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Hydroxylations of substituted naphthalenes by *Escherichia coli* expressing aromatic dihydroxylating dioxygenase genes from polycyclic aromatic hydrocarbon-utilizing marine bacteria

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Abstract

Bioconversion experiments of various mono- or di-substituted naphthalenes such as dimethylnaphthalenes were carried out using the cells of *Escherichia coli* that expressed aromatic dihydroxylating dioxygenase genes (*phnA1A2A3A4* and *phdABCD*) from polycyclic aromatic hydrocarbon-utilizing marine bacteria, *Nocardioides* sp. KP7 and *Cycloclasticus* sp. A5, respectively. We found that the former dioxygenase PhnA1A2A3A4 had broad substrate preference for these compounds and often was able to hydroxylate their methyl groups. Specifically, 1,4-dimethylnaphthalene was predominantly bioconverted into 1,4-dihydroxymethylnaphthalene.

Keywords: Polycyclic aromatic hydrocarbon; Aromatic dihydroxylating dioxygenase; Dimethylnaphthalene; *Cycloclasticus*; *Nocardioides*

1. Introduction

Marine Biotechnology Institute has isolated marine bacteria that grow on polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and anthracene as the sole carbon source [1,2]. The bacteria contained an actinomycete *Nocardioides* sp. strain KP7 and *Cycloclasticus* sp. strain A5 that is a member of the γ subdivision of the *Proteobacteria*. Genes coding for PAH dihydroxylating dioxygenases, which are the first enzymes for PAH degradation, were isolated from these marine bacteria, and designated the *phdABCD* genes (from *Nocardioides* sp. KP7) [3] and the *phnA1A2A3A4* (from *Cycloclasticus* sp. A5) [4]. *phdA* and *phnA1* encoded the large (α) subunit of the oxygenase component that is an iron-sulfur protein. *phdB* and *phnA2* coded for its small (β) subunit. *phdC* and *phnA3* encoded a ferredoxin, and *phdD* and *phnA4* coded for a ferredoxin reductase. The encoded proteins PhdA and PhdB exhibited moderate (less than 60%) sequence identity to the large and small subunits of other aromatic dihydroxylating dioxygenases [3]. PhnA1 and PhnA2 also exhibited moderate (less than 62%) sequence identity to the corresponding proteins of the other dioxygenases [4]. The cells of recombinant *Escherichia coli*, into which the *phdABCD* or *phnA1A2A3A4* genes were introduced and expressed, were able to biotransform phenanthrene and naphthalene into their *cis*-dihydrodiol forms, i.e., *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene and *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, respectively [4,5]. *E. coli* expressing *phdABCD* was further shown to convert various tricyclic (fused) aromatic compounds, e.g., fluorene, dibenzofuran, dibenzothiophene, carbazole, acridine, and phenanthridine, into their *cis*-dihydrodiol forms or subsequent dehydrated products [6]. On the other hand, the *E. coli* cells expressing *phnA1A2A3A4* were able to hydroxylate biphenyl and diphenylmethane, which were not accepted as the substrates for PhdABCD [4,6]. Here, we compare the catalytic functions between PhnA1A2A3A4 and PhdABCD in the case using various substituted naphthalenes such as dimethylnaphthalenes, several of which are abundantly included in crude oil, as the substrates, and find that the former dioxygenase has broad substrate preference for these compounds and often can hydroxylate their methyl groups.

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2. Experimental

2.1. Plasmids and bacterial strains

Plasmid pPhnA, in which the *phnA1A2A3A4* genes were inserted into *E. coli* vector pBluescript II SK+, was previously described [4]. Plasmid pHA171, in which the *phdABCD* genes were inserted into *E. coli* vector pT7-7, was previously described [3].

2.2. Conversion experiments

E. coli JM109 and *E. coli* BL21(DE3) [7] were used as hosts for plasmids pPhnA and pHA171, respectively, and were cultured in an LB medium [7] including ampicillin (Ap; 150 μg/ml) at 30 °C while reciprocally shaking at 120 rpm (rotation per minute) for 6–7 h until the absorbance at OD 600 nm had reached approximately 1. Eight milliliters of this culture was inoculated into 100 ml of an M9 medium [7] containing 150 μg/ml of Ap, 0.4% (w/v) glucose, 0.5 mM (final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG), and 10 mg of each substrate in a Sakaguchi flask at 30 °C while reciprocally shaking at 120 rpm for 2 days.

The substrates used in this study were purchased from Sigma–Aldrich Chemical Co. The respective substrates were dissolved in small volume of dimethyl sulfoxide (DMSO) and added to the culture.

2.3. Preparation of 1-naphthoic acid methyl ester, 2-naphthoic acid methyl ester, and 4-methyl-1-naphthoic acid methyl ester

1-Naphthoic acid, 2-naphthoic acid, and 4-methyl-1-naphthoic acid (each 500 mg) were stirred in 5% HCl–MeOH (20 ml) for 12 h at room temperature. After concentrated to dryness, the residue was chromatographed on silica gel column chromatography (hexane–EtOAc = 5:1) to afford the corresponding methyl ester. The yields were all quantitative.

