Regio- and Stereospecific Oxidation of Fluorene, Dibenzofuran, and Dibenzothiophene by Naphthalene Dioxygenase from *Pseudomonas* sp. Strain NCIB 9816-4

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The regio- and stereospecific oxidation of fluorene, dibenzofuran, and dibenzothiophene was examined with mutant and recombinant strains expressing naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4. The initial oxidation products were isolated and identified by gas chromatography-mass spectrometry and nuclear magnetic resonance spectrometry. Salicylate-induced cells of *Pseudomonas* sp. strain 9816/11 and isopropyl- β -D-thiogalactopyranoside-induced cells of *Escherichia coli* JM109(DE3)(pDTG141) oxidized fluorene to (+)-(3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrofluorene (80 to 90% relative yield; >95% enantiomeric excess [ee]) and 9-fluorenol (<10% yield). The same cells oxidized dibenzofuran to (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran (60 to 70% yield; >95% ee) and (3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran (30 to 40% yield; >95% ee). Induced cells of both strains, as well as the purified dioxygenase, also oxidized dibenzothiophene to (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene (84 to 87% yield; >95% ee) and dibenzothiophene sulfoxide (<15% yield). The major reaction catalyzed by naphthalene dioxygenase with each substrate was stereospecific dihydroxylation in which the *cis*-dihydrodiols were of identical regiochemistry and of *R* configuration at the benzylic center adjacent to the bridgehead carbon atom. The regiospecific oxidation of dibenzofuran differed from that of the other substrates in that a significant amount of the minor *cis*-3,4-dihydrodiol regioisomer was formed. The results indicate that although the absolute stereochemistry of the *cis*-diene diols was the same, the nature of the bridging atom or heteroatom influenced the regiospecificity of the reactions catalyzed by naphthalene dioxygenase.

Fluorene, dibenzofuran, dibenzothiophene, and carbazole are structural analogs which differ only in the type of atom bridging the two aromatic rings. These compounds are constituents of fossil fuels such as crude oil and coal tar derivatives and are often present in complex mixtures with other polycyclic aromatic compounds (PACs). The widespread use of coal and petrochemicals for industrial processes (coal gasification, wood treatment with creosote, etc.) and their accidental release have led to PAC-contaminated environments with potential health risks (14, 21, 42). Fundamental research examining the reaction sequences and the specificity of enzymes utilized by microorganisms to metabolize or transform individual hydrocarbons and PACs provides a basis for certain bioremediation strategies (7, 20, 40, 43) and also can identify alternative routes for the preparation of specific hydroxylated products derived from PACs (11, 50, 53, 58).

Bacterial aromatic ring dioxygenases are multicomponent enzymes that exhibit extremely broad substrate specificities and often are capable of initiating the catabolism of substrates which themselves do not support strain growth (20). In addition to the relaxed substrate specificity observed in asymmetric *cis*-dihydroxylation, certain dioxygenases, such as naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4, catalyze monooxygenation (19, 51, 55, 68), sulfoxidation (3, 37), *O*- and *N*-dealkylation (36, 48), and desaturation (19, 48, 67) reactions for a number of aromatic and benzocyclic compounds. These oxidation reactions are thought to be catalyzed by the same strong oxidizing species formed from the interaction of oxygen with mononuclear iron in the large (α)

subunit of the terminal oxygenase with the diversity of reactions being dependent on interactions between the active site of the oxygenase and the structure of the substrate (19).

The regio- and stereochemistry of the initial oxidation products formed from PACs by NDO and other dioxygenases are of interest for several reasons. First, knowledge of the regiospecificity of PAC oxidation could allow the prediction of the degradative sequences which lead to the formation of dead-end products that could exhibit increased solubility and/or toxicity (9, 44). Second, the enantiomeric composition and purity of the initial oxidation products (i.e., *cis*-diols) are of interest since subsequent pathway enzymes can be enantioselective. The dioxygenase-catalyzed formation of novel *cis*-diol enantiomers from low-molecular-weight PACs (50) indicates the potential of different soil bacteria to yield both *cis*-diol enantiomers through the initial *cis*-dihydroxylation of prochiral substrates. This finding, coupled with the enantioselective nature of certain *cis*-dihydrodiol dehydrogenases (2, 12a, 28), as well as the enantioselective metabolism of acetamide pesticide stereoisomers (45) and chiral intermediates of linear alkylbenzenes (62), illustrates the importance of examining both the formation and subsequent metabolism of chiral intermediates when studying the biodegradation of environmental pollutants. Finally, studies of the regio- and stereospecific oxidation of aromatic compounds can reveal new chiral intermediates in high enantiomeric purity that can serve as synthons in the environmentally benign, enantiocontrolled preparation of value-added, biologically active natural products (5, 6, 12, 26, 27, 60, 63).

Fluorene, dibenzofuran, and dibenzothiophene are substrates for initial dioxygenation and subsequent transformation by organisms possessing naphthalene-degrading (or similar) enzymes (8, 10, 11, 23, 33, 35, 39, 41, 59). *Sphingomonas*

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yanokuyae B8/36 (31) oxidized dibenzofuran (8) and dibenzothiophene (35, 39) to their respective *cis*-1,2-dihydrodiols, as major products. However, the initial product(s) resulting from the *cis*-dihydroxylation of fluorene has not been characterized, and the enantiomeric compositions of the *cis*-dihydrodiols of fluorene, dibenzofuran, and dibenzothiophene have yet to be determined. The oxidation of fluorene and other PACs by a recombinant *Pseudomonas aeruginosa* strain expressing NDO from *Pseudomonas putida* G7 has been reported previously (57). Fluorene was oxidized to 9-fluorenol and 9-fluorenone (combined yield, ~30% [56]) and phenolic regioisomers of fluorene and of 9-hydroxyfluorene, the latter apparently resulting from the dehydration of their 1,2- and 3,4-dihydrodiols (57). The relative yields of the initial dihydrodiol regioisomers of fluorene and 9-fluorenol have not been reported.

