibit the highest turnover frequency (> 1000 $min^{-1}$) of any CYP enzyme and this overcomes one of the major hurdles to the use of these enzymes as chemical catalysts.[6, 10] Wild-type P450Bm3 (WT) turns over most substrates, other than fatty acids, at desultory rates but it is a versatile platform for designing monooxygenase biocatalysts via protein engineering.[5, 6, 11-15]

It has been shown that the addition of perfluorocarboxylic acids (PFCs) as inert decoy molecules enhanced the ability of WT P450Bm3 to hydroxylate benzenes and short chain alkanes. This approach allowed the enzyme to efficiently generate compound I, the active species for substrate oxidation.[16-18] In addition, generic accelerator variants of P450Bm3 which enhance the activity but maintain the product selectivity of the WT enzyme have been developed.[11, 19-21] It has been proposed this behaviour arises from these variants being in a catalytically ready conformation, such that substrate-induced changes to the structure play a less significant role in promoting the electron transfer steps.[11, 21]

In order to facilitate the oxidation of smaller substrates such as benzene and cyclohexane by P450Bm3, we selected PFCs as decoy molecules in combination with the rate enhancing variant KT2 (A191T/N239H/I259V/A276T/L353I).[11, 21] It was expected that the PFCs–which have shorter alkyl chains than those of the natural substrates–would also bind to the KT2 active site and facilitate the initiation the catalytic cycle and the activation of molecular oxygen. The combination of the PFC decoy molecule and rate enhancing variant KT2 would therefore enhance the oxidation of these unnatural substrates over that of the WT enzyme in combination with decoy molecules and the KT2 variant alone. We initially investigated the hydroxylation of benzene and cyclohexane by P450Bm3 KT2 using PFC8, PFC9 and PFC10.[22]

As reported previously the addition of PFC8, PFC9 or PFC10 improved the activity of the turnovers of WT P450Bm3 with cyclohexane and benzene.[22] These turnovers yielded phenol and cyclohexanol as the sole products. Both were identified by co-elution experiments with standards using GC-MS (Fig. 1 and Fig. S1). PFC10 itself induced the highest activity of NADPH oxidation in WT P450Bm3 followed by PFC9 and then PFC8 (Table 1). This trend in NADPH oxidation activity was followed for the different PFC molecules in the presence of cyclohexane and benzene. Addition of PFC9 increased the coupling efficiency with the WT to a greater extent than PFC10 for both substrates (Table 1 and Fig. 1).[22] Overall the combined effects of increased activity and coupling resulted in the largest improvements in the product formation rate for PFC9 and PFC10 over PFC8 (Table 1). The maximal augmentation of the activity upon addition of a decoy molecule was 170-fold for cyclohexane and 225-fold for benzene.

The KT2 variant increased the NADPH oxidation activity, coupling efficiency and hence the product formation rate with both substrates, in line with what has been reported previously with other substrates (Table 1 and Fig. 1).[11, 21] Using the KT2 variant resulted in a 12-fold improvement in benzene oxidation activity and

[a] *Department of Chemistry, University of Adelaide, SA 5005, Australia*
[b] *Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan*
[c] *Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford, OX1 3QR, UK*
** To whom correspondence should be addressed. stephen.bell@adelaide.edu.au*
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**Table 1** *In vitro* turnover activity data for the P450Bm3 variants and decoy molecule combinations with benzene and cyclohexane as substrates and in the absence of substrate (decoy). N = NADPH turnover rate. C = coupling efficiency (%). PFR = Product formation rate. Rates are in nmol.(nmol P450)$^{-1}$.min$^{-1}$. All data are reported as the mean of at least three experiments.