2.4. Extraction and HPLC analysis of the converted products

To extract the converted products as well as the substrates, an equal volume of methanol (MeOH) to that of the culture medium was added to the co-culture of the transformed cells of *E. coli*, and mixed for 30 min. After centrifuging to remove the cells, the liquid phase was analyzed by high-pressure liquid chromatography (HPLC) or used for further purification of the converted products. The liquid phase (80 μl) was applied to HPLC in an XTerra C_{18} column (4.6 mm × 150 mm, Waters) with a photodiode array detector (model L-7455, Hitachi). Development was at a flow rate of 1 ml/min with solvent A (5% acetonitrile (CH_3CN) and 20 mM phosphoric acid) for 3 min, then by a linear gradient from solvent A to solvent B (95% CH_3CN and 20 mM phosphoric acid) for 15 min, and finally with solvent B for 10 min, the maximum absorbance being monitored in the range of 200–500 nm.

2.5. Purification and identification of the converted products

The liquid phase (1000 ml), which had been obtained by the procedure just described, was concentrated *in vacuo* and extracted with ethyl acetate (EtOAc; 500 ml × 2). The resulting organic layer was concentrated *in vacuo* and analyzed by thin-layer chromatography (TLC) on silica gel (E. Merck 60F-254 0.25-mm silica gel plates). The formed products were purified by column chromatography on Silica Gel 60 (20 mm × 250 mm, Merck). Their structures were analyzed by mass spectral data {MS [electric impact (EI)] and HRMS (EI); Jeol DX505W}, nuclear magnetic resonance (NMR) spectral data (400 MHz, Bruker AMX400) and specific rotation ($[\alpha]_D$) spectral data (Rudolph Research Analytical AUTOPOL IV).

2.5.1. 8-Methyl-1,2-dihydro-naphthalene-1,2-diol (**1**: product converted from 1-methyl-naphthalene)

The crude EtOAc extract (110.8 mg) was subjected to column chromatography (hexane–EtOAc = 1:1) to yield 7.3 mg of **1** (yield 5.9%). HRMS (EI) calcd. for $C_{11}H_{12}O_2$ (M^+), 176.0838; found 176.0846. $[\alpha]_D^{22}$ +90.8 (*ca.* 1.0, MeOH). ^{1}H NMR ($CDCl_3$) δ: 2.36 (s, 3H), 4.49 (m, 1H), 4.72 (dd, 1H, $J = 1.5$, 5.0 Hz), 5.76 (ddd, 1H, $J = 1.5$, 1.5, 9.8 Hz), 6.33 (dd, 1H, $J = 2.8$, 9.8 Hz), 6.89 (d, 1H, $J = 7.4$ Hz), 7.01 (d, 1H, $J = 7.7$ Hz), 7.13 (dd, 1H, $J = 7.4$, 7.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 18.3 (C-9), 66.3 (C-8), 70.5 (C-7), 125.1 (C-4), 127.3 (C-5), 129.1 (C-3), 130.8 (C-2), 130.8 (C-6), 131.9 (C-4a), 132.2 (C-8a), 137.3 (C-1).

2.5.2. 7-Methyl-1,2-dihydro-naphthalene-1,2-diol (**2**: product converted from 2-methyl-naphthalene)

The crude EtOAc extract (103.0 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 3.3 mg of **2** (yield 2.7%). HRMS (EI) calcd. for $C_{11}H_{12}O_2$ (M^+), 176.0838; found 176.0836. ^{1}H NMR ($CDCl_3$) δ: 2.29 (s, 3H), 4.29 (dd, 1H, $J = 4.0$, 5.0 Hz), 4.58 (d, 1H, $J = 5.0$ Hz), 5.92 (dd, 1H, $J = 4.0$, 9.8 Hz), 6.43 (d, 1H, $J = 9.8$ Hz), 6.94 (d, 1H, $J = 7.6$ Hz), 7.02 (d, 1H, $J = 7.6$ Hz), 7.28 (brs, 1H). ^{13}C NMR ($CDCl_3$) δ: 21.4 (C-9), 67.9 (C-7), 70.8 (C-8), 126.9 (C-4), 127.5 (C-6), 128.2 (C-1), 128.8 (C-4a), 129.0 (C-3), 129.0 (C-5), 135.5 (C-8a), 138.5 (C-2).

2.5.3. 5,8-Dimethyl-1,2-dihydro-naphthalene-1,2-diol (**3**) and (4-hydroxymethyl-naphthalene-1-yl)-methanol (**4**) (products converted from 1,4-dimethyl-naphthalene)

The crude EtOAc extract (138.3 mg) was subjected to column chromatography (hexane–EtOAc = 3:1) to yield 4.0 mg of **3** (yield 3.3%) and 65.0 mg of **4**. (yield 59.0%). **3**: HRMS (EI) calcd. for $C_{12}H_{14}O_2$ (M^+), 190.0994; found 190.0982. ^{1}H NMR ($CDCl_3$) δ: 2.23 (s, 3H), 2.32 (s, 3H), 4.45 (m, 1H), 4.71 (dd, 1H, $J = 1.5$, 5.4 Hz), 5.76 (m, 1H), 6.56 (dd, 1H, $J = 2.7$, 9.8 Hz), 6.91 (d, 1H, $J = 8.0$ Hz), 6.97 (d, 1H, $J = 8.0$ Hz). ^{13}C NMR δ: 18.3 (C-10), 18.9 (C-9), 70.4 (C-7), 66.8 (C-8), 127.3 (C-5), 130.8 (C-3), 130.9 (C-2), 130.9 (C-6), 130.9 (C-4a), 132.0 (C-4), 132.2 (C-8a), 134.9 (C-1).

4: HRMS (EI) calcd. for $C_{12}H_{12}O_2$ (M^+), 188.0838; found 188.0837. ^{1}H NMR ($CDCl_3$) δ: 4.94 (m, 4H), 5.28 (dd, 2H, *J* = 1.3, 5.2 Hz), 7.50 (d, 2H, *J* = 3.4 Hz), 7.54 (m, 2H), 8.09 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 61.3 (C-9, 10), 123.8 (C-2, 3), 124.2 (C-5, 8), 125.5 (C-6, 7), 130.8 (C-4a, 8a), 137.0 (C-1, 4).