The oxidation of fluorene and its structural *O*-, *S*-, and *N*-analogs (dibenzofuran, dibenzothiophene, and carbazole) has been used to compare the regio- and stereospecific oxidations catalyzed by NDO and to determine differences attributable to the structures of substrates that differ only in the single atom (or heteroatom) bridging the two aromatic rings of each compound. We have examined the oxidation of fluorene, dibenzofuran, and dibenzothiophene by mutant and recombinant strains which express NDO from *Pseudomonas* sp. strain NCIB 9816-4 and report the yields, regiochemistry, absolute stereochemistry, and enantiomeric purity of the isolated initial metabolites.

MATERIALS AND METHODS

Bacterial strains and growth. *Pseudomonas* sp. strain 9816/11 is a mutant derived from *Pseudomonas* sp. strain NCIB 9816-4 which lacks active *cis*-1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (19, 71). *Escherichia coli* JM109 (DE3)(pDTG141) contains the structural genes (*nahAaAbAcAd*) for NDO from NCIB 9816-4 on plasmid pT7-5 (65). *E. coli* JM109(DE3)(pT7-5) was used in control experiments. All organisms were grown and NDO expression was induced as described previously (51, 53).

Biotransformation of fluorene, dibenzofuran, and dibenzothiophene. Induced cells of strain 9816/11 were resuspended in 200 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.3) (optical density at 600 nm of 2.0 [7.5 g of cells (wet weight) per liter]) and transferred to 2.8-liter Fernbach flasks. Fluorene, dibenzofuran, and dibenzothiophene (0.1% [wt/vol]) dissolved in *N,N*-dimethylformamide (final concentration, 0.2%) were added individually as substrates, and pyruvate (0.3%) was provided as an energy source. Flasks and their contents were incubated at 30°C with shaking (220 rpm) for 24 h. Excess substrate was removed by filtration through glass wool. Cells were removed by centrifugation, and compounds in the supernatants were isolated as described below. Isopropyl- β -thiogalactopyranoside (IPTG)-induced cells of *E. coli* JM109(DE3)(pDTG141) and JM109(DE3)(pT7-5) were incubated as described above except that biotransformations containing approximately 15 g (wet weight) of cells per liter were conducted in 500-ml volumes, substrates were added at 0.05%, and 0.5% pyruvate was provided as the energy source. The biotransformation of dibenzothiophene by purified NDO was conducted as described previously (19).

Product isolation, identification, and analysis. Supernatants from biotransformation reactions were extracted with ethyl acetate and analyzed by thin-layer chromatography (TLC) (53). Metabolites were isolated by preparative TLC with chloroform-acetone (8:2) or radial dispersion chromatography (RDC) (55). RDC was typically performed on 2-mm-thick silica with a chloroform-acetone step gradient (0 to 60% acetone increased in 20% steps over 40 min) at a flow rate of 8 ml/min. Specific rotations ($[\alpha]_D$), chiral stationary phase-high-performance liquid chromatography, proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR, respectively) spectrometry, and high-resolution mass spectrometry were performed as previously reported (53, 55). Two-dimensional NMR experiments for the determination of direct ^1H - ^{13}C connectivity (HMQC) and long-range (two- and three-bond) ^1H - ^{13}C connectivity (HMBC) (4) and 600-MHz ^1H NMR were performed on a Bruker AMX600 spectrometer. Gas chromatography-mass spectrometry (GC-MS) was performed as described previously (51). Phenyl boronic acid (PBA) (25) and methane boronic acid (MBA) derivatives were prepared by the addition of 1 equivalent of PBA or MBA to isolated *cis*-diols (1 to 2 mg) dissolved in 0.5 ml of acetone. Diastereomeric boronate esters were prepared with (–)-(*S*)- and (+)-(*R*)-2-[1-methoxyethyl]-phenylboronic acid [(–)-(*S*)- and (+)-(*R*)-MPBA, respectively] as described previously (54). The MPBA esters were filtered through small plugs of sodium sulfate with CHCl_3 , concentrated to dryness, and dissolved in 0.6 ml of CDCl_3 (containing 0.05% tetramethylsilane) for ^1H NMR analysis.

RESULTS

Fluorene biotransformation by strains expressing NDO.

Oxidation of fluorene by induced cells of strain 9816/11 gave 32 mg of crude products. TLC analysis showed the presence of fluorene (R_f , 0.7) and two metabolites with R_f values of 0.2 (compound I) and 0.5 (compound II). Both compounds were isolated by preparative TLC to yield 10 mg of compound I and approximately 0.5 mg of compound II. Their physical properties are listed in Table 1. The ^1H and ^{13}C NMR spectra of compound I showed chemical shifts consistent with the structure *cis*-3,4-dihydroxy-3,4-dihydrofluorene. The ^1H NMR chemical shifts of the hydroxymethine (carbinol) protons H-3 and H-4 at 4.50 and 4.69 ppm (Fig. 1A) and their coupling constants of $J_{3,4} = 6.4$ Hz were similar to the values for *cis* protons of vicinal 1,2-dihydrodiols formed from other substrates (24, 28–30, 32, 35). Although compound I dehydrated to phenolic products when analyzed by GC-MS, it gave a stable MBA derivative (M^+ 224 [Table 1]). In addition, high-resolution mass spectrometric analysis of compound I gave an M^+ of 200.0834 ($\text{C}_{13}\text{H}_{12}\text{O}_2$ requires 200.0837) and a mass accurate within 3 millimass units (mmu) for the ^{13}C isotope of the M^+ . Compound II was a minor product (~10% yield) identified as 9-fluorenol by mass and ^1H NMR spectrometry (Table 1) (22, 47, 57).