| | decoy | benzene | | | cyclohexane | | |
|---|---|---|---|---|---|---|---|
| | N | N | C | PFR | N | C | PFR |
| **WT** | - | 29 ± 0.3 | 0.6 ± 0.05 | 0.2 ± 0.02 | 30 ± 2 | 2.2 ± 0.4 | 0.7 ± 0.07 |
| **WT/PFC8** | 35 ± 1 | 71 ± 1 | 3.0 ± 0.2 | 2 ± 0.2 | 212 ± 8 | 31 ± 2 | 65 ± 6 |
| **WT/PFC9** | 56 ± 0.4 | 168 ± 7 | 13 ± 0.6 | 23 ± 1 | 416 ± 7 | 28 ± 2 | 119 ± 8 |
| **WT/PFC10** | 327 ± 11 | 522 ± 6 | 8.6 ± 0.6 | 45 ± 4 | 626 ± 14 | 15 ± 1 | 91 ± 7 |
| **KT2** | - | 77 ± 6 | 3.0 ± 0.2 | 2.3 ± 0.3 | 353 ± 9 | 19 ± 1 | 70 ± 5 |
| **KT2/PFC8** | 83 ± 3 | 220 ± 4 | 4.6 ± 0.4 | 10 ± 1 | 900 ± 19 | 35 ± 4 | 315 ± 36 |
| **KT2/PFC9** | 161 ± 8 | 374 ± 7 | 10 ± 0.3 | 38 ± 1 | 1338 ± 25 | 36 ± 1 | 483 ± 28 |
| **KT2/PFC10** | 699 ± 12 | 847 ± 10 | 8.8 ± 0.8 | 74 ± 1 | 1797 ± 45 | 27 ± 1 | 484 ± 31 |

a 100-fold increase in that of cyclohexane compared WT P450Bm3. The observed activities were lower than the best WT/PFC combinations (Table 1).

Addition of the perfluorinated carboxylic acid decoy molecules to KT2 engendered further enhancement of the oxidation of benzene and cyclohexane. The NADPH oxidation activity, coupling efficiency and product formation rates of all the turnovers increased in the presence of PFCs compared to just KT2. The activity of substrate oxidation by KT2 with the decoy molecules was higher than with the WT enzyme and the PFCs in all cases. The trends in the catalytic parameters of the different PFCs with KT2 mirrored those obtained with the WT; PFC10 induced the highest NADPH oxidation rates but PFC9 resulted in the highest coupling efficiencies (Table 1). As a consequence the product formation rate of KT2 with PFC9 and PFC10 are similar and both are greater than when PFC8 is used. KT2 and PFC10 was the most active combination with benzene. The product formation rate for PFC10 combined with KT2 and benzene was 74 min$^{-1}$. The gain in activity with cyclohexane was even greater with the product formation rate being 484 min$^{-1}$ and 483 min$^{-1}$ with the KT2/PFC10 and KT2/PFC9 combinations. This was four-fold more active than the WT/PFC9 pairing (Table 1 and Fig. 1). For both the WT and KT2 enzyme turnovers the NADPH oxidation rate and coupling efficiency with cyclohexane exceeded that of benzene, resulting in higher activities (Table 1).

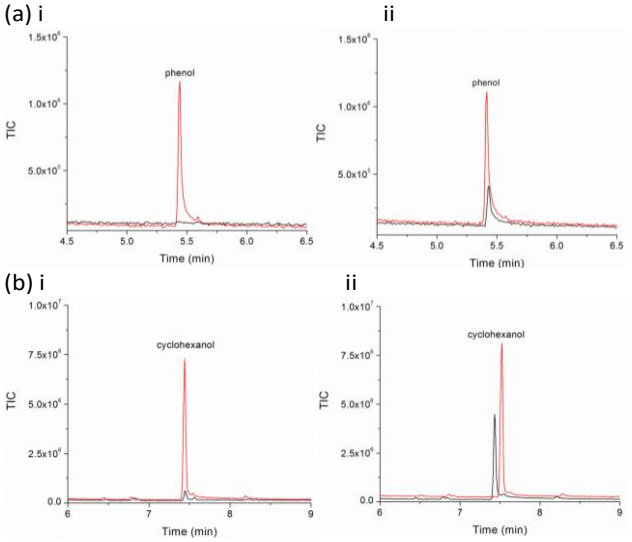



**Fig. 1** GC-MS analysis of the turnovers of (a) benzene and (b) cyclohexane using the WT (i) and KT2 (ii) variants of P450Bm3 with and without the decoy molecule PFC9. In black is the turnover in the absence of the decoy molecule and in red the turnover in the presence of the decoy molecule. In (b) (ii) the turnover of cyclohexane with KT2 with PFC9 has been offset slightly along the x-axis for clarity. The integrations of these peaks and the internal standard are given in Table S1.