*2.5.4. (5-Methyl-naphthalen-1-yl)-methanol (**5**: product converted from 1,5-dimethyl-naphthalene)*

The crude EtOAc extract (134.1 mg) was subjected to column chromatography (hexane–EtOAc = 3:1) to yield 35.5 mg of **5** (yield 32.2%). HRMS (EI) calcd. for $C_{12}H_{12}O$ (M^+), 172.0889, found 172.0892. ^{1}H NMR δ: 2.65 (s, 3H), 5.04 (s, 2H), 7.29 (d, 1H, *J* = 7.0 Hz), 7.37 (dd, 1H, *J* = 7.0, 9.0 Hz), 7.41 (dd, 1H, *J* = 8.0, 9.1 Hz), 7.44 (d, 1H, *J* = 8.0 Hz), 7.90 (d, 1H, *J* = 9.0 Hz), 7.92 (d, 1H, *J* = 9.1 Hz). ^{13}C NMR δ: 19.8 (C-10), 63.7 (C-9), 121.8 (C-8), 124.5 (C-4), 124.9 (C-2), 125.1 (C-7), 125.9 (C-3), 126.6 (C-6), 131.2 (C-8a), 132.9 (C-4a), 134.9 (C-5), 136.7 (C-1).

*2.5.5. (6-Methyl-naphthalen-1-yl)-methanol (**6**: product converted from 1,6-dimethyl-naphthalene)*

The crude EtOAc extract (210.1 mg) was subjected to column chromatography (hexane–EtOAc = 3:1) to yield 40.6 mg of **6** (yield 36.8%). HRMS (EI) calcd. for $C_{12}H_{12}O$ (M^+), 172.0889, found 172.0902. ^{1}H NMR δ: 2.50 (s, 3H), 5.05 (s, 2H), 7.34 (d, 1H, *J* = 8.5 Hz), 7.38–7.39 (2H), 7.62 (s, 1H), 7.69 (dd, 1H, *J* = 2.6, 6.9 Hz), 7.97 (d, 1H, *J* = 8.5 Hz). ^{13}C NMR δ: 21.5 (C-10), 63.6 (C-9), 123.4 (C-8), 124.4 (C-2), 125.4 (C-3), 127.6 (C-5), 127.9 (C-4), 128.5 (C-7), 129.4 (C-8a), 134.0 (C-4a), 135.4 (C-1), 136.1 (C-6).

*2.5.6. (6-Methyl-naphthalene-2-yl)-methanol (**7**) and 3,7-dimethyl-1,2-dihydro-naphthalene-1,2-diol (**8**) (products converted from 2,6-dimethyl-naphthalene)*

The crude EtOAc extract (94.9 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 50.6 mg of **7** (yield 45.9%) and 7.8 mg of **8** (yield 6.4%). **7**: HRMS (EI) calcd. for $C_{12}H_{12}O$ (M^+), 172.0889; found 172.0896. ^{1}H NMR ($CDCl_3$) δ: 2.45 (s, 3H), 4.75 (s, 2H), 7.26 (dd, 1H, *J* = 1.4, 8.4 Hz), 7.36 (dd, 1H, *J* = 1.4, 8.3 Hz), 7.54 (d, 1H, *J* = 1.4 Hz), 7.66 (d, 1H, *J* = 8.4 Hz), 7.67 (d, 1H, *J* = 1.4 Hz), 7.68 (d, 1H, *J* = 8.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 21.7 (C-10), 65.5 (C-9), 125.3 (C-1), 125.3 (C-3), 126.7 (C-5), 127.6 (C-4), 127.6 (C-8), 128.4 (C-7), 131.5 (C-8a), 133.1 (C-4a), 135.5 (C-6), 137.3 (C-2).

8: HRMS (EI) calcd. for $C_{12}H_{14}O_2$ (M^+), 190.0994; found 190.0990. ^{1}H NMR ($CDCl_3$) δ: 1.93 (d, 3H, *J* = 1.2 Hz), 2.27 (s, 3H), 4.04 (d, 1H, *J* = 4.8 Hz), 4.62 (d, 1H, *J* = 4.8 Hz), 6.17 (d, 1H, *J* = 1.2 Hz), 6.85 (d, 1H, *J* = 7.9 Hz), 6.97 (d, 1H, *J* = 7.9 Hz), 7.27 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 20.5 (C-10), 21.4 (C-9), 71.3 (C-7), 71.7 (C-8), 124.2 (C-5), 125.9 (C-4), 127.4 (C-1), 128.8 (C-3), 129.6 (C-4a), 134.4 (C-8a), 135.9 (C-6), 137.3 (C-2).

*2.5.7. 8-Methoxy-1,2-dihydro-naphthalene-1,2-diol (**9**) and 5-methoxyl-1,2-dihydro-naphthalene-1,2-diol (**10**) (products converted from 1-methoxy-naphthalene)*

The crude EtOAc extract (83.9 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 40.6 mg of **9** (yield 69.1%) and 4.8 mg of **10** (yield 4.0%). **9**: HRMS (EI) calcd. for $C_{11}H_{12}O_3$ (M^+), 192.0787, found 192.0768. ^{1}H NMR ($CDCl_3$) δ: 3.77 (s, 3H), 4.40 (m, 1H), 4.96 (d, 1H, *J* = 4.9 Hz), 5.80 (brd, 1H, *J* = 9.5 Hz), 6.30 (dd, 1H, *J* = 2.4, 9.5 Hz), 6.65 (d, 1H, *J* = 7.6 Hz), 6.72 (d, 1H, *J* = 8.2 Hz), 7.17 (dd, 1H, *J* = 7.6, 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ: 55.5 (C-9), 63.2 (C-8), 69.6 (C-7), 110.5 (C-2), 119.6 (C-4), 121.8 (C-8a), 126.7 (C-5), 129.9 (C-3), 131.5 (C-6), 133.1 (C-4a), 157.1 (C-1).

