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Enantioselective Oxidation of 2-Methyl-1-Butanol by Alcohol Oxidase from Methylotrophic Yeasts

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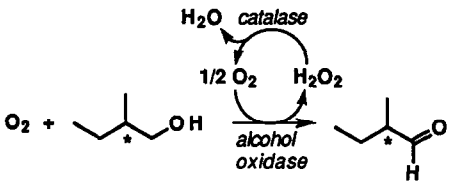
Abstract: The oxidation of racemic 2-methyl-1-butanol by the soluble alcohol oxidase from *Candida boidinii* produced an enantiomeric excess of (*R*)-2-methyl-1-butanol (E = 1.65) as the reaction progressed. This degree of enantioselectivity was not observed with alcohol oxidase isolated from either *Hansenula sp.* or *Pichia pastoris*.

Introduction

Methylotrophic yeasts of the genera *Candida, Pichia, Hansenula,* and *Torulopsis* each produce an alcohol oxidase (AOX) (oxygen oxidoreductase, EC 1.1.3.13) and catalase as major peroxisomal enzymes when grown on methanol.[2] AOX catalyzes the oxidation of alcohols by oxygen to the corresponding aldehyde and hydrogen peroxide. Catalase decomposes the coproduct hydrogen peroxide, which is detrimental to AOX activity[3] and product stability. Substrate specificity in aqueous solution is limited to C_1 - C_5 primary alkanols[4] and aromatic primary alcohols such as benzyl alcohol.[5] AOX activity is usually inhibited by accumulating aldehyde, therefore reactions are often performed in aqueous solutions of amine buffers.[6] The substrate specificity of AOX has been extended to C_6 - C_{11} primary aliphatic alkanols by using aqueous-organic solvents.[7]

We have now examined the enantioselectivity of AOX from several methylotrophic yeasts using 2-methyl-1-butanol (Scheme 1), which has previously been shown to be a substrate of AOX from *Pichia sp.*[4b] and *Pichia pastoris*.[8] Chiral 2-methyl-alkanols are useful as synthons in organic synthesis, and have been prepared enzymatically using lipases, or by chiral reduction of the corresponding aldehyde using baker's yeast.[9] The only prior report of enantioselective alcohol oxidation by an AOX from methylotrophic yeast is for the oxidation of (*R*)- and (*S*)-{1-^{3}H}-ethanol with AOX from *Candida boidinii*,[10] where a 5:1 preference for the removal of the *pro*-1-*R* hydrogen was observed. The chiral center of 2-methyl-1-butanol is α to the site of oxidation, and the influence of this α-chiral center on the enantioselectivity of the oxidation remained to be determined.

Scheme 1



Results and Discussion

The oxidation of racemic 2-methyl-1-butanol (0.15 M) in Tris buffer (0.25 M, pH 8.0) at 27 °C and under 70 psig of oxygen was examined using as catalysts the soluble AOX from *C. boidinii*, *P. pastoris* or *H. sp.*, and soluble *Aspergillus niger* catalase. The ratio of enzyme activities of alcohol oxidase to catalase employed was ca. 1:220. Table 1 summarizes the results of three reactions run to ca. 50 % conversion with the different sources of soluble AOX. The selectivity to 2-methylbutyraldehyde was high for all three reactions (aldehyde ee was not determined). The reaction time varied considerably with the different sources of AOX, even though the reactions employed similar initial concentrations of AOX activity. Reactions using soluble *C. boidinii* AOX afforded reaction mixtures enriched in (*R*)-2-methyl-1-butanol, indicating a preference for oxidation of the *S*-enantiomer. *P. pastoris* and *H. sp.* AOX each showed only a slight preference for oxidation of the *S*-enantiomer (< 3 % $ee_{remaining\ substrate}$ at 50 % conversion). Reactions run using permeabilized whole cells of *P. pastoris* as catalyst resulted in enantioselectivity identical to that observed with the soluble *P. pastoris* AOX (data not shown).

Table 1

source of alcohol oxidase	reaction time (h)	2-methyl-1-butanol conversion (%)	2-methylbutyraldehyde selectivity (%)	(*R*)-2-methyl-1-butanol ee (%)
Candida boidinii	24	52	99	16.6
Pichia pastoris	47	51	97	1.6
Hansenula sp.	95	54	100	2.4

A time course for the oxidation of racemic 2-methyl-1-butanol to 2-methylbutyraldehyde using soluble *C. boidinii* AOX is depicted in Figure 1. The dependence of $ee_{remaining\ substrate}$ for (*R*)-2-methyl-1-butanol on conversion of racemic 2-methyl-1-butanol is illustrated by Figure 2, where oxidations were performed using AOX from *C. boidinii* (three separate reactions), *Hansenula. sp.*, and *P. pastoris*. Reactions utilizing *C. boidinii* AOX clearly show an enantioselectivity over a range of conversion of 2-methyl-1-butanol which was not observed with either of the other sources of AOX. At 82 % conversion of substrate, a 47.6 % $ee_{remaining\ substrate}$ was obtained with *C. boidinii* AOX. A calculated enantiomeric ratio (*E*) of 1.65, generated using equations developed by Sih and coworkers[11] for quantitative treatment of enzyme-catalyzed kinetic resolution data, provided the best fit for the three sets of data for reactions run with *C. boidinii* AOX (Figure 2).