Encouraged by these results we decided to assess if the combined decoy molecule/generic accelerator mutant combinations would work on substituted benzenes. The oxidation of toluene and anisole by P450Bm3 have both been reported to be highly selective (>90%) for the *ortho*-hydroxylated product.[20, 22, 23] The turnovers of toluene generated *p*-cresol and benzyl alcohol as minor products (<9% total product) while 4-methoxyphenol (<10%) is the sole minor product identified for anisole oxidation.[22, 23] The biocatalytic oxidations of xylenes by P450Bm3 have also been reported; the turnover of *p*-xylene is documented to generate only 2,5-dimethylphenol.[24] *m*-Xylene oxidation produced 2,4-dimethylphenol (87%), 2,6-dimethylphenol (11%) and 3-methylbenzylalcohol (2%).[25] The P450Bm3 catalysed oxidation of *o*-xylene was more complex yielding five products. The major product is 2-methylbenzylalcohol (47%) with 2,3- and 3,4-dimethylphenol being generated as two of the minor products, 27% and 10%, respectively. The other two minor metabolites arise from a methyl shift resulting in formation of 2,6-dimethylphenol (8%) and the dearomatisation product, 6,6-dimethylcyclohexa-2,4-dienone (8%).[25] The analysis of the turnovers of these substrates with the PFCs and KT2 would be a more stringent test to assess if these mutant and decoy molecule combinations would alter the product distribution as well as heighten the activity.

All five substrates were tested with WT and KT2 P450Bm3 with PFC8 through to PFC10. Despite the larger size of these substituted benzenes, a similar pattern of activity enhancement was observed with the decoy molecules. The turnovers containing the PFC10 decoy molecule induced the highest activity of NADPH oxidation and those with PFC9 were the more efficiently coupled. In certain instances, e.g. with toluene, the faster rates of NADPH oxidation with PFC10 resulted in higher product formation rates while in others the higher coupling obtained with PFC9 resulted in the maximum activity, e.g. *p*-xylene with KT2/PFC9 (Table 2). In all cases the greatest product formation rates were obtained with KT2/PFC9 or KT2/PFC10. The rate enhancement over the WT/PFC partnerships arose mainly from increased NADPH oxidation activity rather than enhanced coupling (Table 2).

The oxidation of both toluene and anisole was faster than that of benzene for all the enzyme/decoy molecule combinations tested. This was due to increases in the coupling efficiency and the rate of NADPH oxidation. The maximum enhancements in the rate

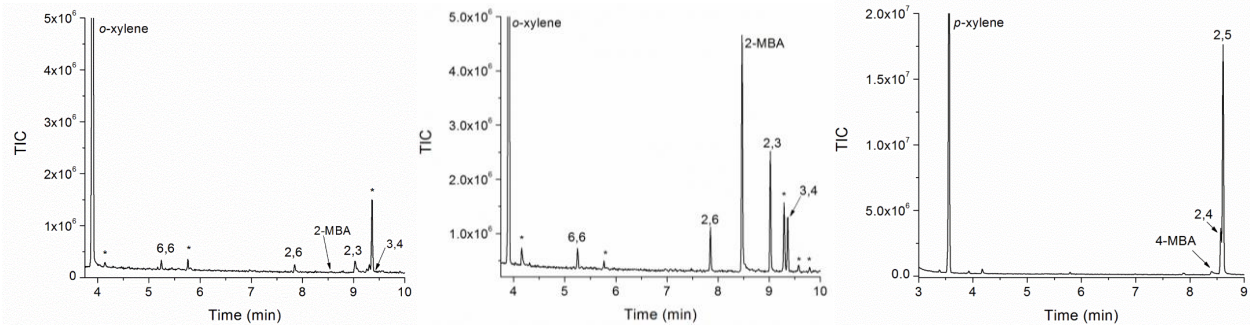
**Table 2** *In vitro* turnover activity data for the P450Bm3 variants and decoy molecule combinations with toluene, anisole, *p*-, *m*- and *o*-xylene. N = NADPH turnover rate. C = coupling efficiency (%). PFR = Product formation rate. Rates are in nmol.(nmol P450) $^{-1}$.min$^{-1}$. All data are reported as the mean of at least three experiments.

