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Substrate and Inhibitor Spectra of Ethylbenzene Dehydrogenase: Perspectives on Application Potential and Catalytic Mechanism

Daniel Knack,[a] Corina Hagel,[a] Maciej Szaleniec,[b] Agnieszka Dudzik,[b] Aleksander Salwinski,[b] and Johann Heider[a]

Laboratory for Microbial Biochemistry, Philipps University of Marburg, Marburg, Germany,[a] and Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Cracow, Poland[b]

Ethylbenzene dehydrogenase (EbDH) catalyzes the initial step in anaerobic degradation of ethylbenzene in denitrifying bacteria, namely, the oxygen-independent hydroxylation of ethylbenzene to (*S*)-1-phenylethanol. In our study we investigate the kinetic properties of 46 substrate analogs acting as substrates or inhibitors of the enzyme. The apparent kinetic parameters of these compounds give important insights into the function of the enzyme and are consistent with the predicted catalytic mechanism based on a quantum chemical calculation model. In particular, the existence of the proposed substrate-derived radical and carbocation intermediates is substantiated by the formation of alternative dehydrogenated and hydroxylated products from some substrates, which can be regarded as mechanistic models. In addition, these results also show the surprisingly high diversity of EbDH in hydroxylating different kinds of alkylaromatic and heterocyclic compounds to the respective alcohols. This may lead to attractive industrial applications of ethylbenzene dehydrogenase for a new process of producing alcohols via hydroxylation of the corresponding aromatic hydrocarbons rather than the customary procedure of reducing the corresponding ketones.

Ethylbenzene is a monocyclic aromatic hydrocarbon compound commonly used in industrial chemical processes, along with benzene, toluene and xylene (together called "BTEX"). In the environment, BTEX compounds are readily degraded, even in the absence of oxygen (2, 6, 9, 25, 33). Degradation of ethylbenzene in anoxic environments proceeds via one of two different possible pathways, which are initiated at the methylene carbon (C-1) of the side chain: addition to fumarate, as known from the sulfate-reducing bacterium EbS7 (17), or oxygen-independent hydroxylation in nitrate-reducing bacteria (1, 10, 23, 25). The initial reaction of the latter degradation pathway is catalyzed by the molybdenum/iron sulfur/heme enzyme ethylbenzene dehydrogenase (EbDH) and proceeds stereospecifically to yield (*S*)-1-phenylethanol as a product (14, 15, 18, 24). Subsequently, a specific alcohol dehydrogenase oxidizes (*S*)-1-phenylethanol to acetophenone (11, 19), which is then carboxylated at the methyl group by a complex ATP-dependent enzyme, yielding benzoylacetate (13, 24). After activating this beta-oxo acid intermediate to a coenzyme A (CoA)-thioester and thiolytic cleavage to acetyl-CoA and benzoyl-CoA, the pathway converges the benzoyl-CoA pathway of anaerobic aromatic degradation (2, 6, 10).

The initial enzyme of the pathway, EbDH, belongs to the dimethyl sulfoxide reductase family of molybdenum enzymes (subfamily II) (21) and is a heterotrimer of three subunits. The α subunit (96 kDa) carries a *bis*-molybdopterin cofactor, which is part of the active site, and a Fe_4S_4 cluster. The β subunit (43 kDa) carries three Fe_4S_4 and one Fe_3S_4 clusters, whereas the γ subunit (23 kDa) contains a heme b cofactor (16). The reaction mechanism of EbDH consists of two alternating half-reactions: (i) hydroxylation of the substrate to the respective secondary alcohol, accompanied by reduction of the Mo-cofactor from the Mo(VI) to the Mo(IV) state, and (ii) reoxidation of the Mo-cofactor by transferring two electrons via the iron-sulfur clusters to the heme and then to an external electron acceptor (16). A hypothetical catalytic mechanism for substrate hydroxylation was recently proposed based on quantum chemical modeling and initial data on the reactivity with different substrate analogs (26, 28–31). In short, two electrons and a proton at the C-1 position of the ethyl group are believed to be abstracted by the Mo-cofactor to form a transient carbocation intermediate, which reacts with a hydroxyl group derived from water to form the alcohol (Fig. 1), (26). Structural data of the enzyme show that the binding pocket near the active site of the enzyme is quite large and has a hydrophobic character (16). For that reason, it is not surprising that many structural analogs of ethylbenzene, are hydroxylated as well, and structurally similar compounds carrying an ethyl- or propyl-group adjacent to an aromatic or heteroaromatic ring appear to be efficiently hydroxylated by EbDH (27, 28).

In the present study we present a kinetic analysis of the reaction of EbDH with a large number of substrate analogs and inhibitors (for structures, see Fig. S1 and S2 in the supplemental material). These studies simultaneously provide a basis for deeper understanding of the reaction mechanism of EbDH and an initial overview of potential biotransformation applications based on this enzyme. An especially attractive aspect of biotechnological applications is the possibility of generating alcohols by hydroxylating the respective hydrocarbon analogs rather than by the standard technique of reducing the respective ketone analogs (11, 12).

