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TETRAHEDRON: *ASYMMETRY*

Synthesis of (*S*)-1-(4-hydroxyphenyl)alcohols by eugenol dehydrogenase from *Pseudomonas fluorescens* E118

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Abstract

(*S*)-1-(4-Hydroxyphenyl)ethanol and (*S*)-1-(4-hydroxyphenyl)propanol were synthesized with enantiomeric excesses of 96.6% and 95.2%, respectively, from the corresponding 4-alkylphenols by eugenol dehydrogenase from *Pseudomonas fluorescens* E118. The enantioselectivity of the enzyme was shown to be pH-dependent. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

(*S*)-1-(4-Hydroxyphenyl)alcohols [(*S*)-4-(1-hydroxyalkyl)phenols] **2** are employed in the synthesis of anti-ulcer[1] and anti-inflammatory[2] agents and liquid crystal materials.[3] The chemical access to enantiomerically enriched forms of 1-(4-hydroxyphenyl)alkanols is based on either a classical preferential crystallization of the phthalate ester salts after protection of the phenolic moiety as a formaldehyde acetal,[4] or the treatment of the *O*-protected 4-hydroxyphenyl alkyl ketones with the enantiomers of chlorodiisopinocampheylborane followed by deprotection.[5] However, due to the necessity of chiral additives and auxiliaries, and a protection and deprotection step, enzymatic one-step preparations under mild reaction conditions have attracted increased interest. So far, two enzymatic syntheses of chiral 1-(4-hydroxyphenyl)alcohols[6–8] using 4-alkylphenol methylhydroxylases from two *Pseudomonas putida* species have been described, however, these enzymes have the tendency to dehydrogenate the alcohol further to the corresponding ketones, and in one case additionally 4-vinylphenol was formed as a by-product,[6] leading to the requirement of additional separation steps during product isolation. We describe here a new enzymatic method for the synthesis of (*S*)-1-(4-hydroxyphenyl)alcohols using eugenol dehydrogenase from *Pseudomonas fluorescens* E118, which has been previously purified and

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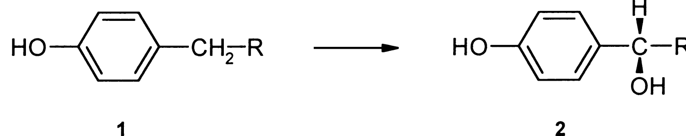
characterized.[9] This enzyme has been shown to catalyze the dehydrogenation of a wide range of 4-hydroxybenzylic structures including the conversion of 4-alkylphenols (**1**) to the corresponding 1-(4-hydroxyphenyl)alcohols after addition of an artificial electron acceptor such as phenazine methosulfate, $K_3Fe(CN)_6$, 2,6-dichlorophenol-indophenol or cytochrome *c* as the enzyme cofactor. Since the hydroxylation of prochiral 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols introduces an asymmetric carbon in the alkyl side chain, we have examined the (pro-)stereoselectivity of this enzyme.



2. Results and discussion

Eugenol dehydrogenase from *Pseudomonas fluorescens* E118 converted a number of 4-alkylphenols to give products whose absolute configurations were (*S*), as shown by HPLC using a Shimadzu LC-6A with a Chiracel OB column (4.6×250 mm, Daicel, Tokyo, Japan) and *n*-hexane:*n*-propanol (9:1, v/v) as eluent at a flow rate of 0.2 ml/min and 30°C, monitored at 280 nm. The retention times of 1-(4-hydroxyphenyl)ethanol were 23.7 min for the (*S*)-enantiomer and 27.0 min for the (*R*)-enantiomer, and the (*S*)- and (*R*)-forms of 1-(4-hydroxyphenyl)propanol eluted after 41.5 min and 45.9 min, respectively. The substrate specificity was determined in a photometric activity test in a quartz cuvette containing 1 μg of the enzyme purified as described,[9] 75 μM 2,6-dichlorophenol indophenol, 150 μM phenazine methosulfate and 10 mM of different 4-alkylphenols in 1 ml 100 mM potassium phosphate, pH 7.0. The reaction was followed by measuring the reduction of 2,6-dichlorophenol indophenol at 600 nm using a Shimadzu UV 1200 spectrophotometer. Since the enzyme activity decreased with the increasing length of the alkyl side chain or the introduction of branched alkyl chains in the substrates (4-ethylphenol 100% relative activity, 4-*n*-propylphenol 96.6%, 4-*n*-butylphenol 84.6%, 4-*n*-hexylphenol 48.7%, 4-*n*-octylphenol 17.9%, 4-isopropylphenol 7.7%, 4-*sec*-butylphenol 15.4%), we have confined our bioconversion studies here to the conversion of 4-ethylphenol and 4-*n*-propylphenol.

