Appl Microbiol Biotechnol (2011) 90:147–157
DOI 10.1007/s00253-010-3064-7

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Bioconversion of substituted naphthalenes and β-eudesmol with the cytochrome P450 BM3 variant F87V

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Received: 19 September 2010 / Revised: 5 December 2010 / Accepted: 6 December 2010 / Published online: 24 December 2010

Abstract Bioconversion of various substituted naphthalenes that contain 1-methoxy- and 1-ethoxy-naphthalenes, methylnaphthalenes, dimethylnaphthalenes, and naphthalenecarboxylic acid *methyl* esters were performed using recombinant *Escherichia coli* cells, which expressed the gene coding for a cytochrome P450 BM3 variant F87V (P450 BM3 (F87V)) that was N-terminally fused to an archaeal peptidyl–prolyl *cis-trans* isomerase. In addition, bioconversion experiments with the same substrates were carried out using those that expressed the *phnA1A2A3A4* genes for a polycyclic aromatic hydrocarbon (PAH)-dihydroxylating dioxygenase, which originated from a PAH-utilizing marine bacterium *Cycloclasticus* sp. strain A5. Consequently, a variety of mono-hydroxylated derivatives were generated from these substituted naphthalenes. Oxidative aryl coupling was found to produce a novel compound 4,4′-diethoxy-[2,2′]-binaphthalenyl-1,1′-diol from 1-ethoxynaphthalene with the *E. coli* cells expressing the P450 BM3 (F87V) gene. This recombinant *E. coli* was further shown to introduce the hydroxyl group *regio*- and *stereo*-specifically into a sesquiterpene β-eudesmol.

Keywords P450 BM3 · Cytochrome P450 · Peptidyl–prolyl *cis-trans* isomerase · Dihydroxylating dioxygenase · Naphthalene · β-eudesmol

Dedicated to Dr. Miho Nodate who died on January 2, 2006.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-010-3064-7) contains supplementary material, which is available to authorized users.

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Introduction

Hydroxylation reactions on inactive carbon atoms in organic molecules, which contain terpenes (isoprenoids), aromatic compounds, and alkyl compounds, are notable among a series of enzymatic reactions, since organic chemistry is substantially difficult to mediate such reactions. Cytochromes P450 (P450s) can occasionally perform such hydroxylation reactions with *regio*- and *stereo*-specific manners (Isin and Guengerich 2007; Nodate et al. 2006). Therefore, P450s have the potential to be important biocatalysts for the synthesis of industrially important organic molecules that are difficult to synthesize chemically. However, P450s can readily lose their stability as catalysts, and require one (eukaryote-type) or two (bacterium- or mitochondrion-type) addition-

al proteins to transfer electrons from NAD(P)H to the heme domain of a P450 protein (Hannemann et al. 2007). Among numerous P450s, P450 BM3 derived from *Bacillus megaterium* was found to have a unique structure, i.e., it was a natural fusion enzyme composed of a P450 part and a eukaryote-type NADPH-P450 reductase domain (Narhi and Fulco 1986). This enzyme has been one of the best studied bacterial P450s, since it is a relatively stable P450 monooxygenase that hydroxylates long-chain fatty acids (12–20 carbons) (Boddupalli et al. 1990; Li and Poulos 1999; Ruettinger et al. 1989). Protein engineering approaches on P450 BM3 have been carried out extensively (Cirino and Arnold 2002; Li et al. 2000; Ost et al. 2000). In particular, the effect of its Phe87 on substrate and reaction specificity has attracted much ongoing attention (Dietrich et al. 2009; Graham-Lorence et al. 1997; Li and Poulos 1997; Li et al. 2001, 2000; Oliver et al. 1997). Structural analysis on the P450 BM3 heme domain revealed that the phenyl side-chain of Phe87 extends into the heme pocket and is positioned above the porphyrin plane (Li and Poulos 1997). The variant (mutant) of P450 BM3 with a Phe87Val (F87V) substitution [P450 BM3 (F87V)] has often been used to examine changes in substrate preference or reaction specificity (Graham-Lorence et al. 1997; Li et al. 2001, 2000). P450 BM3 (F87V) was shown to epoxidate arachidonic acid (14*S*, 15*R*)-specifically (Graham-Lorence et al. 1997), and to hydroxylate β-ionone to form 4-hydroxy-β-ionone (Urlacher et al. 2006). This P450 variant was also found to possess bioconversion activity towards various aromatic compounds, e.g., it converted polycyclic aromatic hydrocarbons, fluorene and 9-methylanthracene, to 9-fluorenol and 9-anthracenemethanol, respectively (Li et al. 2001), and converted phenolic compounds, 2,6-dichlorophenol and 2-(benzyloxy)phenol to their corresponding hydroquinones (Sulistyaningdyah et al. 2005).

Peptidyl–prolyl *cis-trans* isomerases (PPIases) catalyze the *cis-trans* isomerization of the proline imide bond in polypeptides, which may affect the folding rate of proteins (Fischer and Schmid 1999). The FK506 binding protein (FKBP) family is one of three PPIase families and sensitive to the immuno-suppressant, FK506 (Fischer and Schmid 1999). Archaeal FKBPs were shown to possess not only PPIase activity to accelerate the rate of rotation of the peptidyl–prolyl bond, but also act as molecular chaperone, enhancing the refolding of incorrectly folded proteins to prevent protein aggregation (Furutani et al. 2000; Ideno et al. 2001). The archaeal FKBPs were also reported to suppress the heat aggregation of foreign proteins synthesized in *Escherichia coli* (Ideno et al. 2002). Ideno et al. (2004) showed that the FKBP-type PPIase, which was isolated from a hyperthermophilic archaeon *Thermococcus* sp. strain KS-1 (Ideno et al. 2001), enhanced the generation of soluble and active forms from three aggregate-prone proteins, an archaeal rhodansese (thiosulfate sulfurtransferase) and mouse scFV- and Fab-antibody fragments, when the PPIase C terminus was fused to the proteins. On the other hand, these polypeptides formed insoluble aggregates, when expressed alone in *E. coli* (Ideno et al. 2004). In the present study, we construct a fusion protein of this PPIase with the P450 BM3 (F87V), and evaluate its stability in *E. coli* cells. Furthermore, bioconversion experiments with various substituted naphthalenes are carried out using *E. coli* cells that express this fusion gene in order to generate a sustainable source of mono-hydroxylated naphthalene derivatives.