MATERIALS AND METHODS

Enzyme preparation. Ethylbenzene dehydrogenase (EbDH) was purified from "*Aromatoleum aromaticum*" EbN1 grown anaerobically on ethylbenzene as described before (18, 23, 25). Crude extract preparation and purification where performed under aerobic conditions in the presence of ferricenium tetrafluoroborate, which preserves enzyme activity as de-

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Address correspondence to Johann Heider, heider@biologie.uni-marburg.de.
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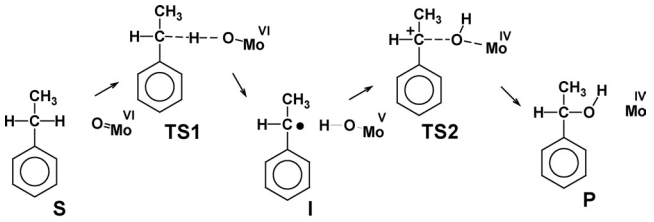

FIG 1 Proposed catalytic mechanism of ethylbenzene hydroxylation by EbDH (29). The molybdenum cofactor of EbDH is represented only by the Mo atom with its oxo or hydroxyl ligands. The respective oxidation states are indicated. S, substrate state; TS1, transition state 1; I, intermediate state; TS2, transition state 2; P, product state.

scribed previously (16). Protein concentrations where determined by the Coomassie dye binding assay (3).

Kinetic measurements. Enzymatic activity was determined in quartz cuvettes by a photometric test as described before (28). Purified EbDH was added to the reaction mixture (100 mM Tris-Cl [pH 7.5] with 200 μM ferricenium-tetrafluoroborate electron acceptor), and the photometric test was started by the addition of the substrate (stock solutions in tert-butanol as nonreactive solvent). The reduction of ferricenium was followed at 290 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) or 300 nm ($\varepsilon = 3.60\ mM^{-1}\ cm^{-1}$). Kinetic parameters were determined by nonlinear fitting of the enzyme activities obtained at varied substrate concentrations to the standard Michaelis-Menten equation or modified Michaelis-Menten equation including substrate inhibition (see Fig. S3 in the supplemental material), using the GraphPad Prism software package.

Inhibition kinetic parameters were calculated from kinetic data with the native substrate ethylbenzene at up to three different inhibitor concentrations and controls without inhibitor. The obtained activities were nonlinearly fitted against the equations for different modes of inhibition (competitive, uncompetitive, mixed) using the program LEONORA (4). Choosing plausible inhibition models for each inhibitor, the overall quality of fitting and the obtained inhibition constants K_{ic} (competitive inhibition) and/or K_{iu} (uncompetitive inhibition) were evaluated. If a model was far off the experimental data or produced very high standard deviations (>50%), very high absolute values, or negative values for either K_{ic} or K_{iu}, it was regarded as implausible.

Liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS) analysis. Products of enzymatic reactions were analyzed on an Agilent 1100 system with DAD and APCI-MS detectors. Products were identified by comigration with authentic standards, if available. Otherwise, they were identified by their mass spectra. The reaction mixtures were routinely analyzed in a normal-phase mode on a chiral Daicel Chiracel OB-H column (250 by 4.6 mm, 5 μm), using *n*-hexane–isopropanol mobile phases (between 95:5% and 70:30%) and MS detection in a positive SCAN/SIM ion mode (+APCI). Ionized species were stabilized by post-column addition of 20 mM NH_4CH_3COO in 75% isopropanol-water. The post-column additive mixture ratio with the mobile phase from the column was 1:1. Only the reaction mixture of indanole was analyzed in a reversed-phase mode on a Zorbax SB-CN column (2.1 × 100 mm, 3.5 μm), using a mobile phase comprised of water (A) and methanol (B) and a gradient program for separation of substrate, product and ferrocene (20 to 60% methanol with initial 3 min hold up at 20% of methanol). The MS detection was performed in a negative ion mode, using an API-ES ion source.

Gas chromatography-MS (GC-MS) analysis. Products of enzyme reactions were analyzed on a Trace DSQ 1000 Thermo Finnigan gas chromatograph with a mass spectrometer, using an electron ionization detector. The separations were conducted on a Cyclodex-B column (60 m, 0.25 mm [inner diameter], 0.25 μm) The analyses were carried out in a split mode (1:20) with helium as a carrier gas and 1-μl injections. The temperature program was adjusted to the polarity and volatility of particular compounds but was basically comprised of a 2-min temperature hold at 60°C or 100°C, followed by a slow temperature gradient (3 to 7°C/min) up to 180°C with a final 5- to 10-min temperature hold at 180°C.

RESULTS

Kinetic properties of ethylbenzene analogs. In the present study, the apparent kinetic parameters of 33 ethylbenzene analogs were determined. The conversion products of 14 substrate analogs had been identified previously as reported (28, 27), and the rest of the products were confirmed by LC (with standard compounds) or LC-MS. Only the products formed from 2-ethyl-1-*H*-indene needed to be confirmed by additional GC-MS data. For a better comparison of the kinetic data, previously reported substrates (28) as well as new substrates were measured with the highest possible concentration of the electron acceptor ferricenium tetrafluoroborate (saturated solution at 200 μM). Therefore, the measured values represent the highest experimentally accessible values for substrate turnover under practical conditions.

Effects of extended side chains. We determined the apparent kinetic parameters of several propyl-substituted substrate analogs and compared them to the rates of the ethyl-substituted analogs (Table 1; see Fig. S4 in the supplemental material). *n*-Propylbenzene showed a 4-fold lower apparent rate and a 70-fold-higher apparent K_m [K_m (app)] value than ethylbenzene, resulting in a 250-fold-lower catalytic efficiency (Table 1). In analogy, all three *n*-propylphenol isomers as well as the heterocyclic compounds 2-*n*-propylthiophene and 2-*n*-propylfurane showed ca. 8- to 9-fold lower apparent conversion rates than their ethyl-substituted analogs (Table 1). The measured K_m (app) values of the propyl-substituted analogs were 1.6- to 2-fold higher than those of the ethyl-substituted analogs, resulting in 15- to 18-fold-lower catalytic efficiencies of the propyl-substituted analogs. The kinetic data of the *ortho*- and *meta*-isomers of *n*-propylphenol were not determined because of the very low activities obtained with these substrates.

