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Biotransformation of Various Substituted Aromatic Compounds to Chiral Dihydrodihydroxy Derivatives

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The biotransformation of four different classes of aromatic compounds by the *Escherichia coli* strain DH5α(pTCB 144), which contained the chlorobenzene dioxygenase (CDO) from *Pseudomonas* sp. strain P51, was examined. CDO oxidized biphenyl as well as monochlorobiphenyls to the corresponding *cis*-2,3-dihydro-2,3-dihydroxy derivatives, whereby oxidation occurred on the unsubstituted ring. No higher substituted biphenyls were oxidized. The absolute configurations of several monosubstituted *cis*-benzene dihydrodiols formed by CDO were determined. All had an *S* configuration at the carbon atom in *meta* position to the substituent on the benzene nucleus. With one exception, the enantiomeric excess of several 1,4-disubstituted *cis*-benzene dihydrodiols formed by CDO was higher than that of the products formed by two toluene dioxygenases. Naphthalene was oxidized to enantiomerically pure (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. All absolute configurations were identical to those of the products formed by toluene dioxygenases of *Pseudomonas putida* UV4 and *P. putida* F39/D. The formation rate of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene was significantly higher (about 45 to 200%) than those of several monosubstituted *cis*-benzene dihydrodiols and more than four times higher than the formation rate of *cis*-benzene dihydrodiol. A new gas chromatographic method was developed to determine the enantiomeric excess of the oxidation products.

The aerobic bacterial degradation of nonactivated aromatic compounds is usually initiated by dioxygenases that incorporate two hydroxyl groups into the aromatic substrate; the products of such reactions are chiral *cis*-dihydrodihydroxy derivatives, which also are called *cis*-dihydrodiols (10). The oxidation of aromatic compounds to *cis*-dihydrodiols is of special interest for biotechnological as well as chemical applications, because single *cis*-dihydrodiols have been shown to be valuable building blocks for stereoselective synthesis of biologically active molecules containing multiple chiral centers (6, 8, 12, 16, 18, 22).

Several multicomponent dioxygenases that dihydroxylate aromatic compounds have been described (7). Toluene dioxygenases (TDO), naphthalene dioxygenase (NDO), and biphenyl dioxygenase (BDO) are the best known members of this class of dioxygenases (12). The components of these oxygenase complexes have been purified, and the encoding genes have been cloned and expressed in *Escherichia coli* (12). Experiments on substrate specificity showed that TDO from *Pseudomonas putida* F39/D and *P. putida* UV4 oxidize monosubstituted benzenes, except fluorobenzene, to 3-substituted *cis*-benzene dihydrodiols with an *S* configuration at the C-1 atom (3, 4, 15). BDO from *Pseudomonas* sp. strain LB400 dihydroxylates and dechlorinates chlorinated biphenyls (11) to the respective 2,3 or 3,4 *cis*-diols, whereby dihydroxylation and dechlorination happen on both phenyl rings. NDO from *Pseudomonas* sp. strain NCIB 9816 has a relaxed substrate specificity and catalyzes the dioxygenation of naphthalene to (+)-*cis*-(1*R*,2*S*)dihydroxy-1,2-dihydronaphthalene. Many related 2- and 3-ring aromatic and hydroaromatic compounds are also substrates and are turned over to the respective *cis*-diols (14). A mutant of the not-so-well-characterized *Pseudomonas fluorescens* N3 was used in the bioconversion of several naphthalene derivatives to the corresponding *cis*-dihydrodiols on a milligrams-to-grams scale (2). A recent review lists more than 140 diols that have been described to date (12). Although only a few of them have been used as synthons, the further development of the area depends on the discovery of novel dioxygenase reactions and on the development of biotechnological processes to produce the different metabolites.

Werlen et al. recently described the cloning and expression in *E. coli* DH5α(pTCB144) of the chlorobenzene dioxygenase (CDO) of *Pseudomonas* sp. strain P51 (21). Analysis of the genes showed that CDO is a three-component aromatic ring dioxygenase. It consists of the gene products of *tcbAa*, encoding the large subunit of the terminal oxygenase; *tcbAb*, encoding the small subunit; *tcbAc*, encoding the ferredoxin; and *tcbAd*, encoding the NADH reductase. Homology comparisons indicate that these genes and gene products are most closely related to those of TDO of *P. putida* F1 (*todC1C2BA*) and are distantly related to those of NDO and BDO (21). Here we report the ability of the CDO system of the recombinant *E. coli* DH5α(pTCB144) to oxidize different classes of aromatic compounds to the corresponding *cis*-dihydrodiols. We characterized each of the 48 reaction products as thoroughly as possible. The CDO system turned out to be well suited for the production of dihydrodiols, and further work to scale up production is under way.