10: HRMS (EI) calcd. for $C_{11}H_{12}O_3$ (M^+), 192.0787, found 192.0786. ^{1}H NMR ($CDCl_3$) δ: 3.77 (s, 3H), 4.28 (dd, 1H, *J* = 4.2, 4.8 Hz), 4.57 (d, 1H, *J* = 4.8 Hz), 5.96 (dd, 1H, *J* = 4.2, 9.7 Hz), 6.77 (d, 1H, *J* = 7.9 Hz), 6.87 (d, 1H, *J* = 9.7 Hz), 7.08 (d, 1H, *J* = 7.8 Hz), 7.19 (dd, 1H, *J* = 7.8, 7.9 Hz). ^{13}C NMR ($CDCl_3$) δ: 55.7 (C-9), 67.7 (C-6), 70.9 (C-5), 110.8 (C-2), 119.6 (C-4), 120.3 (C-8a), 123.1 (C-8), 127.3 (C-7), 129.2 (C-3), 138.2 (C-4a), 155.1 (C-1).

*2.5.8. 7,8-Dihydroxy-7,8-dihydro-naphthalene-1-carboxylic acid methyl ester (**11**: product converted from 1-naphthoic acid methyl ester)*

The crude EtOAc extract (59.8 mg) was subjected to column chromatography (hexane–EtOAc = 1:1) to yield 8.2 mg of **11** (yield 6.9%). HRMS (EI) calcd. for $C_{12}H_{12}O_4$ (M^+), 220.0736; found 220.0713. ^{1}H NMR ($CDCl_3$) δ: 3.88 (s, 3H), 4.49 (m, 1H), 5.00 (m, 1H), 5.97 (m, 1H), 6.35 (dd, 1H, *J* = 2.9, 8.3 Hz), 7.21 (d, 1H, *J* = 7.0 Hz), 7.28 (dd, 1H, *J* = 7.0, 7.8 Hz), 7.63 (d, 1H, *J* = 7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 52.6 (C-10), 65.1 (C-8), 68.8 (C-7), 125.6 (C-5), 128.7 (C-3), 129.0 (C-2), 130.3 (C-1), 131.0 (C-4), 12133.1 (C-4a), 133.1 (C-6), 135.3 (C-8a), 168.4 (C-9).

*2.5.9. 7,8-Dihydroxy-7,8-dihydro-naphthalene-2-carboxylic acid methyl ester (**12**: product converted from 2-naphthoic acid methyl ester)*

The crude EtOAc extract (109.7 mg) was subjected to column chromatography (hexane–EtOAc = 1:1) to yield 8.8 mg of **12** (yield 7.4%). HRMS (EI) calcd. for $C_{12}H_{12}O_4$ (M^+), 220.0736; found 220.0735. ^{1}H NMR ($CDCl_3$) δ: 3.84 (s, 1H), 4.14 (ddd, 1H, *J* = 4.4, 5.4, 5.6 Hz), 4.52 (dd, 1H, *J* = 5.4, 6.7 Hz), 4.83 (d, 1H, *J* = 5.6 Hz), 5.25 (d, 1H, *J* = 6.7 Hz), 6.17 (dd, 1H, *J* = 4.4, 9.6 Hz), 6.57 (d, 1H, *J* = 9.6 Hz), 7.25 (d, 1H, *J* = 7.4 Hz), 7.81 (dd, 1H, *J* = 1.7, 7.4 Hz), 8.04 (d, 1H, *J* = 1.7 Hz). ^{13}C NMR ($CDCl_3$) δ: 52.0 (C-10), 65.9 (C-7), 69.5 (C-8), 126.2 (C-4), 126.9 (C-5), 127.1 (C-1), 128.1 (C-2), 128.3 (C-3), 133.2 (C-6), 136.9 (C-4a), 138.2 (C-8a), 166.2 (C-9).

*2.5.10. 4-Hydroxymethyl-naphthalene-1-carboxylic acid methyl ester (**13**: product converted from 4-methyl-1-naphthoic acid methyl ester)*

The crude EtOAc extract (77.5 mg) was subjected to column chromatography CH_2Cl_2–MeOH = 100:1) to yield 42.0 mg of **13** (yield 38.9%). HRMS (EI) calcd. for $C_{13}H_{12}O_3$ (M^+), 216.0787; found 216.0795. ^{1}H NMR δ: 3.93 (s, 3H), 5.13 (s, 2H), 7.51 (ddd, 1H, *J* = 1.2, 8.1, 8.3 Hz), 7.52 (d, 1H, *J* = 7.4 Hz), 7.54 (ddd, 1H, *J* = 1.1, 8.1, 8.1 Hz), 8.01 (dd, 1H, *J* = 1.1, 8.3 Hz), 8.07 (d, 1H, *J* = 7.4 Hz), 8.86 (dd, 1H, *J* = 1.2, 8.1 Hz). ^{13}C NMR δ: 52.2 (C-11), 63.3 (C-10), 123.2 (C-3), 123.6 (C-5), 126.5 (C-6), 126.5

(C-8), 127.3 (C-1), 127.5 (C-7), 129.7 (C-2), 131.2 (C-4a), 131.4 (C-8a), 141.4 (C-3), 168.0 (C-9).

