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Note

Biocatalytic Synthesis of Dihydroxynaphthoic Acids by Cytochrome P450 CYP199A2

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CYP199A2, a bacterial P450 monooxygenase from *Rhodopseudomonas palustris*, was found to exhibit oxidation activity towards three hydroxynaphthoic acids. Whole cells of the recombinant *Escherichia coli* strain expressing CYP199A2 efficiently catalyzed the regioselective oxidation of 1-, 3-, and 6-hydroxy-2-naphthoic acids to produce 1,7-, 3,7-, and 6,7-dihydroxynaphthoic acid respectively. These results suggest that CYP199A2 might be a useful oxidation biocatalyst for the synthesis of dihydroxynaphthoic acids.

Key words: CYP199A2; cytochrome P450; hydroxylation; naphthoic acid; oxidation

Dihydroxynaphthoic acids are important chemicals found in organisms and they exhibit biological activities. For example, 1,4-dihydroxy-2-naphthoic acid is an intermediate in the menaquinone biosynthetic pathway in various microorganisms.[1] Also, 1,4-dihydroxy-2-naphthoic acid has been reported to stimulate the growth of bifidobacteria and thus to improve conditions in the human intestine.[2] Recently, an alternative menaquinone biosynthetic pathway was found in some microorganisms, including *Helicobacter pylori*, *Campylobacter jejuni*, and *Streptomyces coelicolor*.[3] In this alternative pathway, 5,8-dihydroxy-2-naphthoic acid (1,4-dihydroxy-6-naphthoic acid) was identified as an intermediate, instead of 1,4-dihydroxy-2-naphthoic acid.[4] On the other hand, 6,7-dihydroxy-2-naphthoic acid was reported to exhibit inhibitory potency towards HIV-1 integrase.[5] Derivatives of 3,5-, 3,6-, and 3,7-dihydroxynaphthoic acids have been found to exhibit inhibitory potency towards $pp60^{c\text{-}src}$ tyrosine kinase.[6] Furthermore, dihydroxynaphthoic acids and their derivatives should find use in numerous industrial applications, including dyes and optical materials.[7,8]

Cytochrome P450 monooxygenases (P450s) regio- and stereoselectively introduce one oxygen atom derived from molecular oxygen into organic compounds under mild reaction conditions. P450s catalyze a variety of reactions, including hydroxylation of aliphatic and aromatic carbons, oxidation of organic nitrogen and sulfur, epoxidation, and Baeyer-Villiger oxidation.[9,10] Hence they are of considerable interest as biocatalysts for the synthesis of industrial chemicals and pharmaceuticals.[11–13]

In this paper, we report a synthetic approach to dihydroxynaphthoic acids using CYP199A2, a bacterial P450 from *Rhodopseudomonas palustris*, as an oxidation biocatalyst. We have found that CYP199A2 oxidized 2-naphthoic acid to 7- and 8-hydroxy-2-naphthoic acids (Fig. 1A).[14] CYP199A2 was also reported to exhibit oxidation activity towards a variety of aromatic carboxylic acids, including *para*-substituted benzoic acids, indolecarboxylic acids, and a quinolinecarboxylic acid.[15–17] Here we examined the oxidation activity of CYP199A2 towards hydroxynaphthoic acids and investigated the application of the enzyme in the synthesis of dihydroxynaphthoic acids.

The *CYP199A2* gene was coexpressed with the palustrisredoxin gene from *R. palustris* and the putidaredoxin reductase gene from *Pseudomonas putida* to provide the redox partners of CYP199A2 in *Escherichia coli*.[14,17] Whole cells of the recombinant *E. coli* strain were prepared as described previously.[14,17] Whole-cell assays were performed as substrates with 1-, 3-, and 6-hydroxy-2-naphthoic acids, which are commercially available (Wako Pure Chemicals, Osaka, Japan), and with 7- and 8-hydroxy-2-naphthoic acids, which were biocatalytically synthesized using CYP199A2.[14] The other structural isomers, 4- and 5-hydroxy-2-naphthoic acids, are commercially unavailable. The reaction mixture (250 µl) contained whole cells of the recombinant *E. coli* strain (50 g of wet cells per liter), a substrate (1 mM), dimethyl sufoxide (1% v/v), and potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10% v/v). The reactions were performed in 1.5-ml microtubes at 30 °C with vigorous shaking. High-performance liquid chromatography (HPLC) analysis was performed using a HPLC system (1100 series, Agilent, Palo Alto, CA) with an XTerra MS C18 IS column (4.6 mm × 20 mm; particle size, 3.5 µm; Waters, Milford, MA), as described previously.[14] In brief, the reaction mixture was acidified by the addition of HCl (pH 1.5–2), and was extracted with ethyl acetate (1 ml). The extract (10 µl) was injected into the HPLC system. Mobile phases A and B were composed of an acetonitrile/methanol/potassium phosphate buffer (10 mM, pH 2.7) mixture at a ratio of 2.5:2.5:95 and of acetonitrile, respectively. The samples were eluted with 0% B for 3 min, followed by a linear gradient of 0% to 70% B for 9 min at a flow rate of 1 ml min^{-1}. Compounds were detected spectrophotometrically at 220 and at 250 nm. The reaction products were isolated by the HPLC system with a fraction collector (1200 series, Agilent) and an XTerra MS C18 column

