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Stereochemistry of 1-(4′-hydroxyphenyl)ethanol produced by hydroxylation of 4-ethylphenol by *p*-cresol methylhydroxylase

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Enzymic hydroxylation of 4-ethylphenol by (*a*) *Pseudomonas putida* and (*b*) highly purified *p*-cresol methylhydroxylase gave optically active 1-(4′-hydroxyphenyl)-ethanol. The products were transformed into the phenolic methyl ethers and shown to contain 69.5% and 65.6%, respectively, of the (*S*)-(−)-isomer. The stereochemistry of the reaction is discussed in terms of three distinct steps occurring at the active site of the enzyme.

p-Cresol methylhydroxylase is an unusual flavocytochrome, which has been isolated from *Pseudomonas putida* (N.C.I.B. 9869), as well as from a number of other bacteria (Hopper & Taylor, 1977; Keat & Hopper, 1978). The enzyme is not a monooxygenase, since molecular oxygen is not incorporated into the products, but acts by dehydrogenating *p*-cresol and related alkyl-substituted phenols, followed by hydration to the corresponding alcohol which is then further dehydrogenated to the carbonyl compound. Among the many interesting and uncommon properties of the enzyme is the fact that the prosthetic group of its flavoprotein subunit is 8α-(*O*-tyrosyl)-FAD, the first example of this novel covalently bound flavin (McIntire *et al.*, 1981).

We report here some unexpected findings on the chirality of the 1-(4′-hydroxyphenyl)ethanol produced from the oxidation of 4-ethylphenol by this enzyme.

Materials and methods

Chemicals and enzymes

3,5-Dimethylphenol, 4-ethylphenol, and 4-hydroxyacetophenone were from Aldrich Chemical Co., and silica gel t.l.c. plates with fluorescent indicator were from Analtech Inc. Catalase (from beef liver, 23mg/ml in a suspension containing 0.1% thymol) and phenazine methosulphate were from Sigma Chemical Co. The silica gel (40μm particle diameter) for 'flash chromatography' was purchased from the JT Baker Chemical Co. (*R,S*)-1-(4′-Hydroxyphenyl)ethanol was synthesized by reducing 4-hydroxyacetophenone with $NaBH_4$ in methanol. Diazomethane was prepared by the procedure of de Boer & Backer (1963) and (*S*)-(−)-α-methoxy-α-trifluorophenylacetyl chloride according to Dale *et al.* (1969). The highly purified enzyme, *p*-cresol methylhydroxylase from *Pseudomonas putida* strain 9869 grown on 3,5-dimethylphenol, was isolated by the method of Keat & Hopper (1978).

Isolation of 1-(4′-hydroxyphenyl)ethanol from whole cells

Pseudomonas putida (N.C.I.B. 9869) grown on nutrient agar slants was inoculated into 100ml of a medium containing 0.01% sodium succinate and 0.03% 3,5-dimethylphenol (Keat & Hopper, 1968). After 48h of aerobic growth at 30°C with shaking, the cells were transferred to 1 litre of the same medium without succinate and, after 8h additional growth at 30°C, were again transferred to 11 litres of the same medium. The cells were aerated for 15h at 30°C; 0.01% succinic acid was then added and the growth continued for 3h longer, at which time turbidimetry indicated a cell density of 0.6mg/ml. The cells were harvested by centrifugation, resuspended in 1 litre of 43mM-phosphate buffer, pH 7.0, and placed in an orbital shaker with enough 4-ethylphenol to give a concentration of 0.03% and incubated at 30°C. The formation of 1-(4′-hydroxyphenyl)ethanol (**1**) was monitored by h.p.l.c. analysis, using a 5μm octadecylsilane column (4.6mm × 250mm) with methanol/water (1:1, v/v) as solvent, a flow rate of 1ml/min, and an observation wavelength of 254nm. After 4h an

additional 0.3g of 4-ethylphenol was added and incubation continued for 22h. At that time an additional 0.3g of ethylphenol was added. Incubation was halted after a total of 44h. H.p.l.c. analysis indicated that the yield of **1** did not change after 22h.