Effects of multiple bonds in substituted benzenes. Since the presence of additional multiple bonds in substrate analogs should affect the reactivity of EbDH, we tested two new substrate analogs with C-3 side chains containing either a double bond (3-phenyl-1-propene) or a triple bond (3-phenyl-1-propyne) between C-2 and C-3. Both compounds were indeed hydroxylated ~3-fold faster than ethylbenzene (and 12-fold faster than propylbenzene, the corresponding analog with a single bond) (Table 1; see Fig. S5 in the supplemental material). None of these alternative substrates showed the extremely low apparent K_m value of ethylbenzene (0.45 μM) (28), but the obtained apparent K_m values of 16 and 17 μM were still lower than that of *n*-propylbenzene (30 μM) (Table 1).

Effects of other substituents. Previous data have shown that *ortho*-, *meta*-, and *para*-substituted ethylbenzene isomers react differently (28). Among the *para*-substituted substrates, 4-ethylphenol, 4-ethylanisol (4-methoxyethylbenzene), and 4-ethylaniline showed ca. 2.5- to 5-fold-higher apparent maximum activities than ethylbenzene (Table 1), whereas 4-ethyltoluene, 1,4-diethylbenzene, and 4-ethylbiphenyl showed lower apparent maximum activities (ca. 40 to 60%), and 4-fluoroethylbenzene was converted only very slowly (15%) (Table 1). The K_m (app) values of all *para*-substituted substrates were higher than that of ethylbenzene and ranged from a 6-fold-higher value for 1,4-diethylbenzene up to a 160-fold-higher value for 4-ethylphenol. The *ortho*-isomers of ethylphenol and ethylaniline were converted faster than the re-

TABLE 1 Kinetic parameters of EbDH substrates[a]

Substrate	Substrate	Relative k_{cat}	Apparent		k_{cat}/K_m (s^{-1} M^{-1})
			K_m (μM)	K_i (mM)	
1	Ethylbenzene	1.0	0.45 ± 0.19	NA	2.8×10^6
Substrates with extended alkyl substituents					
2	*n*-Propylbenzene	$(2.6 \pm 0.21) \times 10^{-1}$	30 ± 9.7	NA	1.1×10^4
3	2-*n*-Propylphenol	$(6.3 \pm 0.25) \times 10^{-2}$	ND	NA	NA
4	3-*n*-Propylphenol	$(3.1 \pm 0.41) \times 10^{-2}$	ND	NA	NA
5	4-*n*-Propylphenol	$(6.7 \pm 0.38) \times 10^{-1}$	99 ± 23	NA	8.3×10^3
6	2-*n*-Propylfurane	$(1.5 \pm 0.31) \times 10^{-1}$	41 ± 4.3	NA	4.5×10^3
7	2-*n*-Propylthiophene	$(4.8 \pm 0.71) \times 10^{-1}$	25 ± 6.5	2.4 ± 0.14	2.4×10^4
Substrates with substituents containing multiple bonds					
8	3-Phenyl-1-propene	2.8 ± 0.078	17 ± 1.3	NA	2.0×10^5
9	3-Phenyl-1-propyne	3.4 ± 0.27	16 ± 4.9	NA	2.6×10^5
Substrates with *ortho*-, *meta*-, and *para*-substituents					
10	2-Ethylphenol	$(4.4 \pm 0.15) \times 10^{-1}$	18 ± 4.4	NA	3.0×10^4
11	3-Ethylphenol	$(1.9 \pm 0.19) \times 10^{-1}$	9.3 ± 2.3	NA	2.5×10^4
12	4-Ethylphenol	4.8 ± 0.21	15 ± 3.0	NA	3.9×10^5
13	2-Ethylaniline	1.5 ± 0.058	84 ± 14	NA	2.2×10^4
14	3-Ethylaniline	$(2.5 \pm 0.064) \times 10^{-1}$	17 ± 2.2	NA	1.8×10^4
15	4-Ethylaniline	2.7 ± 0.28	16 ± 3.6	0.36 ± 0.13	2.1×10^5
16	2-Ethyltoluene	$(3.9 \pm 0.38) \times 10^{-2}$	ND	NA	NA
17	3-Ethyltoluene	$(1.0 \pm 0.11) \times 10^{-1}$	ND	NA	NA
18	4-Ethyltoluene	$(5.0 \pm 0.28) \times 10^{-1}$	3.0 ± 0.91	NA	2.1×10^5
19	1,4-Diethylbenzene	$(5.7 \pm 0.14) \times 10^{-1}$	2.8 ± 0.48	NA	2.5×10^5
20	4-Ethylanisol	2.8 ± 0.48	2.9 ± 1.2	0.071 ± 0.032	1.2×10^6
21	4-Ethylbiphenyl	$(4.0 \pm 0.73) \times 10^{-1}$	3.3 ± 0.92	0.006 ± 0.0016	1.5×10^5
22	4-Fluorethylbenzene	1.5×10^{-1}	ND	NA	NA
23	2-Ethylfurane	1.3 ± 0.12	25 ± 6.8	NA	6.6×10^4
24	2-Ethylthiophene	4.1 ± 0.19	12 ± 1.6	2.5 ± 0.71	4.2×10^5
Bicyclic substrates					
25	Indane	1.4 ± 0.11	9.3 ± 2.6	NA	1.7×10^5
26	5-Hydroxyindane	1.4 ± 0.87	37 ± 7.6	NA	4.6×10^4
27	5-Methoxyindane	1.5 ± 0.27	23 ± 6.8	0.15 ± 0.064	7.8×10^4
28	5-Aminoindane	1.9 ± 0.12	34 ± 5.8	NA	7.2×10^4
29	Coumaran	$(6.0 \pm 0.43) \times 10^{-1}$	20 ± 6.1	NA	3.7×10^4
30	Indoline	3.1×10^{-1}	ND	NA	NA
31	1,2,3,4-Tetrahydronaphthalene	5.0×10^{-2}	ND	NA	NA
32	1,2,3,4-Tetrahydronaphth-6-ol	4.0×10^{-2}	ND	NA	NA
33	2-Ethyl-1*H*-indene	8.3 ± 0.56	2.0 ± 0.41	0.11 ± 0.025	5.1×10^6

