Initial Reactions in the Oxidation of 1,2-Dihydronaphthalene by *Sphingomonas yanoikuyae* Strains

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The substrate oxidation profiles of *Sphingomonas yanoikuyae* B1 biphenyl-2,3-dioxygenase and *cis*-biphenyl dihydrodiol dehydrogenase activities were examined with 1,2-dihydronaphthalene and various *cis*-diols as substrates. *m*-Xylene-induced cells of strain B1 oxidized 1,2-dihydronaphthalene to (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene as the major product (73% relative yield). Small amounts of (+)-(*R*)-2-hydroxy-1,2-dihydronaphthalene (15%), naphthalene (6%), and α -tetralone (6%) were also formed. Strain B8/36, which lacks an active *cis*-biphenyl dihydrodiol dehydrogenase, formed (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene (51%), in addition to (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (44%) and (+)-(*R*)-2-hydroxy-1,2-dihydronaphthalene (5%). The *cis*-biphenyl dihydrodiol dehydrogenase of strain B1 oxidized both enantiomers of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, but only the (+)-(1*S*,2*R*)-enantiomers of *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene and *cis*-1,2-dihydroxy-3-phenylcyclohexa-3,5-diene. The results show that biphenyl dioxygenase expressed by *S. yanoikuyae* catalyzes dioxygenation, monooxygenation, and desaturation reactions with 1,2-dihydronaphthalene as the substrate, and *cis*-biphenyl dihydrodiol dehydrogenase catalyzes the enantioselective dehydrogenation of (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene and (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-3-phenylcyclohexa-3,5-diene.

The initial reaction in the metabolism of biphenyl by *Sphingomonas yanoikuyae* B1 (21) (formerly identified as a *Beijerinckia* sp. [14]) is catalyzed by biphenyl 2,3-dioxygenase (BPO), which adds both atoms of molecular oxygen and two hydrogen atoms to biphenyl to form (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-3-phenylcyclohexa-3,5-diene [(+)-*cis*-biphenyl-2,3-dihydrodiol (14, 41)]. (+)-*cis*-Biphenyl-2,3-dihydrodiol is oxidized to 2,3-dihydroxybiphenyl by *cis*-biphenyl dihydrodiol dehydrogenase (BDDH) (14). In contrast, cells of strain B8/36, a mutant lacking BDDH activity, accumulate *cis*-dihydrodiols from biphenyl and several other aromatic substrates [e.g., 6,7-dihydro-5*H*-benzocycloheptene (28), phenanthrene (19), anthracene (19), benz[*a*]anthracene (12, 20, 24), benz[*a*]pyrene (12), acenaphthylene (34), dibenzothiophene (22), and dibenzofuran (10)]. In addition, cells expressing BPO catalyze the sulfoxidation of dibenzothiophene, the desaturation of phenetole, and the O-dealkylation of anisole to yield dibenzothiophene sulfoxide, ethenylxybenzene, and phenol, respectively (22, 27). Each of these activities is also catalyzed by naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 (13, 23, 27, 38).

The *cis*-dihydrodiol dehydrogenases expressed by toluene-, naphthalene-, and biphenyl-utilizing strains also have relaxed substrate specificity (2, 22, 24, 26, 33). For example, purified toluene and naphthalene dihydrodiol dehydrogenases from *Pseudomonas putida* F1 (33) and *Pseudomonas* sp. strain 119 (26) oxidize toluene, naphthalene, biphenyl, phenanthrene, and anthracene *cis*-dihydrodiols to their respective catechols. Similarly, partially purified BDDH from strain B1 oxidizes (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene, *cis*-3,4-dihydroxy-3,4-dihydrodibenzothiophene, and (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene to their respective catechols (22, 24). However, *cis*-naphthalene dihydrodiol dehydrogenases

from strain 119 and *P. putida* NCIMB 8859 are the only *cis*-dihydrodiol dehydrogenases studied in detail for their ability to catalyze the enantioselective oxidation of specific *cis*-diol enantiomers (2, 17). *cis*-Naphthalene dihydrodiol dehydrogenase from strain 119 catalyzes the 1*R*,2*S*-selective NAD⁺-dependent oxidation of racemic *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-1,2-dihydroxy-1,2-DHN), which leads to the resolution of (–)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2-DHN (17). Similarly, whole cells of strain NCIMB 8859 incubated with racemic *cis*-1,2-diols of indan, DHN, and benzocycloheptane catalyze the kinetic resolution of single *cis*-1,2-diol enantiomers (2).