The cells were harvested by centrifugation at 16000*g* and the supernatant solution was extracted twice with 1 litre of diethyl ether. The combined extracts were dried over Na_2SO_4, concentrated to an oil *in vacuo* (35°C), and the remaining ether was removed with a stream of N_2. This yielded 0.93g of a whitish waxy substance. T.l.c. indicated that **1**, 4-ethylphenol, and 4-hydroxyacetophenone were the major products. 4-Ethylphenol was extracted with 10ml of petroleum spirit (b.p. 40–60°C), and 4-hydroxyacetophenone with 10ml of chloroform, leaving 108mg of a white solid. After a second extraction with 30ml of petroleum spirit to remove traces of 4-ethylphenol, there remained 63mg of pure **1**. Its purity was established by t.l.c. and h.p.l.c. The material isolated comigrated with authentic **1**. N.m.r. (^2H_2O) δ (p.p.m.) 1.5 (d, 3, $-CH_3$), 4.5–5 (q, 4, C-*H*; partially obscured by H^2HO), 6.92 and 7.35 (2×d, 4, Ar-*H*). Mass spectrum, *m/z* (relative abundance): 138 (M^+, 7.5), 123 (24.5), 121 (14), 120 (100), 119 (20.5), 95 (11), 94 (6), 91 (24.5), 77 (7.5), 65 (9.5), 63 (6). Authentic **1**: 138 (M^+, 7.5), 123 (22.5), 121 (13), 120 (100), 119 (21.5), 95 (10), 94 (5), 91 (22), 77 (7), 65 (10.5), 63 (5).

Production of 1-(4'-hydroxyphenyl)ethanol by p-cresol methylhydroxylase

To 150ml of an aerobic solution of 50mM-glycine buffer, pH9.5, were added 92mg of 4-ethylphenol, 11.4mg of KCN, 90mg of phenazine methosulphate, 2.3mg of crystalline catalase, and 20mg of highly purified *p*-cresol methylhydroxylase. Phenazine methosulphate served as the electron acceptor in the dehydrogenation, catalase was added to destroy the H_2O_2 produced in the reaction, and KCN introduced to regenerate protein -SH groups oxidized to disulphide during the reaction by cyanolysis. The solution was incubated at 30°C in air with shaking. Samples (2ml) were removed at 5min, 1h, and 2h, acidified, and extracted with 2ml of diethyl ether. The extracts were dried over Na_2SO_4, the ether was removed under a stream of N_2, and the products formed were subjected to h.p.l.c. analysis on an octadecylsilane column, using propan-1-ol/water (1:1, v/v) as the mobile phase, a flow rate of 3ml/min, and a wavelength of detection of 280nm. After 2h, the reaction was stopped by acidification to pH3.0 with H_2SO_4, to prevent further oxidation of the alcohol to the ketone.

The solution was extracted twice with 250ml of diethyl ether and the combined extracts were dried and concentrated at 40°C, as before, yielding a green oil. Methanol was added to give a volume of 0.5ml, followed by chloroform to 5ml total volume. The product was purified by flash chromatography (Still *et al.*, 1978). The column used was 4cm×24cm and contained silica gel particles of 40μm size. The eluting solution was chloroform/methanol (20:1, v/v) and the flow rate 64ml/min. Fractions 1–14 were 10–15ml each; subsequent fractions were 5ml each. Fractions 23–62 contained **1**, well separated from 4-ethylphenol. After evaporation of the solvent *in vacuo*, a red solid was obtained.

Flash chromatography and concentration of the solvent were repeated with chloroform/diethyl ether (1:17, v/v) as eluant, yielding a yellow solid, which was dissolved in 2ml of ether. Hexane was added to incipient turbidity and the solution was allowed to crystallize at 0°C. The light yellow crystals were dissolved in 2ml of methanol, decolorized with charcoal at 55°C, and the charcoal was removed by centrifugal filtration. After evaporation of the methanol in a stream of Ar, the residue obtained was recrystallized, as above, yielding 25mg of pure **1** as a white solid.