The polycyclic aromatic hydrocarbon (PAH)-dihydroxylating dioxygenase genes (*phnA1A2A3A4*) were isolated from a PAH-utilizing marine bacterium *Cycloclasticus* sp. strain A5 (Kasai et al. 2003). The *phnA1*, *A2*, *A3*, and *A4* genes encoded the large (α) subunit of the oxygenase component that is an iron-sulfur proteins, its small (β) subunit, a ferredoxin, and a ferredoxin reductase, respectively (Kasai et al. 2003). Recombinant *E. coli* cells that expressed the *phnA1A2A3A4* genes were shown to exhibit bioconversion capacity of a series of *methyl*- and dimethyl- naphthalenes to generate their *cis*-dihydrodiols or hydroxymethylated derivatives (Shindo et al. 2007a). In the present study, we also indicate that *cis*-dihydrodiols generated from substituted naphthalenes are specifically dehydrated by treatment with 10% HCl/methanol to produce mono-hydroxylated naphthalene derivatives.

Materials and methods

Bacterial strains, plasmid, and genetic manipulation

E. coli DH5α (*ECOS* Competent *E. coli* DH5α; Nippon Gene, Tokyo, Japan) was utilized as the hosts for DNA manipulations. *E. coli* BL21 (DE3) (Nippon Gene) and JM109 (Nippon Gene) were used for the functional expression of the P450 BM3 (F87V) gene and of the *phnA1A2A3A4* genes, respectively. Plasmid pPhnA was described for the expression of the *phnA1A2A3A4* genes (Kasai et al. 2003). Polymerase chain reaction (PCR) amplifications were performed using KOD plus DNA polymerase version 2 (Toyobo, Osaka, Japan) and a thermal cycler (Applied Biosystems, Foster City, CA). Typical thermal conditions for PCR were 94°C/2 min, 25 cycles of (94°C/15 s, 61°C/30 s, 68°C/4 min). Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, CA) or Takara Bio (Ohtsu, Japan). Ligation-Convenience Kit (Nippon Gene) was also used. Plasmid DNA was prepared with a QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Nucleotide sequences were confirmed with Bigdye terminator cycle sequencing ready

reaction kit version 3.1 (Applied Biosystems) and a model 3730 DNA analyzer (Applied Biosystems). All recombinant DNA experiments were carried out according to the suppliers' manuals or Sambrook and Russell (2001).

Construction of plasmids

A 0.91 kb fragment of the FKBP-type PPIase gene (accession no. AB012209) of a hyperthermophilic archaeon, *Thermococcus* sp. KS-1 (deposit no. JCM11816), was amplified by PCR from its genome DNA (Ideno et al. 2004) using primer I, 5'-GGCCATGGGAAAAGTTGAAGCTGGTGAT-3' (the underline indicates the *Nco*I site), and primer II, 5′-CCACTAGTAGCTTCTGAGTCCTCCTC-3′ (underline, *Spe*I). A 1.3 kb fragment of the P450 BM3 variant F87V (P450 BM3 (F87V); accession no. of original P450 BM3: J04832) was obtained by PCR from the plasmid containing this gene (Li et al. 2000) using primer III, 5′-GGAGCTCACAATTAAAGAAATGCCTCAGCC-3′ (underline, *Sac*I), and primer IV, 5′-CGCCTCGAGTTAAT GATGATGATGATGATGCCCAGC-3′ (underline, *Xho*I). In order to construct a linker with a thrombin cleavage site between the 3′-terminal end of the PPIase gene and the N terminus of the P450 BM3 (F87V) gene, we synthesized the oligonucleotide fragment Throm-F (5′- GGACTAG TCTGGTTCGGCGTGGATCC CATATG GAATTCGG -3′) and its complementary strand (Thromb-R; 5′-CCGAA TTC CATATG GGATCCACGCCGAACCAGAC TAGTCC-3′), which were then hybridized. Throm-F contains *Spe*I (ACTAGT), *Bam*HI (GGATCC), *Nde*I (CATATG), and *Eco*RI (GAATTC) restriction sites (underlines). The 0.91-kb PPIase gene fragment and this Thromb-FR fragment were digested with *Nco*I and *Spe*I, and with *Spe*I and *Eco*RI, respectively, and tandemly ligated into the *Nco*I-*Eco*RI site of vector pET21d (Novagen, Madison). The 1.3-kb P450 BM3 (F87V) gene fragment was digested with *Sac*I and *Xho*I, and ligated into the corresponding site of this constructed plasmid. The final constructed plasmid was named pFusionF87V, the structure of which is shown in Fig. 1. Similarly, plasmids pFusionF87V (3 aa) and pFusionF87V (18 aa) with different linker length were constructed (Fig. 1). As a control plasmid, pET21dF87V was also constructed by inserting the *Sac*I-*Xho*I-digested P450 BM3 (F87V) fragment into the corresponding site of pET21d.

Cultivation of bacteria and expression analysis

E. coli BL21 (DE3) carrying plasmid pFusionF87V, pFusionF87V (3 aa), pFusionF87V (18 aa), or pET21dF87V was pre-cultured at 30°C with shaking in 3 ml of an LB medium (Sambrook and Russell 2001) containing 100 μg/ml of ampicillin (Ap). After 4 h of cultivation, 3 ml of the pre-culture was inoculated into 40 ml of an LB medium containing 100 μg/ml of Ap, and cultured in the same way. After 3 h of cultivation, isopropyl β-D-thiogalactopyranoside (IPTG; 0.5 mM at final concentration) were added to the culture, and cultivation was continued by rotary shaking for 16 h at 30°C. The cells were collected, suspended in 2 ml of 10 mM sodium phosphate buffer (pH 8.0), and disrupted by sonication on ice. After centrifugation of the cell homogenate (32,000 g, 30 min), the resulting supernatant was called soluble fraction. The precipitate was washed twice with Hepes-KOH buffer (pH 6.8) containing 4% Triton X-100 for 30 min, and harvested by centrifugation (16,200 g, 20 min). The suspension of this precipitate was called inclusion-body fraction. The protein solutions were analyzed by SDS-PAGE with Coomassie brilliant blue (CBB) R250 staining. The soluble fraction from *E. coli* BL21 (DE3) carrying pFusionF87V or pET21dF87V was also used to measure substrate hydroxylation activity with a NADPH-regeneration system with some modification of the method described (Sulistyaningdyah et al. 2005). Each of the two soluble fractions (0.2– 0.4 mg as the total proteins), which were dissolved in 0.1 M sodium phosphate buffer (pH 8.0), were mixed with 0.5 mM (at final concentration) of sodium lauric acid (the substrate) and 0. 2 μM (at final concentration) of NADPH, and decrease at absorbance at 340 nm was monitored, before and after heat treatment at 60°C for 20 min.