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MATERIALS AND METHODS

Microorganisms, growth conditions, and biotransformation procedure. Single colonies of *E.* coli DH5α(pTCB144) were taken from agar plates and transferred to Erlenmeyer flasks containing 250 ml of sterile Luria-Bertani medium (17) with 50 mg of ampicillin/liter. Cultures were incubated at 25°C on a shaker (110 rpm) for 36 h, by which time the optical density at 578 nm (OD_{578}) had reached levels between 3.5 and 4.0. The cultures were then centrifuged at 20,000 × *g* for 12 min. The supernatant was removed, and cells were washed with M9 mineral salts medium (17). After the wash, the cells were resuspended in M9 medium supplemented with 1 mM glucose, and the OD_{578} was adjusted to approximately 1.0. These cells were used for biotransformation incubations as follows. One hundred milliliters of the washed suspension was transferred to 500-ml Erlenmeyer flasks. Aromatic substrates were added from stock solutions in methanol (35 mM) prior to bacterial suspension. Methanol was allowed to evaporate. Final substrate concentrations were 0.35 mM. Experiments for determination of product-building rates were performed with 1.0 mM concentrations of the substrate. Samples were taken approximately every 45 min and centrifuged. The supernatants were frozen until needed for analysis. Biphenyl, which was known to be transformed by CDO (21), was used as a positive control for all experiments. Experiments were carried out at 25°C.

P. putida F39/D was kindly provided by D. T. Gibson and S. M. Resnick (University of Iowa). It is a mutant of *P. putida* F1 that lacks *cis*-dihydrodiol dehydrogenase activity (9, 24). The mutant was used for comparative experiments in order to determine the absolute configurations of transformation products formed by *E.* coli. DH5α(pTCB144). *P. putida* F39/D was grown at 30°C in M9 medium with 5 mM pyruvate in the presence of toluene vapor. Biotransformations were carried out using the procedure described above for *E.* coli DH5α(pTCB144), but at 30°C and with 5 mM pyruvate instead of 1 mM glucose.

Analytical procedures. (i) Rate determination. The formation of metabolites was measured by high-pressure liquid chromatography (HPLC). Samples of the supernatants from the biotransformation incubations (2 ml) were filtered through 0.20-μm-pore-size membrane filters (FP 030/3; Schleicher & Schuell, Dassel, Germany) or centrifuged at 25,000 × *g* for 2 min and analyzed on a Waters 625 LC HPLC system equipped with a WISP 700 autosampler and a 901 photodiode array detector (Waters Millipore Corp., Milford, Mass.). Separation was done on a C_{18} reversed-phase column (Macherey-Nagel, Düren, Germany) as described elsewhere (21). In order to determine product-building rates, we quantified the transformation products of benzene, fluorobenzene, and naphthalene with the help of authentic standards. The transformation products of toluene and of ethyl-, chloro-, and bromobenzene were acidified, and the resulting phenols were analyzed and quantified accordingly.

(ii) GC-MS. For gas chromatography-mass spectrometry (GC-MS) analysis, *cis*-dihydrodiols were extracted from the supernatant of the incubation mixture with an equal volume of ethyl acetate. The ethyl acetate extract was dried with sodium sulfate and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was dissolved in 100 μl of *N,N*-dimethylformamide (DMF). One hundred microliters of a solution of recrystallized *n*-butylboronic acid (approximately 500 μg of *n*-butylboronic acid/ml dissolved in DMF) was added, and the mixture was heated to 70°C for 15 min to form the *n*-butylboronate derivatives (BB derivatives). The free hydroxy groups of the BB derivatives of the transformation products of the monohydroxybiphenyls, phenol, cresols, and benzyl alcohol were then derivatized with *N,O-bis*(trimethylsilyl)-trifluoroacetamide (TMS) as described elsewhere (13). Samples of the BB derivatives were diluted with cyclohexane (at least 15-fold), 0.5 μl of the diluted samples was injected at 50°C onto a PS09 fused silica capillary column (length, 15m; inner diameter, 0.25 mm; film thickness, 0.25 μm), and the column temperature was increased to 250°C at 10°C/min. Mass spectra were obtained with an ITD 800 (ion trap detection) mass spectrometer (Finnigan, MAT, San Jose, Calif.) coupled with an HRGC 5160 Mega Series gas chromatograph (Carlo Erba Instruments, Milan, Italy).

The enantiomers were separated and quantified by GC-MS. A Tribrid double-focusing magnetic sector hybrid mass spectrometer (VG Analytical, Manchester, United Kingdom) was used. The enantiomers were separated as their BB derivatives on a 25% *t*-butyldimethylsilylated-β-cyclodextrin column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm), obtained from BGB Analytik AG (Rothenfluh, Switzerland). Samples of the BB derivatives were diluted with cyclohexane (at least 15-fold), and 0.5 μl of the diluted samples was injected on-column at 60°C. The column temperature was programmed as follows: 15°C/min to 160°C, 3°C/min to 230°C, and 20°C/min to 250°C. All samples were analyzed by electron ionization (EI+, 70 eV) using full-scan monitoring (m/z = 50 to 250 or 50 to 400). The enantiomeric excess (EE) was defined as $(A_1 - A_2)/(A_1 + A_2) \times 100$, where A_1 and A_2 were the peak areas of the BB derivatives of the two *cis*-dihydrodiol enantiomers, and A_1 was the larger peak area.