Assays

Optical rotations were determined using a Perkin-Elmer model 141 polarimeter. Gas chromatographic separations were carried out using a 15m-fused silica capillary column, 0.32mm i.d., with a bonded phase (0.25μm thickness) of DB-1701. Injector temperature was 250°C, detector temperature 280°C, column temperature 190°C. Injection conditions were 3min isothermal, then 2°C/min temperature rise. Melting points were taken on a Kofler block and were corrected. Reverse phase h.p.l.c. was performed using a Beckman Instrument Inc. Ultraspherogel ODS (octadecylsilane) derivatized column, 4.6mm ×250mm; 5μm particle size. Separation of the (*R*)- and (*S*)-1-(4'-hydroxyphenyl)ethyl acetate was carried out on a 4.6mm×250mm 'Pirkle column', which contained (*R*)-(−)-(3,5-dinitrobenzyl)-phenylglycine ionically bound to 'γ-aminopropyl silanized' silica gel particles of 5μm size (Pirkle & Finn, 1981) and was obtained from the Regis Chemical Co. Correction for the area for overlapping peaks was performed using the method described by Snyder (1972).

Results

Characterization of the product from Pseudomonas putida cells

This sample of **1** was characterized and identified by comparison with an authentic (±)- sample,

using t.l.c., h.p.l.c., and n.m.r. spectroscopy. The mass spectrum showed a molecular ion (M^+) at m/z 138 ($C_8H_{10}O_2$ requires m/z 138). The material had $[\alpha]_D^{20}$ −28.8° (*c* 0.84, water). $[\alpha]_D^{20}$ −34.88° (*c* 0.89, ethanol), and m.p. 138–145°C. Since the pure racemic material has m.p. 132–133°C (Mitsui & Imaizumi, 1954), this indicated a mixture of the (±) and (−) compound. The alcohol **1** has never been obtained in an optically active form. In order to determine the absolute configuration and optical purity of the product, it was converted into the *O*-methyl ether of the phenol.

Methylation with diazomethane gave 1-(4′-methoxyphenyl)ethanol, **2**, identical by t.l.c. with an authentic (±)-sample. The product **2** had $[\alpha]_D^{20}$ −24.20° (*c* 0.83, ethanol). The alcohol 1-(4′-methoxyphenyl)ethanol **2** was first resolved by Balfe *et al.* (1946) and found to have $[\alpha]_D^{20}$ +27.18° (neat). The same alcohol **2** was prepared with $[\alpha]_D^{20}$ +19.40° (ethanol) by Cervinka & Fusek (1968) (see also Cervinka, 1965) by asymmetric reduction of the corresponding ketone, and was assigned the (*R*) configuration. Okamoto *et al.* (1975) obtained the alcohol **2** by resolution, with $[\alpha]_D^{18}$ +15.02° (neat), and calculated its maximum rotation to be +45.2° (neat), by using an n.m.r. shift reagent method.

The product isolated in the present work, (−)-**2**, with $[\alpha]_D^{20}$ −24.20° (ethanol), is thus of the (*S*)-configuration shown, and therefore the initial product (−)-**1** must also possess the (*S*)-configuration (Fig. 1).

In order to determine the enantiomeric composition (optical purity) of the product (*S*)-(−)-**2**, the alcohol was treated with the acid chloride of optically pure (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetic acid **3** (Dale *et al.*, 1969) to give a mixture of the diastereomeric esters **4** (*S,S*) and **5** (*R,S*). These could be separated by gas chromatography which showed the product **2** to consist of the (*S*)-(−) isomer and the (*R*)-(+) isomer in the ratio of 69.5:30.5.

Characterization of the product formed by p-cresol methylhydroxylase

The sample of alcohol **1** produced by the pure enzyme was characterized by t.l.c. (silica gel; chloroform/methanol, 10:1, v/v) and had $[\alpha]_D^{20}$ −30.77° (*c* 0.90, ethanol). Its m.p. of 131–138°C similarly indicated it to be a mixture of the (±) and (−) compounds. Methylation with diazomethane gave compound **2** [identified by t.l.c. in comparison with an authentic (±)-sample], with a rotation $[\alpha]_D^{20}$ −22.58° (*c* 0.59, ethanol). Derivatization (Dale *et al.*, 1969) to form the diastereomeric esters with (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetic acid, **3**, followed by gas chromatographic analysis, showed the product to consist of the (*S*)-(−) isomer and the (*R*)-(+) isomer in the ratio of 65.6:34.4.