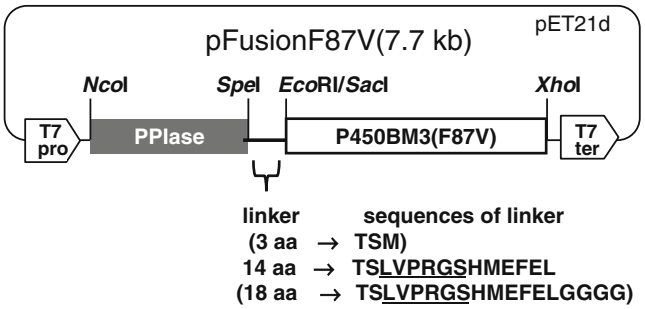


Fig. 1 Structure of plasmid pFusionF87V. pFusionF87V is the plasmid containing the linker sequence for 14 amino acid residues (*aa*), which includes a thrombin cleavage site (*underlined*). For comparison, two similar plasmids containing the linker sequences for 3 and 18 aa were constructed, and designated pFusionF87 (*3 aa*) and pFusionF87 (*18 aa*), respectively

Bioconversion experiments

E. coli BL21 (DE3) carrying plasmid pFusionF87V was grown in an LB medium including Ap (100 μg/ml) at 37°C with rotary shaking for 4 h until the absorbance at OD 600 nm reached approximately 0.8. Five ml of this culture was inoculated into 500 ml of an LB medium including Ap (100 μg/ml), 5-aminolevulinic acid hydrochloride (5-ALA;

80 μg/ml), ammonium iron (II) sulfate (0.1 mM), and IPTG (0.1 mM) in an Erlenmeyer flask with baffle, and cultured at 20°C for 20 h by rotary shaking. The cells were collected by centrifugation, and resuspended in 100 ml of sodium phosphate buffer (50 mM, pH 7.2) containing 5% glycerol in an Erlenmeyer flask containing baffles or a Sakaguchi flask. Each substrate dissolved in dimethyl sulfoxide (DMSO) was added at the final concentration of 1 mM to the cell suspension, and bioconversion was performed by cultivation at 25°C for 1– 2 days while rotary shaking.

E. coli JM109 carrying plasmid pPhnA was cultured in an LB medium including150 μg/ml of Ap at 30°C with shaking for 6– 7 h until the absorbance at OD 600 nm reached approximately 1. Eight milliliters of this culture was inoculated into 100 ml of an M9 medium (Sambrook and Russell 2001) including150 μg/ml of Ap, 0.4% glucose, 0.5 mM of IPTG, and 1 mM of each substrate in a Sakaguchi flask, and bioconversion was performed with cultivation at 30°C with rotary shaking for 2 days.

The substrates used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO), Wako Pure Chemical Industries (Osaka, Japan) or Tokyo Chemical Industry Co. (Tokyo, Japan).

Extraction and HPLC analysis of products

Five hundred microliters of the reaction mixture liquid, as described above, was added to an equal volume of ethyl acetate, and shaken to facilitate the extraction of products into the organic layer. After centrifugation, the organic phase was analyzed by high pressure liquid chromatography (HPLC; Waters 2695, Waters Corp., Milford, MA) equipped with an on-line photodiode array detector (Waters 2996). An aliquot of the organic phase (10 μl) was applied to HPLC and separated using an XTerra MS C_{18} column (4.6×100 mm, Waters), and a flow rate of 1 ml/min was used, with solvent A (5% acetonitrile (CH_3CN) and 20-mM phosphoric acid) for 3 min, then a linear gradient from solvent A to solvent B (95% CH_3CN and 20-mM phosphoric acid) for 25 min, and finally with solvent B for 15 min, the maximum absorbance being monitored in the range of 200–500 nm.

Purification and identification of products

The liquid phase containing the reaction mixture (200 ml; 100 ml×2) was extracted with ethyl acetate (EtOAc; 200 ml×2 times). The resulting organic layer was concentrated *in vacuo*, and analyzed by thin-layer chromatography on silica gel (E. Merck 60F-254, 0.25-mm silica gel plates). The products were purified by column chromatography on Silica Gel 60 (20 mm (diameter)×250 mm (length), Merck). To elucidate the structures of these products high-resolution mass spectral data (HREI-MS (Jeol DX505W; Jeol, Tokyo, Japan) or HRAPCI-MS (Jeol JMS-T100LP)), and nuclear magnetic resonance (NMR) spectral data (400 MHz, Bruker AMX400) were acquired.

Chromatographic and spectroscopic data of products

Chromatographic and spectroscopic data of the individual products (1–11, 13, 14, 16–21) are described in the Electronic supplemental material section.

Results

Expression of the P450 BM3 variant F87V gene in *E. coli*

The P450 BM3 variant F87V (P450 BM3 (F87V)) gene was N-terminally fused to the archaeal PPIase via the linker sequences for 3, 14, and 18 amino acid residues to construct pFusionF87V (3 aa), pFusionF87V, and pFusionF87V (18 aa), respectively (Fig. 1). These plasmids were individually expressed in *E. coli* BL21 (DE3). The synthesized fusion proteins were detected as the predominant proteins in the respective soluble fractions (Fig. 2a, lanes 2–4), while these proteins were present as the low abundance bands in the inclusion-body fractions (Fig. 2b, lanes 2–4). The soluble protein from *E. coli* carrying plasmid pFusionF87V (18 aa) (Fig. 2a, lane 4) was less abundant compared with those from pFusionF87V (3 aa) and pFusionF87V (Fig. 2a, lanes 2, 3). In contrast, the *E. coli* cells expressing the P450 BM3 (F87V) gene not fused to the PPIase gene, which harbored plasmid pET21dF87V, synthesized the gene product in the inclusion-body fraction dominantly (Fig. 2a, b, lane 1).

