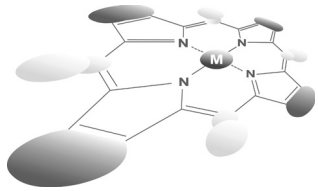
Journal of Porphyrins and Phthalocyanines
J. Porphyrins Phthalocyanines 2015; **19**: 329–334
DOI: 10.1142/S1088424615500145

Published at http://www.worldscinet.com/jpp/



Highly efficient hydroxylation of gaseous alkanes at reduced temperature catalyzed by cytochrome P450BM3 assisted by decoy molecules

Norifumi Kawakami[a], Zhiqi Cong[b], Osami Shoji*[b◊] and Yoshihito Watanabe*[c]

[a] *Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, 223-8522 Yokohama, Japan*
[b] *Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan*
[c] *Research Center for Materials Science Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan*

Dedicated to Professor Shunichi Fukuzumi on the occasion of his retirement

Received 9 November 2014
Accepted 4 December 2014

ABSTRACT: Cytochrome P450BM3 functions as a small-alkane hydroxylase upon the addition of perfluorocarboxylic acids (PFs) as decoy molecules. The coupling efficiency (product formation rate per NADPH consumption rate) for the hydroxylation of small alkanes was improved by reducing the reaction temperature to 0 °C.

KEYWORDS: cytochrome P450, gaseous alkanes, perfluorocarboxylic acids, decoy molecules.

INTRODUCTION

Hydroxylation of small alkanes catalyzed by enzymes has been recognized as an important process for producing precursors of fine chemicals and feedstocks. Several small alkane hydroxylases, such as methane monooxygenase and omega alkane hydroxylase AlkB, have been widely studied in the context of industrial applications [1–6]. In addition, a variety of engineered enzymes that catalyze the hydroxylation of small alkanes has also been successfully prepared by site-directed and/or random mutagenesis [7, 8]. For example, cytochrome P450BM3, which catalyzes the hydroxylation of fatty acids having an alkyl chain length from 12 to 20 (C12–20) carbon units, has been engineered to catalyze the hydroxylation of gaseous alkanes such as ethane, propane, and butane [9–11]. Although wild-type P450BM3 selectively catalyzes the hydroxylation of long-alkyl-chain fatty acids (Fig. 1b, left), our research group and Zilly *et al.* recently reported that even wild-type P450BM3 can catalyze the hydroxylation of small alkanes in the presence of a series of perfluorocarboxylic acids (PFs) with alkyl chain lengths from 5 to 14 (PFC5–14); the latter perfluorinated compounds were named "decoy molecules" (Fig. 1b, right) [12, 13]. According to the reaction mechanism of fatty acid hydroxylation by P450BM3 (Fig. 1b, left), fatty acid binding induces a positive redox potential shift of the heme iron by removing a ligated water molecule, which is crucial for initiating the catalytic cycle. Molecular oxygen is then reductively activated by utilizing two electrons from NADPH to form an oxoferryl(IV) porphyrin π cation radical (so-called Compound I), which is responsible for the hydroxylation of substrates (Fig. 1) [14–17]. We found that because PFs have structures that are similar to those of fatty acids, it is also possible to initiate the catalytic cycle to form Compound I [12]; however, the PFs are not hydroxylated because of the high dissociation energy of C–F bonds. By adding small alkanes to the reaction mixture, the produced Compound I can hydroxylate the small alkanes instead of PFs (Fig. 1b, right). Interestingly, the catalytic activities of the alkane hydroxylation depend on the alkyl chain length of the PFs. For example,

◊ SPP full member in good standing

*Correspondence to: Osami Shoji, email: shoji.osami@a.mbox.nagoya-u.ac.jp; Yoshihito Watanabe, email: p47297a@nucc.cc.nagoya-u.ac.jp, tel: +81 52-789-3557, fax: +81 52-789-2953