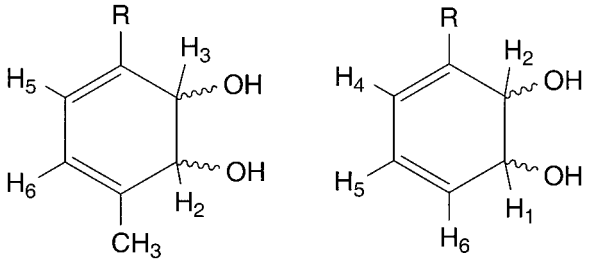

FIG. 1. Designation of the protons of mono- and 1,4-disubstituted *cis*-benzene dihydrodiols, formed by CDO.

(iii) ^{1}H NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ASX 400-MHz NMR spectrometer at ambient temperature. The solvents were methanol-d_4 or acetonitrile-d_3. Chemical shifts are given in parts per million relative to tetramethylsilane (at 0 ppm).

(iv) Protein determination. Protein contents were determined by the method of Bradford (5) with bovine serum albumin as the standard.

(v) Optical rotations. Optical rotations were measured in methanol solutions with a Polarimeter 241 (Perkin-Elmer International Inc., Rotkreuz, Switzerland).

Isolation of products. In order to isolate some of the transformation products in the milligram range, the supernatants of the incubation mixtures (1.5 to 2.5 liters) were extracted twice with 1/2 volume of ethyl acetate. Prior to the extraction, a sufficient amount of Na_2SO_4 was added in order to improve the transfer of the *cis*-dihydrodiols into the organic phase and to prevent the formation of emulsions. The ethyl acetate was evaporated in a rotary evaporator to dryness, and the remains were redissolved in methanol and filtered (FP 030/3; pore size, 0.2 μm; Schleicher & Schuell). Samples of the filtered methanol extracts (500 μl) were injected on a semipreparative C_{18} column (ET 250/21, Nucleosil 100-7; Macherey-Nagel). Compounds were eluted by running a linear gradient from 70% (vol/vol) water and 30% methanol to 30% water and 70% methanol in 40 min.

Chemicals. Monochlorobiphenyls and 4,4′-dichlorobiphenyl were obtained from Johnson & Matthey (Karlsruhe, Germany); 2,2′-dichlorobiphenyl and 2,2′,6-trichlorobiphenyl were obtained from Promochem (Wesel, Germany); 4-chlorotoluene, 2,2-bis(4-chlorophenyl)-1,1-dichlorethane (DDE), pyrene, 1,1-bis(4-chlorophenyl)-2,2,2-trichlorethane (DDT), 4-biphenylcarbonitrile, all dihydroxybiphenyls, 1,4-dichlorobenzene, 1,2,3-trichlorobenzene, dibenzofuran (DBF), diphenylether, *cis*-1,2-dihydrocatechol, 2,4-dichlorophenoxyacetic acid, and 2-hydroxydiphenylmethane were obtained from Aldrich (Buchs, Switzerland); 4-fluoroaniline, dibenzo-*p*-dioxin, and 2,6-dichlorobiphenyl were obtained from Socochim (Lausanne, Switzerland); 4-bromo-2-methylphenol, all tetrachlorobenzenes, 2,3-dichlorobiphenyl, and 2,4,5-trichlorobiphenyl were obtained from Riedel de Häen (Seelze, Germany); 2-bromo-4-methylphenol was obtained from Janssen Chimica (Geel, Belgium). 3-Chlorodibenzofuran was a gift from Hauke Harms (Swiss Federal Institute for Environmental Sciences and Technology [EAWAG]). Standards of (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene, (±)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene, (+)-*cis*-(2*R*,3*S*)-dihydroxy-2,3-dihydrobiphenyl, and (−)-*cis*-(2*S*,3*R*)-dihydroxy-2,3-dihydrobiphenyl were gifts from S. M. Resnick and D. T. Gibson. All other chemicals were obtained from Fluka (Buchs, Switzerland).

RESULTS

Identification of reaction products. Dihydrodiol metabolites were identified by GC-MS of their respective BB derivatives and, when possible, by ^{1}H NMR spectroscopy. BB derivatives of *cis*-dihydrodiol isomers were separated on a GC column with an achiral stationary phase and could be distinguished from those of *trans*-dihydrodiols because of significant mass differences between their molecular ions (19).

