Pergamon

*Bioorganic & Medicinal Chemistry Letters*, Vol. 5, No. 13, pp. 1383-1388, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00228-6

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

Dawn S. Clark, Shimona Geresh[1] and Robert DiCosimo*

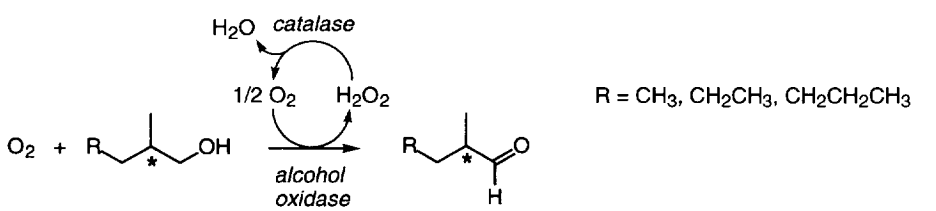
*DuPont Central Research and Development Department, Experimental Station, P.O. Box 80328, Wilmington, Delaware 19880-0328*

**Abstract:** The oxidation of several racemic 2-methyl-1-alkanols by the alcohol oxidase from *Candida boidinii*, *Hansenula sp.*, *Pichia pastoris*, and *Torulopsis methanothermo* afforded reaction mixtures enriched in (*R*)-2-methyl-1-alkanol at incomplete conversions. The alcohol oxidase from *C. boidinii* exhibited the highest enantioselectivity, with the greatest enantiomeric excess obtained for (*R*)-2-methyl-1-pentanol (90.6 % ee at 76 % conversion; $E = 4.8$).

## Introduction

Chiral 2-methyl-1-alkanols are useful as synthons in organic synthesis, and have been prepared enzymatically using lipases,[2] or by chiral reduction of the corresponding aldehyde[2a] or 2-substituted allyl alcohol[3] using baker's yeast. We have previously reported that oxidation of racemic 2-methyl-1-butanol by the alcohol oxidase (AOX, EC 1.1.3.13) from the methylotrophic yeast *Candida boidinii* produced an enantiomeric excess of the remaining alcohol ($E = 1.6$) as the reaction progressed.[4] The AOX from the methylotrophic yeasts *Candida boidinii*, *Hansenula sp.*, *Pichia pastoris*, and *Torulopsis methanothermo* have now each been examined as catalysts for the enantioselective oxidation of several 2-methyl-1-alkanols (Scheme 1). AOX catalyzes the oxidation of the alcohols by oxygen to the corresponding aldehydes and hydrogen peroxide.[5] Catalase is included in reaction mixtures to decompose hydrogen peroxide, which is detrimental to AOX activity[6] and product stability. AOX activity was inhibited by accumulating aldehyde, therefore reactions were performed in aqueous solutions of Tris buffer.[7] The substrate specificity of each of the AOXs has been reported to be limited to $C_1$ - $C_5$ alkanols,[8] which limited the range of chiral substrates which could be screened with these enzymes.

Scheme 1



## Results and Discussion

The enantioselectivity of methylotrophic yeast AOXs for oxidation of racemic 2-methyl-1-butanol (0.150 M), 2-methyl-1-pentanol (0.050 M), and 2-methyl-1-hexanol (0.020 M) in Tris buffer and under oxygen pressure was examined, and the results are listed in Table 1. The ratio of enzyme activities of AOX to catalase employed was ca. 1:220. Reactions run using either AOX immobilized on oxirane acrylic beads or permeabilized whole cells as catalyst resulted in enantioselectivities identical to that observed with the soluble AOX (data not shown).