*2.5.11. 3,8-Dimethyl-1,2-dihydro-naphthalene-1,2-diol (**14**: product converted from 1,6-dimethyl-naphthalene)*

The crude EtOAc extract (210.2 mg) was subjected to column chromatography (hexane–EtOAc = 1:1) to yield 31.2 mg of **14** (yield 25.6%). HRMS (EI) calcd. for $C_{12}H_{14}O_2$ (M^+), 190.0994; found 190.0972. $[\alpha]_D^{22}$ −20.1 (*ca.* 0.5, MeOH). ^{1}H NMR (DCl_3) δ: 1.97 (s, 3H), 2.41 (s, 2H), 4.40 (m, 1H), 4.80 (d, 1H, $J = 4.6$ Hz), 6.18 (s, 1H), 6.87 (d, 1H, $J = 7.4$ Hz), 7.01 (d, 1H, $J = 7.9$ Hz), 7.16 (dd, 1H, $J = 7.4$, 7.9 Hz). ^{13}C NMR ($CDCl_3$) δ: 18.3 (C-9), 18.8 (C-10), 67.1 (C-8), 72.5 (C-7), 122.6 (C-5), 124.2 (C-4), 129.0 (C-3), 129.1 (C-2), 131.9 (C-8a), 133.0 (C-4a), 137.0 (C-1), 138.2 (C-6).

2.5.12. Spectroscopic data of 4-hydroxymethyl-1-naphthoic acid

HRMS (EI) calcd. for $C_{12}H_{10}O_3$ (M^+), 202.0630; found 202.0610. ^{1}H NMR (CD_3OD) δ: 5.14 (s, 2H), 7.59 (2H), 7.64 (d, 1H, $J = 7.4$ Hz), 8.14 (dd, 1H, $J = 2.5$, 7.2 Hz), 8.17 (d, 1H, $J = 7.4$ Hz), 8.94 (dd, 1H, $J = 2.4$, 7.0 Hz). ^{13}C NMR (CD_3OD) δ: 63.1 (C-10), 124.2 (C-3), 124.8 (C-8), 127.3 (C-7), 127.5 (C-6), 128.1 (C-5), 128.8 (C-1), 130.8 (C-2), 132.7 (C-4a), 132.8 (C-8a), 143.4 (C-4), 171.1 (C-9).

3. Results

3.1. Biotransformation of the substituted naphthalenes

Respective substituted naphthalenes (Fig. 1) were biotransformed through the co-cultivation with the cells of *E. coli* carrying plasmid pPhnA, which express the PAH dihydroxylating dioxygenase genes from *Cycloclasticus* sp. strain A5 [4]. Several of the above substrates (Fig. 1) were biotransformed through the co-cultivation with the cells of *E. coli* carrying plasmid pHA171, which express the PAH dihydroxylating dioxygenase genes from *Nocardioides* sp. strain KP7 [3]. The products were analyzed by chromatographic and spectroscopic methods (Fig. 1).

3.2. Structural analysis of products biotransformed by aromatic dihydroxylating dioxygenase from Cycloclasticus sp. A5

3.2.1. A product converted from 1-methyl-naphthalene

The molecular formula of the compound (**1**) converted with *E. coli* (pPhnA) was determined to be $C_{11}H_{12}O_2$ by HRMS (EI) data. Analysis of double quantum filtered correlation spectroscopy (DQF COSY) and ^{1}H detected multiple quantum coherence (HMQC) spectra showed that **1** was a dihydrodiol derivative of 1-methylnaphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-4 (δ 6.89) to C-5 (δ 127.3) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.33) to H-8 (δ 4.49). The absolute configurations of C-1 and C-2 in **1** were confirmed to be *R* and *S*, respectively, by $J_{1,2}$ (5.0 Hz) and $[\alpha]_D$ [+90.8 (*ca.* 1.0, MeOH)] values [8]. Thus, **1** was identified as (1*R*,2*S*)-8-methyl-1,2-dihydro-naphthalene-1,2-diol.

3.2.2. A product converted from 2-methylnaphthalene

The molecular formula of the compound (**2**) converted with *E. coli* (pPhnA) was determined to be $C_{11}H_{12}O_2$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **2** was a dihydrodiol derivative of 2-methylnaphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-5 (δ 6.43) to C-4 (δ 126.9) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.43) to H-8 (δ 4.58). Thus, **2** was identified as (1*R*,2*S*)-7-methyl-1,2-dihydro-naphthalene-1,2-diol.

3.2.3. Products converted from 1,4-dimethyl-naphthalene

The molecular formula of the compound (**3**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{14}O_2$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **3** was a dihydrodiol derivative of 1,4-dimethylnaphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-9 (δ 2.33) and H-5 (δ 6.56) to C-8a (δ 132.2) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.56) to H-8 (δ 4.71). Thus, **3** was identified as (1*R*,2*S*)-5,8-methyl-1,2-dihydro-naphthalene-1,2-diol. This was a novel compound.

The molecular formula of the compound (**4**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O_2$ by HRMS (EI) data. The ^{1}H and ^{13}C NMR spectra of **4** showed that both methyl functions in 1,4-dimethylnaphthalene (substrate) were oxidized to the corresponding primary alcohols. Thus, **4** was identified to be (4-hydroxymethyl-naphthalene-1-yl)-methanol.