The identification of compound I as the 3,4-dihydrodiol of fluorene was based on two-dimensional ^1H - ^{13}C -correlated HMQC and HMBC experiments (not shown) which allowed the assignment of the ^{13}C and ^1H NMR chemical shifts reported in Table 1. The 3,4-diol regiochemical assignment was confirmed by the long-range carbon-proton connectivities observed between the H-1 diene proton (6.30 ppm) and the C-9 benzylic carbon (38.5 ppm) in the HMBC experiment. No long-range correlations between the latter signal (C-9) and the carbinol signal H-3 (4.50 ppm) or H-4 (4.69 ppm) were observed; these correlations would be expected for a 1,2-dihydrodiol. ^1H NMR analysis (Fig. 1A) and GC-MS of the MBA derivative of compound I did show the presence of a minor dihydrodiol regioisomer which was tentatively identified as *cis*-1,2-dihydroxy-1,2-dihydrofluorene. The minor diol was observed by GC-MS at <10% of the total ion current area relative to compound I.

IPTG-induced cells of JM109(DE3)(pDTG141) oxidized fluorene to yield 260 mg of an off-white solid material. TLC analysis showed the presence of two products with R_f values of 0.2 and 0.5. RDC (4-mm-thick silica) was used to isolate the major product (174 mg of a white solid; R_f , 0.2), which had mass and ^1H NMR spectral properties identical to those of compound I formed by strain 9816/11. This material gave an $[\alpha]_D$ of +214 (c 0.56, methanol). The less polar material (31 mg) was concentrated, applied to a 2-mm-thick silica RDC plate, and eluted with 5% acetone-chloroform (flow rate, 7 ml/min) to yield 18 mg of a compound with physical properties identical to those of compound II (Table 1). A minor compound detected in this sample by GC-MS had an M^+ at m/z 182 and spectral characteristics consistent with a hydroxyfluorene isomer. The major oxidation products formed by NDO with fluorene were (+)-*cis*-3,4-dihydroxy-3,4-dihydrofluorene and 9-fluorenol as shown in Fig. 2A. Fluorene oxidation products were not observed in control experiments with strain JM109(DE3)(pT7-5).

Dibenzofuran biotransformation by strains expressing NDO.

Oxidation of dibenzofuran by induced cells of strain 9816/11 gave 128 mg of crude products. While TLC showed the presence of unused dibenzofuran (R_f , 0.7) and a major product with an R_f value of 0.2, GC-MS analysis showed the presence

TABLE 1. Physical properties of the major compounds formed from fluorene, dibenzofuran, and dibenzothiophene by bacterial strains expressing NDO