BB derivatives of *cis*-dihydrodiol enantiomers were separated on a GC column with a chiral stationary phase. This procedure worked satisfactorily for the determination of EE values for nonracemic mixtures of enantiomers. When we analyzed enantiomers that produced only a single peak in the

TABLE 1. Products formed from substituted biphenyls by whole cells of *E. coli* DH5α(pTCB 144), which contains the CDO of *Pseudomonas* sp. strain P51

Substrate	Relative amt (mol %)[a]	Adsorbance maxima (nm)[b]	*m/z* of prominent ions (relative abundance)[c]	Chemical shifts (δ; ppm) and coupling constants (J, Hz)	EE (%)	Suggested structure and other properties
2-Chlorobiphenyl	90	280	290, M^+ +2 (36); 288, M^+ (100); 253 (13); 176 (11); 169 (22); 152 (24); 141 (23)	δH: 2′ = 4.40, 3′ = 4.58, 4′ = 5.89, 5′ = 6.07, 6′ = 6.00, 3–6 = 7.25–7.43 J: 2′, 3′ = 5.8, 2′,4′ = 1.0, 3′,4′ = 2.9, 3′, 5′ = 2.3, 3′, 6′ = 0.5, 4′, 5′ = 9.6, 4′, 6′ = 1.0, 5′,6′ = 5.4	ND[d]	(+)-*cis*-2′,3′-Dihydroxy-2′,3′-dihydro-2-chlorobiphenyl mp = 118–120° $[\alpha]_D$ = +120°
	10	ND	290, M^+ +2, (34); 288, M^+ (100); 253 (16); 176 (18); 169 (22); 141 (28)		ND[d]	*cis*-Dihydroxydihydro-2-chlorobiphenyl[e]
3-Chlorobiphenyl	100	303	290, M^+ +2 (32); 288, M^+ (100); 253 (28); 204 (19); 188 (10); 176 (29); 169 (25); 152 (44); 141 (57)	δH: 2′ = 4.35, 3′ = 4.47, 4′ = 5.84, 5′ = 6.04, 6′ = 6.42, 2,4–6 = 7.25–7.59 J: 2′, 3′ = 2.7, 4′,5′ = 9.7, 4′,6′ = 0.9, 5′,6′ = 5.6	ND[d]	(+)-*cis*-2′,3′-Dihydroxy-2′,3′-dihydro-3-chlorobiphenyl mp = 135°C $[\alpha]_D$ = +135°
4-Chlorobiphenyl	100	303	290, M^++2 (31); 288, M^+ (100); 253 (29); 204 (30); 188 (15); 176 (32); 169 (27); 152 (47); 141 (58)		>98	(+)-*cis*-2′,3′-Dihydroxy-2′,3′-dihydro-4-chlorobiphenyl mp = 136–139°C $[\alpha]_D$ = +119°
2-Aminobiphenyl	100	280	269, M^+ (12); 212 (88); 185 (20); 169 (23); 168 (100); 167 (61)		>98	*cis*-Dihydroxydihydro-2-aminobiphenyl[e]
2-Hydroxybiphenyl	100	294	342, M^+ (8); 269 (35); 258 (15); 242 (65); 73 (100)		ND	*cis*-Dihydroxydihydro-2-hydroxybiphenyl[e]
3-Hydroxybiphenyl	ND	288	342, M^+ (17); 269 (42); 258 (19); 242 (69); 73 (100)		ND	*cis*-Dihydroxydihydro-3-hydroxybiphenyl[e]
	ND	305	342, M^+ (18); 269 (43); 258 (18); 242 (65); 73 (100)		ND	*cis*-Dihydroxydihydro-3-hydroxybiphenyl[e]
4-Hydroxybiphenyl	100	310	342, M^+ (19); 269 (43); 258 (18); 242 (67); 73 (100)		ND	*cis*-Dihydroxydihydro-4-hydroxybiphenyl[e]
4-Biphenyl carbonitrile	100	248	279, M^+ (100); 222 (25); 195 (55); 179 (28); 167 (87); 151 (27)		ND	4-(*cis*-Dihydroxy-cyclohexadienyl)-benzonitrile[e]

[a] Relative to the total amount of products; calculated by integration of GC-MS chromatograms (TIC, *m/z* = 90 to 400). ND, not determined.
[b] By HPLC diode array.
[c] The *m/z* values of the prominent ions of the mass spectra of the BB derivatives are given. Additionally, hydroxybiphenyldihydrodiols were derivatized with TMS. Relative abundance, in parentheses, is given as a percentage.
[d] GC-MS analysis of the BB derivatives gave a single peak. Since no standards were available, the EE could not be determined.
[e] Exact isomeric structure unknown.

chromatogram and for which we could not obtain standards, the method did not allow us to decide whether the single peak was the result of a pure enantiomer with a very high EE or whether it resulted from lack of separation. For such cases, EE values were not reported.

Absolute configurations of some of the products formed by CDO were determined by comparison of the retention times and mass spectra of the BB derivatives of the products with those of authentic standards or with those of the products formed by *P. putida* F39/D, which had been published previously. The optical rotations of products that were isolated in sufficient amounts were measured, and the absolute configurations of the products were assigned by comparison of the optical rotations to published data. The transformation products of the hydroxybiphenyls were identified by the corresponding TMS derivatives of the BB derivatives. Figure 1 shows our usage of nomenclature to specify the protons of dihydrodiol compounds.