3.2.4. Products converted from 1,5-dimethyl-naphthalene

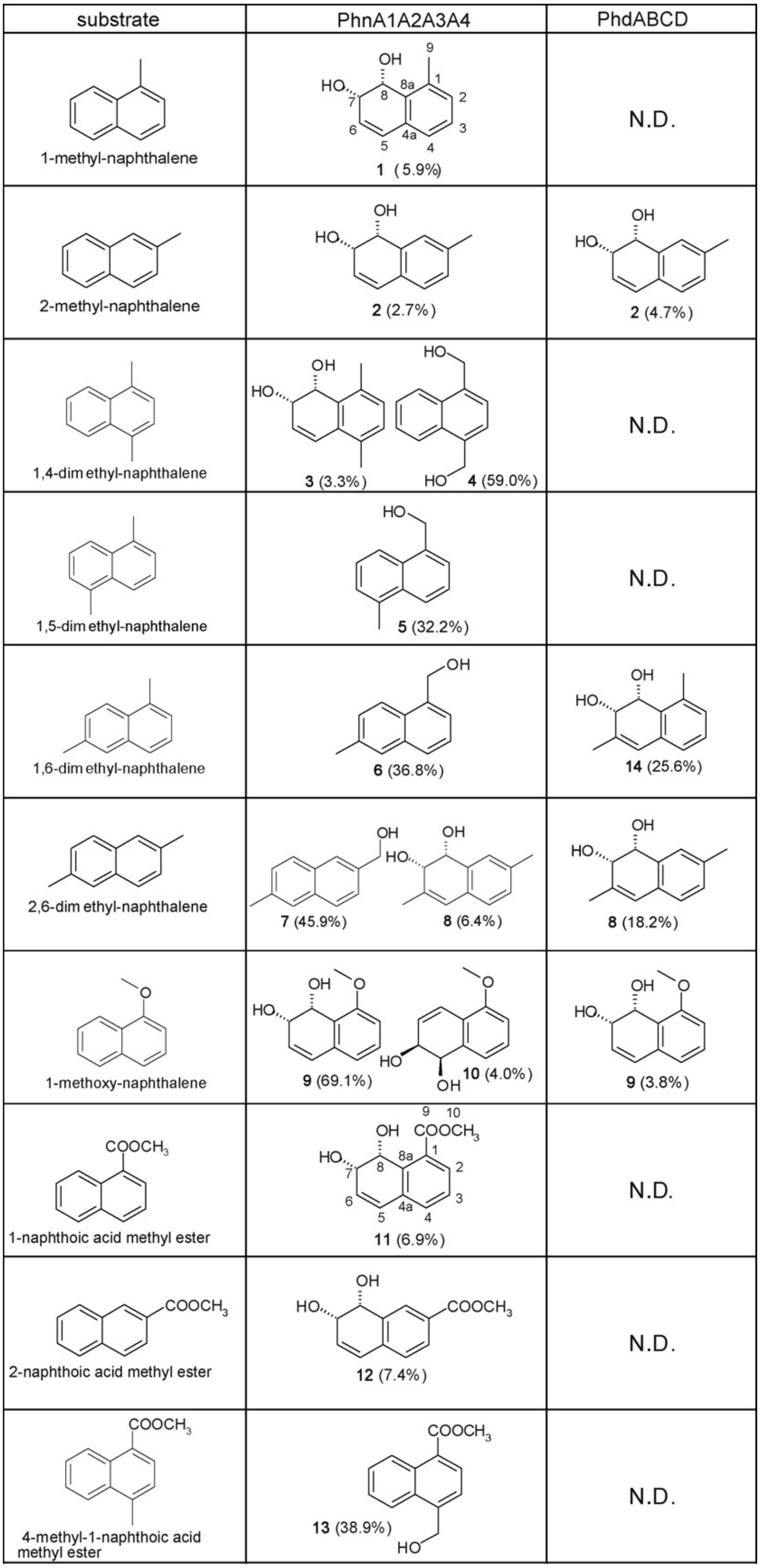
The molecular formula of the compound (**5**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O$ by HRMS (EI) data. The ^{1}H and ^{13}C NMR spectra of **5** showed that one methyl function in 1,5-dimethyl-naphthalene (substrate) were oxidized to the corresponding primary alcohol. Thus, **5** was identified to be (5-methyl-naphthalen-1-yl)-methanol.

3.2.5. Products converted from 1,6-dimethylnaphthalene

The molecular formula of the compound (**6**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O$ by HRMS (EI) data. The ^{1}H and ^{13}C NMR spectra of **6** showed that one methyl function in 1,5-dimethyl-naphthalene (substrate) were oxidized to the corresponding primary alcohol. The oxidized methyl was confirmed to be C-9 by the observation of ^{1}H–^{13}C long range couplings from H-9 (δ 5.05) to C-1 (δ 135.4), C-2 (δ 124.4), and C-8a (δ 129.4). Thus, **6** was identified to be (6-methyl-1-naphthalen-1-yl)-methanol.

3.2.6. Products converted from 2,6-dimethyl-naphthalene

The molecular formula of the compound (**7**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O$ by HRMS (EI) data. The ^{1}H and ^{13}C NMR spectra of **7** showed that one methyl function in 2,6-dimethyl-naphthalene (substrate) were oxidized



N.D.: Not Detected

Fig. 1. Bioconversion of various substituted naphthalenes through the cells of *Escherichia coli* carrying plasmid pPhnA that expressed *phnA1A2A3A4* from *Cycloclasticus* sp. A5 or plasmid pHA171 that expressed phdABCD from *Nocardioides* sp. KP7. Percent value in the parentheses represents the yield of the products purified.

to the corresponding primary alcohol. Thus, **7** was identified to be (6-methyl-naphthalen-2-yl)-methanol.