For each of the bioconversions of 40 mM and 75 mM 4-ethylphenol and 60 mM 4-propylphenol, 5 mg of the purified enzyme was used in a 3 h reaction in 20 ml 100 mM potassium phosphate buffer, pH 7.0, containing 10 mM phenazine methosulfate. Bioconversions were monitored by HPLC using an ODS C18 column (4.6×150, MS Instruments, Tokyo, Japan) and a methanol:H_2O:acetic acid (45:52:3, v/v/v) eluent at 1 ml/min, detected at 280 nm, and the enantiomeric excesses (e.e.) were determined from the ratio of the peak areas obtained by the above-mentioned chiral stationary phase. 4-Ethylphenol (40 mM) was converted quantitatively in a batch reaction to 40 mM (*S*)-1-(4-hydroxyphenyl)ethanol (Fig. 1A) with an enantiomeric excess of 96.6%. Due to a relatively high toxicity and inhibition effect on the enzyme, 4-propylphenol was added in three portionwise feeding steps of 20 mM each after 0, 1 and 2 h, finally leading to 60 mM (*S*)-1-(4-hydroxyphenyl)propanol (Fig. 1 B) with 95.2% e.e. Using a similar fed-batch approach for 4-ethylphenol added in three steps of 25 mM each, finally 74 mM (*S*)-1-(4-hydroxyphenyl)ethanol were accumulated with 96.6% e.e. In all of these bioconversions, neither ketones nor 4-vinylphenol, the latter of which could have only been formed in the reaction of 4-ethylphenol, were detected as by-products.

1-(4-Hydroxyphenyl)ethanol and 1-(4-hydroxyphenyl)propanol were isolated by ethyl acetate extraction, evaporation and silica gel chromatography (Wakogel C300, Wako, Osaka, Japan) using ben-

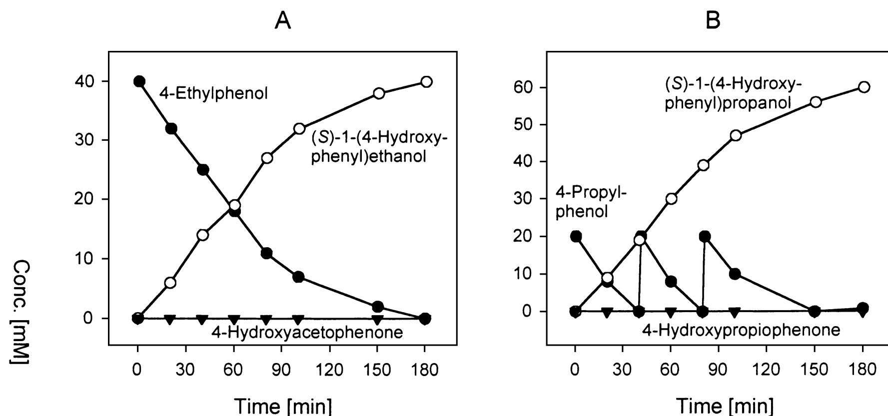


Figure 1. Time course of the conversion of 4-ethylphenol (A) and 4-propylphenol (B) by eugenol dehydrogenase from *Pseudomonas fluorescens* E118. The reactions were carried out at 30°C in 20 ml 100 mM potassium phosphate buffer, pH 7.0, containing 10 mM phenazine methosulfate and 5 mg of the purified enzyme, and followed by HPLC. 4-Ethylphenol was converted in a batch reaction, and 4-propylphenol in a fed-batch reaction