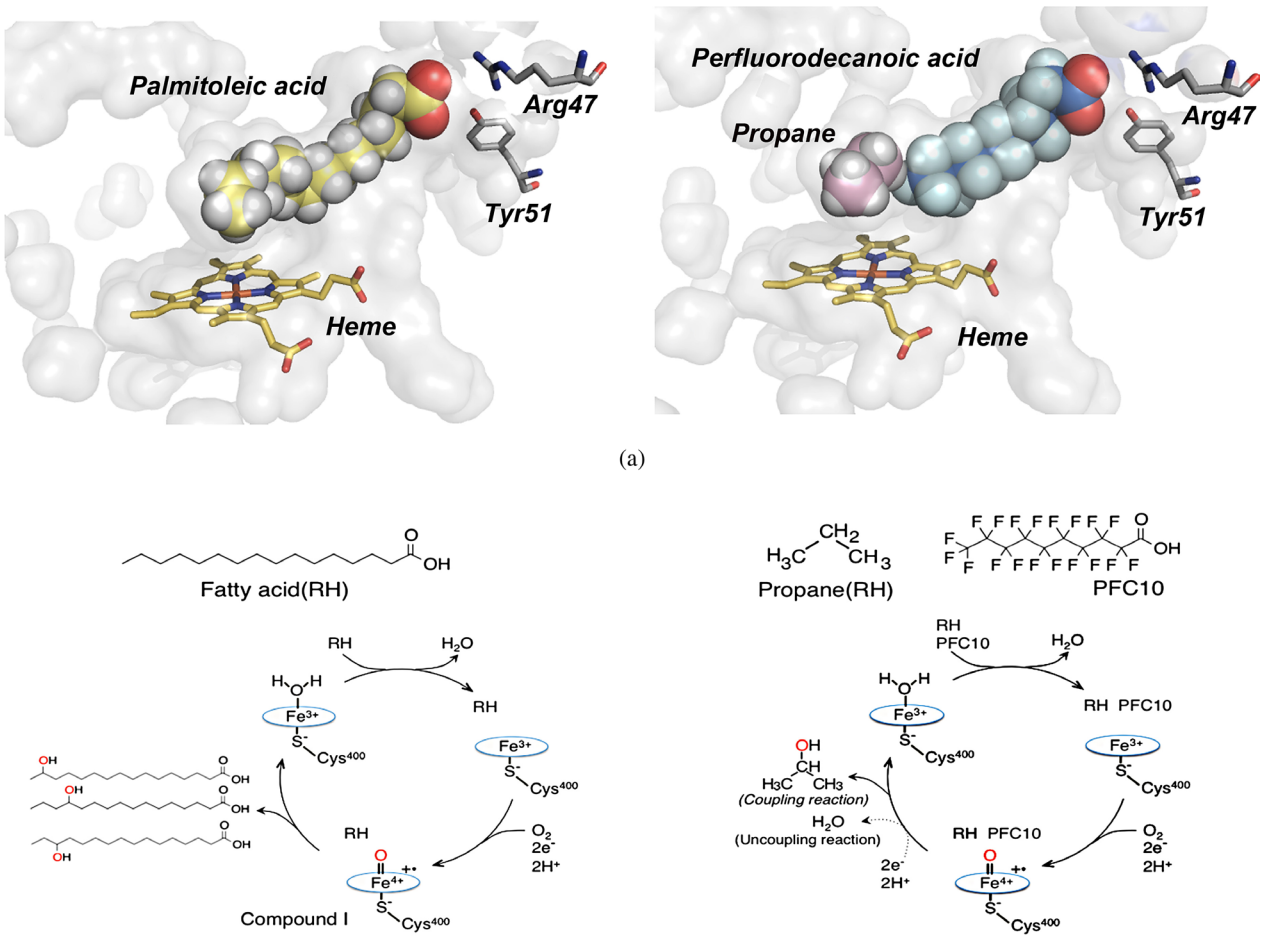


Fig. 1. (a) The active site of P450BM3 containing palmitoleic acid (left; PDB code: 1FAG) and the plausible active-site structure of P450BM3 containing PFC10 and propane (right). (b) General reaction mechanism of P450BM3 (left) and the reaction mechanism of propane hydroxylation catalyzed by P450BM3 with perfluorodecanoic acid (PFC10) as a decoy molecule (right). In the presence of PFC10, although uncoupling reaction produces water molecule (dotted line in the reaction cycle), propane is converted to 2-propanol (solid line in the reaction cycle)