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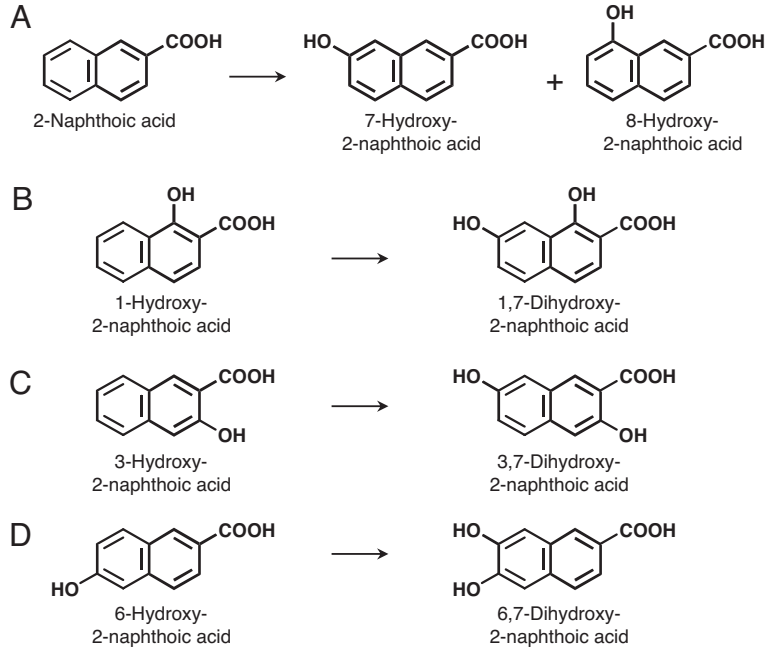

Fig. 1. Biocatalytic Oxidation of 2-Naphthoic Acid (A), 1-Hydroxy-2-naphthoic Acid (B), 3-Hydroxy-2-naphthoic Acid (C), and 6-Hydroxy-2-naphthoic Acid (D) by CYP199A2.

(4.6 mm × 250 mm; particle size, 3.5 μm; Waters), as described previously.[14] Mass analysis was performed using a Thermo Finnigan LTQ FT (Waltham, MA) with an electrospray ionization source.[14] NMR analysis was performed using a Bruker ADVANCE600 (Billerica, MA).[14] The protein concentration was measured using a Coomassie protein assay kit (Pierce, Rockford, IL) with a bovine serum albumin standard.[18] The P450 concentration was measured based on CO-reduced difference spectra using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm.[19]

We examined the oxidation activity of CYP199A2 towards 1-, 3-, and 6-hydroxy-2-naphthoic acids using the whole cells. As shown in Fig. 2, HPLC analysis of the reactions of CYP199A2 with 1-, 3-, and 6-hydroxy-2-naphthoic acids showed one major peak besides the substrate peak. These new peaks were not detected following reactions with recombinant *E. coli* cells carrying the void vector without the *CYP199A2* gene. The compounds corresponding to the new peaks were confirmed to be monooxygenation products of the hydroxynaphthoic acids based on precise determination of their mass values. Further, the products were analyzed by ^{1}H NMR, ^{13}C NMR, and two-dimensional NMR spectroscopy. In the correlation spectroscopy (COSY) spectrum of the monooxygenated 1-hydroxy-2-naphthoic acid product corresponding to a peak at 7.1 min in Fig. 2A, ^{1}H-^{1}H couplings between H-3 and H-4 and between H-5 and H-6 were observed, suggesting that the substrate was oxidized at the C-7 position. In the heteronuclear multiple bond correlation (HMBC) spectrum, ^{1}H-^{13}C long-range couplings from H-5 to C-4, C-8a, and C-7 were observed. Based on these observations, this product was identified as 1,7-dihydroxy-2-naphthoic acid (Fig. 1B). Similarly, in the COSY spectrum of the monooxygenated 3-hydroxy-2-naphthoic acid product corresponding to a peak at 6.3 min (Fig. 2B), only one ^{1}H-^{1}H coupling between H-5 and H-6 was observed, suggesting that the substrate was oxidized at the C-7 position. In the HMBC spectrum, ^{1}H-^{13}C long-range couplings from H-5 to C-4, C-8a, and C-7 were observed. Based on these observations, this product was identified as 3,7-dihydroxy-2-naphthoic acid (Fig. 1C). Furthermore, in the COSY spectrum of the monooxygenated 6-hydroxy-2-naphthoic acid product corresponding to a peak at 6.1 min (Fig. 2C), only one ^{1}H-^{1}H coupling between H-3 and H-4 was observed, suggesting that the substrate was oxidized at the C-7 or the C-8 position. In the HMBC spectrum, ^{1}H-^{13}C long-range couplings from H-5 to C-4, C-8a, and C-7 and from H-8 to C-1, C-4a, and C-6 were observed. Based on these observations, this product was identified as 6,7-dihydroxy-2-naphthoic acid (Fig. 1D).