The soluble fractions prepared from the *E. coli* BL21 cells carrying plasmids pFusionF87V and pET21dF87V were further used to measure substrate hydroxylation activity by a NADPH-regeneration system. Each of the two soluble fractions (0.2–0.4 mg as the total proteins) were mixed with the substrate (sodium lauric acid) and NADPH, and NADPH-regeneration activity was measured, before and after heat treatment at 60°C for 20 min. Remaining activity in the soluble fractions after the heat treatment was as follows: *E. coli* (pFusionF87V), 43.9%±14.3%; *E. coli* (pET21dF87V), 27.7%±3.0%. This result indicates that the fusion protein of P450 BM3 (F87V) with the PPIase is superior to the P450 BM3 (F87V) without the PPIase in the heat treatment. Thus, *E. coli* BL21 (DE3) carrying pFusionF87V was used in the following experiments.

Biotransformation of the substituted naphthalenes

A variety of substituted naphthalenes (Fig. 3) were biotransformed through co-cultivation with the cells of

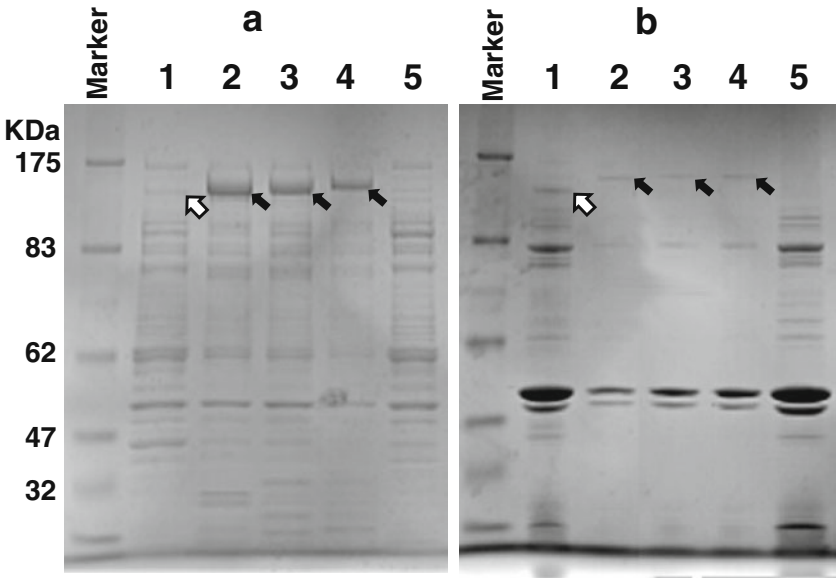


Fig. 2 Expression of the hybrid genes encoding P450 BM3 (F87V) that was N-terminally fused to the PPIase via the linkers of 3, 14, and 18 aa. The results of SDS-PAGE analysis of the soluble fraction and inclusion-body fraction are shown in (**a**) and (**b**), respectively. Proteins were extracted from *Escherichia coli* cells that carry plasmids, pET21dF87V (*lane 1*), pFusionF87V (*3 aa*; *lane 2*), pFusionF87V (pFusionF87V (*14 aa*); *lane 3*), pFusionF87V (*18 aa*; l*ane 4*), and pET21d (*lane 5*; the control vector). Expected molecular masses of the synthesized proteins are represented with *arrows* (137 kDa) for the P450 proteins fused to the PPIase or with *white arrows* (119 kDa) for the P450 protein without PPIase

E. coli carrying plasmid pFusionF87V, which was responsible for the expression of the P450 BM3 (F87V) gene, and with those carrying plasmid pPhnA, which conferred the expression of the PAH-dihydroxylating dioxygenase genes from *Cycloclasticus* sp. A5 (Kasai et al. 2003). *cis*-Dihydrodiols synthesized with the latter recombinant *E. coli* were treated with 10% HCl/methanol for 5 min to enable dehydration reaction to proceed. Converted compounds were analyzed by chromatographic and spectroscopic methods to elucidate their structures (Fig. 3).

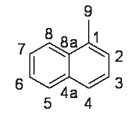
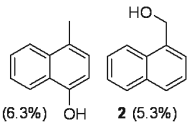
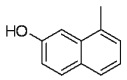
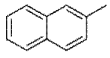
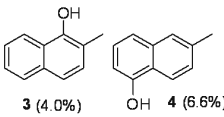
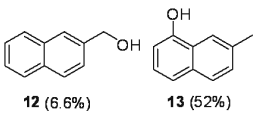
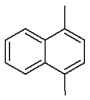
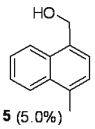
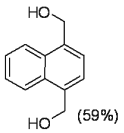
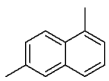
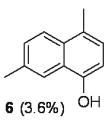
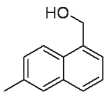
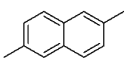
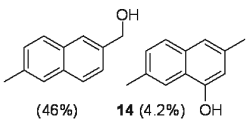
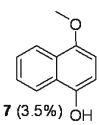
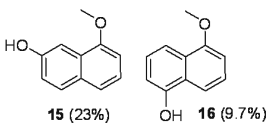
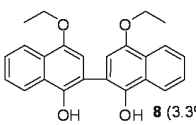
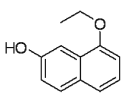
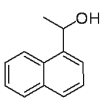
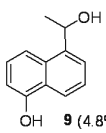
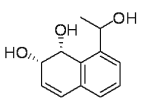
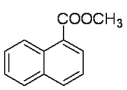
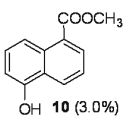
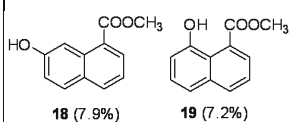
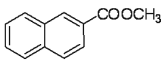
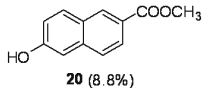
Compounds converted from 1-methylnaphthalene with P450 BM3 (F87V) The molecular formula of **1** was determined to be $C_{11}H_{10}O$ from HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **1** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 4-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks between H-2 (δ 7.13) and H-3 (δ 6.72) and from H-5 (δ 8.21) to H-8 (δ 7.94). Thus, **1** was identified to be 4-methylnaphthalen-1-ol. The molecular formula of **2** was determined to be $C_{11}H_{10}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **2** showed that the *methyl* functions in 1-methylnaphthalene (substrate) were oxidized to the corresponding primary alcohols. Thus, **2** was identified to be naphthalen-1-yl-methanol.