The molecular formula of the compound (**8**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{14}O_2$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **8** was a dihydrodiol derivative of 2,6-dimethyl-naphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-10 (δ 1.93) to C-7 (δ 71.3). Thus, **8** was identified as (1*R*,2*S*)-3,7-dimethyl-1,2-dihydro-naphthalene-1,2-diol.

3.2.7. A product converted from 1-methoxy-naphthalene

The molecular formula of the compound (**9**) converted with *E. coli* (pPhnA) was determined to be $C_{11}H_{12}O_3$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **9** was a dihydrodiol derivative of 1-methoxy-naphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-4 (δ 6.65) to C-5 (δ 126.7) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.30) to H-8 (δ 4.96). Thus, **9** was identified as (1*R*,2*S*)-8-methoxy-1,2-dihydro-naphthalene-1,2-diol.

The molecular formula of the compound (**10**) converted with *E. coli* (pPhnA) was determined to be $C_{11}H_{12}O_3$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **10** was a dihydrodiol derivative of 1-methoxy-naphthalene. The regiochemical assignment of the 5,6-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-4 (δ 7.08) to C-5 (δ 70.9) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 4.57) to H-8 (δ 6.87). Thus, **10** was identified as (1*R*,2*S*)-5-methoxy-1,2-dihydro-naphthalene-1,2-diol.

3.2.8. A product converted from 1-naphthoic acid methyl ester

The molecular formula of the compound (**11**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O_4$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **11** was a dihydrodiol derivative of 1-naphthoic acid methyl ester. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-4 (δ 7.21) to C-5 (δ 125.6) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.35) to H-8 (δ 5.00). Thus, **11** was identified as (7*S*,8*R*)-7,8-dihydroxy-7,8-dihydro-naphthalene-1-carboxylic acid methyl ester.

3.2.9. A product converted from 2-naphothoic acid methyl ester

The molecular formula of the compound (**12**) converted with *E. coli* (pPhnA) was determined to be $C_{12}H_{12}O_4$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **12** was a dihydrodiol derivative of 2-naphthoic acid methyl ester. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-5 (δ 6.57) to C-4 (δ 126.2) and ^{1}H–^{1}H vicinal spin networks from H-5 (δ 6.57) to H-8 (δ 4.52). Thus, **12** was identified as (7*S*,8*R*)-7,8-dihydroxy-7,8-dihydro-naphthalene-2-carboxylic acid methyl ester.

3.2.10. A product converted from 4-methyl-1-naphthoic acid methyl ester

The molecular formula of the compound (**13**) converted with *E. coli* (pPhnA) was determined to be $C_{13}H_{12}O_3$ by HRMS (EI) data. The ^{1}H and ^{13}C NMR spectra of **13** showed that the methyl function at C-4 in 4-methyl-1-naphthoic acid methyl ester (substrate) was oxidized to the corresponding primary alcohol. Thus, **13** was identified to be 4-hydroxymethyl-naphthalene-1-carboxylic acid methyl ester.

3.3. Structural analysis of products biotransformed by aromatic dihydroxylating dioxygenase from Nocardioides sp. KP7

3.3.1. A product converted from 1,6-dimethylnaphthalene

The nine substrates used for the bioconversion with *E. coli* (pPhnA) were tested for bioconversion with *E. coli* (pHA171). Bioconversion was achieved only on 2-methyl-naphthalene (yield 4.7%), 1,6-dimethyl-naphthalene (yield 25.6%), 2,6-dimethyl-naphthalene (yield 18.2%), and 1-methoxy-naphthalene (yield 3.8%) (Fig. 1). The converted compounds of 2-methyl-naphthalene, 2,6-dimethyl-naphthale, and 1-methoxy-naphthalene were identified to be **2**, **8**, and **9**, respectively, by the comparison with a authentic sample on TLC, HPLC, and ^{1}H NMR spectrum.

The molecular formula of the converted product (**14**) from 2,6-dimethyl-naphthalene was determined to be $C_{12}H_{14}O_2$ by HRMS (EI) data. Analysis of DQF COSY and HMQC spectra showed that **14** was a dihydrodiol derivative of 1,6-dimethyl-naphthalene. The regiochemical assignment of the 7,8-diol was confirmed by the long range ^{1}H–^{13}C coupling from H-10 (δ 1.93) to C-7 (δ 72.5). Thus, **14** was identified as (1*R*,2*S*)-3,7-dimethyl-1,2-dihydro-naphthalene-1,2-diol. This was a novel compound.

4. Discussion

To our knowledge, PAH- or naphthalene-dihydroxylating dioxygenases are the enzymes that convert naphthalene and several or some of its substituents into their *cis*-dihydrodiol forms. For example, naphthalene dioxygenase derived from *Pseudomonas fluorescens* N3 that was able to grow on naphthalene as the sole carbon source converted various mono-substituted naphthalenes such as 1-methylnaphtalene, 2-methylnaphtalene, 1-methoxynaphthalene and 2-methoxynaphthalene into their *cis*-dihydrodiols [9,10]. Naphthalene dioxygenase (NidABEF) derived from *Rhodococcus opacus* TKN14 that grows on *o*-xylene as the sole carbon source was able to convert various dimethyl-naphthalenes, i.e., 1,2-dimethyl-naphthalene, 1,4-dimethyl-naphthalene, 1,5-dimethyl-naphthalene and 2,3-dimethyl-naphthalene, as well as naphthalene, 1-methyl-naphthalene and 2-methyl-naphthalene, into their *cis*-dihydrodiol forms [11]. The results obtained with *E. coli* expressing the dioxygenase genes (*phdABCD*) from *Nocardioides* sp. KP7 (Fig. 1) are convincing in such a sense. PhnA1A2A3A4 from *Cycloclasticus* sp. A5 was able to transform the tested mono-substituted naphthalenes,