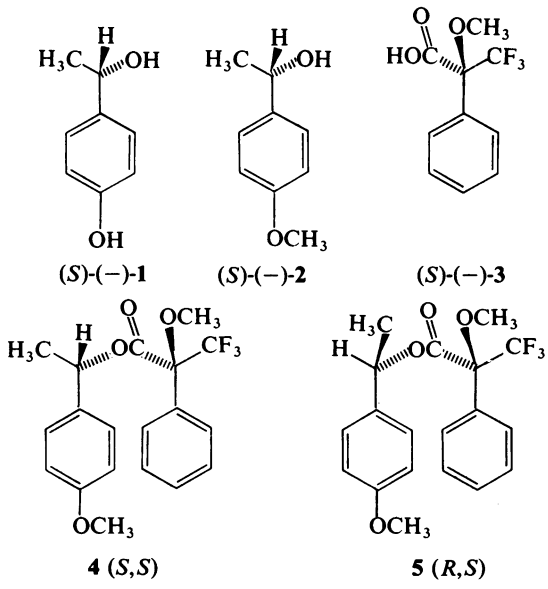


Fig. 1. *Structures for (S)-1-(4′-hydroxyphenyl)ethanol, (S)-(−)-1, the corresponding methyl ether, (S)-(−)-2, the derivatizing acid (S)-α-methoxyl-α-trifluoromethylphenylacetic acid, (S)-(−)-3, and the two diastereomers analysed by g.l.c., 4 (S,S) and 5 (R,S)*

Approx. 1–2mg of **2** was also acetylated with 150μl each of acetic anhydride and pyridine (4h at 60°C). The separation ofthe (*R*) and (*S*) derivatives of this ester on the Pirkle column is shown in Fig. 2. Integration of the peaks showed the presence of (*S*)-(−) and (*R*)-(+) isomers in the ratio of 69.9:30.1, in agreement with the results of gas-chromatographic analysis.

Discussion

The oxidation of 4-ethylphenol to 4-hydroxyacetophenone by *p*-cresol methylhydroxylase can be separated into three distinct and potentially stereospecific steps (Scheme 1). The first step is the removal of a prochiral hydrogen from 4-ethylphenol, the second step is the addition of water (Hopper, 1978) to produce (*R*)- or (*S*)-1-(4′-hydroxyphenyl)ethanol (**1**), and the third is the oxidation of the (*R*) or (*S*) form of **1** to the ketone. Of these three reactions step 2, the hydration of the putative quinone methide to **1**, seemed the easiest to study from the standpoint of stereospecificity, since it involved readily available materials and the product could be easily converted to a compound of known physical and stereochemical properties. The experimental design was to methylate **1** to the anisole derivative with diazomethane

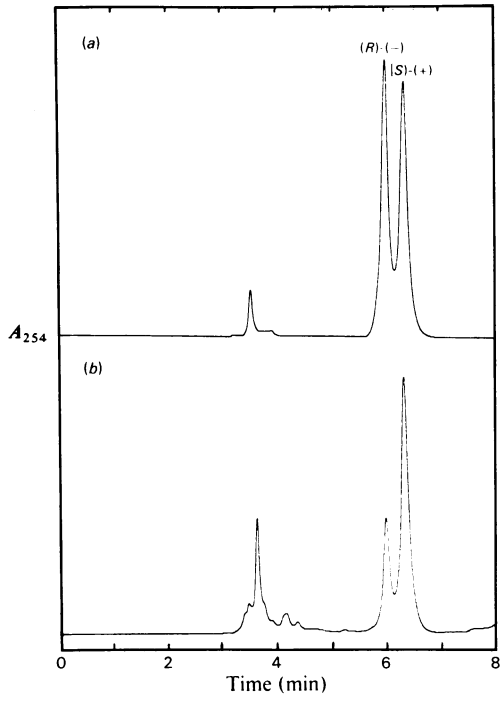


Fig. 2. *Separation of species of 1-(4′-methoxyphenyl)ethyl acetate on the 'Pirkle column'*