Compounds converted from 2-methylnaphthalene with P450 BM3 (F87V) The molecular formula of **3** was determined to be $C_{11}H_{10}O$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **3** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 1-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks between H-3 (δ 7.24) and H-4 (δ 7.38) and from H-5 (δ 7.77) to H-8 (δ 8.12). Thus, **3** was identified to be 2-methylnaphthalen-1-ol. The molecular formula of **4** was determined to be $C_{11}H_{10}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **4** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 5-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-6 (δ 6.74) to H-8 (δ 7.34), and ^{1}H–^{13}C long-range couplings from H-1 (δ 7.58) and H-6 (δ 6.74) to C-4a (δ 122.5) and from H-7 (δ 7.29) to C-5 (δ 151.4). Thus, **4** was identified to be 6-methylnaphthalen-1-ol.

A compound converted from 1,4-dimethylnaphthalene with P450 BM3 (F87V) The molecular formula of **5** was determined to be $C_{12}H_{12}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **5** showed that one *methyl* functions in 1,4-dimethylnaphthalene (substrate) were oxidized to its corresponding primary alcohol. Thus, **5** was identified to be (4-methylnaphthalen-1-yl)-methanol.

A compound converted from 1,6-dimethylnaphthalene with P450 BM3 (F87V) The molecular formula of **6** was determined to be $C_{12}H_{12}O$ by HRAPCI-MS data. The ^{1}H

Fig. 3 Bioconversion of various substituted naphthalenes through the cells of *Escherichia coli* carrying plasmid pFusionF87V or pPhnA. Percent value in the parentheses represents the yield of the purified products, which was calculated from the amounts recovered after purification. It was shown for the first time in the present study that the compounds with number (*1–10*) were produced by P450 BM3 (F87V). The compounds without number below the chemical structures of the products from *E. coli* (pPhnA) were already reported (Shindo et al. 2007a)

substrate	pFusionF87V: P450BM3(F87V)	pPhnA: PhnA1A2A3A4
 1-methylnaphthalene	 **1** (6.3%) **2** (5.3%)	 **11** (18%)
 2-methylnaphthalene	 **3** (4.0%) **4** (6.6%)	 **12** (6.6%) **13** (52%)
 1,4-dimethylnaphthalene	 **5** (5.0%)	 (59%)
 1,6-dimethylnaphthalene	 **6** (3.6%)	 (37%)
 2,6-dimethylnaphthalene	not converted	 (46%) **14** (4.2%)
 1-methoxynaphthalene	 **7** (3.5%)	 **15** (23%) **16** (9.7%)
 1-ethoxynaphthalene	 **8** (3.3%)	 **17** (30%)
 α-methyl-1-naphthalenemethanol	 **9** (4.8%)	 **A** (8.1%)
 1-naphthalenecarboxylic acid, methyl ester	 **10** (3.0%)	 **18** (7.9%) **19** (7.2%)
 2-naphthalenecarboxylic acid, methyl ester	not converted	 **20** (8.8%)

and ^{13}C NMR spectra of **6** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 4-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin coupling between H-2 (δ 7.05) and H-3 (δ 6.70), and ^{1}H–^{13}C long-range couplings from H-5 (δ 7.97) to C-4 (δ 151.0). Thus, **6** was identified to be 4,7-dimethylnaphthalen-1-ol.

A compound converted from 1-methoxynaphthalene with P450 BM3 (F87V) The molecular formula of **7** was determined to be $C_{11}H_{10}O_2$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **7** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 4-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin coupling between H-2 (δ 6.58) and H-3 (δ 6.67), and ^{1}H–^{13}C long-range couplings from H-2 to C-4 (δ 145.1) and from H-3 and H-9 (δ 3.89) to C-1 (δ 149.9). Thus, **7** was identified to be 4-methoxynaphthalen-1-ol.

A compound converted from 1-ethoxynaphthalene with P450 BM3 (F87V) The molecular formula of **8** was determined to be $C_{24}H_{22}O_4$ by HRAPCI-MS data. Since only 12 signals were observed in the ^{13}C-NMR spectrum of **8**, compound **8** was confirmed to possess a dimer structure. In addition, the presence of one phenolic OH function to each naphthalene ring of **8** was also proposed from the ^{1}H and ^{13}C NMR spectra. The regiochemical assignments of the 4-OH and 4′-OH, and the linkage of C-3–C-3′ were confirmed by the observation of ^{1}H–^{13}C long-range couplings from H-2 (H-2′) (δ 6.92) to C-4 (C-4′) (δ 142.6), and NOE between H-2 (H-2′) and H-9 (H-9′) (δ 4.20). Thus, **8** was determined to be 4,4′-diethoxy-[2,2′]-binaphthalenyl-1,1′-diol. Compound **8** was a novel compound according to CAS data base.

A compound converted from α-methyl-1-naphthalenemethanol with P450 BM3 (F87V) The molecular formula of **9** was determined to be $C_{12}H_{12}O_2$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **9** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 5-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-2 (δ 7.64) to H-4 (δ 8.14) and from H-6 (δ 6.80) to H-8 (δ 7.55), and ^{1}H–^{13}C long-range couplings from H-3 (δ 7.39) to C-1 (δ 142.7) and C-4a (δ 126.4) and from H-6 (δ 6.80) to C-4a. Thus, **9** was identified to be 5-(1-hydroxy-ethyl)-naphthalen-1-ol.