Boyd and associates have shown that *P. putida* UV4 oxidizes 1,2-dihydronaphthalene (DHN) to (+)-(*R*)-1-hydroxy-1,2-DHN, (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (*cis*-1,2-dihydroxy-1,2,3,4-THN), naphthalene, and (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN. Studies with deuterium-labelled substrates indicated that (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN formation occurred through reaction sequences involving (i) naphthalene or (ii) 1-hydroxy-1,2-DHN and 1,5,6-trihydroxy-1,2,5,6-THN as intermediates (1, 4–6). Mutant and recombinant strains expressing NDO from NCIB 9816-4 and toluene dioxygenase (TDO) from strain F1 also catalyze multiple reactions with DHN (38). For example, *P. putida* strain F39/D expressing TDO oxidizes DHN to (+)-(*R*)-1-hydroxy-1,2-DHN, (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-THN, naphthalene, and (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN by reactions involving monooxygenation, dioxygenation, and desaturation (38). In contrast, mutant and recombinant strains expressing NDO from *Pseudomonas* sp. NCIB 9816-4 oxidize DHN to naphthalene, (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN and (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN. 1-Hydroxy-1,2-DHN was not formed by NDO, and no evidence for a triol intermediate in the formation of (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN by NDO or TDO has been obtained (38).

Since the desired biological activities of chiral therapeutics are often associated with only a single enantiomer, most new drug candidates, as well as many existing racemic drugs, are

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now being developed as single-enantiomer formulations. The increasing demand for single-enantiomer forms of chiral drugs has been accompanied by an increased interest in enzyme-catalyzed enantiospecific reactions (37). Bacterial dioxygenase-mediated *cis*-dihydroxylation reactions have yielded a variety of homochiral *cis*-diols which have been used in the efficient enantioselective synthesis of a diverse array of natural and unnatural products with biological activity (8, 15). Recent studies have shown that toluene and naphthalene dioxygenases produce certain *cis*-diols (2, 28, 38), benzylic alcohols (40), and chiral sulfoxides (3, 23) of opposite stereochemistry. In addition, the *cis*-diol dehydrogenases from naphthalene- and toluene-utilizing strains may have use in the enantiospecific resolution of *cis*-diols (2, 17). While the biphenyl-oxidizing strains of *S. yanoikuyae* exhibit broad specificity in the oxidation of polycyclic aromatic hydrocarbons (12, 19, 25, 34), relatively little data is available concerning their potential to oxidize benzocyclic aromatic compounds (28) and whether the substrate oxidation profile of BPO is more similar to that of TDO or NDO.

The present study was initiated to examine the types of reactions catalyzed by strain B8/36 expressing BPO with DHN and to determine the enantioselectivity of the BDDH from strain B1 with various *cis*-diols, including those formed from DHN. We report here the regio- and stereospecific oxidation of DHN by BPO, the enantioselective dehydrogenation of *cis*-diols by BDDH, and the initial reactions involved in DHN metabolism by *S. yanoikuyae*.

MATERIALS AND METHODS

Chemicals. ($\pm$)-1-Hydroxy-1,2-DHN, ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN, and ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN were synthesized by methods previously described (7, 17, 39). Analytical standards of (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN and (-)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN were prepared by incubation of *Pseudomonas* sp. strain 9816/11 with 1,2-DHN (18, 38). *S. yanoikuyae* B8/36 was used to prepare (+)-*cis*-biphenyl dihydrodiol (14). *Pseudomonas* sp. strain C250 was used to prepare (-)-*cis*-biphenyl dihydrodiol (29). (-)-(5*S*)-2-(1-Methoxyethyl)-phenyl boronic acid (MPBA) was prepared as previously described (31). Other chemicals were of the highest purity commercially available.