[a] The relative k_{cat} is the relative turnover number of every EbDH substrate in comparison to ethylbenzene. The absolute k_{cat} values for ethylbenzene ranged from 0.14 to 1.4 s^{-1} depending on the enzyme batch. The apparent K_m and K_i values were determined using the program GraphPad Prism. The apparent k_{cat} values were calculated using the molecular mass of EbDH (165 kDa). ND, not determined; NA, not applicable.

spective *meta* isomers, whereas this trend was reversed for *ortho*- and *meta*-ethyltoluene (Table 1). The K_m (app) values of all possible isomers were only determined for the ethylphenols and ethylanilines, because the measured activities of the other compounds were too low to obtain reliable kinetic data. The lowest K_m (app) values were recorded for 3-ethylphenol and 3-ethylaniline, respectively, whereas the highest K_m (app) values were recorded for 2-ethylaniline (Table 1). The apparent k_{cat}/K_m values showed that the catalytic efficiencies for the *ortho*- and *meta*-isomers are quite similar, whereas the *para*-isomers are converted with significantly higher efficiencies (Table 1; see also Fig. S6 in the supplemental material).

Bicyclic substrates. We identified the bicyclic aromatic compound indane as an additional substrate to be hydroxylated by EbDH and tested related analogs for their activities. Indane itself exhibited a higher apparent maximum activity compared to ethylbenzene (143%), whereas its K_m (app) value was 20-fold higher. Indane derivatives containing a methoxy-, amino-, or hydroxy-substituent at the 5 position were converted as fast as indane (5-hydroxyindane and 5-methoxyindane [Table 1]; see also Fig. S7 in the supplemental material) or slightly faster (5-aminoindane; Table 1). Their K_m (app) values were slightly higher (2.5- to 4-fold) than that of indane (Table 1). Coumaran (2,3-dihydrobenzofuran), an *O*-heterocyclic analog of indane, showed a 2-fold-lower

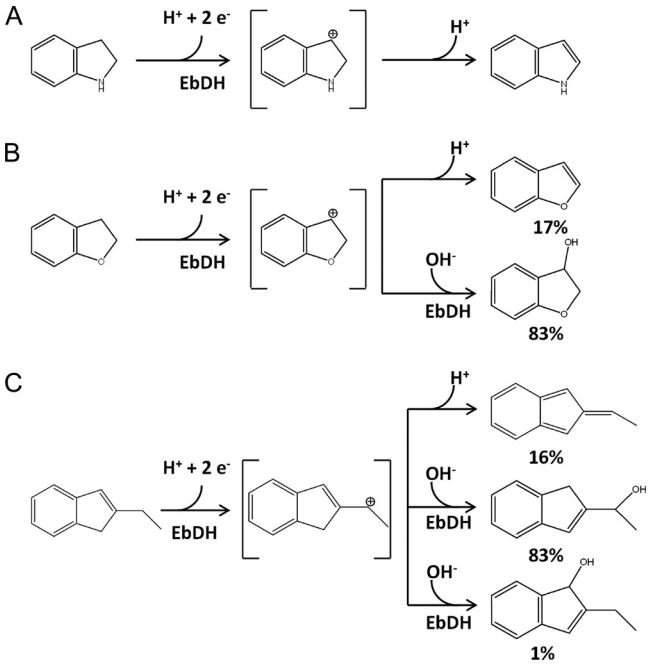

FIG 2 Reactions of some bicyclic substrates with EbDH. (A) Conversion of indoline to a dehydrogenated product (indol). (B) Conversion of coumaran to two products: benzofuran as a dehydrogenated side product and 3-hydroxycoumaran as the main product. (C) Conversion of 2-ethyl-1*H*-indene to three products: a dehydrogenated hydrocarbon compound (top) and two alcohols with hydroxyl groups on C-1 of the ethyl substituent (main product, middle) or on the cyclopentadiene ring (trace amounts, bottom).

enzymatic activity and slightly higher K_m (app) value as indane. Product analysis by LC-MS and GC-MS showed that benzofuran was generated as a second oxidized product in addition to 3-hydroxycoumaran (see below). The *N*-heterocyclic indane analog indoline also reacted with EbDH, but its kinetic parameters could not be determined properly because UV-absorbing products and nonenzymatic side reactions obscured the measurements. In this case, the only detectable product was indol, whereas no evidence for a hydroxylated product was obtained by LC-MS analysis (see below). Indene was identified as another substrate of EbDH, although this was only based on detection of the hydroxylation product by LC-MS and GC-MS analyses (data not shown), while the conversion rate was too low to be measured via the photometric assays. Indene was also identified as a strong inhibitor of the enzyme (see below). Furthermore, weakly converted bicyclic substrates of EbDH, 1,2,3,4-tetrahydronaphthalene and 1,2,3,4-tetrahydronaphth-6-ol, were detected and showed apparent enzymatic activities of ca. 5 and 40%, respectively, compared to ethylbenzene (Table 1).