the highest catalytic activities for propane and butane hydroxylation reactions were observed in the presence of PFC10 and PFC9, respectively. Therefore, we concluded that the PFs partially occupy the active site of P450BM3, leaving a small amount of space that was suitable for accommodating small alkanes (Fig. 1a, right). Although small alkanes are hydroxylated in the presence of PFs, the coupling efficiency (product formation rate per NADPH consumption rate) was low: 18 and 55% for propane and butane hydroxylation reactions, respectively. As reported previously [18], Compound I, generated from P450BM3 in the presence of PFs, is also reduced back to the resting state through a further two-electron reduction leading to the production of H_2O. This unproductive consumption of Compound I competes with the hydroxylation of small alkanes (Fig. 1b, right) [12, 19]. By increasing the propane pressure from 8 kPa to 0.5 MPa, the coupling efficiency was improved to 50% [19], indicating that the concentration of small alkanes is a key parameter that affects coupling efficiency. We reasoned that the coupling efficiency could be improved if the enzymatic reaction was performed at lower temperature because this would lead to an increase in the concentration of gaseous alkanes in the aqueous solution. We herein report that the effect of temperature on the catalytic activity and coupling efficiency of propane and butane hydroxylation reactions catalyzed by wild-type P450BM3 in the presence of PFs is indeed significant.

EXPERIMENTAL

Materials

P450BM3 was prepared according to the method previously reported [12]. The concentration of the enzyme was determined by CO-difference spectra. The details of purification of P450BM3 are shown in below.

E. coli cells expressing P450BM3 suspended in 20 mM Tris-HCl (pH 7.4) are disrupted by using an ultrasonicator at 4 °C. After removing cell debris by centrifugation, the supernatant was applied to DE-52 column (*Whatman).* The proteins weakly bound to DE-52 are washed out with 20 mM Tris-HCl containing 50 mM KCl (pH 7.4) and tightly bound proteins including P450BM3 are eluted with the Tris-buffer containing 250 mM KCl. The P450BM3 fraction was collected and further purified by DEAE 650S (TOSOH). P450BM3 was eluted with the Tris-HCl buffer with KCl concentration gradient from 0 to 120 mM. To remove the bound substrates, NADPH was added immediately before applying to gel filtration column, Sephacryl S-300 (GE Healthcare), equilibrated with 20 mM Tris buffer with 100 mM KCl (pH 7.4) and the P450BM3 fraction was collected. All chemical reagents were purchased from commercial sources and used without further purification. *o*-Cresol, *m*-cresol, and perfluorononanoic acid (PEC9) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Benzylalcohol, *p*-cresol, and perfluorooctanoic acid (PFC8) were purchased from Sigma-Aldrich Co., (St. Louis, MO). Toluene were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). The following chemicals were purchased from WAKO Pure Chemical Industries, Ltd (Osaka, Japan): benzene, and perfluorodecanoic acid (PFC10). Gaseous alkanes are purchased from Taiyo Nippon Sanso Corp. NADPH was purchased from Nakalai tesque, Inc.

Measurement

UV-vis spectra were recorded on a Shimadzu UV-2400 PC spectrophotometer. GC analysis was performed by GC2014 (Shimadzu Corp.) with Rtx-1 column (Restek Corp.). HPLC analysis was performed using an Inertsil® ODS-3 column (4.6 mm × 250 mm; GL Sciences, Inc., Tokyo, Japan) installed on a Shimadzu SCL-10AVP system controller equipped with Shimadzu LC-10ADVP pump systems, a Shimadzu RF-10AXL fluorescence spectrometer, a Shimadzu CTO-10AVP column oven, and a Shimadzu DGU-12A degasser.