zene:methanol 95:5 (v/v) as eluent with yields of 63% and 66%, respectively. The product identity was confirmed by GC–MS and ^{1}H NMR. GC–MS spectra were recorded in a Trio-1 mass spectrometer (Raleigh, USA) connected with a 5890 Hewlett–Packard gas chromatograph (Palo Alto, USA) plus DB-1 capillary column (J&W Scientific, Tokyo, Japan) using helium as carrier gas and a temperature program of 1 min at 50°C and 50–250°C at 15°C/min. NMR spectra were obtained from a Bruker WM-360 high field NMR spectrometer (Billerica, USA) with methanol-d_4 as solvent and tetramethylsilane as internal standard. The mass spectrum of the ethanol product showed peaks at *m/z* 138 (M^+, 22.2% relative abundance), 123 (84.4%), 121 (16.7%), 120 (100%), 95 (55.6%), 94 (15.5%), 91 (71.1%), 77 (48.9%), 65 (37.8%) and 63 (17.8%) consistent with the MS pattern of authentic 1-(4-hydroxyphenyl)ethanol.[6] The *m/z* values of the (*S*)-propanol were 152 (M^+, 8.9%), 134 (33.3%), 133 (24.4%), 123 (100%), 107 (13.3%), 94 (44.4%), 91 (11.1%) and 77 (48.9%). The GC–MS data indicated the incorporation of an oxygen in the aliphatic carbon chain at the carbon adjacent to the aromatic ring. ^{1}H NMR spectra of both products indicated a methine proton adjacent to the aromatic ring and methylene protons at the α position of the aliphatic carbon chain. The chemical shifts δ for the (*S*)-ethanol product were 1.39 (d, 3, CH_3), 4.72 (q, 1, C–*H*), 6.74 and 7.18 ppm (2 d, 4, Ar–*H*), and the protons of the product of 4-propylphenol appeared at chemical shifts δ of 0.85 (d, 3, CH_3), 1.76 and 1.81 (2 d, 2, CH_2), 4.41 (q, 1, C–*H*), 6.72 and 7.13 ppm (2 d, 4, Ar–*H*). Besides identical molecule ion masses, the MS and ^{1}H NMR spectra of reference compounds bearing the alcohol group at the distal aliphatic carbon, 2-(4-hydroxyphenyl)-1-ethanol and 3-(4-hydroxyphenyl)-1-propanol, clearly differed from our bioconversion products, excluding the possibility of the hydroxylation of the distal aliphatic carbon. The specific rotation $[\alpha]_D^{20}$ of the isolated (*S*)-ethanol product, measured in a Jasco DIP-1000 digital polarimeter (Easton, USA), was −44.8 (*c* 1.74, ethanol) in agreement with the value of the almost enantiomerically pure (*S*)-form $[\alpha]_D^{20}$ −47.5 (*c* 4.98, ethanol).[6] The specific rotation $[\alpha]_D^{20}$ of the isolated (*S*)-propanol was −36.7 (*c* 1.35, ethanol).

The enantiomeric excess of the products depended on the pH of the bioconversion mixture with the highest e.e. values reached at pH 7.0 (Fig. 2). This is likely due to the pH-dependent enantioselectivity of the enzyme, because the enantiomeric excesses of the products were not affected by acid and base treatment. A pH-dependent stereoselectivity has not been reported so far for similar 4-alkylphenol methylhydroxylases.[6–8] The enantioselectivities of eugenol dehydrogenase were in the range of the

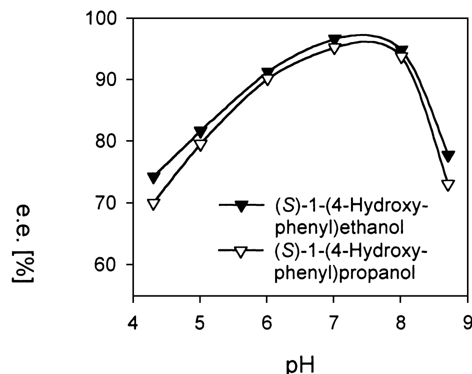


Figure 2. Effect of the pH on the enantioselectivity of eugenol dehydrogenase from *Pseudomonas fluorescens* E118. The reaction mixture was composed as described in the legend of Fig. 1 except for using potassium phosphate buffer of different pH. The reaction was stopped after 3 h, and e.e. values were determined by chiral HPLC

analogous bioconversions catalyzed by 4-alkylphenol methylhydroxylases, which resulted in 31–94% e.e. for the (*S*)-alcohol[6,7] and 92–98% e.e. for the (*R*)-alcohol,[8] depending on the kind of alkyl rest. On the other hand, the 4-alkylphenol methylhydroxylases were only enantioselective with cytochrome *c*, but almost not with phenazine methosulfate, leading to a nearly racemic mixture of the alcohol products when phenazine methosulfate was used. This contrasts with eugenol dehydrogenase, which was highly enantioselective with phenazine methosulfate, and the enantioselectivity of which did not depend on the kind of electron acceptor as tested with phenazine methosulfate, $K_3Fe(CN)_6$, 2,6-dichlorophenol-indophenol and cytochrome *c*. The asymmetric bioconversion described here has advantages compared to previous enzymatic preparations with regard to productivity, yield and absence of by-products.

Acknowledgements

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