Substrates yielding multiple products. Among the bicyclic substrates tested as new EbDH substrates, coumaran and indoline yielded unexpected oxidized products containing a double bond between C-2 and C-3 of the heterocyclic ring, which were produced either in small amounts compared to the "usual" hydroxylated product (for coumaran), or as the sole detected product (for indoline) (Fig. 2). A similar observation was made with the substrate analog 2-ethyl-1*H*-indene, which was among the compounds yielding the highest measurable enzymatic activities with EbDH. This substrate was converted to two main products as characterized by GC-MS: 83% of the substrates were hydroxylated at the ethyl substituent, whereas 16% were dehydrogenated to a hydrocarbon with an additional double bond, as shown in Fig. 2. In addition, trace amounts of a third conversion product hydroxylated at the methylene carbon of the cyclopentadiene ring were identified. Kinetic analysis suggested a 2-fold higher apparent k_{cat} value for this compound than the highest measurable activity (because of substrate inhibition, see inhibitor section below) and a quite low K_m value of 2 μM, resulting in the highest catalytic efficiency value of all known EbDH substrates.

Kinetics of EbDH inhibitors. We determined the inhibitory kinetic parameters of several previously identified (28) and new inhibitors of EbDH to contribute to further characterization of its reaction mechanism (Table 2).

Alcohols. The products of ethylbenzene or 2-ethylnaphthalene conversion, (*S*)-1-phenylethanol and 1-(2-naphthyl)ethanol, were tested exemplarily as product inhibitors of the enzyme. The observed inhibitory effects of the alcohol products fitted to a competitive inhibition model of the enzyme (4, 5, 28). The K_{ic} values obtained for those inhibitors are quite low, ranging from 26.7 μM for (*S*)-1-phenylethanol to 3.2 μM for 1-(2-naphthyl)ethanol (Fig. 2).

Methylated compounds. Another group of EbDH inhibitors

TABLE 2 Kinetic parameters of EbDH inhibitors[a]

No.	Inhibitor	Inhibition type	Mean ± SD	
			K_{ic} (μM)	K_{iu} (μM)
1	(*S*)-1-Phenylethanol	Competitive	27 ± 11	NA
2	1-(2-Naphthyl)ethanol	Competitive	3.2 ± 0.40	NA
3	Toluene	Competitive	3.4 ± 1.1	NA
4	2-Methylfurane	Competitive	(4.5 ± 1.1) × 10^2	NA
5	2-Methylthiophene	Mixed	2.6 ± 0.72	(3.2 ± 0.95) × 10^3
6	Styrene	Competitive	1.5 ± 0.34	NA
7	Indene	Competitive	0.38 ± 0.12	NA
8	Indol	Competitive	(1.1 ± 0.45) × 10^2	NA
9	2-Ethylbenzenethiol	Mixed	0.04 ± 0.01	19 ± 6.6
10	1,2-Diethylbenzene	Competitive	0.4 ± 0.1	NA
11	4-Ethylpyridine	Mixed	14 ± 3.3	4.1
12	Anisole	Mixed	0.9 ± 0.1	(2.4 ± 0.33) × 10^2

[a] Inhibition was tested by a standard assay with ethylbenzene as the substrate. The inhibition type and the inhibition kinetic parameters K_{ic} and K_{iu} were determined by nonlinear fitting of the experimental data to the respective equations using the software LEONORA (4). NA, not applicable.

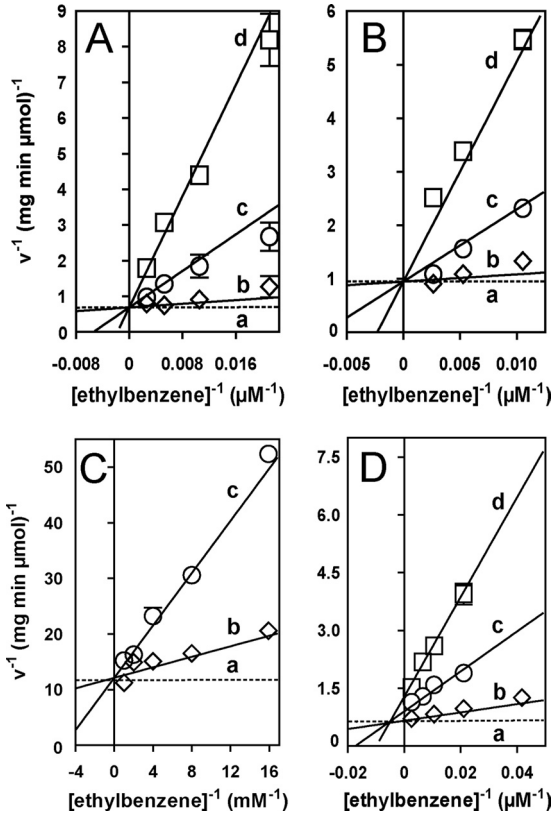

FIG 3 Double-reciprocal plots of inhibition kinetic data. (A) Data for 1-(2-naphthyl)ethanol: no inhibitor (a, dotted lines without symbols) and in the presence of 0.095 mM (b, diamonds), 0.95 mM (c, circles), or 2.9 mM (d, squares) 1-(2-naphthyl)ethanol. (B) Data for toluene: no inhibitor (a, dotted lines without symbols) and in the presence of 0.095 mM (b, diamonds), 0.95 mM (c, circles), or 2.9 mM (d, squares) toluene. (C) Data for indene: no inhibitor (a, dotted lines without symbols) and in the presence of 0.1 mM (b, diamonds) or 0.5 mM (c, circles) indene. (D) Data for anisole: no inhibitor (a, dotted lines without symbols) and in the presence of 19 μM (b, diamonds), 95 μM (c, circles), and 240 μM (d, squares) anisole.

were methyl substituted substrate analogs, such as toluene (Fig. 3), 2-methylfurane, and 2-methylthiophene. Compared to ethyl-substituted compounds, these methyl-substituted compounds were never hydroxylated at the C-1 position of their substituent but acted as mixed-type or competitive inhibitors (Table 2). Their K_{ic} values ranged from a low value for toluene (3.4 μM) to much higher values for methylfurane and methylthiophene (448 and 258 μM, respectively).