Organisms and growth conditions. The biphenyl-utilizing *S. yanoikuyae* strain B1 (14, 21) and the mutant strain B8/36, lacking BDDH activity, were grown in 2.8-liter Fernbach flasks containing 800 ml of mineral salts medium (MSB [36]; pH 7.2) supplemented with 0.2% (wt/vol) pyruvate and 0.02% (wt/vol) yeast extract. Both strains were grown in the presence of *m*-xylene vapor. *m*-Xylene induces the biphenyl degradative enzymes in strains B1 and B8/36 (25). Cultures were incubated at 30°C on a rotary shaker (200 rpm) and harvested in the late exponential phase of growth by centrifugation (9,000 $\times$ g, 10 min, 4°C). The cells were washed once with 50 mM sodium-potassium (Na-K) phosphate buffer (pH 7.2) and suspended in the same buffer for biotransformation experiments.

Biotransformation of DHN, ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN, and ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN. Flasks contained the following: 400, 100, or 50 ml of induced cells suspended to a turbidity of 2.6 to 3.0 at 600 nm; 0.2% (wt/vol) pyruvate; and 0.05% (wt/vol) substrate. After 20 to 24 h of incubation (30°C, 200 rpm), cells were removed by centrifugation, and the clarified supernatants were extracted as described previously (32).

Time course studies were conducted in duplicate with 50-ml reaction volumes. At designated intervals, 1-ml samples were centrifuged (microcentrifuge, 20,000 $\times$ g, 5 min), and the supernatants were spiked with 10 μ l of a 10% (vol/vol) *n*-hexadecane solution in ethyl acetate, extracted three times with 0.5 ml of ethyl acetate, and dried with Na₂SO₄ prior to analysis. Transformations with ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN as the substrate were conducted individually for various time points, with 5-ml reaction volumes.

Analytical methods. Thin-layer chromatography (TLC) was performed with 0.2-mm-thick silica gel sheets and preparative layer chromatography (PLC) was performed with 0.5-, 1.0-, or 2.0-mm-thick silica gel plates (Silica gel 60 F₂₅₄; E. Merck Industries, Inc., Gibbstown, N.J.). The developing solvent was chloroform-acetone (8:2). ($\pm$)-1-Hydroxy-1,2-DHN was purified by radial dispersion chromatography (RDC) with 1-mm-thick silica gel plates by using chloroform (5 ml/min) to elute residual naphthalene and methanol to elute 1-hydroxy-1,2-DHN. Compounds were visualized by observing the quenching of fluorescence under shortwave UV light (254 nm).

Results from gas chromatography-mass spectrometry (GC-MS), ¹H nuclear magnetic resonance (NMR), and specific rotations were obtained as previously described (32). GC for time course experiments was performed under conditions

previously described (32) by using a Hewlett-Packard model 5890 series II gas chromatograph equipped with a flame ionization detector and a model 3396A series II integrator. One-microliter samples (containing 0.01% hexadecane as internal standard) were injected with a split ratio of approximately 30:1, and attenuation, area rejection, and threshold were set at -1, 300, and -2, respectively. Normalized compound percentages were determined with the following two equations: (i) normalized peak area of each compound = area of compound/area of hexadecane standard and (ii) normalized compound percentage = (normalized compound peak area/sum of all normalized substrate and product peak areas) $\times$ 100.

Chiral stationary-phase-high-performance liquid chromatography (CSP-HPLC) was performed with Chiralcel OJ and OB-H columns (25 cm by 4.6 mm; Chiral Technologies, Exton, Pa.) as previously described (32). The Chiralcel OJ column was used to resolve (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN, (-)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2-DHN, (-)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN, and (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-THN, which eluted with retention times of 31.0, 34.0, 23.2, and 17.6 min, respectively. The Chiralcel OB-H column was used to determine the enantiomeric purity of 2-hydroxy-1,2-DHN; (-)-(5*S*)-2-hydroxy-1,2-DHN and (+)-(5*R*)-2-hydroxy-1,2-DHN eluted at 13.0 and 13.8 min, respectively.