We also examined the oxidation activity of CYP199A2 towards 7- and 8-hydroxy-2-naphthoic acids, which are products of 2-naphthoic acid oxidation by CYP199A2. CYP199A2 did not exhibit any activity towards 7-hydroxy-2-naphthoic acid. In contrast, the recombinant *E. coli* strain expressing CYP199A2 consumed 60% of 8-hydroxy-2-naphthoic acid in 180 min, and HPLC analysis of the reaction showed several small peaks besides the substrate peak (data not shown). We also confirmed that the recombinant *E. coli* cells carrying the void vector did not consume 8-hydroxy-2-naphthoic acid. We have reported that whole cells expressing CYP199A2 produced 0.29 mM 7-hydroxy-2-naphthoic acid and 0.44 mM 8-hydroxy-2-naphthoic acid from 1 mM 2-naphthoic acid.[17] The apparently insufficient conversion yield might be attributed partly to further transformation of 8-hydroxy-2-naphthoic acid by CYP199A2.

We examined the time courses of the synthesis of 1,7-, 3,7-, and 6,7-dihydroxynaphthoic acids by the CYP199A2 whole-cell catalyst. As shown in Fig. 3A, the CYP199A2 whole-cell catalyst (50 g of wet cells per liter) converted 1 mM 1-hydroxy-2-naphthoic acid to

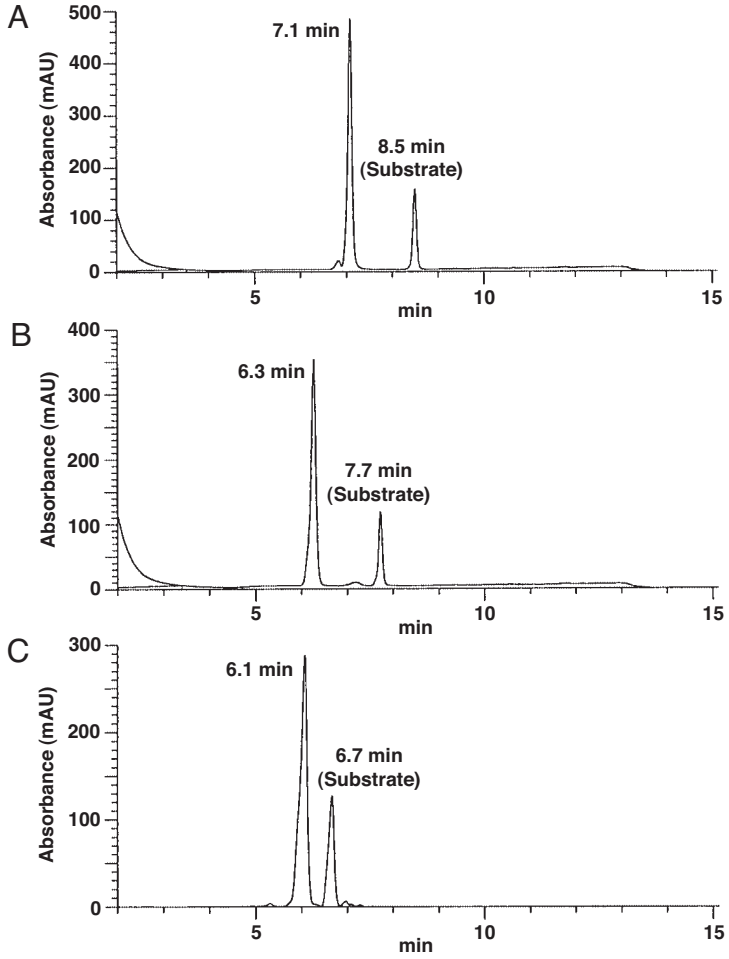

Fig. 2. HPLC Chromatograms of the Reactions of CYP199A2 with 1-Hydroxy-2-naphthoic Acid (A), 3-Hydroxy-2-naphthoic Acid (B), and 6-Hydroxy-2-naphthoic Acid (C).