Gaseous alkane hydroxylation reactions

Reaction mixture containing 500 nM P450BM3, 100 μM PFs was prepared in the gaseous alkane saturated buffer at 20 or 0 °C. The reaction temperature was kept by water bath and iced water for the reaction at 20 and 0 °C, respectively. PFs for stock solution were dissolved in DMSO and adjusted to 20 mM. Gaseous alkane saturated buffer was also prepared at each temperature. Immediately after adding 5 mM NADPH (for the long-term reaction, 20 mM of NADPH was used), the reaction mixture was pressured by a balloon containing gaseous alkane and molecular oxygen with the ratio of 4 to 1. The remaining air in the reaction vessel was replaced by making gas flow from balloon to the out *via* the vessel for 1 min. One hour later, the amount of remaining NADPH was estimated by measuring the absorbance at 340 nm.

Propanol and butanol detection

After one-hour reaction, 1 mL of reaction mixture was transferred to new glass vial containing 0.15 g of sodium nitrite for derivatization, and then 1 mM 3-pentanol was added as internal standard. Before putting the sample on ice, 1 mL of hexane (for propanol) or dichloromethane (for butanol) were added. To the resulting reaction mixture, 150 μL of aqueous solution of sulfuric acid (20%) was gently added. The glass vial was kept on ice for 15 min. The derivatized products, 2-propylnitrite or 2-butylnitrite, were extracted and analyzed by Gas Chromatography (GC). The analytical conditions were as follows: column temperature, 323 K (holding 5 min) to 523 K (25 K/min, holding 3 min); the injection port and FID detector temperature were 523 K; carrier gas, helium. The products were identified based on the retention time of authentic samples. The concentration of the products was determined by using the ratio of peak area of the product to the area of the internal standard (3-pentanol).

Hydroxylation of benzene

Benzene hydroxylation by P450BM3 was carried out in 20 mM Tris-HCl (pH = 7.4) buffer containing 100 mM KCl at 0 °C for 1 h in the presence of 0.5 μM P450BM3, 10 mM benzene, 5 mM NADPH, and 100 μM PFC9. PFC9 was dissolved in DMSO and added to the reaction mixture. After 1 h reaction, a solution of hydrochloric acid (1 M) was added to the reaction mixture to quench the reaction followed by neutralization with a solution of KOH (1 M). The resulting solution was filtrated and analyzed by reverse phase HPLC. The HPLC analytical conditions were as follows: flow rate, 0.5 mL/min; acetonitrile/water = 1/1; column temperature, 30 °C; monitoring absorption at 210 and 270 nm. Phenol was identified using authentic samples. Reaction was performed at least three times.

Hydroxylation of toluene

The reaction was carried out in the same manner as for the hydroxylation of benzene. The reaction was evaluated by reverse phase HPLC. Products were identified using authentic samples.

NADPH consumption

Immediately after preparing reaction mixture containing 0.5 μM P450BM3, 10 mM benzene, and 100 μM perfluorinated carboxylic acids, reaction was initiated by the addition of 5 mM NADPH. The absorbance of NADPH at 340 nm was monitored. The concentration of the NADPH was estimated using its molar extinction coefficient at 340 nm, 6220 $M^{-1} \cdot cm^{-1}$.

Table 1. The catalytic activity of propane and butane hydroxylations by P450BM3 with PFs at 0 °C and 20 °C. The amount of 2-propanol and 2-butanol after 1 h reaction are given in [μM]. The amount of NADPH consumed and the coupling efficiency are given in [μM] and [Product]/[NADPH consumed] × 100, respectively