Transformation of substituted biphenyls. CDO dihydroxylated biphenyl and several monosubstituted biphenyls (Table 1). Biphenyl was oxidized to enantiomerically pure (+)-*cis*-(2*R*,3*S*)-dihydroxy-2,3-dihydrobiphenyl. Oxidation of mono-

TABLE 2. Products formed from substituted benzenes by whole cells of *E. coli* DH 5α(pTCB 144), which contains the CDO of *Pseudomonas* sp. strain P51

Substrate	Relative amt (mol%)[a]	Adsorbance maxima (nm)[b]	*m/z* of prominent ions (relative abundance)[c]	Suggested structure
1,2,3-Trichlorobenzene	100	277	284, M^++4 (11); 282, M^++2 (35); 280, M^+ (37); 247 (46); 245 (81); 210 (9); 198 (100); 196 (23); 133 (32)	1,2,3-Trichlorobenzene-*cis*-dihydrodiol[c]
1,2,4-Trichlorobenzene	100	283	284, M^+ +4 (30); 282, M^+ +2 (99); 280, M^+ (100); 247 (45); 245 (74); 212 (10); 210 (39); 133 (96); 57 (24)	1,2,4-Trichlorobenzene-*cis*-dihydrodiol[d]
1,2,3,4-Tetrachlorobenzene	100	ND	318, M^+ +4 (9); 316, M^+ +2 (17); 314, M^+, (15); 281 (60); 279 (61); 246 (38); 244 (68); 218 (50); 216 (100); 57 (76)	*cis*-1,2-Dihydroxy-3,4,5,6-tetrachlorocyclohexa-3,5-diene
Benzonitrile	88	282	203, M^+ (57); 119 (77); 103 (100); 91 (67); 76 (60)	*cis*-Dihydroxy cyclohexadienecarbonitrile[d]
	12	ND	203, M^+ (67); 146 (25); 119 (77); 103 (100); 91 (67)	
Benzyl chloride	100	257	228, M^+ +2 (34); 226, M^+ (100); 191 (46); 177 (27); 169 (30); 142 (36); 134 (23); 107 (69); 91 (28); 79 (22)	*cis*-Dihydroxydihydrobenzyl chloride[d]
Benzyl cyanide	98.8	262	217, M^+ (20); 191 (39); 177 (51); 160 (36); 134 (70); 117 (61); 107 (100); 91 (90); 77 (61)	1-(*cis*-1,2-Dihydroxy-3,5-cyclohexadienyl)-acetonitrile[e]
	0.6	ND	217, M^+ (22); 191 (36); 177 (52); 160 (36); 117 (68); 107 (100); 91 (79); 77 (56)	2-(*cis*-2,3-Dihydroxy-4-6-cyclohexadineyl)-acetonitrile[e]
	0.6	ND	217, M^+ (17); 191 (43); 177 (61); 160 (49); 117 (50); 107 (100); 91(93); 77 (52)	3-(*cis*-3,4-Dihydroxy-1,5-cyclohexadienyl)-acetonitrile[e]
Diphenylmethane	100	267	268, M^+ (84); 191 (53); 184 (28); 177 (57); 121 (40); 91 (100)	*cis*-Dihydroxydihydrodiphenymethane[d]

[a] Relative to the total amount of products; calculated by integration of GC-MS chromatograms (total ion current, m/z = 90 to 400).
[b] By HPLC. ND, not determined.
[c] The m/z values of the prominent ions of the mass spectra of the BB derivatives are given. Relative abundance, in parentheses, is given as a percentage.
[d] Exact isomeric structure unknown.
[e] Any of the benzyl cyanide products may have one of these structures.

chlorobiphenyls occurred on the unsubstituted ring at the 2,3 position. Measurements of the optical rotations of the monochlorinated *cis*-biphenyl dihydrodiol products confirmed that these products were nonracemic (Table 1). Several other monosubstituted biphenyls were oxidized by CDO. Higher substituted biphenyls (2,3- 2,6-, 2,2′-, and 4,4′-dichlorobiphenyl; 2,2′,6- and 2,4,5-trichlorobiphenyl; 2,2′-, 4,4′-, and 2,5- dihydroxybiphenyl) were not transformed by CDO. Neither dihydroxylation nor dechlorination products were detected by GC-MS analysis.

TABLE 3. Rates of formation of *cis*-dihydrodiols by CDO

Substrate	Formation rate (nmol/min · mg of protein^{-1}) of product
Benzene	4.3
Toluene	9.1
Ethylbenzene	7.6
Fluorobenzene	8.7[a]
Chlorobenzene	8.7[a]
Bromobenzene	15.9
Naphthalene	22.8

[a] Formation rates for the products of chloro- and bromobenzene are the rates for the major isomer products.

TABLE 4. EE of 1,4-disubstituted *cis*-benzene dihydrodiols formed by several dioxygenases

Substrate	EE (%) of the dioxygenation product formed by:		
	P. putida UV4 TDO[a]	*P. putida* F39/D TDO	CDO[b]
4-Fluorotoluene	83	90	49
4-Chlorotoluene	15	10	77[c]
4-Bromotoluene	37	26	77
4-Iodotoluene	80	80	98
1-Chloro-4-iodobenzene	15	ND[d]	67

[a] Data taken from reference 3.
[b] *Pseudomonas* sp. strain P51 CDO, cloned into *E. coli* DH5α(pTCB 144).
[c] EE of the major isomer.
[d] ND, not determined.