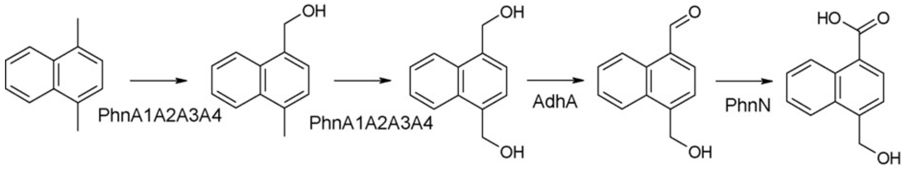


Fig. 2. Proposed pathway for synthesizing 4-hydroxymethyl-1-naphthoic acid from 1,4-dimethyl-naphthalene through the cells of recombinant *E. coli*.

i.e., 1-methyl-naphthalene, 2-methyl-naphthalene, 1-methoxy-naphthalene and 2-naphthoic acid methyl ether, into their *cis*-dihydrodiol forms (Fig. 1). Whereas, this dioxygenase tended to hydroxylate the methyl group in the case using di-substituted naphthalenes such as dimethyl-naphthalenes and 4-methyl-1-naphthoic acid methyl ether as the substrates (Fig. 1). Specifically, in the case using 1,4-dimethyl-naphthalene as the substrate, 1,4-dihydroxymethyl-naphthalene was generated as the predominant product (Fig. 1). When threefold 1,4-dimethyl-naphthalene was used as the substrate, we observed the generation of small amounts of 1-hydroxymethyl-4-methyl-naphthalene (data not shown). Thus, this monohydroxylated compound is likely to be utilized again as the substrate for PhnA1A2A3A4 to produce the dihydroxylated form (Fig. 2). The results suggest that this dioxygenase enzyme has very broad substrate preference for mono- or di-substituted naphthalenes. We previously isolated alcohol dehydrogenase genes (*adhA* and *adhB*) from *R. opacus* TKN14 and *Rhodococcus erythropolis* PR4, respectively [12] and an aldehyde dehydrogenase gene (*phnN*) from *Shingomonas* sp. 14DN61 that grows on 1,4-dimethyl-naphthalene as the sole carbon source [13]. The cells of *E. coli* carrying plasmid pPhnN-AdhA-TKN14 or pPhnN-AdhB that expressed the *adhA* or *adhB* and *phnN* genes were able to convert 1,4-dihydroxymethyl-naphthalene into 4-hydroxymethyl-1-naphthoic acid [12], whose spectroscopic data are shown in Section 2. The proposed pathway for synthesizing this product from 1,4-dimethyl-naphthalene is shown in Fig. 2. The hydroxylated products synthesized biotechnologically in the present study may be promising as starting materials for synthesizing any industrially useful chemicals.

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References

[1] T. Iwabuchi, Y. Inomata-Yamauchi, A. Katsuta, S. Harayama, J. Mar. Biotechnol. 6 (1998) 86–90.
[2] Y. Kasai, H. Kishira, K. Syutsubo, S. Harayama, Environ. Microbiol. (2001) 246–255.
[3] A. Saito, T. Iwabuchi, S. Harayama, J. Bacteriol. 182 (2000) 2134–2141.
[4] Y. Kasai, K. Shindo, S. Harayama, N. Misawa, Appl. Environ. Microbiol. 69 (2003) 6688–6697.
[5] H.-K. Chun, Y. Ohnishi, N. Misawa, K. Shindo, M. Hayashi, S. Harayama, S. Horinouchi, Biosci. Biotechnol. Biochem. 65 (2001) 1774–1781.
[6] K. Shindo, Y. Ohnishi, H.-K. Chun, H. Takahashi, M. Hayashi, A. Saito, K. Iguchi, K. Furukawa, S. Harayama, S. Horinouchi, N. Misawa, Biosci. Biotechnol. Biochem. 65 (2001) 2472–2481.
[7] J. Sambrook, D.W. Russell, Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
[8] G. Bestetti, D. Bianchi, A. Bosetti, P.D. Gennaro, E. Galli, B. Leoni, F. Pelizzoni, G. Sello, Appl. Microbiol. Biotechnol. 44 (1995) 306–313.
[9] P.D. Gennaro, G. Sello, G. Bestetti, Res. Microbiol. 148 (1997) 355–364.
[10] P.D. Gennaro, G. Sello, D. Bianchi, P. D'Amico, J. Biol. Chem. 272 (1997) 30254–30260.
[11] T. Maruyama, M. Ishikura, H. Taki, K. Shindo, H. Kasai, M. Haga, Y. Inomata, N. Misawa, Appl. Environ. Microbiol. 71 (2005) 7705–7715.
[12] X. Peng, H. Taki, S. Komukai, M. Sekine, K. Kanoh, H. Kasai, S.-K. Choi, S. Omata, S. Tanikawa, S. Harayama, N. Misawa, Appl. Microbiol. Biotechnol. 71 (2006) 824–832.
[13] X. Peng, K. Shindo, K. Kanoh, Y. Inomata, S.-K. Choi, N. Misawa, Appl. Microbiol. Biotechnol. 69 (2005) 141–150.