Diastereomeric MPBA esters were prepared (31) in order to determine the relative yield, absolute stereochemistry, and enantiomeric excess (ee) of a mixture of *cis*-1,2-dihydroxy-1,2,3,4-THN and *cis*-1,2-dihydroxy-1,2-DHN. The ¹H NMR chemical shifts observed for the mixture of esters formed with (-)-(5*S*)-MPBA were compared with those determined previously with individual diols of known chirality (31). In addition, ¹H NMR analysis (CDCl₃, 360 MHz) of the (-)-(5*S*)-MPBA esters of ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN and ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN (3:1 mixture) showed that the methoxy signals of the *cis*-(1*R*,2*S*)-enantiomers of the DHN- and THN-diols were well resolved ($\Delta\delta$, 61 ppb) at 3.166 and 3.105 ppm, respectively. The methoxy signals of the esters formed with the *cis*-(1*S*,2*R*)-enantiomers of the DHN- and THN-diols were observed at 3.217 and 3.221 ppm, respectively, but were not well resolved ($\Delta\delta$, 4 ppb). However, the methyl signals of the (-)-(5*S*)-MPBA esters of the *cis*-(1*S*,2*R*)-enantiomers of the DHN- and THN-diols were well resolved ($\Delta\delta$, 29 ppb) at 1.374 and 1.345 ppm, respectively.

Preparation of cell extracts. Induced cells of strain B1 were suspended in 50 mM 3-[*N*-morpholino]-propanesulfonic acid (MOPS) buffer (pH 8.0) containing 1 mM dithiothreitol (1 g [wet weight]/2.5 ml of buffer) and disrupted by three passages through a chilled French pressure cell (20,000 lb/in²). Cell debris was removed by centrifugation (100,000 $\times$ g, 60 min, 4°C), and the soluble supernatant was used as a cell extract for enzyme assays.

***cis*-Biphenyl dihydrodiol dehydrogenase assay.** *cis*-Biphenyl dihydrodiol dehydrogenase activity was measured spectrophotometrically at 340 nm by monitoring the reduction of NAD⁺. Reaction mixtures (1 ml) contained Na-K phosphate buffer (pH 7.0), 2.6 mM NAD⁺, 0.33 mM *cis*-diol substrate, and 0.3 to 50 U of enzyme (10 to 100 μ l of cell extract). Reactions were initiated by the addition of the *cis*-diol substrate, and assays were conducted in triplicate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1.0 nmol of NADH per min, and specific activities are reported as units per milligram of protein. Protein concentrations were determined by the method of Bradford (6a).

RESULTS

Oxidation of DHN by strain B1. *m*-Xylene-induced cells of strain B1 oxidized DHN to three major products detected by TLC with *R_f* values of 0.2, 0.5, and 0.8. GC-MS analysis showed four products (compounds I, II, III, and IV) with the properties listed in Table 1. Compounds I (41.5 mg) and II (4.3 mg) were purified by PLC. Compound I (*R_f*, 0.2) had a specific rotation of [α]_D -33 (c 0.42, methanol) and spectral characteristics identical to those of synthetic *cis*-1,2-dihydroxy-1,2,3,4-THN. These data identify compound I as (-)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN (38). GC-MS and ¹H NMR spectra of compound II were consistent with those of 2-hydroxy-1,2-DHN (16). The identity of compound II as 2-hydroxy-1,2-DHN was confirmed by a two-dimensional H,H-correlated spectroscopy (COSY) experiment which showed coupling between the hydroxymethine (carbinol) signal (H-2, 4.48 ppm) and the benzylic protons (H-1, 3.06 ppm) and the adjacent alkene proton (H-3, 6.13 ppm) (Fig. 1). Compound II (*R_f*, 0.5) had a specific rotation of [α]_D +137 (c 0.12, chloroform). Boyd and coworkers (6) have reported the preparation of (+)-(5*R*)-2-hydroxy-1,2-DHN ([α]_D +267, chloroform) through an asymmetric synthesis. On the basis of its dextrorotatory property, the absolute stereochemistry of compound II was assigned an *R*-con-

TABLE 1. Physical properties of the oxidation products formed from DHN by *S. yanoikuyae* B1 and B8/36