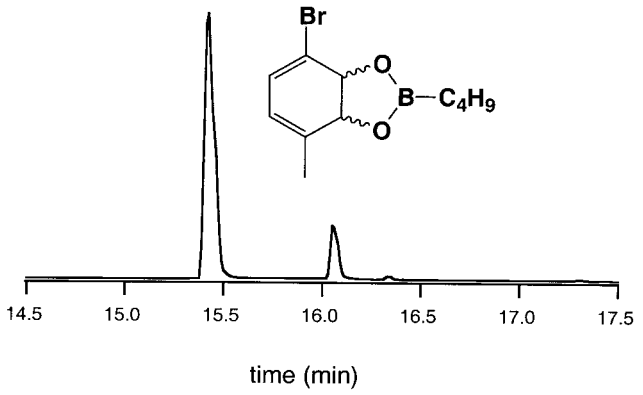

FIG. 2. Determination of the enantiomeric composition of *cis*-4-bromo-2,3-dihydroxy-1-methyl-cyclohexa-4,6-diene, which is formed by oxidation of 4-bromotoluene by CDO. Enantiomers were separated as their BB derivatives. The mass spectra of the enantiomers were identical. The major enantiomer was formed with an EE of 77%, which was calculated by integration of the peak areas of the major and minor enantiomer. The isomeric structure of the product was determined by ^{1}H NMR analysis.

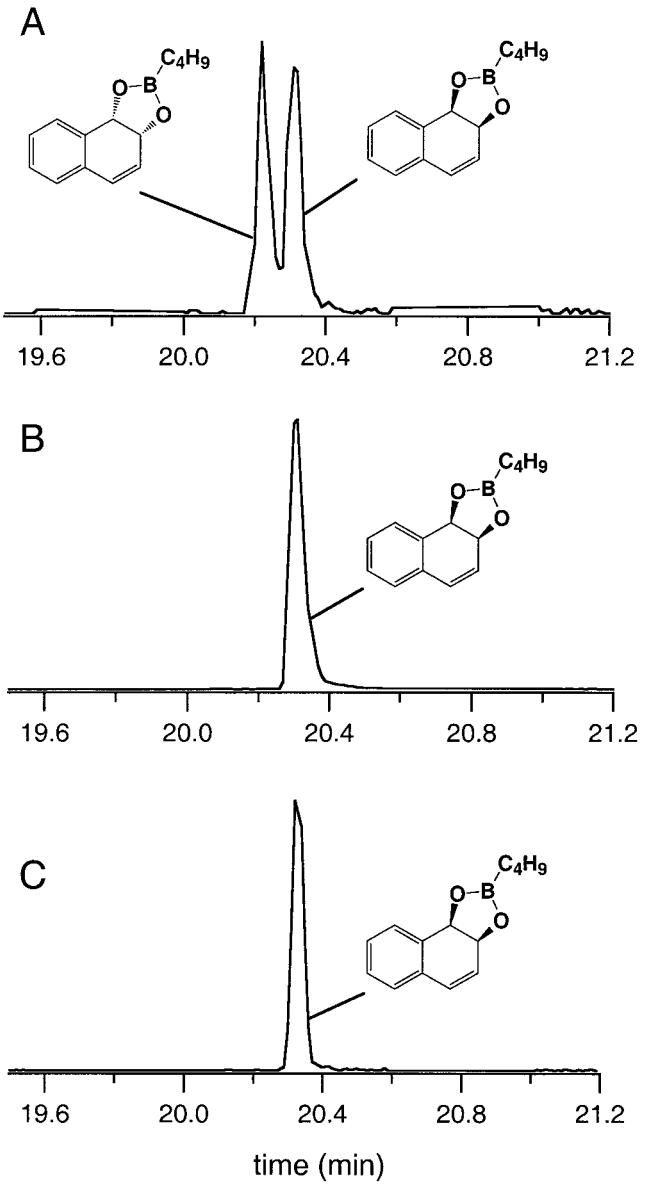

FIG. 3. GC-MS analysis and determination of the absolute configuration of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. (A) Separation of the BB derivatives of an authentic standard of (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene. The mass spectra of the two compounds were identical. (B) BB derivative of an authentic standard of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. (C) BB derivative of the oxidation product formed from naphthalene by CDO. The BB derivatives in panels B and C had identical retention times and identical mass spectra.