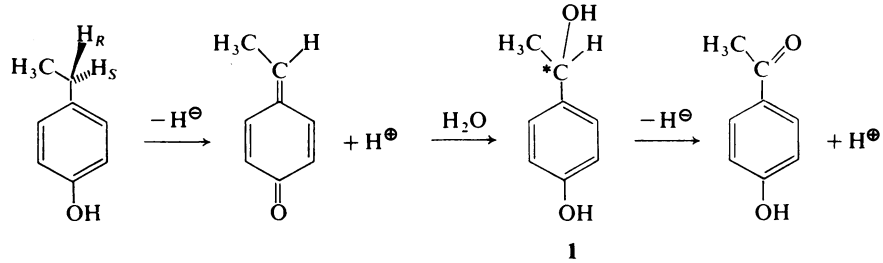
(*a*) Separation of (*R*,*S*)-1-(4′-methoxyphenyl)ethyl acetate; (*b*) separation of 1-(4′-methoxyphenyl)ethyl acetate derived from the enzymic synthesis. The solvent system used was hexane/propan-2-ol (97:3, v/v), the flow rate 1 ml/min, the eluant was monitored at 254nm and the experiment was performed at room temperature. The peaks between 3 and 4.5min are oxidation and breakdown products of the original alcohol **1**, and contaminants derived from phenazine methosulphate. The (*R*)-(+) isomer has been shown to be the first peak eluted (Kasai *et al.*, 1983). The integrated values are 49.5% (*R*)-(+) and 50.5% (*S*)-(−) for (*a*) and 30.1% (*R*)-(+) and 69.9% (*S*)-(−) for (*b*). The α value [retention volume of the (*S*) isomer minus the void volume divided by the retention volume of the (*R*) isomer minus the void volume] is 1.11 (literature value 1.12; Kasai *et al.*, 1983).

and then convert the latter to one or both of the diastereomeric esters by reaction with (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetyl chloride. The two esters were then to be separated by g.l.c. and characterized. Alternatively, the anisole derivative could also be acetylated to yield 1-(4-hydroxyphenyl)ethyl acetate, the two stereoisomers of which may be readily separated in a Pirkle column, on which the (*S*) isomer is retained more than the (*R*) form (Kasai *et al.*, 1983).

The finding that the conversion of 4-ethylphenol to 1-(4′-hydroxyphenyl)ethanol by *p*-cresol methylhydroxylase is only partially stereospecific is somewhat unexpected but by no means unique. It was demonstrated over 30 years ago that the formation of acetylmethylcarbinol from acetaldehyde by carboxylase is only partly stereospecific (Singer, 1952). Since then a number of other examples of this have been reported (Dickerson & Dalziel, 1967; Byers, 1978; Rétey *et al.*, 1978; Marletta *et al.*, 1981).

The three possibly stereospecific steps in the processing of phenols by the enzyme are the initial dehydrogenation, the hydration of the hypothetical quinone methide intermediate, and the dehydrogenation of the alcohol to the carbonyl (Scheme 1). If only the last step was not entirely stereospecific, one would not expect racemization of the alcohol **1**, because reversal of the last step, i.e. reduction of the acetophenone into the alcohol, does not occur with the pure enzyme. We must therefore assume that one or both of the prior steps yields a partly racemized product. If one of the preceding steps, e.g. the hydration step, lacked stereospecificity, yielding equal amounts of the (*R*) and (*S*) isomers of **1**, finding predominantly the (*S*)-(−) isomer in the isolated product could be explained by a preferential oxidation of (*R*)-(+)-isomer to the 4-hydroxyacetophenone.