	Propane 2-propanol, μM[a,b]	NADPH consumption, μM[a,b]	Coupling efficiency, %[a]	Butane 2-butanol, μM[a,b]	NADPH consumption, μM[a,b]	Coupling efficiency, %[a]	*ee*, *S*%	No alkanes NADPH consumption, μM[a,b]
PFC8 0 °C	160 ± 20 (320)	590 ± 130 (1180)	27 ± 5	1110 ± 220 (2220)	1320 ± 60 (2640)	84 ± 12	18	200 ± 30 (400)
PFC8 20 °C	500 ± 80 (1000)	1930 ± 70 (3860)	26 ± 5	2170 ± 310 (4340)	3180 ± 430 (6360)	68 ± 1	14	400 ± 110 (800)
PFC9 0 °C	660 ± 120 (1320)	1160 ± 170 (2320)	57 ± 10	2620 ± 230 (5240)	2730 ± 110 (5460)	96 ± 9	20	410 ± 180 (820)
PFC9 20 °C	1310 ± 270 (2620)	4190 ± 100 (8380)	31 ± 7	2820 ± 480 (5640)	3610 ± 580 (7220)	78 ± 2	14	2100 ± 60 (4200)
PFC10 0 °C	480 ± 40 (960)	1110 ± 160 (2220)	44 ± 5	1140 ± 50 (2280)	1510 ± 80 (3020)	75 ± 1	16	660 ± 80 (1320)
PFC10 20 °C	1620 ± 80 (3240)	4240 ± 70 (8480)	38 ± 2	2830 ± 360 (5660)	4050 ± 290 (8100)	70 ± 6	16	3860 ± 130 (7720)

[a]Values are averages ± standard deviation calculated from three different experiments. [b]Values in the parentheses are turnover frequency (nmol/hour/nmol-P450BM3).

RESULTS AND DISCUSSION

We first investigated whether the use of P450BM3 in conjunction with PFs catalyzed the hydroxylation of propane and butane gas at 0 °C (Table 1) and confirmed that, even at this low temperature, the P450BM3–PF complexes functioned as a monooxygenase to selectively yield 2-propanol and 2-butanol, respectively. To understand the effect of reaction temperature, hydroxylation of propane and butane was also carried out at 20 °C and the coupling efficiency and hydroxylation rate were compared with those measured at 0 °C [20]. The coupling efficiency of propane hydroxylation improved slightly from 38 to 44% in the presence of PFC10, upon reducing the reaction temperature, whereas the hydroxylation rate decreased from 3240 to 960 nmol/h/nmol-P450BM3. Similarly, a small improvement in the coupling efficiency and a decrease in the catalytic activity were observed for butane hydroxylation in the presence of PFC10 at 0 °C. The coupling efficiency for propane hydroxylation upon lowering the temperature showed a more significant improvement, from 31 to 57%, in the presence of PFC9. Furthermore, the coupling efficiency of butane hydroxylation was also drastically improved at lower temperature, from 78 to 96%, in the presence of PFC9. Notably, butane hydroxylation with PFC9 at 0 °C resulted in almost complete coupling (96%) while maintaining a catalytic activity comparable to that measured for the reaction performed at 20 °C. The uncoupling reaction in the absence of alkane gas was also dramatically suppressed, from 4200 to 820 turnover frequency, by lowering the temperature (Table 1), suggesting that P450BM3 was activated when the PFC9 and gaseous alkanes occupied the active-site space simultaneously. Although the uncoupling reaction in the absence of alkane gas was also suppressed in the case of PFC10 (from 7720 to 1320 turnover frequency), the coupling efficiency was not much improved. We reasoned that PFC10 is too effective for initiating the generation of active species even at 0 °C. Because PFC10 alone was sufficient enough for the generation of active species, the uncoupling reaction was predominant even in the presence of alkane gas.