Com- pound	Sub- strate	GC-MS data ^a		Chemical shifts (δ) ^b		Relative yield (%) ^c	Identification
		R _t (min)	Ion fragment <i>m/z</i> values (% relative intensity)	¹ H NMR	¹³ C NMR		
I	FLN ^d	8.15 (MBA)	224 (100), 223 (66), 209 (11), 182 (71), 181 (34), 165 (58), 164 (26), 153 (60), 152 (48), 139 (6), 127 (6), 115 (8), 76 (18)	7.52 (d, <i>J</i> = 7.4 Hz, H-5), 7.45 (d, <i>J</i> = 7.2 Hz, H-8), 7.27 (t, <i>J</i> = 7.4 Hz, H-7), 7.15 (t, <i>J</i> = 7.4 Hz, H-6), 6.30 (dd, <i>J</i> = 9.5 Hz, 2.5 Hz, H-1), 5.92 (dd, <i>J</i> = 9.5 Hz, 2.5 Hz, H-2), 4.69 (d, <i>J</i> = 6.4 Hz, H-4), 4.50 (dd, <i>J</i> = 6.4 Hz, 3.5 Hz, H-3), 3.41 (s, H-9 [2H])	144.66 (C-4b), 144.30 (C- 8a), 140.00 (C-9a), 138.55 (C-4a), 132.89 (C-2), 127.13 (C-7), 125.29 (C- 6), 124.61 (C-8), 123.17 (C-1), 120.08 (C-5), 71.23 (C-3), 65.26 (C-4), 38.51 (C-9)	80–90	<i>cis</i> -3,4-Dihydroxy-3,4- dihydro-9H-fluorene
II	FLN	7.14	182 (84), 181 (100), 165 (14), 152 (45), 151 (15), 126 (5), 76 (26)	7.66 (d, <i>J</i> = 8.9 Hz, 4H), 7.39 (t, <i>J</i> = 18.0 Hz, 2H), 7.32 (td, <i>J</i> = 7.4 Hz, 1.0 Hz, 2H), 5.60 (d, <i>J</i> = 7.6 Hz, 1H)	ND	9–18	9-Fluorenol
III	DBF ^e	8.25	202 (3), 184 (100), 155 (10), 128 (29), 102 (11), 92 (10)	7.24-7.71 (m, H-6,7,8,9), 6.51 (dd, <i>J</i> = 10.0 Hz, 2.6 Hz, H-4), 6.09 (ddd, <i>J</i> = 10.0 Hz, 2.6 Hz, 1.0 Hz, H-3), 4.93 (dd, <i>J</i> = 6.2 Hz, 0.9 Hz, H-1), 4.62 (dt, <i>J</i> = 6.2 Hz, 2.7 Hz, H-2)	155.79 (C-5a), 153.10 (C- 9b), 136.83 (C-3), 128.32 (C-9a), 124.93 (C-8), 124.08 (C-7), 120.28 (C- 9), 117.03 (C-4), 115.30 (C-4a), 112.11 (C-6), 71.58 (C-2), 65.23 (C-1)	60–70	<i>cis</i> -1,2-Dihydroxy-1,2- dihydrodibenzofuran
IV	DBF	8.41	202 (<2), 184 (100), 155 (9), 128 (20), 102 (7), 92 (7)	7.24-7.71 (m, H-6,7,8,9), 6.61 (dd, <i>J</i> = 9.7 Hz, 2.6 Hz, H-1), 5.87 (ddd, <i>J</i> = 9.7 Hz, 2.4 Hz, 0.8 Hz, H-2), 4.76 (dd, <i>J</i> = 6.4 Hz, 0.6 Hz, H-4), 4.70 (dt, <i>J</i> = 6.4 Hz, 2.5 Hz, H-3)	156.47 (C-5a), 155.88 (C- 4a), 130.76 (C-2), 126.11 (C-9a), 125.44 (C-7), 123.99 (C-8), 120.38 (C- 9), 117.38 (C-1), 114.95 (C-9b), 112.43 (C-6), 72.45 (C-3), 65.96 (C-1)	30–40	<i>cis</i> -3,4-Dihydroxy-3,4- dihydrodibenzofuran
V	DBT ^f	10.35	218 (16), 200 (100), 184 (11), 172 (54), 171 (59), 146 (9), 127 (30)	7.87–7.94 (m, 2H), 7.40 (td, <i>J</i> = 7.5 Hz, 1.2 Hz, 1H), 7.32 (td, <i>J</i> = 7.5 Hz, 1.2 Hz, 1H), 6.54 (dd, <i>J</i> = 9.8 Hz, 2.6 Hz, 1H), 6.03 (ddd, <i>J</i> = 10.7 Hz, 2.6 Hz, 1.0 Hz, 1H), 4.95 (t, <i>J</i> = 6.0 Hz, 1H), 4.63– 4.59 (m, 1H), 4.20 (d, <i>J</i> = 8.1 Hz, 1H, [OH]), 4.06 (d, <i>J</i> = 6.0 Hz, 1H, [OH])	140.76 (C), 140.15 (C), 136.85 (C), 135.08, 131.91 (C), 125.96, 125.60, 123.93, 123.07, 120.95, 71.72, 66.23	84–87	<i>cis</i> -1,2-Dihydroxy-1,2- dihydrodibenzothiophene
VI	DBT	10.71	200 (88), 184 (100), 172 (56), 171 (88), 168 (16), 153 (16), 139 (34)	8.0 (d, <i>J</i> = 7.7 Hz, 2H), 7.82 (d, <i>J</i> = 7.7 Hz, 2H), 7.61 (td, <i>J</i> = 7.5 Hz, 1.1 Hz, 2H), 7.51 (td, <i>J</i> = 7.5 Hz, 1.1 Hz, 2H)	ND	9–14	Dibenzothiophene sulfoxide

^a R_s and ion fragmentation patterns were determined under the conditions described in the text. MBA or PBA in parentheses indicates that the diols were analyzed (GC-MS) as their MBA or PBA derivatives. Where two R_s are given, *m/z* data are listed respectively.

^b Determined at 360 MHz (¹H NMR) or 90 MHz (broad-band-decoupled ¹³C NMR) in a deuterated solvent (d₆-acetone for compounds I, and III to V; CDCl₃ for compounds II and VI). ¹H NMR spectra of compounds I, III, and IV were recorded following deuterium exchange of the hydroxyl protons by addition of 50 μ l of deuterium oxide. Chemical shift multiplicities are abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; ddd, doublet of doublet of doublets; dt, doublet of triplets; td, triplet of doublets. Coupling constants (*J* values) are given in hertz. Chemical shift assignments for compounds I, III, and IV (numbered as in Fig. 2) are based on ¹H-¹³C correlated HMQC and HMBC experiments; otherwise, ¹³C chemical shifts of quaternary carbons are designated by "(C)". ND, not determined.

^c Determined by gravimetric analysis of isolated compounds and integration of the magnitudes of ¹H NMR signals and from the total ion current areas of compounds in GC-MS chromatograms of extracts and/or PBA derivatives.

^d FLN, fluorene.

^e DBF, dibenzofuran.

^f DBT, dibenzothiophene.