To our knowledge, this is the first report of the preparation of an enantiomerically-enriched α-substituted primary alkanol using a methylotrophic yeast alcohol oxidase. Although an *E* of 1.65 is relatively low for an enantioselective reaction, and the $ee_{remaining\ substrate}$ does not exceed 95 % at some practical conversion of 2-methyl-1-butanol, the fact that *C. boidinii* AOX can catalyze an enantioselective oxidation may make possible the development of a new route for the preparation of chiral alcohols or their derivatives. Screening a selection of chiral primary alcohols with *C. boidinii* AOX may result in higher ee than that obtained for 2-methyl-1-butanol, as may the screening of sources of AOX other than methylotrophic yeasts. Using AOX for the preparation of chiral alcohols is a simple reaction to perform, and would not require enzyme cofactors (as in the case of alcohol dehydrogenase) or the preparation and hydrolysis of esters (as when using lipases). Screening of additional chiral alcohols with *C. boidinii* AOX is underway, and a determination of the ee of the resulting aldehydes will also be performed.

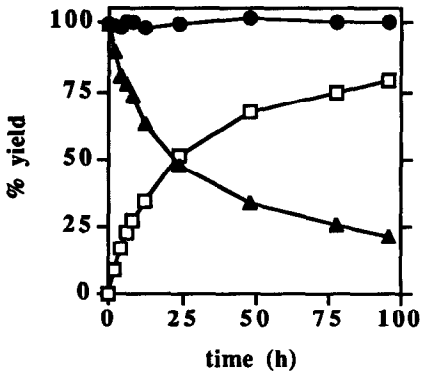


Figure 1. Time course for oxidation of 2-methyl-1-butanol (0.15 M) in Tris buffer (0.25 M, pH 8.0) using *C. boidinii* alcohol oxidase (29.3 IU/mL) and *A. niger* catalase (6,478 IU/mL); 2-methyl-1-butanol (▲), 2-methylbutyraldehyde (❑), mass balance (●).

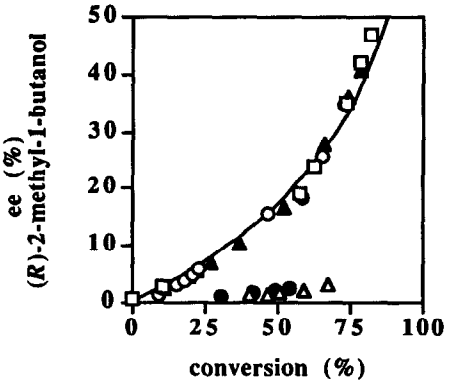


Figure 2. Enantiomeric excess (remaining substrate) versus conversion of 2-methyl-1-butanol using *C. boidinii* (29.3 IU/mL) (❑, ○, ▲; three separate runs), *P. pastoris* (30.0 IU/mL) (△), *H. sp.* (28.0 IU/mL) (●), and calculated $E = 1.65$ (line).

Experimental Section

Materials. (*S*)-(-)-2-methyl-1-butanol (99 %) was obtained from Kodak; (*R*)-(+)-2-methyl-1-butanol was not commercially available. All other chemicals were obtained from commercial sources and used as received. *Canidida boidinii, Pichia pastoris* and *Hansenula sp.* alcohol oxidase (EC 1.1.3.13), *Aspergillus niger* catalase (EC 1.11.1.6), and Equine Liver alcohol dehydrogenase (EC 1.1.1.1) were purchased from Sigma and used without further purification. *Pichia pastoris* GTS 115 (*his4*) was obtained from Phillips Petroleum Co. (NRRL Y-15851), and grown according to a published procedure.[12] *P. pastoris* whole cells required permeabilization prior to use as catalyst; cells were permeabilized by mixing a suspension of 10 wt % wet cells in 0.2 % (w/v) benzalkonium chloride (50 mM phosphate buffer, pH 7.0) at 27 °C for 60 min, then washing the permeabilized cells with phosphate buffer (50 mM, pH 7.0).[13]

Assays. Alcohol oxidases were initially assayed for activity using a coupled enzyme assay procedure. A 3-mL quartz cuvette containing 2.45 mL of phosphate buffer (0.1 M, pH 7.5) and 90 μL of 11.28 mM β-nicotinamide adenine dinucleotide (reduced form) in water was capped with a rubber septum, and the solution bubbled with oxygen for three minutes. To the cuvette was then added 300 μL of a solution of 3.4 units alcohol dehydrogenase/mL phosphate buffer (0.1 M, pH 7.5), and 100 μL of a solution containing ca. 0.17 IU of alcohol oxidase in this same buffer. The assay solution was briefly mixed and 60 μl of 2.5 M ethanol (in same phosphate buffer) was added. The rate of decomposition of β-NADH was measured by the change in absorbance at 340 nm ($\varepsilon = 6.22$ L mM^{-1} cm^{-1}) for 180 s at 27 °C. Catalase activity was measured using a reported enzyme assay which measures the decomposition of hydrogen peroxide.[14]