Compound	TLC (R_f) ^a	R_t (min) ^b	Mass spectrum [M^+ , m/z of fragment ions (% relative intensity)]	¹ H NMR chemical shifts (δ) ^c	Product identification	Relative % yield of product ^d	
						Strain B1	Strain B8/36
I	0.2	13.2	M^+ 164, 146 (62), 118 (100)	¹ H (acetone- d_6) δ 1.80–1.87 (m, 1H), 1.98–2.09 (m, 1H), 2.67–2.75 (m, 1H), 2.82–2.83 (m, 1H), 3.71–3.72 (s, 1H), 3.92–3.97 (m, 1H), 4.60 (d, J = 3 Hz, 1H), 7.06–7.08 (m, 1H), 7.14–7.17 (m, 2H), 7.39–7.42 (m, 1H)	<i>cis</i> -1,2-Dihydroxy-1,2,3,4-tetra-DHN	73	44
II	0.5	10.75	M^+ 146 (100), 128 (93), 115 (84), 102 (16), 91 (22), 63 (33)	¹ H (CDCl ₃) δ 3.04–3.06 (d, J = 5.8 Hz, 1H), 4.46–4.50 (m, 1H), 6.11–6.15 (dd, J = 4.4 Hz, J = 9.5 Hz, 1H), 6.55–6.57 (d, J = 3.7 Hz, 1H), 7.09–7.26 (m, 4H)	2-Hydroxy-1,2-DHN	15	5
III	0.8	8.3	M^+ 128 (100)	ND ^e	Naphthalene	6	— ^f
IV	0.8	11.0	M^+ 146 (63), 118 (100), 90 (73), 63 (21), 39 (24)	ND	α -Tetralone	6	—
V	0.2	12.7	M^+ 162 (23), 144 (29), 132 (6), 128 (117), 116 (100), 103 (14), 90 (23)	ND	<i>cis</i> -1,2-Dihydroxy-1,2-DHN	—	51

^a TLC R_f values were determined in chloroform-acetone (8:2).^b R_t , retention time determined by GC-MS as described in text.^c ¹H NMR chemical shifts (δ) were recorded in the deuterated solvents listed at 360 MHz. Chemical shift multiplicities, coupling constants (J values in hertz), and proton integrations are listed in parentheses.^d Determined after 20 h of incubation from GC-MS peak area integrations of total ion current chromatograms.^e ND, not determined.^f —, not detected (<1%).

figuration. Since the concentration of compound II used for the determination of $[\alpha]_D$ was low, the magnitude of the rotation was considered tenuous. However, CSP-HPLC analysis indicated that the enantiomeric purity of compound II was quite high (>92% ee). Compounds III and IV had retention times and mass spectra identical to those of authentic naphthalene and α -tetralone standards, respectively. GC-MS analysis indicated that the relative yields, at 20 h, of compounds I, II, III, and IV, based on peak area integrations of total ion current (tic) chromatograms, were 73, 15, 6, and 6%, respectively.

Oxidation of DHN by strain B8/36. *m*-Xylene-induced cells of strain B8/36 oxidized DHN to three products detected by TLC with R_f values of 0.2, 0.5, and 0.8. GC-MS analysis showed two major products and one minor product eluting at 12.7 min (M^+ 162) and 13.2 min (M^+ 164) and 10.75 min (M^+ 146), respectively. GC-MS analysis of the PLC-purified material with an R_f value of 0.2 indicated the presence of two compounds. These two products had mass spectral characteristics of compounds I and V listed in Table 1. Compounds I and V had GC retention times, mass, and ¹H NMR spectra (17, 18) consistent with authentic standards (38). These results indicate that the products of the DHN transformation by strain B8/36 were *cis*-1,2-dihydroxy-1,2,3,4-THN (compound I), 2-hydroxy-1,2-DHN (compound II), and *cis*-1,2-dihydroxy-1,2-DHN (compound V). The relative percent yields of compounds I, II, and V at 20 h, based on GC-MS peak area integrations of tic chromatograms, were 44, 5, and 51%, respectively.

The enantiomeric composition and absolute configuration of compounds I and V formed by strain B8/36, at 24 h were determined by ¹H NMR analysis of the PLC-purified mixture

(R_f 0.2) derivatized with (–)-(S)-MPBA (31). The ¹H NMR spectrum (CDCl₃) of the MPBA esters showed two chemical shifts corresponding to the OMe signals of the diastereomers formed by derivatization of (–)-(S)-MPBA with (–)-(1R,2S)-*cis*-1,2-dihydroxy-1,2,3,4-THN (3.105 ppm) and (+)-(1R,2S)-*cis*-1,2-dihydroxy-1,2-DHN (3.166 ppm) (Fig. 2). The absence of both the methoxy and methyl signals corresponding to the (S)-MPBA esters of the (1S,2R)-enantiomers of *cis*-1,2-dihydroxy-1,2-DHN and *cis*-1,2-dihydroxy-1,2,3,4-THN, accompanied by resolved racemate data, indicated that the (1R,2S)-enantiomers of compounds I and V were of high enantiomeric purity (>95% ee). The relative yields of compounds I and V, determined from the magnitude of the integrals, were 78 and 22%, respectively.