Unsaturated compounds. A separate group of inhibitors was identified in the present study as ethylbenzene analogs with a double bond between C-1 and C-2 of the side chain. This included the open-chain compound styrene, as well as analogous bicyclic compounds such as indene (Fig. 3) or indol. All of these compounds acted as competitive inhibitors. Styrene and indene showed extremely low K_{ic} values of <2 μM, whereas indole showed only a weak inhibitory effect with a K_{ic} of 107 μM.

Other inhibitory substrate analogs. Among the many analyzed ethyl-substituted substrate analogs, a few compounds were identified as inhibitors of EbDH: 2-ethylbenzenethiol was identified as a strong mixed-type inhibitor with a very low K_{ic} of 0.04 μM and a K_{iu} of 18.6 μM, whereas 1,2-diethylbenzene was identified as competitive inhibitor with a low K_{ic} of 0.4 μM. 4-Ethylpyridine was a reasonable mixed-type inhibitor with a K_{ic} of 13.6 μM and a K_{iu} of 4.1 μM, although the isomeric 3-ethylpyridine was converted as a substrate analog without inhibitory properties (28). Another strong inhibitor acting as a structural analog of ethylbenzene was anisole (methoxybenzene). It showed a mixed-type inhibition with a low K_{ic} value of 0.9 μM and a rather high K_{iu} of 240 μM (Table 2, Fig. 2).

Compounds exhibiting substrate inhibition. Some substrates tested during the present study showed a clear substrate inhibitory effect (see Fig. S8 in the supplemental material). Among these compounds, 4-ethylbiphenyl showed a very low substrate inhibition constant (5.5 μM) close to the apparent K_m value (3.3 μM). The substrate inhibition constants for 4-ethylanisol, 5-methoxyindane, and 2-ethyl-1*H*-indene were in the range of 71 to 108 μM (Table 1; see Fig. S8 in the supplemental material). The highest K_i values were found for 2-ethylthiophene and 2-*n*-propylthiophene (2,400 to 2,500 μM). Some of these substrates contain both an ethyl substituent, which is hydroxylated, and a second functional group present in other known inhibitors of EbDH. Examples are 4-ethylanisol and 5-methoxyindane, which contain an inhibitory methoxy-group as in anisole, and 2-ethyl-1*H*-indene, which contains the molecular scaffold of the inhibitor indene (see Fig. S1 in the supplemental material).

DISCUSSION

Potential application aspects. We demonstrate in the present study that EbDH turns over 33 different substrates with reasonable kinetic parameters for potential applications, making it a highly interesting candidate for a novel biotransformation route of alcohol synthesis from aromatic hydrocarbons. The electron acceptor ferricenium tetrafluoroborate can principally be regenerated electrochemically to achieve the high conversion yields needed for industrial applications (20, 22, 28) and make the process more economical. Using EbDH to generate alcohols offers some advantages compared to established biotransformation procedures such as reduction of ketones by alcohol dehydrogenases [for example, (*S*)-1-phenylethanol-dehydrogenase] (11, 12) or by oxygen-dependent hydroxylation by mono- or dioxygenases (32). In contrast to those enzymes, no expensive NADH is needed, and no oxygen needs to be supplied, which limits most oxygenase-based biotransformation reactions (32). Furthermore, the enzyme has already been shown to be highly enantioselective for many substrates (27). To conclude, the properties of EbDH make it a very promising target for establishing a new biotransformation process, which may be further advanced by procedural tuning (immobilization of the enzyme or whole-cell process) or protein engineering by mutagenesis.

Kinetic properties of ethylbenzene derivatives. One of the principle aims of the present study was to investigate the conversion of alternative substrates by EbDH and to establish correlations to their chemical properties. We can ascribe several patterns of EbDH reactivity associated with chemical categories of substrates, as follows.

(i) Substrates with *n*-propyl rather than ethyl substituents were predicted by quantum chemical calculations to have a higher activation energy, which should result in lower apparent k_{cat} values (M. Szaleniec, unpublished). This fits the experimental data which showed 4- to 9-fold lower k_{cat} values of the *n*-propyl-substituted substrates relative to the corresponding ethyl-substituted analogs.

The *n*-propyl-substituted substrates also show increased K_m (app) values, indicating steric hindrance of binding as an additional determinant for their lower reactivity.

(ii) Substrates with a double or a triple bond adjacent to the C-1 atom in their alkyl substituents showed a dramatic increase of activity compared to the saturated substrate (3-phenyl-1-propene and 3-phenyl-1-propyne versus *n*-propylbenzene). This correlates to better mesomeric stabilization of the predicted radical and carbocation intermediates in the proposed catalytic mechanism (26, 29). The K_m (app) values of both unsaturated compounds are lower than that of *n*-propylbenzene, which may reflect better binding to the active site by loss in flexibility (29).

(iii) The differences in apparent k_{cat} values of *para*-substituted ethylbenzenes are mainly determined by the electronic properties of the substituents (resonance and inductive effects) and only to a much smaller extent by steric factors. These substituent effects can be explained with the Hammett σ^+ constants that describe their electron donating/withdrawing effects on benzene rings (7, 8). The activity of EbDH tended to increase with substituents with more negative Hammett σ^+ constants that help to stabilize the radical and/or carbocation intermediate species in the proposed catalytic mechanism and vice versa (28, 29). The observed K_m (app) values appear to correlate with the polarity of the substituents instead of their resonance or inductive effects, suggesting that substrates with apolar substituents bind better to the enzyme than those with polar ones.