| | WT | WT +PFC9 | WT +PFC10 | KT2 | KT2 +PFC9 | KT2 +PFC10 |
|---|---|---|---|---|---|---|
| **Toluene** | | | | | | |
| N | 22 ± 0.9 | 355 ± 3 | 622 ± 8 | 68 ± 2 | 549 ± 7 | 822 ± 12 |
| C | 1.6 ± 0.5 | 40 ± 1 | 25 ± 2 | 9.7 ± 3 | 41 ± 1 | 33 ± 1 |
| PFR | 0.3 ± 0.1 | 144 ± 3 | 158 ± 10 | 7 ± 2 | 225 ± 8 | 271 ± 9 |
| **anisole** | | | | | | |
| N | 122 ± 2 | 262 ± 2 | 581 ± 3 | 189 ± 12 | 399 ± 9 | 729 ± 16 |
| C | 1.4 ± 0.1 | 18 ± 3 | 11 ± 0.5 | 7.5 ± 1 | 19 ± 2 | 12 ± 0.1 |
| PFR | 2.0 ± 0.1 | 48 ± 9 | 64 ± 3 | 14 ± 2 | 76 ± 6 | 87 ± 2 |
| ***p*-xylene** | | | | | | |
| N | 63 ± 5 | 687 ± 7 | 943 ± 7 | 267 ± 10 | 1010 ± 33 | 1080 ± 8 |
| C | 19 ± 2 | 56 ± 4 | 41 ± 4 | 34 ± 0.9 | 54 ± 2 | 42 ± 2 |
| PFR | 12 ± 2 | 386 ± 26 | 394 ± 35 | 90 ± 1 | 549 ± 22 | 460 ± 26 |
| ***m*-xylene** | | | | | | |
| N | 33 ± 0.6 | 546 ± 3 | 698 ± 8 | 178 ± 5 | 830 ± 7 | 1050 ± 12 |
| C | 3.5 ± 0.4 | 55 ± 1 | 40 ± 0.4 | 26 ± 2 | 57 ± 2 | 43 ± 2 |
| PFR | 1.1 ± 0.1 | 302 ± 5 | 278 ± 4 | 46 ± 4 | 476 ± 15 | 455 ± 12 |
| ***o*-xylene** | | | | | | |
| N | 29 ± 1 | 284 ± 5 | 468 ± 5 | 106 ± 2 | 738 ± 18 | 1020 ± 16 |
| C | 1.9 ± 0.1 | 53 ± 2 | 28 ± 2 | 30 ± 3 | 45 ± 1 | 34 ± 1 |
| PFR | 0.6 ± 0.06 | 149 ± 8 | 131 ± 6 | 32 ± 2 | 334 ± 17 | 346 ± 10 |

of product formation, over WT P450Bm3, were approximately 900-fold for toluene and 40-fold for anisole (Table 2 and Table S2). The larger gain with toluene predominantly arose from a more significant increase in the coupling efficiency when compared to the anisole turnovers. The best KT2/PFC combinations resulted in product formation rates which were almost double that of the optimal WT/PFC pairings. The oxidation of toluene and anisole resulted in the generation of *o*-cresol (94-95%) and *o*-methoxyphenol (88-92%), respectively (Fig. S2, S3 and Table S3). Benzyl alcohol and *p*-cresol from toluene and 4-methoxyphenol from anisole were observed as the minor metabolites in these turnovers. The product distributions were virtually unchanged when compared to the WT enzyme (Scheme 1, Fig. S2, Table S3).