The differences between the products formed from DHN by strains B8/36 and B1 were presumably due to an active BDDH in strain B1. To test this hypothesis, *m*-xylene-induced cells of strains B1 and B8/36 were incubated with DHN, and product formation was determined as a function of time. Strain B1 oxidized DHN to (–)-(1R,2S)-*cis*-1,2-dihydroxy-1,2,3,4-THN (major product) and 2-hydroxy-1,2-DHN, with naphthalene and (+)-(1R,2S)-*cis*-1,2-dihydroxy-1,2-DHN observed as transient intermediates (Fig. 3A). In contrast, strain B8/36 oxidized DHN to (+)-(1R,2S)-*cis*-1,2-dihydroxy-1,2-DHN and (–)-(1R,2S)-*cis*-1,2-dihydroxy-1,2,3,4-THN as major products; 2-hydroxy-1,2-DHN was observed as a minor product. Small amounts of naphthalene were detected at 1 and 2 h (2 to 8%) but not after 4 h (Fig. 3B). Naphthalene was detectable at <1% (GC-MS) in the DHN used in this study.

Oxidation of (±)-*cis*-1,2-dihydroxy-1,2-DHN and (±)-*cis*-dihydroxy-1,2,3,4-THN by strains B1 and B8/36. Kinetic studies

FIG. 1. Two-dimensional ^1H , ^1H -COSY spectrum of compound II (CDCl_3 , 600 MHz) formed by BPO. Correlations between the hydroxymethine (carbinol) signal (H-2, 4.48 ppm) and the benzylic protons (H-1, 3.06 ppm) and the adjacent alkene proton (H-3, 6.13 ppm) confirm the structure of the metabolite as 2-hydroxy-1,2-DHN.

of the oxidation of ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN and ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN by strain B1 were conducted to examine the enantioselectivity of BDDH. After 5 h, an enrichment of (–)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2-DHN was observed concomitant to the disappearance of ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN. Although preferential oxidation of the (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN was apparent, complete removal of ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN (to <2.5 $\mu\text{g/ml}$) was observed within 20 h.

In a similar experiment, strain B1 oxidized ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN to a single product (compound VI) with an R_f value of 0.55 by TLC. Compound VI was isolated by PLC and had the following characteristics: GC-MS, retention time (R_t) of 10.75 min, molecular ion [M^+ (% relative intensity)] at m/z 162 (41) with major fragment ions at m/z 144 (61), 133 (11), 118 (97), 105 (9), and 90 (100); ^1H NMR (CDCl_3) δ 2.02

to 2.08 (m, 1H), 2.50 to 2.56 (m, 1H), 3.06 to 3.16 (m, 2H), 4.38 (dd, $J = 5.5$ Hz, $J = 13.5$ Hz, 1 H), 7.24 to 7.28 (m, 1H) 7.33 to 7.37 (m, 1H), 7.51 to 7.55 (m, 1H), 8.05 (d, $J = 7.8$ Hz, 1H). These data suggested that compound VI was 2-hydroxy-1-tetralone. CSP-HPLC analysis of remaining *cis*-1,2-dihydroxy-1,2,3,4-THN indicated that after 24 h, only (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN was present (>98% ee) (Fig. 4). Strain B8/36 oxidized ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN to less than 8.0% 2-hydroxy-1-tetralone after 24 h (Fig. 4). Controls incubated without cells remained unchanged after 24 h.

The substrate specificity of BDDH in cell extracts prepared from strain B1 was also examined by measuring NAD^+ reduction in the presence of various *cis*-diols (Table 2). The highest BDDH activity was measured with (+)-*cis*-biphenyl-2,3-dihydrodiol as the substrate. No activity was observed with (–)-*cis*-biphenyl-2,3-dihydrodiol or (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-

FIG. 2. Expanded region of the ^1H NMR spectrum of $(-)-(S)$ -MPBA esters of the mixture (R_f 0.2) obtained from DHN by strain B8/36. The chemical shifts at 3.166 and 3.105 ppm correspond to the methoxy signals of the diastereomeric esters of $(+)-(1R,2S)$ -*cis*-1,2-dihydroxy-1,2-DHN and $(-)-(1R,2S)$ -*cis*-1,2-dihydroxy-1,2,3,4-THN, respectively.