FIG. 1. ^1H NMR spectra of the isolated dihydrodiols formed from fluorene and dibenzofuran by strain 9816/11 expressing NDO. (A) Spectrum of (+)-(3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrofluorene (compound I) (Fig. 2); (B) spectrum of a mixture containing (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran (compound III) (Fig. 2) and (3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran (compound IV) (Fig. 2) in a ratio of 7:3, respectively. The spectra were recorded at 600 MHz in d_6 -acetone.

of dibenzofuran and four compounds with M^+ at m/z 184 (retention time [R_t], 7.41 min), 184 (R_t , 8.11 min), 202 (R_t , 8.31 min), and 202 (R_t , 8.38 min) with product distributions of 3, 3, 61, and 21%, respectively. The M^+ of the latter two compounds at m/z 202 suggested that they were dibenzofuran dihydrodiols. The major product was isolated by preparative TLC and gave 95 mg of a white solid. ^1H NMR chemical shifts indicated the presence of a major dihydrodiol (compound III) and a minor dihydrodiol (compound IV), which were formed in a 7:3 ratio (Fig. 1B). The ^{13}C NMR spectrum of this mixture showed 24 carbon resonances representing compounds III and IV (Table 1).

The ratio of the two compounds, and the fact that the 600-MHz ^1H NMR chemical shifts of compounds III and IV were well resolved (Fig. 1B), allowed structural and chemical shift assignments of both compounds in the mixture by analysis of the spectra from one- and two-dimensional NMR experiments. Compound III was identified as *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran on the basis of ^1H - ^{13}C connectivities observed in HMQC and HMBC experiments (not shown). The 1,2-dihydrodiol regiochemistry of compound III was supported by the HMBC ^1H - ^{13}C connectivities observed between the H-4 proton (6.51 ppm) and the bridgehead C-4a (115.30 ppm) and C-9b (153.10 ppm) carbons and between the H-1 carbinol signal (4.93 ppm) and the bridgehead C-9b, C-4a, and C-9a (128.32 ppm) carbon resonances. Compound IV was similarly identified as *cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran. The 3,4-dihydrodiol regiochemistry of compound IV was supported by the HMBC ^1H - ^{13}C connectivities observed between the H-4 carbinol signal (4.76 ppm) and the bridgehead C-4a (155.88 ppm) and C-9b (114.95 ppm) carbons, while the H-1 proton signal (6.61 ppm) showed long-range connectivity with the signals of the three bridgehead carbons at the C-9b, C-4a, and C-9a (126.11 ppm) positions.

IPTG-induced cells of *E. coli* JM109(DE3)(pDTG141) oxidized dibenzofuran to yield 204 mg of crude products. The major product observed by TLC had an R_f of 0.2 and was isolated by RDC. Fractions containing the major product were combined to give 168 mg of a white solid. ^1H NMR analysis showed the presence of two metabolites with chemical shifts identical to those of compound III and compound IV formed by strain 9816/11. The magnitude of the integrals of the ^1H

NMR chemical shifts corresponding to the alkene protons indicated that compounds III and IV were formed by JM109 (DE3)(pDTG141) in a ratio of approximately 6:4. These compounds were of identical absolute stereochemistry and enantiomeric purity to those formed by strain 9816/11 (see below and Table 2). The major oxidation products formed by NDO with dibenzofuran are listed in Table 1 and shown in Fig. 2B. Dibenzofuran oxidation was not observed in control experiments with JM109(DE3)(pT7-5).

Dibenzothiophene biotransformation by strains expressing NDO. Oxidation of dibenzothiophene by induced cells of strain 9816/11 yielded 62 mg of crude products. TLC analysis showed the presence of unused dibenzothiophene (R_f , 0.7), a major metabolite with an R_f of 0.2 (compound V), and a minor product with an R_f of 0.5 (compound VI). GC-MS analysis of the crude products showed the presence of dibenzothiophene and three compounds with M^+ at m/z 218 (R_t , 10.35 min), 200 (R_t , 10.75 min), and 200 (R_t , 10.83 min) and relative yields of approximately 16, 74, and 10%. The last two compounds were not well resolved. Compound V (36 mg) and compound VI (6 mg) were isolated by preparative TLC, and their physical properties are listed in Table 1. The ^{13}C and ^1H NMR spectra of compound V showed chemical shifts and proton coupling constants consistent with the structure (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene previously described (35). The 1,2-regiochemistry of compound V was confirmed by showing that the UV absorption spectra of the dihydrodiol and its acid-catalyzed dehydration product(s) were identical to those of the *cis*-1,2-dihydrodiol (35) but distinct from those of the *cis*-3,4-dihydrodiol of dibenzothiophene reported by Mahaffey (39). High-resolution mass spectrometry of compound V gave an M^+ of 218.03955 ($\text{C}_{12}\text{H}_{10}\text{O}_2\text{S}$ requires 218.04015) and a mass accurate within 3 mmu for the ^{13}C isotope of the M^+ . In addition to the *cis*-1,2-dihydrodiol of dibenzothiophene, analysis of compound V by ^1H NMR and of its PBA derivative by

FIG. 2. Major oxidation products formed from fluorene (A), dibenzofuran (B), and dibenzothiophene (C) by NDO from *Pseudomonas* sp. strain NCIB 9816-4. The yields and names of compounds I to VI are given in the text and Table 1. The absolute configurations of the *cis*-dihydrodiols are given in Table 2.