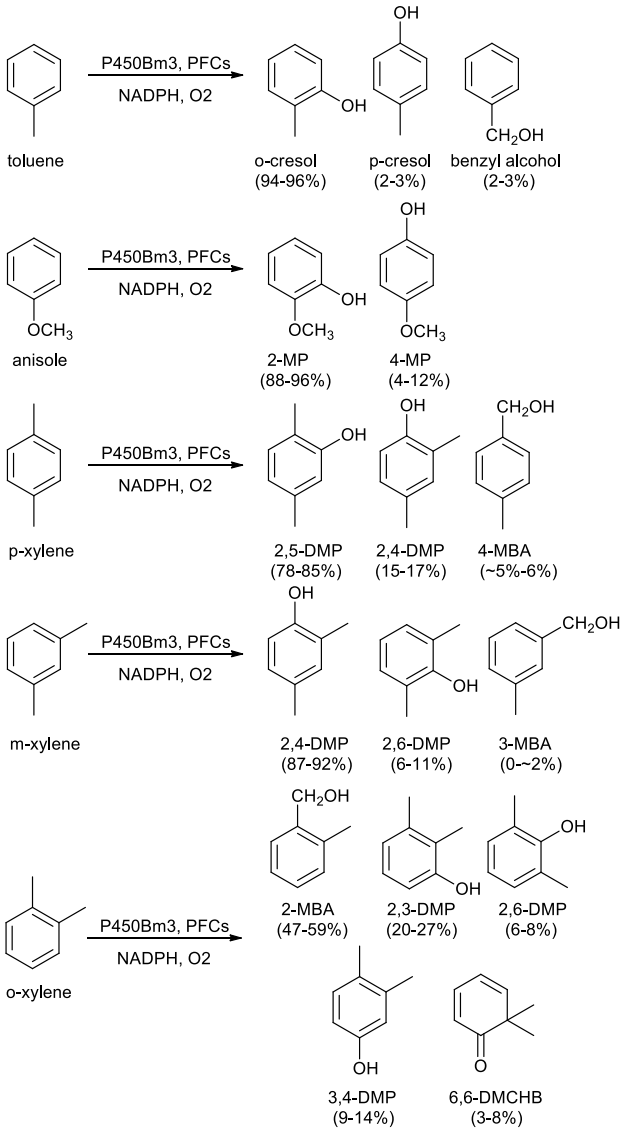
The oxidation of all the xylene substrates was enhanced using the decoy molecule/generic rate accelerator combinations. The maximum activity enhancements over the WT enzyme for the three xylene substrates were achieved with the KT2/PFC9 or KT2/PFC10 combination (*p*-xylene, 45-fold; *m*-xylene, 430-fold; *o*-xylene, 575-fold, Table 2 and Fig, S4). In addition the product formation rates observed were higher than those achieved with toluene. The product distributions observed across all the turnovers were comparable to those reported for the WT enzyme and the KT2 variant suggesting that the partnering of a decoy molecule and the rate enhancing variant do not significantly alter the interactions of the substrate with the enzyme's active site amino acids and therefore it's orientation in the substrate binding pocket (Scheme 1, Fig. S2 and S3 and Table S3). For example the ratios of the five products formed by the WT enzyme and *o*-xylene, including those arising from a shift in a methyl group, are similar to those obtained with the KT2/PFC10 combination, which has the highest activity (Fig. 2 and Table S3).

One point of difference to the previously reported data for *p*-xylene was that three products were observed in the GC-MS analysis of all the turnovers (Fig. 2, Fig. S5 and Table S3).[24] These corresponded to 4-methylbenzyl alcohol (0-6%), 2,4- and 2,5-dimethylphenol (15-17% and 77-85%, respectively). The 2,4-dimethylphenol product must arise via the NIH shift of a methyl group an activity that is also observed with *o*-xylene (Scheme S1).[25]




(a) i (a) ii b)

**Fig. 2** GC-MS analysis of the turnovers of (a) *o*-xylene using (i) WT P450Bm3 and (ii) KT2 and PFC10, the products are labelled; 2-methylbenzylalcohol (2-MBA), 2,3-dimethylphenol (2,3), 3,4-dimethylphenol (3,4), 2,6-dimethylphenol (2,6), and 6,6-dimethylcyclohexa-2,4-dienone (6,6).(b) (i) *p*-xylene using the KT2 and PFC8, the products are labelled 2,5-dimethylphenol (2,5), 2,4-dimethylphenol (2,4) and 4-methylbenzyl alcohol (4-MBA) Impurities are labelled *.




**Scheme 1** The products formed from the turnovers of P450Bm3 enzyme/decoy molecule combinations with benzene substrates. Abbreviations used; 2-methoxyphenol (2-MP), 4-methoxyphenol (4-MP), 2,5-dimethylphenol (2,5-DMP), 2,4-dimethylphenol (2,4-DMP), 4-methylbenzyl alcohol (4-MBA), 2,6-dimethylphenol (2,6-DMP), 3-methylbenzylalcohol (3-MBA), 2-methylbenzylalcohol (2-MBA), 2,3-dimethylphenol (2,3-DMP), 3,4-dimethylphenol (3,4-DMP) and 6,6-dimethylcyclohexa-2,4-dienone (6,6-DMCHD).

In conclusion we have shown that the combination of a rate accelerating mutant and a decoy molecule is a simple and efficient method to improve the activity of P450Bm3 without altering the product distribution. The improvements were greatest for cyclohexane and the KT2/PFC decoy molecule combinations seem to be well suited for the oxidation of smaller alkanes. This approach provides additional evidence for multiple binding sites in P450Bm3 [26] and could be used to drive substrate oxidation by CYP enzymes at high activities and improved product yields. This would enable the generation of the required levels of product for synthetic chemistry applications for reactions which show promising regio- and stereoselectivity with WT or mutant forms of the enzyme. It could even be applied to the non-physiological reactions recently described for the this versatile enzyme.[13]

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