A compound converted from 1-naphthalenecarboxylic acid methyl ester with P450 BM3 (F87V) The molecular formula of **10** was determined to be $C_{12}H_{10}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **10** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 5-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-2 (δ 8.18) to H-4 (δ 8.47) and from H-6 (δ 6.87) to H-8 (δ 8.45), and ^{1}H–^{13}C long-range couplings from H-3 (δ 7.50) to C-1 (δ 127.0) and C-4a (δ 125.0) and from H-6 (δ 6.87) to C-4a. Thus, **10** was identified to be 5-hydroxynaphthalene-1-carboxylic acid *methyl* ester.

A compound converted from 1-methylnaphthalene with PhnA1A2A3A4 The molecular formula of **11** was determined to be $C_{11}H_{10}O$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **11** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 7-OH was confirmed by the observation of ^{1}H–^{1}H spin networks of H-2 (δ 7.29)–H-3 (δ 7.23)–H-4 (δ 7.64), and H-5 (δ 7.76)–H-6 (δ 7.10)–H-8 (δ 7.29), and ^{1}H–^{13}C long-range couplings from H-9 (δ 2.61) to C-8a (δ 133.8) and from H-5 to C-7 (δ 153.3) and C-8a. Thus, 11 was identified to be 8-methylnaphthalen-2-ol. It is likely that 11 was synthesized by dehydration from the direct enzyme product (1*R*,2*S*)-8-*methyl*-1,2-dihydronaphthalene-1,2-diol (Shindo et al. 2007a). This *cis*-dihydrodiol was also reported to be produced in *E. coli* that expressed the naphthalene dioxygenase genes isolated from *Rhodococcus opacus* TKN14 (Maruyama et al. 2005).

Compounds converted from 2-methylnaphthalene with PhnA1A2A3A4 The molecular formula of **12** was determined to be $C_{11}H_{10}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **12** showed that **12** was naphthalen-2-yl-methanol (Otomatsu et al. 2010). This compound was reported to be synthesized by *E. coli* that expressed the *CYP153A13a* (P450balk) gene isolated from a marine bacterium *Alcanivorax borkumensis* SK2 (Nodate et al. 2006; Otomatsu et al. 2010). The molecular formula of **13** was determined to be $C_{11}H_{10}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **13** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 8-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-5 (δ 7.40) to H-7 (δ 6.79), and ^{1}H–^{13}C long-range couplings from H-1 (δ 7.94) to C-4a (δ 133.0) and from H-6 (δ 7.23) to C-4a and C-8 (δ 150.8). Thus, **13** was identified to be 7-methylnaphthalen-1-ol. It is likely that **13** was synthesized by dehydration from the direct enzyme product (1*R*,2*S*)-7-*methyl*-1,2-dihydronaphthalene-1,2-diol (Shindo et al. 2007a). This *cis*-dihydrodiol was also reported to be produced in *E. coli* that expressed the naphthalene dioxygenase genes of *R. opacus* TKN14 (Maruyama et al. 2005) and in *E. coli* that expressed the PAH-dihydroxylating dioxygenase genes isolated from a

marine bacterium *Nocardioides* sp. strain KP7 (Shindo et al. 2007a).

A compound converted from 2,6-dimethylnaphthalene with PhnA1A2A3A4 The molecular formula of **14** was determined to be $C_{12}H_{12}O$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **14** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 4-OH was confirmed by the observation of ^{1}H–^{1}H spin networks of H-5 (δ 7.86)–H-7 (δ 7.29)–H-8 (δ 7.62), and ^{1}H–^{13}C long-range couplings from H-9 (δ 2.42) to C-1 (δ 119.6), C-2 (δ 134.7), and C-3 (δ 110.8). Thus, **14** was identified to be 3,7-dimethylnaphthalen-1-ol. It is likely that **14** was synthesized by dehydration from the direct enzyme product (1*R*,2*S*)-3,7-dimethyl-1,2-dihydronaphthalene-1,2-diol (Shindo et al. 2007a). This *cis*-dihydrodiol was also reported to be produced in *E. coli* that expressed the PAH-dihydroxylating dioxygenase genes of *Nocardioides* sp. KP7 (Shindo et al. 2007a).

Compounds converted from 1-methoxynaphthalene with PhnA1A2A3A4 The molecular formula of **15** was determined to be $C_{11}H_{10}O_2$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **15** showed that **15** was 8-methoxynaphthalen-2-ol (Chun et al. 2001). This compound was reported to be synthesized by *Streptomyces lividans* cells that expressed the PAH-dihydroxylating dioxygenase genes of *Nocardioides* sp. KP7 (Chun et al. 2001). The molecular formula of 16 was determined to be $C_{11}H_{10}O_2$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of 16 showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 5-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-6 (δ 6.84) to H-8 (δ 7.85) and from H-2 (δ 6.84) to H-4 (δ 7.74), and ^{1}H–^{13}C long-range couplings from H-3 (δ 7.39) to C-1 (δ 155.4) and C-4a (δ 125.3) and from H-6 to C-4a and C-5 (δ 151.2). Thus, **16** was identified to be 5-methoxynaphthalen-1-ol. It is likely that **15** and **16** were synthesized by dehydration from the direct enzyme products (1*R*,2*S*)-8-methoxy-1,2-dihydronaphthalene-1,2-diol and (1*R*,2*S*)-5-methoxy-1,2-dihydronaphthalene-1,2-diol, respectively (Shindo et al. 2007a).

A compound converted from 1-ethoxynaphthalene with PhnA1A2A3A4 The *cis*-dihydrodiol, which was generated with this recombinant *E. coli* as the direct enzyme product, was determined to exhibit the molecular formula of $C_{12}H_{14}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of this compound were shown to be a dihydrodiol derivative of the substrate. The regiochemical assignment of the 7,8-diol was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-2(δ 6.76) to H-4 (δ 6.70) and from H-5 (δ 6.36) to H-8 (δ 5.04), and ^{1}H–^{13}C long-range couplings from H-5 to C-4 (δ 119.5). Thus, this dihydrodiol was identified to be 8-ethoxy-1,2-dihydronaphthalene-1,2-diol. The molecular formula of **17**, which was produced by the acid treatment of the dihydrodiol, was determined to be $C_{12}H_{12}O_2$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **17** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 7-OH was confirmed by the observation of ^{1}H–^{1}H spin network of H-5 (δ 7.70)–H-6 (δ 7.10)–H-8 (δ 7.59), and ^{1}H–^{13}C long-range couplings from H-8 to C-1 (δ 153.6). Thus, 17 was identified to be 8-ethoxynaphthalen-2-ol.