(iv) The effects of *ortho*-substituted substrates on EbDH activity were either not significant or much smaller than those of the correlated *para*-substituted isomers. This may be explained by an increasing importance of steric factors on the reactivity of the neighboring ethyl group (26, 28).

(v) No resonance stabilization of the intermediates is expected for *meta*-substituted substrates, a finding consistent with the observation that they were converted at slower rates than the corresponding *ortho*- and *para*-isomers. The K_m (app) values for *ortho*- and *meta*-substituted substrates did not show a consistent trend.

Bicyclic compounds as a new chemical category of substrates. It is hypothesized that the fast hydroxylation of the bicyclic hydrocarbon indane by EbDH may be associated with two factors: (i) the almost planar structure of the aliphatic ring, possibly mimicking the geometry of the transition state and (ii) the presence of two potential hydroxylation sites. The fixed geometry of the hydroxylation site in form of the cyclopentene ring may lower the activation energies of both predicted transition states of the mechanistic model as indicated by quantum chemical calculations (M. Szaleniec, unpublished data). Surprisingly, substituted indane derivatives with a methoxy-, amino-, or hydroxy-substituent at C-5 (equivalent to *para*-substituted ethylbenzenes) were not converted faster than indane. This indicates that indane may mimic the transition state so well that no further effect is achieved by additional stabilizing substituents. In contrast, a stimulatory substituent effect on substrate hydroxylation was observed for 1,2,3,4-tetrahydronaphth-6-ol relative to 1,2,3,4-tetrahydronaphthalene, which is hydroxylated with a rather poor activity. Because the aliphatic cyclohexene ring in 1,2,3,4-tetrahydronaphthalene is not planar like the cyclopentene ring of indane, it should not mimic the transition state as well.

Substrates serving as mechanistic models. For the three substrates coumaran, indoline and 2-ethyl-1*H*-indene, unexpected dehydrogenated reaction products were observed instead of or in addition to the "usual" hydroxylated products. The predicted carbocation intermediates of these compounds allow to split off a proton rather than accept a hydroxyl group, because the resulting products are stabilized by relocating double bonds. This is consistent with the proposed mechanism of EbDH (26), making these compounds valuable mechanistic models for the enzyme. The kinetic parameters obtained for coumaran conversion suggest that the O-heterocyclic ring has only a weak effect on the affinity to the enzyme or the stabilization of the reaction intermediates, compared to indane. However, the small amounts of a dehydrogenated side product detected in addition to the hydroxylated main product suggest that the carbocation intermediate of coumaran can also be stabilized by deprotonation (26). The other heterocyclic indane derivative, indoline, even yielded exclusively the dehydrogenated derivative indol as a detectable product, suggesting that no hydroxyl rebound occurs at all. This is consistent with the expected instability of a hypothetical carbocation intermediate from indoline, which should be deprotonated to indol much faster than converted to the alcohol product. Another substrate yielding multiple products was the ethyl-substituted indene derivative 2-ethyl-1*H*-indene, which was hydroxylated at the ethyl side chain to 83% or dehydrogenated to a hydrocarbon product with an additional double bond to 16%. This last example shows most clearly that the predicted carbocation intermediate in the proposed reaction mechanism of EbDH apparently reacts either by "rebounding" with the hydroxyl ion bound at the Mo-cofactor or by eliminating a proton, giving rise to the observed reaction products. Traces of a third product hydroxylated at the cyclopentadiene ring of 2-ethyl-1*H*-indene are consistent with low levels of an analogous product formed from indene and suggest that 2-ethyl-1*H*-indene may bind to the active site in an alternative conformation, generating the carbocation in the cyclopentadiene ring rather than the ethyl group. The two alternative regiospecific activations of 2-ethyl-1*H*-indene (at the ethyl substituent or at C-3 of the cyclopentadiene ring, respectively) would yield the identical dehydrogenated product.

Properties of inhibitors of EbDH. (i) EbDH is subject to relatively weak product inhibition by secondary alcohols, as already described for (*S*)-1-phenylethanol (28) and established here with 1-(2-naphthyl)ethanol, the product formed from the alternative substrate 2-ethylnaphthalene. As expected for product inhibition, both compounds are competitive inhibitors. Compared to (*S*)-1-phenylethanol, 1-(2-naphthyl)ethanol inhibited the enzyme more efficiently, possibly because the bulky, hydrophobic 1-(2-naphthyl)ethanol fits better into the EbDH binding pocket.

(ii) Another group of EbDH inhibitors are methyl-substituted substrate analogs such as toluene, 2-methylfurane and 2-methylthiophene, which mimic closely the structure of ethylbenzene. Toluene and 2-methylfurane show purely competitive inhibition, whereas 2-methylthiophene showed mixed inhibition. The competitive effect suggests that these substrates bind tightly to the active site, while being unable to be converted. A recent quantum chemical calculation (Szaleniec, unpublished) showed that the conversion of methyl-substituted aromatic rings to a carbocation intermediate is energetically more unfavorable than that of ethyl- or propyl-substituted rings, and therefore may not occur under physiologic conditions. Toluene shows a quite low K_{ic} value, as expected based on its structural similarity to ethylbenzene, whereas methylated heterocyclic compounds were much weaker inhibitors. An additional uncompetitive inhibition component

observed with 2-methylthiophene is matched with substrate inhibitory effects of 2-ethyl- and 2-*n*-propylthiophene (see below) and indicates that the sulfur of the heterocycle may affect the functionality of the active site.