Compounds were detected at 220 nm (A and B) and at 250 nm (C). Peaks at 7.1 min in A, at 6.3 min in B, and at 6.1 min in C were found to correspond to 1,7-, 3,7-, and 6,7-dihydroxy-2-naphthoic acid respectively.

1,7-dihydroxy-2-naphthoic acid in 60 min. The initial rate of hydroxylation was estimated to be 26 mol (mol P450)$^{-1}$ min^{-1} (560 nmol (g of wet cells)$^{-1}$ min^{-1}). Similarly, the CYP199A2 biocatalyst converted 1 mM 3-hydroxy-2-naphthoic acid to 3,7-dihydroxy-2-naphthoic acid in 90 min (Fig. 3B). The initial rate of hydroxylation was estimated to be 13 mol (mol P450)$^{-1}$ min^{-1} (290 nmol (g of wet cells)$^{-1}$ min^{-1}). Furthermore, the CYP199A2 biocatalyst produced 0.70 mM 6,7-dihydroxy-2-naphthoic acid from 1 mM 6-hydroxy-2-naphthoic acid in 180 min (Fig. 3C). The initial rate of hydroxylation was estimated to be 4.9 mol (mol P450)$^{-1}$ min^{-1} (110 nmol (g of wet cells)$^{-1}$ min^{-1}). The conversion yield of 6,7-dihydroxy-2-naphthoic acid was relatively low (70%). There is a possibility that an additional reaction(s) might have occurred, since we observed that the reaction generated a brown compound(s) that was not extracted with ethylacetate.

The CYP199A2 whole-cell catalyst oxidized 1-, 3-, and 6-hydroxy-2-naphthoic acids at rates of 26, 13, and 4.9 mol (mol P450)$^{-1}$ min^{-1} respectively. Using the same whole cells, the oxidation rate of 2-naphthoic acid was previously estimated to be 13 mol (mol P450)$^{-1}$ min^{-1}.[17] These results suggest that the hydroxyl group adjacent to the carboxyl group on the hydroxy-2-naphthoic acid molecules does not have unfavorable effect on oxidation activity. For comparison, the well-studied bacterial P450 enzyme, $P450_{cam}$, and the Y96F mutant have shown hydroxylation rates of 0.7 and 99.7 mol (mol P450)$^{-1}$ min^{-1} respectively towards naphthalene *in vitro*,[20] although there has been no report concerning bacterial P450s that exhibit activities towards naphthoic acids and hydroxynaphthoic acids, except for CYP199A2 (and CYP199A1[14]).

In conclusion, we found that CYP199A2 exhibited oxidation activity towards three hydroxynaphthoic acids. Whole cells expressing CYP199A2 efficiently catalyzed the oxidation of 1-, 3-, and 6-hydroxy-2-naphthoic acids to produce 1,7-, 3,7-, and 6,7-dihydroxynaphthoic acid respectively. It is interesting to note that CYP199A2 regioselectively oxidized these hydroxynaphthoic acids at the C-7 position, although this enzyme generated two products, 7- and 8-hydroxy-2-naphthoic acids, from 2-naphthoic acid. CYP199A2 provides a new, easy and environmentally friendly synthetic approach to the dihydroxynaphthoic acids from commercially available inexpensive hydroxynaphthoic acids. This efficient synthetic method might pave the way to new industrial and pharmaceutical applications of these chemicals.

The data concerning mass and NMR analyses are shown below.

1,7-Dihydroxy-2-naphthoic acid: ^{1}H NMR (600 MHz, [D_6]DMSO): $\delta = 7.14$ (dd, $J = 8.7$, 2.0 Hz, 1H; H-6),