FIG. 3. Oxidation of DHN ($\square$) to *cis*-1,2-dihydroxy-1,2,3,4-THN ($\blacksquare$), naphthalene ($\blacktriangle$), *cis*-1,2-dihydroxy-1,2-DHN ($\triangle$), and 2-hydroxy-1,2-DHN ($\circ$) by strains B1 (A) and B8/36 (B). Relative concentrations of metabolites at 20 h were within 4% of plotted values shown at 10 h.

FIG. 4. Oxidation of $(\pm)$ -*cis*-1,2-dihydroxy-1,2,3,4-THN ($\circ$ and $\square$) to 2-hydroxy-1-tetralone ($\bullet$ and $\blacksquare$) by strains B1 (circles) and B8/36 (squares).

THN. BDDH activity was approximately the same for racemic and $(+)-(1R,2S)$ -*cis*-1,2-dihydroxy-1,2-DHN (i.e., 17 and 27% of the maximum rate). Very low BDDH activity ($<5\%$) was observed with racemic *cis*-1,2-dihydroxy-1,2,3,4-THN as the substrate (Table 2).

DISCUSSION

The results summarized in Fig. 5 show that induced cells of *S. yanoikuyae* B8/36 oxidize DHN to $(-)-(1R,2S)$ -*cis*-1,2-dihydroxy-1,2,3,4-THN, $(+)-(1R,2S)$ -*cis*-1,2-dihydroxy-1,2-DHN, and $(+)-(R)$ -2-hydroxy-1,2-DHN, respectively. The high enantiomeric purity of both *cis*-diols ($>95\%$ ee) and the 2-hydroxy-

TABLE 2. Substrate specificity of *cis*-biphenyl dihydrodiol dehydrogenase

Substrate	Sp act (SD) ^a	% Activity
$(+)$ - <i>cis</i> -(1 <i>S</i> ,2 <i>R</i>)-dihydroxy-3-phenylcyclohexa-3,5-diene	48.0 (2.4)	100
$(-)$ - <i>cis</i> -(1 <i>R</i> ,2 <i>S</i>)-dihydroxy-3-phenylcyclohexa-3,5-diene	0	0
$(\pm)$ - <i>cis</i> -1,2-dihydroxy-1,2-dihydronaphthalene	13.2 (1.8)	17
$(+)$ - <i>cis</i> -(1 <i>R</i> ,2 <i>S</i>)-dihydroxy-1,2-dihydronaphthalene	8.0 (1.5)	27
$(\pm)$ - <i>cis</i> -1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene	1.3 (0.9)	2
$(-)$ - <i>cis</i> -(1 <i>R</i> ,2 <i>S</i>)-dihydroxy-1,2,3,4-tetrahydronaphthalene	0	0

^a One unit of activity is the amount of enzyme required to catalyze the formation of 1 nmol of NADH per min. SD, standard deviation.

FIG. 5. Proposed pathway for the oxidation of DHN by cells of strain B8/36 expressing BPO. Chemical designations: I, (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN; II, (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN; III, naphthalene; V, (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN.

1,2-DHN (>92% ee) indicate that the *cis*-dihydroxylation and monohydroxylation reactions with DHN were catalyzed with high stereospecificity. Similar findings were observed with NDO from *Pseudomonas* sp. 9816/11 (38); however, (+)-(1*R*,2*S*)-2-hydroxy-1,2-DHN has not been observed previously as a metabolite of 1,2-DHN. In contrast, strain B1 oxidized DHN to (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN as the major product; the minor products detected were (+)-(1*R*,2*S*)-2-hydroxy-1,2-DHN and α -tetralone. These results and product profiles from kinetic studies suggested that the (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN formed by strain B1 was further oxidized by BDDH, presumably to 1,2-dihydroxynaphthalene, with subsequent metabolism through the naphthalene pathway described by Eaton and Chapman (11). The BDDH in strain B8/36 is not active.