Table 2. The catalytic activity of propane hydroxylation by P450BM3 with PFC10 at 0 and 20 °C for 24 h. The amount of 2-propanol and NADPH consumed after 24 h reaction are given in [μM]. The total tuenover number (TON) and the coupling efficiency are given in nmol/nmol-P450BM3, and [Product]/[NADPH consumed] × 100, respectively

	2-propanol, μM	NADPH consumption, μM	Coupling efficiency, %	Turnover number (TON)
20 °C	2380	14 100	17	4760
0 °C	4920	12 400	40	9840

Given that the solubility of gaseous molecules is inversely proportional to solvent temperature, the concentration of gaseous molecules in the buffer solution increases upon lowering the reaction temperature and thus the coupling efficiency is improved by performing the reaction at 0 °C rather than at 20 °C. In fact, the solubilities of propane and butane in water at 4 °C are reported to be 3.46 and 3.21 M, respectively, which are twice as large as those of propane at 19.8 °C (1.74 M) and butane at 20 °C (1.46 M) [21]. In addition to these results, propane hydroxylation in the presence of PFC9 over a longer period showed a total turnover number (TON) of 9840, with 40% coupling efficiency for 24 h reaction at 0 °C, whereas the same reaction performed at 20 °C had a TON of 4760, with 18% coupling efficiency, indicating that cooling the reaction mixture was also effective with respect to the total amount of product formed (Table 2). The enantioselectivity for the formation of 2-butanol increased slightly, especially in the presence of PFC9 (from 14 to 20% *ee*, Table 1), but the small difference suggests that the orientation of butane in the active site is not significantly affected by the reaction temperature. Although improved butane hydroxylation by engineered P450BM3 at 0 °C was recently described, the effect of temperature upon the enantioselectivity was not reported [22]. To investigate the effect of performing the reaction at 0 °C upon hydroxylation of substrates other than gaseous alkane, we also conducted the hydroxylation reaction with benzene and toluene [23] at 0 and at 20 °C for 1 h (Table 3).

Table 3. The catalytic activity of benzene and toluene hydroxylations by P450BM3 with PFC9 at 0 °C and 20 °C for 1 h. The amount of products after 1 h reaction are given in [μM]. The amount of NADPH consumed and the coupling efficiency are given in [μM] and [Product]/[NADPH consumed] × 100, respectively

Substrate	Product, μM[a]		NADPH consumption, μM[a]	Coupling efficiency, %
	Major	Minor		
Benzene 0 °C	980 ± 180 (Phenol)		3070 ± 20	32 ± 6
Benzene 20 °C	2030 ± 480 (Phenol)		8270 ± 420	25 ± 7
Toluene 0 °C	1830 ± 70 (*o*-Cresol)	280 ± 30 (Benzyl alcohol)	4200 ± 140	50 ± 4
Toluene 20 °C	4100 ± 260 (*o*-Cresol)	510 ± 50 (Benzyl alcohol)	9860 ± 30	47 ± 3

[a]Values are averages ± standard deviation calculated from at least three different experiments.

The coupling efficiency for the hydroxylation of benzene (25%) and toluene (47%) at 20 °C was improved to 32% and 50%, respectively, by reducing the temperature to 0 °C. Because the regioselectivity of toluene hydroxylation was not affected significantly by the reaction temperature, we presume the orientation of toluene in the cavity of P450BM3 at 0 °C would be not much different from that at 20 °C. The improved coupling efficiency observed for the benzene and toluene hydroxylation using PFC9 as a decoy molecule could be attributed to suppression of the uncoupling reaction in the absence of substrate at 0 °C.

Given that the coupling efficiency was clearly improved with gaseous alkane hydroxylation, the improved solubility of gaseous alkanes at 0 °C in the buffer solution together with the suppression of the uncoupling reaction in the absence of substrate at 0 °C contribute to efficient reactions catalyzed by P450BM3 with the assistance of decoy molecules.

CONCLUSION

We have demonstrated that the coupling efficiency of propane and butane hydroxylation catalyzed by the P450BM3-decoy molecule system is significantly improved by performing the reaction at 0 °C. The coupling efficiency for propane and butane reached 57 and 96%, respectively. Conducting the reaction at low temperature is beneficial not only for the coupling efficiency but also for the total amount of product formed through small alkane hydroxylation. Although the turnover rate at 0 °C clearly decreased, performing the reaction at 0 °C could be one of the strategies to improve the efficiency of gaseous alkane hydroxylation catalyzed by P450BM3 with the assistance of decoy molecules.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (S) to Y.W. (24225004), Grant-in-Aid for Scientific Research on Innovative Areas "Molecular Activation Directed toward Straightforward Synthesis" to O.S. (25105724), and a Grant-in-Aid for Young Scientists (A) to O.S. (21685018) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan).