TABLE 2. Determination of absolute configuration and enantiomeric purity by ^1H NMR analysis of diastereomeric MPBA esters^a

Compound	Derivatizing agent	-OCH ₃		-CH ₃		<i>cis</i> -Dihydrodiol absolute configuration ^b
		δ (ppm) CDCl ₃	$\Delta\delta$ (ppb)	δ (ppm) CDCl ₃	$\Delta\delta$ (ppb)	
I	(<i>S</i>)-MPBA	3.121	-112	1.440	+131	(+)-(3 <i>S</i> ,4 <i>R</i>)- <i>cis</i> -3,4-Dihydroxy-3,4-dihydro-9 <i>H</i> -fluorene
I	(<i>R</i>)-MPBA	3.223	— ^c	1.309	—	
III	(<i>S</i>)-MPBA	3.122	-99	1.437	+113	(1 <i>R</i> ,2 <i>S</i>)- <i>cis</i> -1,2-Dihydroxy-1,2-dihydrodibenzofuran
III	(<i>R</i>)-MPBA	3.221	—	1.324	—	
IV	(<i>S</i>)-MPBA	3.174	ND ^d	1.429	+31	(3 <i>S</i> ,4 <i>R</i>)- <i>cis</i> -3,4-Dihydroxy-3,4-dihydrodibenzofuran
IV	(<i>R</i>)-MPBA	ND	—	1.398	—	
V	(<i>S</i>)-MPBA	3.103	-120	1.445	+146	(+)-(1 <i>R</i> ,2 <i>S</i>)- <i>cis</i> -1,2-Dihydroxy-1,2-dihydrodibenzothiophene
V	(<i>R</i>)-MPBA	3.223	—	1.299	—	

^a Chemical shifts (δ) at 360 MHz are reported for the (–)-(*S*)-MPBA esters of *cis*-diols shown in Fig. 2, and $\Delta\delta$ values represent the distance and direction of each shift from the corresponding signal of the boronate ester formed with (+)-(*R*)-MPBA. A negative $\Delta\delta$ value indicates that the signal of interest is upfield from the corresponding signal of the diastereomer formed with the opposite boronic acid; a positive $\Delta\delta$ value means that the signal of interest is downfield from the corresponding signal of the ester formed with the opposite boronic acid. The enantiomeric excess was >95% for all *cis*-diols derivatized with (*S*)-MPBA.

^b The *cis*-diols listed have the same absolute stereochemistry for the hydroxyl-bearing carbon adjacent to the bridgehead carbon; differences in the absolute configurations of compounds I, III, and IV are due to the numbering systems of the fluorene, dibenzofuran, and dibenzothiophene ring systems (Fig. 1).

^c —, not applicable.

^d ND, not determined.

GC-MS showed the presence of a minor *cis*-dihydrodiol (M^+ 304; R_f , 17.82 min) which was tentatively identified as *cis*-3,4-dihydroxy-3,4-dihydrodibenzothiophene (39). The minor dihydrodiol was observed at 2 to 5% of the yield of the major *cis*-1,2-dihydrodiol.

Compound VI was isolated with a 14% relative yield and identified as dibenzothiophene sulfoxide on the basis of its mass and ^1H NMR spectra (Table 1). The *meso* symmetry of this product was apparent in the ^1H NMR spectrum, which showed four aromatic signals, each representing two chemical shift-equivalent protons.

IPTG-induced cells of *E. coli* JM109(DE3)(pDTG141) oxidized dibenzothiophene to yield 188 mg of crude products. TLC analysis showed the presence of two compounds with R_f values of 0.2 and 0.5. Both compounds were isolated by RDC. The major product (146 mg of white solid; R_f , 0.2) had mass and ^1H NMR spectral properties identical to those of compound V formed by strain 9816/11 and an $[\alpha]_D^{25}$ of +224 (c 0.43, methanol). GC-MS analysis of the PBA derivative of compound V showed the presence of approximately 5% of a compound corresponding to the minor *cis*-3,4-dihydrodiol of dibenzothiophene (not shown). The other isolated product (13.7 mg of white solid; R_f , 0.5) had physical properties identical to those of dibenzothiophene sulfoxide (compound VI). The involvement of NDO in the formation of compound VI was confirmed with purified NDO, which oxidized dibenzothiophene to dibenzothiophene sulfoxide with an approximate relative yield of 10% as determined by GC-MS. The major oxidation products formed by NDO with dibenzothiophene are shown in Fig. 2C. Dibenzothiophene was not oxidized in control experiments with strain JM109(DE3)(pT7-5).

Absolute configurations of compounds I, III, IV, and V. The absolute stereochemistry and enantiomeric purity of the *cis*-dihydrodiols formed from fluorene, dibenzofuran, and dibenzothiophene by each strain expressing NDO were determined by ^1H NMR analysis of diastereomeric esters formed with (–)-(*S*)- and (±)-MPBA (54). A consistent trend in the directional shifts of the diagnostic methoxyl and methyl signals of the MPBA esters formed with each of the four *cis*-dihydrodiols was observed. In each case, the chemical shift of the methoxyl signal of the ester formed with (–)-(*S*)-MPBA was located upfield of the methoxyl signal of the ester formed with (+)-(*R*)-MPBA (Table 2). In contrast, the opposite directional shift was observed for the methyl signals of the same esters. The direc-

tional shifts reported in Table 2 are consistent with trends previously observed for a series of *cis*-diols of known stereochemistry and indicate that the absolute configurations of the dihydrodiols formed in this study (compounds I, III, IV, and V) are of *R* configuration at the carbon adjacent to the bridgehead carbon as shown in Fig. 2. Given the *R* configuration at the benzylic carbinol center and their *cis*-relative stereochemistry, the vicinal dihydrodiols formed by NDO have been assigned the absolute configurations listed in Table 2. The ^1H NMR spectra of the (–)-(*S*)-MPBA esters formed with compounds I, III, IV, and V showed no detectable signals corresponding to the chemical shifts observed for the (+)-(*R*)-MPBA esters, indicating that each *cis*-diol was enantiomerically pure (>95% enantiomeric excess).