Oxidations. Into a 3 oz. Fischer-Porter glass reaction vessel equipped with magnetic stir bar was placed 10 mL of an aqueous solution containing racemic 2-methyl-1-butanol (0.15 M), Tris buffer (0.25 M, pH 8.0), and as catalyst either soluble alcohol oxidase (ca. 300 IU) and *A. niger* catalase (ca. 65,000 IU), or 0.34 g of permeabilized *P. pastoris* whole cells. The vessel was sealed and flushed with oxygen, then pressurized with

oxygen at 70 psig (483 kPa) and stirred at 27 °C.

Determination of conversion and enantiomeric excess. The conversions and enantiomeric excess of remaining substrate in oxidation reactions were determined using a Hewlett Packard 5890A gas chromatograph equipped with an ASTEK Chiraldex™ G-TA (γ-cyclodextrin, trifluoroacetyl) capillary column (30 m x 0.25 mm), carrier gas He (25 mL / min) with a split ratio of 100:1 at 30 °C; baseline separation of (*S*)- and (*R*)-2-methyl-1-butanol (24.5 and 25.3 min, respectively) was obtained. Conversions were calculated from the total area of the alcohol peaks relative to the peak area for octane internal standard, and enantiomeric $\text{excess}_{\text{remaining substrate}}$ were obtained from the ratio of peak areas of (*S*)- and (*R*)-2-methyl-1-butanol. Analytical samples were prepared in the following manner: 150 μL of MES [2-(N-morpholino)ethanesulfonic acid] buffer (0.25 M, pH 5.2) was added to 150 μL of reaction mixture (final pH 6.0, liberating the aldehyde from its imine with Tris), then the resulting solution was extracted with 150 μL of 0.05 M octane in methylene chloride and then 150 μL of methylene chloride. The combined organic extracts were analyzed by chiral GC for (*S*)- and (*R*)-2-methyl-1-butanol and 2-methylbutyraldehyde.

References and Notes

1. Present address: Institutes for Applied Research, Ben Gurion University of the Negev, P.O. Box 1025, Beer Sheva 84110, Israel.
2. (a) Van der Klei, I. J.; Harder, W.; Veenhuis, M. *Yeast* **1991**,*7*; 195-209. (b) Woodward, J. R. Biochemistry and Applications of Alcohol Oxidase from Methylotrophic Yeasts. In *Advances in Autotrophic Microbiology and One-Carbon Metabolism*; Codd, G. A., Ed.; Kluwer Academic Publishers: Dordrecht, Netherlands, 1990; pp. 193-225.
3. Geissler, J.; Kroneck, P. M. H.; Ghisla, S. In *Flavins and Flavoproteins*; Walter de Gruyter & Co.; Berlin, 1984, 569-572.
4. (a) Tani, Y.; Miya, T.; Ogata, K. *Agr. Biol. Chem.* **1972**, *36*, 76-83. (b) Patel, R. N.; Hou, C. T.; Laskin, A. I.; Derelanko, P. Microbial Oxidation of Methanol: Properties of Crystallized Alcohol Oxidase from *Pichia Sp.*. In Flavins and Flavoproteins, Massey, V.; Williams, C. H., Eds.; Elsevier North Holland: Amsterdam, Netherlands, 1982; pp 196-201.
5. Duff, S. J. B.; Murray, W. D. *Biotechnol. Bioeng.* **1989**, *34*, 153-159.
6. (a) Duff, S. J. B.; Murray, W. D.; Overend, R. P. *Enzyme Microb. Technol.* **1989**, *11*, 770-775. (b) Shachar-Nishri, Y.; Freeman, A. *Appl. Biochem. Biotechnol.* **1993**, *39/40*, 387-399.
7. Murray, W. D.; Duff, S. J. D. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 202-205.
8. Murray, W. D.; Duff, S. J. B.; Lanthier, P. H., U.S. Patent 4,871,669, October 3, 1989.
9. (a) Hoegberg, H. E. *NATO ASI Ser., Ser. C* **1992**, *381* (Microbial Reagents in Organic Synthesis), 399-410. (b) Barth, S.; Effenberger, F. *Tetrahedron Asymmetry*, **1993**, *4*, 823-833.
10. Cardemil, E. *Biochem. Biophys. Res. Commun.* **1975**, *67*, 1093-1099.
11. Chen, C.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Amer. Chem. Soc.*, **1982**, *104*, 7294-7299.
12. Duff, S. J. B.; Murray, W. D. *Biotechnol. Bioeng.* **1986**, *31*, 44-49
13. Zhang, M.; Wang, H. Y. *Enzyme Microb. Technol.* **1994**, *16*, 10-17.
14. Seip, J. E.; Fager, S. K.; Gavagan, J. E.; Gosser, L. W.; Anton, D. L.; Di Cosimo, R. *J. Org. Chem.*, **1993**, *58*, 2253-2259.

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