A compound converted from α-methyl-1-naphthalenemethanol with PhnA1A2A3A4 The *cis*-dihydrodiol (A), which was generated with this recombinant *E. coli* as the direct enzyme product, was determined to exhibit the molecular formula of $C_{12}H_{14}O_3$ by HRAPCI-MS data. The molecular formula of compound A was determined to be $C_{12}H_{14}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of A showed that this compound was a dihydrodiol derivative of the substrate. The regiochemical assignment of the 7,8-diol was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-2 (δ 7.46) to H-4 (δ 7.04) and from H-5 (δ 6.45) to H-8 (δ 4.85), and ^{1}H–^{13}C long-range couplings from H-5 to C-4 (δ 127.1). Thus, compound A was determined to be 8-(1-hydroxy-ethyl)-1,2-dihydronaphthalene-1,2-diol. The stereochemistry of A was considered to be 1*R*,2*S* according to other examples of the *cis*-dihydrodiols synthesized from naphthalene derivatives (Shindo et al. 2007a). However, the acid treatment of compound A did not generate any stable compounds.

Compounds converted from 1-naphthalenecarboxylic acid methyl ester with PhnA1A2A3A4 The molecular formula of **18** was determined to be $C_{12}H_{10}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **18** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 7-OH was confirmed by the observation of ^{1}H–^{1}H spin network of H-5 (δ 7.79)–H-6 (δ 7.19)–H-8 (δ 8.52), and ^{1}H–^{13}C long-range couplings from H-8 to C-1 (δ 124.4). Thus, **18** was identified to be 7-hydroxynaphthalene-1-carboxylic acid *methyl* ester. The molecular formula of **19** was determined to be $C_{12}H_{10}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **19** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 8-OH was confirmed by the observation of ^{1}H–^{1}H vicinal spin networks from H-2 (δ 8.26) to H-4 (δ 8.05) and from H-5 (δ 7.45) to H-7 (δ 7.17), and phenolic OH at δ 10.51 (hydrogen bond formation).

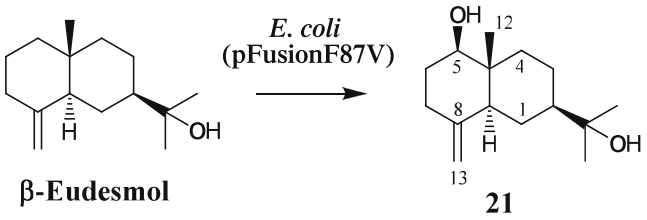


Fig. 4 Bioconversion of β-eudesmol through the cells of *Escherichia coli* carrying plasmid pFusionF87V

Thus, **19** was identified to be 8-hydroxynaphthalene-1-carboxylic acid *methyl* ester. It is likely that **18** and **19** were synthesized by dehydration from the direct enzyme product (7*S*,8*R*)-7,8-dihydroxy-7,8-dihydronaphthalene-1-carboxylic acid *methyl* ester (Shindo et al. 2007a).

A compound converted from 2-naphthalenecarboxylic acid methyl ester with PhnA1A2A3A4 The molecular formula of **20**, which generated after the acid treatment, was determined to be $C_{12}H_{10}O_3$ by HRAPCI-MS data. The ^{1}H and ^{13}C NMR spectra of **20** showed that one phenolic OH was inserted to the naphthalene ring of the substrate. The regiochemical assignment of the 6-OH was confirmed by the observation of ^{1}H–^{1}H spin network of H-5 (δ 7.43)–H-7 (δ 6.91)–H-8 (δ 7.42), and ^{1}H–^{13}C long-range couplings from H-4 (δ 7.83) to C-2 (δ 126.4) and C-5 (δ 120.1). Thus, 20 was identified to be 6-hydroxynaphthalene-2-carboxylic acid *methyl* ester.

A compound converted from β-eudesmol with P450 BM3 (F87V) The molecular formula of **21** was determined to be $C_{15}H_{26}O_2$ by HREI-MS data. The ^{1}H and ^{13}C NMR spectra of **21** showed one alcoholic OH was inserted to the decaline ring of the substrate. The regiochemical assignment of the 5-OH was confirmed by the observation of ^{1}H–^{13}C long-range couplings from H-12 (δ 0.68) to C-5 (δ 79.3) and from H-13 (δ 4.53 and δ 4.77) to C-7 (δ 34.2), and ^{1}H–^{1}H vicinal spin network of H-5 (δ 3.42)–H-6 (δ 1.56 and δ 1.81)–H-7 (δ 2.11 and δ 2.32). The equatorial orientation of 5-OH was proved by the large coupling constant of $J_{5,6b}$ (11.5 Hz). Thus, **21** was identified to be 5-hydroxy-ß-eudesmol [IUPAC name 6-(1-hydroxy-1-*methyl*-ethyl)-8a-*methyl*-4-methylene-decahydro-naphthalen-1-ol] (Fig. 4).