DISCUSSION

The results obtained in the present study indicate that NDO from *Pseudomonas* sp. strain NCIB 9816-4 catalyzes enantio-specific *cis*-dihydroxylation as the major reaction with fluorene, dibenzofuran, and dibenzothiophene (Fig. 2). Recombinant *E. coli* expressing the structural genes for NDO and/or purified enzyme confirmed the involvement of NDO in the reactions catalyzed with these PAC substrates by strain 9816/11. The initial oxidation products formed from dibenzofuran and dibenzothiophene by *S. yanoikuyae* strain B8/36 (31) were also identified as *cis*-1,2-dihydrodiols (8, 35); however, the analogous metabolite from fluorene has not been characterized. The present characterization of the structures and absolute stereochemistry of the isolated *cis*-dihydrodiols formed under identical conditions allows comparison of the trends and differences in the regio- and stereospecific oxidation of these substrates by NDO. In addition to catalyzing the *cis*-dihydroxylation of each substrate, NDO catalyzed the benzylic 9-monohydroxylation of fluorene and the sulfoxidation of dibenzothiophene.

NDO from *Pseudomonas* sp. strain NCIB 9816-4 oxidized fluorene to a major product identified as (+)-(3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrofluorene (compound I) (Fig. 2), the regiochemistry of which was identical to that of the *cis*-1,2-dihydrodiols formed from dibenzofuran and dibenzothiophene by the biphenyl-oxidizing strain B8/36 (8, 35). The absolute configuration of compound I (Table 2) was consistent with the stereochemistry of the *cis*-diols formed by NDO with naphthalene and other related substrates (1, 19, 29, 49, 51, 67). A

minor dihydrodiol, tentatively identified as *cis*-1,2-dihydroxy-1,2-dihydrofluorene (<5% yield) (not shown), was detected by ^1H NMR (Fig. 1A). GC-MS analysis of the isolated dihydrodiol fraction derivatized with MBA also showed the presence of two dihydrodiols of fluorene (in a ratio of approximately 95:5), but dihydrodiols of 9-fluorenol were not detected. The latter were reported as metabolites in fluorene biotransformations by *P. aeruginosa* PAO1(pRE695) (57). The absence of dihydrodiols of 9-fluorenol in the present study could result from the lower yields of 9-fluorenol observed (see below).

The benzylic monooxygenation of fluorene by NDO from *Pseudomonas* sp. strain NCIB 9816-4 (compound II) (Fig. 2) is consistent with its ability to catalyze the monooxygenation of indan and indene (19, 68), 1- and 2-indanone (55), 1*R*- and 1*S*-indanol (36), ethylbenzene (38), 9,10-dihydrophenanthrene, and 9,10-dihydroanthracene (51). The benzylic monooxygenation and sulfoxidation reactions catalyzed by toluene dioxygenase (TDO) from *P. putida* F1 and NDO from *Pseudomonas* sp. strain NCIB 9816-4 have shown distinct differences in the yields and, in some cases, stereochemistry of the products formed by these two dioxygenases (37, 38, 49, 55, 67, 68, 70). These results suggest that the relative contribution of monooxygenation reactions during catalysis is dependent upon the intrinsic characteristics of TDO and NDO, as well as the chemical reactivity and accessibility of the benzylic or sulfide functionalities with the substrate binding and/or catalytic sites of the individual dioxygenases. The finding that strains expressing NDO from *Pseudomonas* sp. strain NCIB 9816-4 oxidized fluorene to 9-fluorenol as a minor product (9 to 18% [Table 1]) is consistent with a recent report of cells expressing NDO, from *P. putida* G7, in which 9-fluorenol and 9-fluorenone accounted for ~30% of the relative product yield (56, 57). The reason for the small differences in the observed yields is not known, but they could be due to experimental protocols or subtle differences in the oxygenase components of the NDOs from strain NCIB 9816-4 and *P. putida* G7. With respect to the latter explanation, the nucleotide sequences of the NCIB 9816-4 genes encoding the large (α) and small (β) subunits of the terminal oxygenase component of NDO (*nahAc* and *nahAd*), show 96.9 and 94.8% identity at the predicted amino acid level with the *nahAc* and *nahAd* genes of *P. putida* G7, respectively (34, 46, 61). The nucleotide sequences of the genes encoding the α and β subunits of the terminal oxygenase of the biphenyl dioxygenase from the polychlorinated biphenyl-degrading strain LB400 (*bphA* and *bphE*) (15) show 95.6 and 99.5% identity at the predicted amino acid level with the isofunctional genes (*bphA1* and *bphA2*, respectively) from strain KF707 (18, 66), yet these closely related biphenyl dioxygenases exhibit dramatic differences in substrate specificity (16, 18). Thus, fine genetic differences could be responsible for the observed differences in the regioselectivities of the NDO-catalyzed oxidations mentioned above (and below).

NDO oxidized dibenzofuran to (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran (compound III) (Fig. 2), which had regio- and stereochemistry identical to those of (3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrofluorene (compound I) and (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene (compound V) formed by NDO as major products from fluorene and dibenzothiophene, respectively. However, the significant yield of (3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran as a minor product (30 to 40% yield; compound IV) (Fig. 2) was unique for dibenzofuran. The corresponding dihydrodiol regioisomers formed by NDO from fluorene and dibenzothiophene were typically <5% of the yield of the major dihydrodiols. The absolute stereochemistry of compound IV was consistent with the facial stereoselectivity observed for *cis*-dihydroxylation re-

FIG. 3. Possible orientations of fluorene (X = C), dibenzofuran (X = O), and dibenzothiophene (X = S) relative to the NDO catalytic site. The open arrow represents a fixed activated oxygen species capable of oxidizing the three substrates in orientation A to yield the major products (compounds I, III, and V) or oxidizing those in orientation B to yield the minor products (compounds II, IV, and VI).

actions catalyzed by NDO (see below). Compounds III and IV were also formed in a ratio of 2:1 by a recombinant strain of *E. coli* expressing NDO from *P. putida* G7 (69). Neither the 2,3-dihydrodiol regioisomer (8) nor products resulting from the formation and subsequent breakdown of the angular diol, 4,4a-dihydroxy-4-hydrodibenzofuran (13, 17, 64), were detected in this study.