Table 1. Enantioselectivity of 2-methyl-1-alkanol oxidations using AOXs from methylotrophic yeasts.

| alkanol | AOX source | time (h) | conversion (%) | aldehyde selectivity (%) | (*R*)-2-methyl-1-alkanol ee (%) | *E* |
|---|---|---|---|---|---|---|
| 2-methyl-1-butanol[a] | *C. boidinii* | 24 | 52 | 99 | 16.6 | 1.6 |
| " | *P. pastoris* | 47 | 51 | 97 | 1.6 | 1.1 |
| " | *H. sp.* | 95 | 54 | 100 | 2.4 | 1.1 |
| 2-methyl-1-pentanol | *C. boidinii* | 24 | 43 | 100 | 40.0 | 4.8 |
| " | *P. pastoris* | 48 | 39 | 96 | 18.4 | 2.1 |
| " | *H. sp.* | 72 | 41 | 100 | 10.0 | 1.5 |
| " | *Torulopsis* | 48 | 19 | 95 | 8.2 | 2.3 |
| 2-methyl-1-hexanol | *C. boidinii* | 72 | 42 | 100 | 30.0 | 3.2 |
| " | *P. pastoris* | 24 | 16 | 96 | 0.4 | 1.1 |
| " | *H. sp.* | 216 | 47 | 100 | 12.2 | 1.5 |

[a] from reference 4.

Oxidation of the three racemic 2-methyl-1-alkanols by each of the AOXs produced reaction mixtures enriched in (*R*)-2-methyl-1-alkanol, indicating a preference for oxidation of the *S*-enantiomer by all four AOXs. The AOX from *P. pastoris*, *Hansenula sp.*, and *T. methanothermo* each showed a significantly lower preference for oxidation of the (*S*)-enantiomer when compared to *C. boidinii* AOX. The dependence of $ee_{remaining\ substrate}$ on the conversion of the racemic 2-methyl-1-butanol, 2-methyl-1-pentanol, and 2-methyl-1-hexanol for oxidations performed using AOX from *C. boidinii* is depicted in Figure 1, together with the calculated enantiomeric ratio (*E*)[9] which provided the best fit for each set of data.

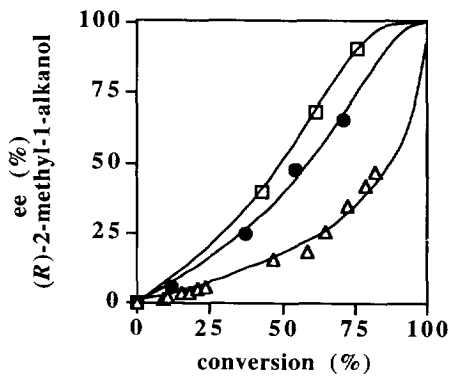



**Figure 1.** Enantiomeric excess (ee, remaining substrate) versus conversion of 2-methyl-1-butanol (Δ) and calculated enantiomeric ratio (*E*) of 1.65 (—), 2-methyl-1-pentanol (□) and calculated *E* of 4.80 (—), and 2-methyl-1-hexanol (●) and calculated *E* of 3.20 (—), using *Candida boidinii* AOX (29.3 IU/mL).

The greatest enantiomeric excess was obtained for the oxidation of 2-methyl-1-pentanol (90.6 % $ee_{substrate}$ at 76 % conversion). No enantioselectivity was observed when using any of the four AOXs to oxidize racemic 3-methyl-1-pentanol, where the chiral center was two carbon atoms removed from the carbinol carbon. The range of 2-alkyl-1-alkanols which could be oxidized by the four AOXs was very limited; oxidation of 2-ethyl-1-hexanol was not detected under reaction conditions identical to those for 2-methyl-1-hexanol. For chiral 2-alkanol oxidation, neither *C. boidinii* nor *P. pastoris* AOX showed any preference for either enantiomer at conversions of up to 33 % of racemic 2-butanol.