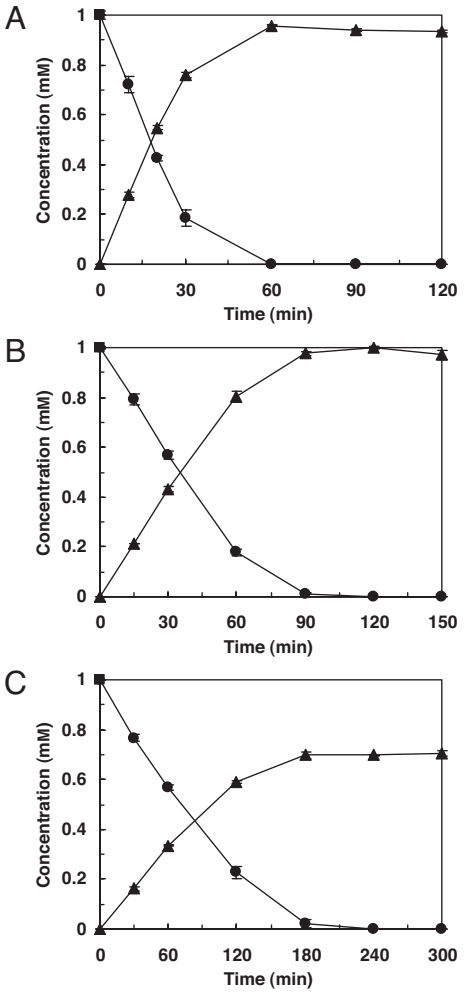

Fig. 3. Synthesis of 1,7-Dihydroxy-2-naphthoic Acid (A), 3,7-Dihydroxy-2-naphthoic Acid (B), and 6,7-Dihydroxy-2-naphthoic Acid (C) by CYP199A2 Whole-Cell Catalyst.

In A, the time courses of 1-hydroxy-2-naphthoic acid consumption (circles) and 1,7-dihydroxy-2-naphthoic acid production (triangles) are shown. In B, the time courses of 3-hydroxy-2-naphthoic acid consumption (circles) and 3,7-dihydroxy-2-naphthoic acid production (triangles) are shown. In C, the time courses of 6-hydroxy-2-naphthoic acid consumption (circles) and 6,7-dihydroxy-2-naphthoic acid production (triangles) are shown. The plot represents the average of three independent experiments, and error bars represent the standard deviation from the mean.

7.27 (d, $J = 8.5$ Hz, 1H; H-4), 7.46 (d, $J = 2.0$ Hz, 1H; H-8), 7.55 (d, $J = 8.5$ Hz, 1H; H-3), 7.73 (d, $J = 8.7$ Hz, 1H; H-5); ^{13}C NMR (600 MHz, [D$_6$]DMSO): $\delta = 113.1$ (C-8), 116.6 (C-2), 126.0 (C-4), 128.6 (C-6), 129.5 (C-3), 134.8 (C-8a), 137.3 (C-5), 139.0 (C-4a), 163.4 (C-7), 163.8 (C-1), 172.4 (C-2′); MS (ESI) (m/z): Calcd for $C_{11}H_7O_4$ $[M - H]^-$: 203.0344, found: 203.0346.

3,7-Dihydroxy-2-naphthoic acid: ^{1}H NMR (600 MHz, [D$_6$]DMSO): $\delta = 7.17$ (dd, $J = 8.8$, 2.4 Hz, 1H; H-6), 7.19 (d, $J = 2.4$ Hz, 1H; H-8), 7.24 (s, 1H; H-4), 7.65 (d, $J = 8.8$ Hz, 1H; H-5), 8.33 (s, 1H; H-1); ^{13}C NMR (600 MHz, [D$_6$]DMSO): $\delta = 110.3$ (C-8), 111.8 (C-4), 116.0 (C-2), 123.2 (C-6), 128.3 (C-5), 128.8 (C-7), 131.1 (C-1), 132.8 (C-4a), 154.4 (C-8a), 154.7 (C-3), 172.6 (C-2′); MS (ESI) (m/z): Calcd for $C_{11}H_7O_4$ $[M - H]^-$: 203.0344, found: 203.0346.

6,7-Dihydroxy-2-naphthoic acid: ^{1}H NMR (600 MHz, [D$_6$]DMSO): $\delta = 7.19$ (s, 1H; H-5), 7.29 (s, 1H; H-8), 7.65 (d, $J = 8.5$ Hz, 1H; H-4), 7.69 (d, $J = 8.5$ Hz, 1H; H-3), 8.28 (s, 1H; H-1); ^{13}C NMR (600 MHz, [D$_6$]DMSO): $\delta = 110.2$ (C-5), 111.7 (C-8), 123.3 (C-3), 125.8 (C-2), 126.5 (C-4), 128.6 (C-8a), 129.3 (C-1), 132.1 (C-4a), 148.4 (C-7), 150.0 (C-6), 168.8 (C-2′); MS (ESI) (m/z): Calcd for $C_{11}H_7O_4$ $[M - H]^-$: 203.0344, found: 203.0345.

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