As an example, arguments in favor of the structure of (+)-*cis*-2′,3′-dihydroxy-2′,3′-dihydro-3-chlorobiphenyl are summarized as follows. The molecular ion of the BB derivative at *m/z* 288 proves the *cis* configuration, since the BB derivative of the *trans*-dihydrodiol would have had a molecular ion at *m/z* 392 (19). The ion at *m/z* 290 ($M^{+\bullet}+2$), with about one-third of the abundance of the molecular ion, indicated the presence of one chlorine atom. The fragmentation could be rationalized as follows. The loss of one chlorine atom leads to the ion at *m/z* 253. The loss of the *n*-butyl group yields the ion at *m/z* 231. The ions at *m/z* 204, 176, and 141 are formed by the loss of the C_4H_9BO moiety and by a successive loss of CO and $Cl^\bullet$. The direct loss of $Cl^\bullet$ from the ion at *m/z* 204 forms the ion at *m/z* 169. The ions at *m/z* 188 and 152 arise from the loss of the $C_4H_9BO_2$ moiety and one molecule of HCl from the molecular ion. ^{1}H NMR analysis of the isolated product proved the exact position of the two hydroxy groups. Signals of the hydroxy protons could not be seen, since the sample was dissolved in CD_3CN. The product had four aromatic protons with a typical 1,3 substitution (23), three olefinic protons, and two dihydrodiol protons (Table 1). This confirms that the dihydroxylation took place on the unsubstituted phenyl moiety. The J-coupling pattern among the olefinic protons, namely J(4′,5′) = 9.7 Hz, J(4′,6′) = 0.9 Hz, and J(5′,6′) = 5.6 Hz, is consistent only with a 2′,3′-dihydroxylation. The NMR spectrum of the oxidation product of 3-chlorobiphenyl (and 2-chlorobiphenyl) agrees with literature data (11); additional J couplings among protons could be assigned here (Table 1). Since the optical rotation of the sample ($[\alpha]_D$) was +135°, the oxidation product of 3-chlorobiphenyl formed by CDO was assigned as (+)-*cis*-2′,3′-dihydroxy-2′,3′-dihydro-3-chlorobiphenyl. GC-MS analysis of the BB derivative of the product on a chiral column gave a single peak.

Transformation of substituted benzenes. CDO oxidized benzene, 1,4-dichlorobenzene, fluorobenzene, 1-chloro-4-iodobenzene, 1-bromo-4-iodobenzene, bromobenzene, styrene, anisol, and ethylbenzene to *cis*-dihydrodiol derivatives (data not shown). Most substrates were dihydroxylated to one *cis*-dihydrodiol isomer. As these products were previously described (12), they were not further investigated. Table 2 shows the mass spectral properties of the BB derivatives of interesting new *cis*-dihydrodiols. The data show that CDO is able to oxidize tri- and tetrachlorobenzene, benzonitrile, benzyl chloride, benzyl cyanide, and diphenylmethane to *cis*-dihydrodiols. CDO oxidized monosubstituted benzenes faster than benzene (Table 3). Compounds with polar substituents, such as phenol,

TABLE 5. Products formed from various aromatic substrates by whole cells of *E. coli* DH 5α(pTCB144), which contains the CDO of *Pseudomonas* sp. strain P51

Substrate	Relative amt (mol%)[a]	Adsorbance maxima (nm)[b]	*m/z* of prominent ions, (relative abundance)[c]	Suggested structure
Dibenzofuran	88	303	268, M^+ (100); 184 (18); 156 (17)	*cis*-1,2-Dihydroxy-1,2-dihydrodibenzofuran
	12	ND	268, M^+ (100); 184 (26); 168 (21)	*cis*-3,4-Dihydroxy-3,4-dihydrodibenzofuran
3-Chlorodibenzofuran	100	ND	304, M^+ +2 (33); 302, M^+ (100); 245 (13); 218 (99); 202 (46); 190 (46)	Chloro-*cis*-dihydroxydrodibenzofuran[d]
Dibenzodioxin	100	293	284, M^+ (90); 227 (12); 200 (78); 184 (100); 172 (28); 156 (12); 128 (36)	*cis*-Dihydroxydihydrodibenzodioxin[d]
Diphenylether	90	278	270, M^+ (100); 213 (27); 186 (55); 170 (42); 158 (23); 141 (36); 120 (65); 77 (90); 65 (92); 51 (78)	*cis*-Dihydroxydihydrodiphenylether[d]
	10	ND	270, M^+ (80); 186 (100); 86 (35); 77 (30)	

[a] Relative to the total amount of products, calculated by integration of GC-MS chromatograms (TIC, m/z = 90 to 400).
[b] By HPLC. ND, not determined.
[c] The m/z values of the prominent ions of the mass spectra of the BB derivatives are given. Relative abundance, in parenheses, is given as a percentage.
[d] Exact isomeric structure unknown.

2,4-dichlorophenoxyacetic acid, benzyl alcohol, aniline, and 4-fluoroaniline did not serve as substrates for CDO. CDO did not oxidize 1,2,3,5-tetrachlorobenzene, 2-hydroxydiphenylmethane, DDE, or DDT.