REFERENCES

1. Merkx M, Kopp DA, Sazinsky MH, Blazyk JL, Muller J and Lippard SJ. *Angew. Chem. Int. Ed.* 2001; **40**: 2782–2807.
2. Lee SJ, McCormick MS, Lippard SJ and Cho US. *Nature* 2013; **494**: 380–384.
3. Koch DJ, Chen MM, van Beilen JB and Arnold FH. *Appl. Environ. Microbiol.* 2009; **75**: 337–344.
4. Nieder M and Shapiro J. *J. Bacteriol.* 1975; **122**: 93–98.
5. Staijen IE, van Beilen JB and Witholt B. *Eur. J. Biochem.* 2000; **267**: 1957–1965.
6. Jiang Y and Dalton H. *Biochim. Biophys. Acta, Gen. Subj.* 1994; **1201**: 76–84.
7. Bell SG, Stevenson JA, Boyd HD, Campbell S, Riddle AD, Orton EL and Wong LL. *Chem. Commun.* 2002; 490–491.
8. Xu F, Bell SG, Lednik J, Insley A, Rao ZH and Wong LL. *Angew. Chem. Int. Ed.* 2005; **44**: 4029–4032.
9. Boddupalli SS, Pramanik BC, Slaughter CA, Estabrook RW and Peterson JA. *Arch. Biochem. Biophys.* 1992; **292**: 20–28.
10. Meinhold P, Peters MW, Chen MMY, Takahashi K and Arnold FH. *ChemBioChem* 2005; **6**: 1765–1768.
11. Fasan R, Meharenna YT, Snow CD, Poulos TL and Arnold FH. *J. Mol. Biol.* 2008; **383**: 1069–1080.
12. Kawakami N, Shoji O and Watanabe Y. *Angew. Chem. Int. Ed.* 2011; **50**: 5315–5318.
13. Zilly FE, Acevedo JP, Augustyniak W, Deege A, Hausig UW and Reetz MT. *Angew. Chem. Int. Ed.* 2011; **50**: 2720–2724.
14. Rittle J and Green MT. *Science* 2010; **330**: 933–937.
15. Makris TM, von Koenig K, Schlichting I and Sligar SG. *J. Inorg. Biochem.* 2006; **100**: 507–518.
16. Murataliev MB and Feyereisen R. *Biochemistry* 1996; **35**: 15029–15037.
17. Perera R, Sono M, Raner GM and Dawson JH. *Biochem. Biophys. Res. Commun.* 2005; **338**: 365–371.
18. Pons N, Pipino S and De Matteis F. *Biochem. Pharmacol.* 2003; **66**: 405–414.
19. Kawakami N, Shoji O and Watanabe Y. *Chem. Sci.* 2013; **4**: 2344–2348.
20. We found that air was still remained in the reaction vessel in our previous method [12]. Incomplete replacement of air with small alkane causes the low concentration of small alkanes in the reaction mixture. By thoroughly replacing the remaining air by small alkanes, we obtained the higher coupling efficiency of propane and butane hydroxylation than those of the previous results [12].
21. Yalkowsky SH, He Y and Jain P. *Handbook of Aqueous Solubility Data*, 2nd ed., CRC Press: Boca Raton, 2010; pp 75 and 127.
22. Staudt S, Muller CA, Marienhagen J, Boing C, Buchholz S, Schwaneberg U and Groger H. *Beilstein Journal of Organic Chemistry* 2012; **8**: 186–191.
23. Shoji O, Kunimatsu T, Kawakami N and Watanabe Y. *Angew. Chem. Int. Ed.* 2013; **52**: 6606–6610.