For oxidations of racemic 2-methyl-1-butanol with *C. boidinii* AOX in aqueous buffer, the observed ee for the product 2-methylbutyraldehyde was significantly lower than a calculated value based on the ee of remaining 2-methyl-1-butanol; this $ee_{product}$ dropped to almost zero at 50 % conversion. It was determined that Tris buffer added to aqueous reaction mixtures to reduce product inhibition of AOX (by formation of an imine/hemiaminal with 2-methylbutyraldehyde) was responsible for racemization of the aldehyde. The *C. boidinii* AOX was therefore immobilized on oxirane acrylic beads, and the reaction repeated in hexane (to limit product inhibition in the absence of Tris) using the immobilized AOX and catalase immobilized on 4% beaded agarose. The observed $ee_{product}$ and calculated $ee_{product}$ for (*S*)-2-methylbutyraldehyde at 8 % conversion of 2-methyl-1-butanol in hexane were 19.4 % and 22.0 %, respectively, demonstrating that the *S*-enantiomer of the product was produced in excess, as expected. The rate of oxidation of 2-methyl-1-butanol in hexane was ten-fold greater than in aqueous buffer under the same reaction conditions; the increased rate was most likely due to the increased solubility of oxygen in hexane.

It has been previously reported that for some enzymatic reactions run in non-aqueous media, the degree of enantioselectivity may change, or even be reversed, by changing the solvent.[10] Typically, major changes in enantioselectivity have often been observed when changing from a hydrophobic solvent to a hydrophilic solvent, or by changing the activity of water in a given solvent. In the present case, *C. boidinii* AOX immobilized on oxirane beads was completely inactive in the hydrophilic solvents tetrahydrofuran, acetonitrile and *tert*-butanol, but remained active in the hydrophobic solvents hexane, methylene chloride and toluene. No difference in the enantioselectivity of 2-methyl-1-butanol oxidations in hexane or toluene were observed when compared to reactions run in aqueous buffer (Table 2).

Table 2. Enantioselectivity of 2-methyl-1-butanol oxidation using immobilized *C. boidinii* AOX in organic solvents.

| solvent | time (h) | 2-methyl-1-butanol conversion (%) | 2-methylbutyraldehyde selectivity (%) | (*R*)-2-methyl-1-butanol ee (%) | *E* |
|---|---|---|---|---|---|
| hexane | 48 | 84 | 100 | 39.3 | 1.6 |
| toluene | 24 | 35 | 100 | 10.8 | 1.7 |

Prior to this work, the only reports of enantioselective alcohol oxidation by an AOX from a methylotrophic yeast was for the oxidation of (*R*)- and (*S*)-{1-$^3$H}-ethanol with *C. boidinii* AOX, where a preference for the removal of the *pro*-1-*R* hydrogen was observed.[11] We have determined that the AOXs from methylotrophic yeasts catalyze the oxidation of several short-chain 2-methyl-1-alkanols with relatively low enantioselectivity. Similarly, a small *E* (1.6) for the oxidation of racemic glycidol by *Hansenula polymorpha* AOX has recently

been reported.[12] Low enantioselectivities, together with the limited range of substrates which can be oxidized, limits the practical application of methylotrophic yeast alcohol oxidases for the preparation of chiral 2-alkyl-1-alkanols or their derivatives. For resolution of racemic 2-alkyl-1-alkanols by lipase-catalyzed esterification with vinyl acetate in dichloromethane,[2b] the observed enantioselectivity for 2-methyl-1-pentanol and 2-methyl-1-hexanol ($E$ = 5.9 and 8.7, respectively) was only slightly greater than that produced by *C. boidinii* AOX, but $E$ increased markedly with increasing chain length of the alkanol.[2a-d] Identification of an alcohol oxidase which has both a broad substrate specificity and enantioselectivities comparable to alternative enzymatic resolution methods currently available could result in a method for the preparation of chiral 2-alkyl-1-alkanols which would not require cofactor regeneration (as in the case of alcohol dehydrogenases) or the preparation or hydrolysis of esters (when using) lipases, and thereby offer a preferred method for selected resolutions.