Strains expressing NDO oxidized dibenzothiophene to (+)- (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene (~85% yield; compound V) (Fig. 3). This dihydrodiol was previously identified as the major product from dibenzothiophene oxidation by *S. yanoikuyae* B8/36, and the specific rotation reported (35) indicates that the dihydrodiol was of the same 1*R*,2*S* absolute configuration as compound V formed in the present study (Table 2). Mahaffey showed that strain B8/36 also formed small but significant amounts of a minor dihydrodiol identified as *cis*-3,4-dihydroxy-3,4-dihydrodibenzothiophene (39). ^1H NMR and GC-MS analyses of compound V formed by NDO also showed the presence of a compound tentatively identified as *cis*-3,4-dihydroxy-3,4-dihydrodibenzothiophene (2 to 5% yield) (not shown).

In this study, strains expressing NDO catalyzed the sulfoxidation of dibenzothiophene to form dibenzothiophene sulfoxide (compound VI) (Fig. 2) in yields of 10 to 14%. The involvement of NDO in the sulfoxidation reaction was confirmed with purified enzyme. No dibenzothiophene sulfone was observed in biotransformations with whole cells or purified NDO. Recent studies reported that *P. aeruginosa* PAO1(pRE695) expressing NDO from *P. putida* G7 catalyzed sulfoxidation of 3-methylbenzothiophene but not of dibenzothiophene (57). Reasons for this apparent difference in the substrate specificity of NDO expressed in strain PAO1(pRE695) are not clear at the present time, but it could result from differences in experimental conditions and/or the NDOs themselves (see above). However, the sulfoxidation of dibenzothiophene by NDO from *Pseudomonas* sp. strain NCIB 9816-4 is consistent with the ability of purified NDO and TDO to catalyze the sulfoxidation of aryl alkyl sulfides to chiral sulfoxides (37). In addition, Allen et al. have reported dioxygenase-catalyzed sulfoxidation of aryl alkyl and diaryl sulfides and thioacetals by *P. putida* UV4 and the naphthalene-utilizing strain NCIMB 8859 (3).

The higher relative yield and identical regio- and stereochemistry of the major oxidation products formed by NDO from fluorene, dibenzofuran, and dibenzothiophene (Fig. 2) suggest that each substrate is accommodated preferentially in the NDO active site (i.e., substrate binding and/or catalytic site) in a similar orientation. Attack by a single invariant form of activated dioxygen on each of the three substrates, as illustrated in Fig. 3A, could account for the asymmetric *cis*-dihydroxylation reactions, which result in the formation of chiral

cis-dihydrodiols (compounds I, III, and V) (Fig. 2). A suboptimal fit occurring by a 180° rotation of fluorene, dibenzofuran, and dibenzothiophene into the NDO substrate binding site (Fig. 3B) could account for the minor products (compounds II, IV, and VI) (Table 1). In this orientation, the heteroatoms of dibenzofuran and dibenzothiophene or the 9-methylene position of fluorene are in proximity to the site of oxygen attack. This increased accessibility of the activated dioxygen species to the 9-methylene group of fluorene or the bridging heteroatoms of dibenzothiophene and dibenzofuran could account for the formation of 9-fluorenol, dibenzothiophene sulfoxide, and (3*S*, 4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran as minor products through reactions involving monooxygenation, sulfoxidation, and *cis*-dihydroxylation. The 3*S*,4*R* absolute configuration of compound IV, formed as a minor product, is consistent with dioxygenation of dibenzofuran in this orientation. The formation of more dibenzofuran-3,4-dihydrodiol (compound IV) than compounds II and VI is probably due to the unavailability of the oxygen heteroatom for reaction. This fact, coupled with the electronegativity and potential hydrogen-bonding properties of the bridging oxygen atom, could direct the activated oxygen species of NDO to the 3,4-position when dibenzofuran is in the suboptimal orientation shown in Fig. 3B.

The results of the present study indicate that the preferred sites for NDO-catalyzed asymmetric *cis*-dihydroxylation were the 3,4-position of fluorene and the 1,2-positions of dibenzofuran and dibenzothiophene as shown in Fig. 2. A similar conclusion was made with respect to the regiospecific oxidation of the *N*-heterocyclic analog, carbazole, in which 3-hydroxycarbazole was identified as the sole product of oxidation by both NDO and biphenyl dioxygenase and was presumed to form through the dehydration of an unstable *cis*-3,4-dihydrodiol (53). The absolute stereochemistry of the *cis*-dihydrodiols of fluorene, dibenzofuran, and dibenzothiophene formed (Table 2) is consistent with most other examples in which *cis*-dihydroxylation reactions by NDO, from *Pseudomonas* sp. strain NCIB 9816-4, yield enantiopure *cis*-diols of *R* configuration at the benzylic (carbinol) center adjacent to the bridgehead carbon (52).

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