The step in the oxidation of 4-ethylphenol to alcohol **1** most likely to be responsible for the partial racemization is the hydration of the quinone methide. One could readily visualize that



1

Scheme 1. *Reactions catalysed by p-cresol methylhydroxylase*

*, Asymmetric carbon atom.

in the hydration of the enzyme-bound quinone methide intermediate the small water molecule could enter and attack the α-carbon from either the *re* or *si* side, although the structure of the complex may be such that, for steric reasons, the probability of attack from one side is significantly greater than from the other, resulting in an approximate 7:3 ratio in favour of the (*S*)-(−) isomer.

It may be relevant in this context that the 4-ethylphenol is bound to the enzyme far more loosely than is *p*-cresol ($K_D = 2$mM versus 16 μM) and that the same type of relation appears to hold for the two alcohols, **1** versus *p*-hydroxybenzyl alcohol (McIntire, 1983), so that it is conceivable that the quinone methide intermediate is also poorly bound to the active site. If so, the extensive studies of Lehmann (1978) demonstrating that the looser the binding of drugs and other small molecules to a receptor site, the less stereospecific is the binding, would suggest a lack of complete stereospecificity in the hydration step.

Another possibility is that the partially stereoselective step is the initial binding of the 4-ethylphenol to the substrate site of the enzyme. Assuming that the substrate may be bound in either of two possible orientations, with the *pro-R* or the *pro-S* hydrogen atom oriented toward the protein and the other hydrogen atom pointing toward the flavin, and that for steric reasons one type of binding predominates, it is conceivable that this orientation persists through the dehydrogenation and hydration steps and thus predetermines the ratio of the two enantiomers of the alcohol to be produced. We hope to evaluate these possibilities experimentally in a future study.

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References

Balfe, M. P., Evans, A., Kenyon, J. & Nandi, K. N. (1946) *J. Chem. Soc.* 803–807

Byers, L. (1978) *Arch. Biochem. Biophys.* **186**, 335–342

Cervinka, O. (1965) *Coll. Czech. Chem. Commun.* **30**, 1687–1691

Cervinka, O. & Fusek, J. (1968) *Z. Chem.* **8**, 145–146

Dale, J. A., Dull, D. L. & Mosher, H. S. (1969) *J. Org. Chem.* **34**, 2543–2549

de Boer, T. J. & Backer, H. J. (1963) *Org. Syn. Coll.* **4**, 250–253

Dickerson, F. M. & Dalziel, K. (1967) *Biochem. J.* **104**, 165–172

Hopper, D. J. (1978) *Biochem. J.* **175**, 345–347

Hopper, D. J. & Taylor, D. G. (1977) *Biochem. J.* **167**, 155–162

Kasai, M., Froussios, C. & Ziffer, H. (1983) *J. Org. Chem.* **48**, 459–464

Keat, M. J. & Hopper, D. J. (1978) *Biochem. J.* **175**, 649–658

Lehmann, F. P. A. (1978) *Receptors Recognition Ser. A* **5**, 1–77

Marletta, M. A., Srere, P. A. & Walsh, C. (1981) *Biochemistry* **20**, 3719–3723

McIntire, W. (1983) Ph.D. Thesis, University of California, Berkeley

McIntire, W., Edmondson, D. E., Hopper, D. J. & Singer, T. P. (1981) *Biochemistry* **20**, 3068–3075

Mitsui, S. & Imaizumi, S. (1954) *J. Chem. Soc. Jpn.* **75**, 974–976

Okamota, K., Kinoshita, T., Takemura, Y. & Yoneda, H. (1975) *J. Chem. Soc. Perkin 2* 1426–1433

Pirkle, W. H. & Finn, J. M. (1981) *J. Org. Chem.* **46**, 2935–2938

Rétey, J., Smith, E. H. & Zagalak, B. (1978) *Eur. J. Biochem.* **83**, 437–451

Singer, T. P. (1952) *Biochim. Biophys. Acta* **8**, 108–109

Snyder, L. R. (1972) *J. Chromatogr. Sci.* **10**, 200–212

Still, W. C., Kahn, M. & Mitra, A. (1978) *J. Org. Chem.* **43**, 2923–2925