## Experimental Section

*Materials.* 2-Methyl-1-hexanol was purchased from Narchem Corporation. (*S*)-(-)-2-methyl-1-butanol (99 %) was obtained from Kodak; (*R*)-(+)-2-methyl-1-butanol was not commercially available. Neither enantiomer of 2-methyl-1-pentanol or 2-methyl-1-hexanol was commercially available, therefore an enriched mixture of (*R*)-2-methyl-1-pentanol or (*R*)-2-methyl-1-hexanol was prepared by the lipase-catalyzed esterification of the racemic mixture with vinyl acetate in methylene chloride.[2b-c] 2-Methylhexanal was prepared from 2-methyl-1-hexanol according to a reported method[13] as described below. All other chemicals were obtained from commercial sources and used as received. *Candida boidinii*, *Pichia pastoris* and *Hansenula sp.* alcohol oxidase (EC 1.1.3.13), *Aspergillus niger* catalase (EC 1.11.1.6), and peroxidase Type 1 from horseradish (EC 1.11.1.7) were purchased from Sigma and used without further purification. Eupergit® C250L oxirane acrylic beads were purchased from Accurate Chemicals. *Torulopsis methanothermo* (ATCC 20434, cultured in ATCC media 436) whole cells required permeabilization prior to use as catalyst; cells were permeabilized by mixing a suspension of 10 % (w/v) wet cells in 0.2 % (w/v) benzalkonium chloride (50 mM phosphate buffer, pH 7.0) at 27 °C for 1 h, then washing the permeabilized cells with phosphate buffer (50 mM, pH 7.0).[14]

*2-Methylhexanal.* A solution of 1.0 mL (11 mmol) of oxalyl chloride in 25 mL of dry methylene chloride was cooled to -78 °C and 1.7 mL (22 mmol) of dimethyl sulfoxide in 5 mL of methylene chloride was added dropwise. After stirring for two minutes, 1.40 mL (10 mmol) of 2-methyl-1-hexanol in 10 mL of methylene chloride was added dropwise. The resulting solution was stirred for 0.25 h, then 7.0 mL (50 mmol) of dry triethylamine was added in one portion. The resulting solution was allowed to warm to room temperature with stirring for 16 h, then poured into 50 mL of water and extracted with methylene chloride (2 x 50 mL). The combined organic layers were washed with saturated NaCl, dilute HCl (1%), dilute sodium carbonate (5%), and water, then dried over magnesium sulfate and evaporated to afford 1.02 grams (89 % yield) of a clear oil: $^1$H NMR[15] (300 MHz, $CDCl_3$) $\delta$ 9.63 (d, 1H, CHO), 2.30 (m, 1 H, -C*H*CHO), 1.08 (d, 3 H, $-CH_3$), 0.93 (t, 3 H, $-CH_2CH_3$); $^{13}$C NMR $\delta$ 13.5, 14.0, 22.9, 29.3, 30.5, 46.5, 205.2.

*Immobilization of alcohol oxidase on oxirane acrylic beads.* Into a 15 mL polypropylene tube was weighed 950 mg of oxirane acrylic beads (Eupergit® C250L), then 14 mL of bicine buffer (0.10 M, pH 8) was added. The oxirane acrylic beads were suspended in the buffer by briefly mixing, then allowed to settle, and the fine particles which remained suspended were removed by pipet, along with as much of the supernatant which could be removed without disturbing the settled beads. The washing procedure was repeated three times, then a

solution of *C. boidinii* alcohol oxidase (293 IU) in 9.6 mL of bicine buffer (0.10 M, pH 8) was added to the contents of the tube. The resulting mixture was mixed on a rotating platform at room temperature for 24 hours. The supernatant was then removed, and the immobilized enzyme washed three times with phosphate buffer (0.10 M, pH 7.5) prior to assaying for activity (50 IU/gram oxirane beads). The immobilized enzyme was stored in this same wash buffer at 5 °C until use.