(iii) Styrene, indene and indol were newly identified as EbDH inhibitors. All three inhibitors contain a double bond between the C-1 and C-2 atoms of an alkyl substituent of a benzylic ring, and all show a competitive type of inhibition, exhibiting very strong inhibition with low K_{ic} values in case of styrene and indene but only a weak inhibitory effect in case of indole. For all of these compounds, hydroxylation of the C-1 atom involved in the double bond would require very high activation energy, as suggested by quantum chemical calculations (Szaleniec, unpublished). Thus, the inhibitory effect of these compounds probably originates from their structural similarity to the activated radical or carbocation intermediate species in the reaction mechanism. Among them, indene is simultaneously a weak substrate of EbDH, judging from detected traces of a product hydroxylated at the sp^3-hybridized carbon of the cyclopentadiene ring.

(iv) A few ethyl-substituted substrate analogs were identified as inhibitors rather than as substrates of EbDH. Among these is 4-ethylpyridine, a relatively strong mixed type inhibitor of EbDH, whereas the isomeric 3-ethylpyridine is a weak substrate without inhibitory properties (28, 29). This observation is in accordance with different expected resonance stabilizations of the derived carbocation intermediates, which should be energetically accessible for 3-ethylpyridine, but not for 4-ethylpyridine, explaining the competitive inhibition component of 4-ethylpyridine. This behavior was recently confirmed by quantum chemical modeling, demonstrating that 4-ethylpyridine is indeed unable to form a carbocation intermediate (29). The additional uncompetitive inhibition component cannot be explained in detail thus far but may be related to the impairment of function by the heterocyclic thiophene derivatives described before. Another effective competitive inhibitor is 1,2-diethylbenzene, which shows a very low K_{ic} in the range to the K_m (app) for ethylbenzene and again is isomeric to a substrate of EbDH, 1,4-diethylbenzene. As with the inhibitor indene, small amounts of hydroxylated products from 1,2-diethylbenzene were detected by LC-MS analysis, indicating that its inhibitory activity may be explained by occupying the active site while being converted very slowly, probably correlated to steric interference between the two ethyl groups. The thiolated substrate analog 2-ethylbenzenethiol showed a very strong mixed inhibition effect with a very low K_{ic} value and a high K_{iu} value. The kinetic parameters suggest an even better affinity of this inhibitor toward the active site than of ethylbenzene, while the inhibitory effects are apparently correlated with the presence of the thiol ligand extending into the active site. The corresponding substrate analog with a hydroxyl substituent in place of the thiol (2-ethylphenol) is hydroxylated with a decent rate. This is consistent with a postulated resonance stabilization/destabilization hypothesis of the radical and carbocation transition states. (8; Szaleniec, unpublished). Finally, anisole was found to be another mixed-type inhibitor of EbDH with a very low K_{ic} value, which contains a methoxy group as chemical analog of an ethyl ligand that cannot be hydroxylated. The additional uncompetitive inhibition effect cannot be rationalized thus far, but an analogous effect related to uncompetitive inhibition also causes the observed substrate inhibition for the chemical analog 4-methoxyethylbenzene (see below).

Substrate inhibition. Some EbDH substrates described in the present study showed clear substrate inhibition effects at high concentrations. Substrate inhibition is generally analogous to uncompetitive inhibition, because it can only been seen at substrate concentrations that guarantee occupation of the active site. Therefore, the inhibition must be caused by binding of a second molecule to the enzyme-substrate complex (5). In some of the observed cases, the substrates contain an ethyl or propyl substituent to be hydroxylated and a second structural component responsible for the inhibitory effect. Examples are methoxy-substituted substrates such as 4-methoxyethylbenzene or 5-methoxyindane, which show similar substrate inhibition constants. It is interesting that the K_{iu} value of the corresponding monofunctional inhibitor anisole lies in the same order of magnitude, suggesting a similar inhibitory effect of all of these compounds. A similar observation was made for the thiophene derivatives. Both 2-ethyl and 2-propylthiophene show weak substrate inhibition with inhibition constants similar to the K_{iu} value of the inhibitory analog 2-methylthiophene. This supports the notion that binding of a second substrate to the enzyme-substrate complex (uncompetitive inhibition) may be linked to the presence of the methoxy ligand or the thiophene ring, respectively. Another interesting case of substrate inhibition was recorded for 2-ethyl-1*H*-indene, where the inhibitory indene ring structure is combined with an ethyl substituent to be hydroxylated. However, the corresponding unsubstituted inhibitor indene in this case showed only competitive inhibition, which does not account for the apparent binding of a second molecule of 2-ethyl-1*H*-indene to the enzyme-substrate complex. Finally, very strong substrate inhibition was recorded for 4-ethylbiphenyl, leading to the most significant inhibitory effect and the lowest calculated substrate inhibition constant among the measured compounds. Unfortunately, it is technically not possible to identify additional competitive inhibitory effects, which should be expected for some of these substrates, based on the presence of "inhibitory" (e.g., methoxy) ligands next to the catalytically active ethyl or propyl groups.

Conclusions on the catalytic mechanism of EbDH. The observed kinetic behavior of EbDH with 33 substrates and 13 inhibitors allows us to conclude that different factors affect substrate binding and turnover. Based on the results shown, apolar substrates seem to be binding more efficiently to the active site than polar ones, whereas substrates with electron donating substituents (mostly in *para* position relative to the ethyl or propyl group) will increase the reaction rate, probably by stabilizing postulated radical and carbocation intermediates. Unsaturated bonds in the alkyl substituent also contribute to stabilize these transition state intermediates and increase the reactivity of the respective substrates, whereas double bonds including the carbon atom to be hydroxylated prevent the enzymatic turnover. The strong activity measured with bicyclic substrates such as indane indicates a flat conformation of the radical-type first transition state, and the generation of dehydrogenated products instead or additional to hydroxylated ones may be taken as a strong indication for the involvement of a carbocation intermediate in the reaction mechanism.

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