The oxidation of both enantiomers of *cis*-1,2-dihydroxy-1,2-DHN by strain B1 indicated that BDDH is not enantioselective for this substrate; however, BDDH did preferentially oxidize the (1*R*,2*S*)-enantiomer. Thus, the BDDH from strain B1 exhibits stereospecificity similar to that of the *cis*-naphthalene dihydrodiol dehydrogenases from *Pseudomonas* sp. strains 119 and NCIMB 8859, although the latter are truly enantioselective for (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN (2, 17).

Time course studies using strains B1 and B8/36 with ($\pm$)-*cis*-1,2-dihydroxy-1,2,3,4-THN as the substrate indicated that neither enantiomer of *cis*-1,2-dihydroxy-1,2,3,4-THN served as the intermediate in the oxidation of DHN to (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN. However, the (1*S*,2*R*)-enantiomer did serve as a substrate for BDDH of strain B1, forming 2-hydroxy-1-tetralone, leading to the resolution of (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN (>98% ee, 24 h) as illustrated in Fig. 6. The small amount of dehydrogenase activity observed with strain B8/36 suggests that the BDDH mutation is slightly

“leaky” in this strain. Recently, a complementary enantioselective oxidation of *cis*-1,2-dihydroxy-1,2,3,4-THN has been reported for a naphthalene-degrading organism, strain NCIMB 8859. Strain NCIMB 8859 was shown to resolve the enantiomer of *cis*-1,2-dihydroxy-1,2,3,4-THN opposite to that shown in this study (2).

The substrate specificity of BDDH observed with whole-cell transformations was further examined by assaying BDDH activity in cell extracts prepared from induced cells of strain B1 with various chiral and racemic *cis*-diols (Table 2). The results from these assays confirmed the enantioselectivity of BDDH observed in the biotransformation studies. BDDH was able to oxidize both enantiomers of *cis*-1,2-dihydroxy-1,2-DHN, but only the (1*S*,2*R*)-enantiomer of *cis*-1,2-dihydroxy-1,2,3,4-THN.

(+)-(1*R*,2*S*)-2-Hydroxy-1,2-DHN formed by B8/36 appears to be a dead-end product in the oxidation of DHN, since this compound reproducibly accumulates under biotransformation conditions. The transient nature of naphthalene observed in time course studies and the complete conversion of naphthalene (>98%) to (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN observed previously (30) suggest that, like TDO and NDO from *Pseudomonas* sp. strains F39/D and 9816/11 (38), naphthalene is the intermediate in the oxidation of DHN to (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-DHN by BPO in strains B1 and B8/36.

The results obtained from this study show that BPO of *S. yanoikuyae* B1 catalyzes desaturation, allylic hydroxylation, and dioxygenation reactions with DHN (Fig. 5), while BDDH catalyzes the oxidation of both enantiomers of *cis*-1,2-dihydroxy-1,2-DHN and the enantioselective oxidation of (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-THN (Fig. 4 and 6). BPO and BDDH exhibit unique regiospecific monooxygenase and dehydrogenase activities, respectively. BPO catalyzes the stereospecific monooxygenation of DHN to form (+)-(1*R*,2*S*)-2-hydroxy-1,2-DHN, a product that has not been observed with TDO or NDO (6, 38). BDDH catalyzes the oxidation of ($\pm$)-*cis*-1,2-dihydroxy-1,2-DHN and the enantioselective oxidation of (+)-(1*S*,2*R*)-*cis*-1,2-dihydroxy-1,2,3,4-THN. While the stereospecificity of BPO appears similar to that of NDO with respect to *cis*-dihydroxylation of benzocyclic and polycyclic aromatic substrates (e.g., yielding diols of the 1*R*,2*S* configuration), the stereoselectivity of BDDH appears to complement the *cis*-dihydrodiol dehydrogenase activity of the naphthalene-degrading strain NCIMB 8859 (2). These properties suggest that strains B1 and B8/36 may have the potential to supply addi-

FIG. 6. Resolution of (–)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2,3,4-THN. The (1*S*,2*R*)-enantiomer served as a substrate for BDDH of strain B1, forming 2-hydroxy-1-tetralone, and led to the resolution shown as described in the text.

tional chiral precursors for the preparation of biologically active molecules (8, 9, 15, 35).

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