*Assays.* The enzymatic activity of alcohol oxidase was measured using a modification of a previously reported assay.[14] The assay solution contained 10 mg of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1.25 mL methanol, 625 μL peroxidase solution (2 mg/mL in 0.10 M $KH_2PO_4$ buffer, pH 7.5), and 60.6 mL of 0.10 M $KH_2PO_4$ buffer (pH 7.5). This assay solution was prepared immediately prior to use. To a 3-mL quartz cuvette containing 2.50 mL of the above assay solution was then added a solution containing ca. 0.01 IU of soluble AOX in 0.10 M $KH_2PO_4$ buffer (pH 7.5). The rate of oxidation of ABTS was measured by the change in absorbance at 405 nm ($\varepsilon$ = 36.8 L $mM^{-1}$ $cm^{-1}$) for 180 s at 25 °C; assays of immobilized enzymes or permeabilized cells were performed as above with stirring of a suspension of the beads or cells in the cuvette. Catalase activity was measured using a reported enzyme assay which measures the decomposition of hydrogen peroxide at 210 nm.[16]

*Oxidations in Aqueous Buffer.* Into a 3 oz. Fischer-Porter glass reaction vessel equipped with magnetic stir bar was placed 10 mL of an aqueous solution containing either racemic 2-methyl-1-butanol (0.150 M), racemic 2-methyl-1-pentanol (0.050 M), or racemic 2-methyl-1-hexanol (0.020 M) in Tris buffer (0.250 M, pH 8.0), and as catalyst either soluble alcohol oxidase (ca. 300 IU) and *A. niger* catalase (ca. 65,000 IU), or 1.0 g of permeabilized *T. methanothermo* whole cells. The vessel was sealed and flushed with oxygen, then pressurized with oxygen at 70 psig (483 kPa) and stirred at 27 °C.

*Oxidations in Organic Solvents.* The alcohol oxidase immobilized on oxirane acrylic beads was washed with either hexane, toluene, dichloromethane, tetrahydrofuran, acetonitrile, or *tert*-butanol (3 x 10 mL) prior to use in the same solvent. Into a 20 mL thick-walled glass tube was placed 100 mg of the immobilized alcohol oxidase (ca. 5 IU), catalase immobilized on 4% beaded agarose (ca. 3,300 IU) and 2 mL of racemic 2-methyl-1-butanol (0.050 M) in solvent. The tube was sealed with a bottle cap and flushed with oxygen, then pressurized with oxygen to the desired pressure and mixed on a rotary shaker at (250 x 100) rpm at 27 °C. There was no apparent difference in the rate of oxidation when using oxygen at pressures from 14.7 (101 kPa) to 70 psig (483 kPa) (data not shown).

*Determination of Conversion and Enantiomeric Excess.* The conversion and enantiomeric excess of remaining substrate in oxidation reactions were determined by gas chromatography using an ASTEK Chiraldex™ G-TA (γ-cyclodextrin, trifluoroacetyl) capillary column (30 m x 0.25 mm, or 50 m x 0.25 mm). Conversions were calculated from the total area of the alcohol peaks (underivatized) relative to the peak area for octane internal standard, and enantiomeric $excess_{remaining\ substrate}$ were obtained from the ratio of peak areas of (*S*)- and (*R*)-2-methyl-1-butanol, (*S*)- and (*R*)-trifluoroacetyl derivatives of 2-methyl-1-pentanol, and (*S*)- and (*R*)-trifluoroacetyl derivatives of 2-methyl-1-hexanol. Analytical samples were prepared in the following manner: 150 μL of MES [2-(N-morpholino)ethanesulfonic acid] buffer (0.250 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 the 2-methyl-1-alkanols and 2-methyl-1-alkanals.