Discussion

In the present study, we adopted the FKBP-type PPIase derived from a hyperthermophilic archaeon to construct the protein fused to the N terminus of the cytochrome P450 BM3 variant F87V (P450 BM3 (F87V)). This fusion P450 tended to exist as the predominant soluble protein and more stable than the un-fused P450 after heat treatment, when synthesized in recombinant *E. coli* cells. This result suggests that the archaeal PPIase can exhibit its natural functions not only as PPIase but also as a molecular chaperone to prevent protein aggregation (Furutani et al. 2000, Ideno et al. 2001), when fused to the P450 BM3 (F87V) protein. Thus, *E. coli* cells that harbor plasmid pFusionF87V (Fig. 1) were used for the bioconversion of various substituted naphthalenes, which contain 1-methoxy- and 1-ethoxy-naphthalenes, methylnaphthalenes, dimethylnaphthalenes, and naphthalenecarboxylic acid *methyl* esters. This recombinant *E. coli* was found to biotransform these compounds to their monohydroxylated derivatives, except for 2,6-dimethylnaphthalene and 2-naphthalenecarboxylic acid *methyl* ester, as shown in Fig. 3. Specifically, 1-ethoxynaphthalene was converted to a novel compound, 4,4′-diethoxy-[2,2′]-binaphthalenyl-1,1′-diol (Fig. 3, compound 8). This product is likely to be generated via oxidative aryl coupling through a non-enzymatic dimerization process from 4-ethoxynaphthalen-1-ol, which is speculated to be the direct enzyme product, as shown in Fig. 5. Some sesquiterpenes that have physiological functions were also tested towards bioconversion with the *E. coli* cells carrying plasmid pFusionF87V. Consequently, β-eudesmol [2-(4a-*methyl*-8-methylene-decahydro-naphthalen-2-yl)-propan-2-ol] that possesses pharmacologically unique effects (Chiou et al. 1995, Kimura et al. 1991) was found to be hydroxylated in a *regio*- and *stereo*-specific manner, as shown in Fig. 4.

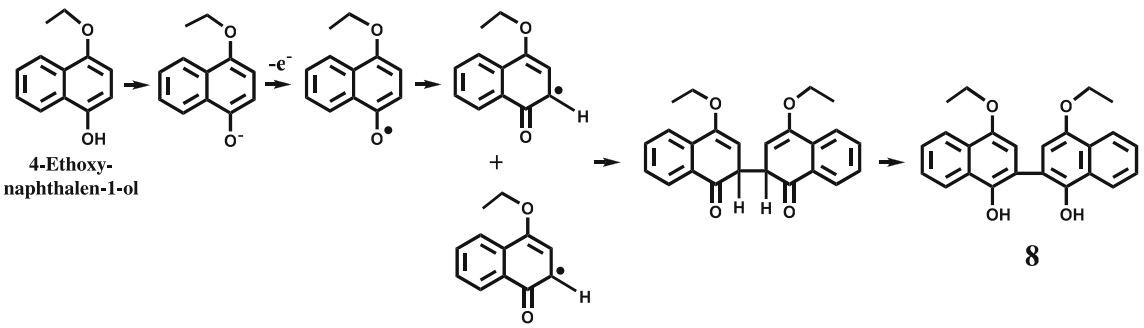


Fig. 5 Proposed synthetic route of compound **8** from the direct enzymatic product, 4-ethoxynaphthalen-1-ol

Previous studies have revealed that P450 BM3 (F87V) can biotransform various aromatic compounds (Li et al. 2001, Sulistyaningdyah et al. 2005). Urlacher et al. (Urlacher et al. 2006) also showed that this P450 converted β-ionone to its 4-hydroxylated derivative. The present study revealed that P450 BM3 (F87V) can introduce the hydroxyl group into various substituted naphthalenes as well as sesquiterpene β-eudesmol. These findings indicate that P450 BM3 (F87V) possess wide substrate affinity (preference) for biocatalytic reactions. Therefore, this P450 F87V variant, particularly that fused to the FKBP-type PPIase, should be promising for biotechnological use as a promiscuous and stable P450 that is easily synthesized in *E. coli*.

We have carried out bioconversion experiments towards a variety of aromatic compounds using recombinant *E. coli* cells, which expressed the genes that code for several aromatic hydrocarbon-dihydroxylating dioxygenases (Kagami et al. 2008, Maruyama et al. 2005, Misawa et al. 2005, Misawa et al. 2002, Shindo et al. 2005, Shindo et al. 2007a, Shindo et al. 2004, Shindo et al. 2007b). Among them, the corresponding enzyme (PhnA1A2A3A4) from a PAH-utilizing marine bacterium *Cycloclasticus* sp. A5, which was synthesized in *E. coli* cells harboring plasmid pPhnA, was found to exhibit broad substrate preference for various substituted naphthalenes (Shindo et al. 2007a). It was shown in the present study that mono-hydroxylated naphthalenes were generated with the acid treatment of the *cis*-dihydrodiols that were synthesized with PhnA1A2A3A4, and can complement the mono-hydroxylated naphthalenes produced with the P450 BM3 (F87V) enzymes (Fig. 3).

We supplemented low concentrations of IPTG from the beginning of culture for the cultivation of *E. coli* cells carrying pFusionF87V or pPhnA in the bioconversion experiments, as shown in the Materials and Methods section. Such culture conditions were here adopted as facile cultivation methods, since their bioconversion efficiency was similar to that of an ordinary IPTG induction method (Sambrook and Russell 2001) (data not shown). Further improvements should be needed in bioconversion methods of *E. coli* (pFusionF87V) and *E. coli* (pPhnA) for practical use, since the yields obtained in the present experiments remained in low levels (Fig. 3).

Compounds including the naphthalene ring in their molecular structure can be candidates of biologically active chemicals, medicines, or other industrial commodities, e.g., propranolol and naphazoline nitrate are medicines. The WalK/WalR (YycG/YycF) two-component system, which is essential for cell viability, is highly conserved and specific to low-GC gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, making it an attractive target for novel antimicrobial compounds. A screening was recently carried out to search WalR inhibitors using a variety of naphthalene derivatives including those produced in the present study. As a result, 4-methoxy-1-naphthol (Fig. 3, compound 7; designated walrycin A) was found to be a specific inhibitor targeting the WalR response regulator (Gotoh et al. 2010). Our trials to produce a large variety of aromatic compounds that are difficult to synthesize chemically seem to supply researchers of medicine or chemical biology with valid screening sources (Gotoh et al. 2010, Rahim et al. 2008).

Acknowledgements The authors thank Dr. Paul D. Fraser, Royal Holloway University of London, for his critical reading of this manuscript. This work was supported in part by the Research and Development Program for New Bio-industry Initiatives (2006–2010) from the Bio-oriented Technology Research Advancement Institution (BRAIN) of Japan. This work was also supported during MBI by New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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