Transformation of substituted toluenes. Many substituted toluenes served as substrates for CDO. Incubation of toluene, 4-fluorotoluene, 4-bromotoluene, and 4-iodotoluene produced one single *cis*-dihydrodiol isomer [(+)-*cis*-(2*R*,3*S*)-dihydroxy-I-methylcyclohexa-4,6-diene and *cis*-4-bromo-2,3-dihydroxy-1-methylcyclohexa-4,6-diene for toluene and 4-bromotoluene, respectively], whereas incubation of 4-chlorotoluene produced all three possible isomers. *E. coli* DH5α(pTCB144) oxidized *para*-substituted toluenes to the same major and minor enantiomers as *P. putida* F39/D, whereby the EE values of the products formed by *E. coli* DH5α(pTCB144) were usually higher (Table 4). The separation by GC of the BB derivatives of *cis*-4-bromo-2,3-dihydroxy-1-methyl-cyclohexa-4,6-diene, which was formed by oxidation of 4-bromotoluene, is shown in Fig. 2.

Transformation of additional aromatic substrates. CDO oxidized several additional aromatic substrates to *cis*-dihydrodiols. Naphthalene was oxidized to enantiomerically pure (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (Fig. 3). It was formed at a rate of 22.8 nmol/min · mg of protein^{-1}, which is much higher than the transformation rates of benzene and several monosubstituted benzenes (Table 3).

Dibenzofuran was oxidized by CDO to two *cis*-dihydrodiol isomers (Table 5). CDO oxidized 3-chlorodibenzofuran, dibenzo-*p*-dioxin, and diphenylether (Table 5) but not 3-methyldiphenylether or polycyclic aromatic hydrocarbons, such as pyrene, anthracene, and phenanthrene.

DISCUSSION

We showed that CDO from *Pseudomonas* sp. strain P 51, which was cloned into *E. coli* DH5α(pTCB144) (21), was able to oxidize various substituted aromatic compounds to *cis*-dihydrodiols. The absolute configurations of several products were determined. They were identical to those of the products formed by TDO and NDO (1, 3, 15, 20, 24, 25). NMR spectra were recorded if enough product (ca. 1 mg) was available. The J-coupling pattern among the olefinic protons was used to assign the positions of the hydroxy groups. For some compounds (e.g. chlorobiphenyls and bromobenzene) most of the long-range J couplings were resolved as well. For products such as dihydrodihydroxytoluene or dihydrodihydroxyfluorobenzene, long-range J couplings manifested themselves in 1H,1H-cosy correlation signals but could not be extracted from the one-dimensional NMR spectra directly.

We were able to separate *cis*-dihydrodiol enantiomers by GC. Advantages of the method were the low detection limit (<0.5 μg of the derivative), the fact that no exhaustive isolation and cleanup was needed, and the fact that derivatization could be done with ordinary laboratory equipment. Several standards of the BB derivatives of racemic or nonracemic mixtures of *cis*-dihydrodiol enantiomers [e.g., (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene (Fig. 3), (±)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene, (±)-*cis*-2,3-dihydroxy-2,3-dihydrobiphenyl, and (±)-*cis*-1,2-indandiol] were tested and could be successfully separated. Even high EE values were quantified. For example, the EE values of the *cis*-dihydrodiol products of fluorobenzene and 4-iodotoluene turned out to be 95 and 98%, respectively. The analyses were reproducible with a relative standard deviation of 3%.

One disadvantage of the method was that a single peak in a gas chromatogram does not automatically prove enantiomeric purity of the measured compound. To this end, standards of both enantiomers would be needed to ensure that in principle they can be separated. Many measurements gave single peaks, e.g., the BB derivatives of *cis*-chlorobenzene dihydrodiols or of the monosubstituted *cis*-biphenyl dihydrodiols. Since authentic standards of both enantiomers of these products were unavail-

able, we could not unequivocally prove enantiopurity. So far, all available standards were separated and quantifications of even high EE values were reproducible. Therefore, it is quite likely that most *cis*-dihydrodiols that eluted as single peaks also are pure enantiomers.

CDO oxidized various 1,4-disubstituted benzenes to two *cis*-dihydrodiol enantiomers. With the exception of the oxidation product of 4-fluorotoluene, the products that were formed by CDO had higher EE values than those formed by TDO of *P. putida* UV4 and *P. putida* F39/D (Table 4). Boyd et al. (4) concluded that the EE of 1,4-disubstituted *cis*-benzene dihydrodiols formed by TDO of *P. putida* UV4 is largely controlled by steric effects. Obviously, there have to be additional parameters that influence the EE of 1,4-disubstituted *cis*-benzene dihydrodiols formed by CDO.

The formation rates of the transformation products of benzene, several monosubstituted benzenes, and naphthalene are given in Table 3. As indicated in an earlier study (21), naphthalene is turned over faster by CDO than benzene and monosubstituted benzenes. The formation rates of the monosubstituted *cis*-benzene dihydrodiols are higher than that of *cis*-benzene dihydrodiol. We assume that the large substituent helps the substrate to fit into the active binding site of the enzyme. However, the differences in the transformation rates between the monosubstituted benzenes cannot be exclusively explained by varying sizes of the substituent. We postulate that the rate of formation of monohalogenated *cis*-benzene dihydrodiols by CDO increases with smaller size and with lower electronegativity of the halogen substituent. Both influences compensate for each other in the case of a chlorine or fluorine substituent.

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