The trifluoracetyl derivatives were prepared by mixing 200 μL of the final organic extract and 150 μL of trifluoroacetic anhydride. The resulting solution was mixed for one minute and then evaporated under nitrogen. The residue was dissolved in 100 μL of methylene chloride and analyzed by chiral GC. The $ee_{product}$ for 2-methylbutyraldehyde was determined by extracting a hexane reaction mixture with water to remove unreacted 2-methyl-1-butanol, then reducing the aldehyde with lithium aluminum hydride and analyzing the resulting 2-methyl-1-butanol.

## References


1. Present address: Institutes for Applied Research, Ben Gurion University of the Negev, P.O. Box 1025, Beer Sheva 84110, Israel.
2. (a) Högberg, 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. (c) Indlekofer, M.; Reuss, M.; Barth, S.; Effenberger, F. *Biocatalysis* **1993**, *7*, 249. (d) Nordin, O.; Hedenström, E.; Högberg, H. E. *Tetrahedron Asymmetry* **1994**, *5*, 785.
3. Ferraboschi, P.; Casati, S.; Santaniello, E. *Tetrahedron Asymmetry* **1994**, *5*, 19.
4. Clark, D. S., Geresh, S.; DiCosimo, R. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1745.
5. (a) Van der Klei, I. J.; Harder, W.; Veenhuis, M. *Yeast* **1991**, *7*, 195. (b) Woodward, J. R. In *Advances in Autotrophic Microbiology and One-Carbon Metabolism*; Codd, G. A., Ed.; Kluwer Academic Publishers: Dordrecht, Netherlands, 1990; pp 193-225.
6. Geissler, J.; Kroneck, P. M. H.; Ghisla, S. In *Flavins and Flavoproteins*; Walter de Gruyter & Co.; Berlin, 1984; pp 569-572.
7. (a) Duff, S. J. B.; Murray, W. D.; Overend, R. P. *Enzyme Microb. Technol.* **1989**, *11*, 770. (b) Shachar-Nishri, Y.; Freeman, A. *Appl. Biochem. Biotechnol.* **1993**, *39/40*, 387.
8. (a) Tani, Y.; Miya, T.; Ogata, K. *Agr. Biol. Chem.* **1972**, *36*, 76. (b) Patel, R. N.; Hou, C. T.; Laskin, A. I.; Derelanko, P. In *Flavins and Flavoproteins*, Massey, V.; Williams, C. H., Eds.; Elsevier North Holland: Amsterdam, Netherlands, 1982; pp 196-201.
9. Chen, C.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Amer. Chem. Soc.* **1982**, *104*, 7294.
10. (a) Tawaki, S.; Klibanov, A. M. *J. Am. Chem Soc.* **1992**, *114*, 1182. (b) Carrea, G.; Ottolina, G.; Riva, S. *Trends Biotechnol.* **1995**, *13*, 63.
11. (a) Cardemil, E. *Biochem. Biophys. Res. Commun.* **1975**, *67*, 1093. (b) Kraus, A.; Simon, H. *Hoppe-Seyler's Z. physiol. Chem.* **1975**, *356*, 1477.
12. Geerlof, A.; van Tol, J. B. A.; Jongejan, J. A.; Duine, J. *Biosci. Biotech. Biochem.* **1994**, *58*, 1028.
13. Mancuso, A. J.; Huang, S. L.; Swern, D. *J. Org. Chem.* **1978**, *43*, 2480.
14. Zhang, M.; Wang, H. Y. *Enzyme Microb. Technol.* **1994**, *16*, 10.
15. Skotnicki, J. S.; Schaub, R. E.; Weiss, M. J.; Dessy, F. *J. Med. Chem.* **1977**, *20*, 1662.
16. Aebi, H. E. In *Methods of Enzymatic Analysis*, Bergmeyer, H. U., Ed., Verlag Chemie: Deerfield Beach, Florida: 1983 (3rd Edition); Vol. III, pp 273-286.


(*Received in USA* 25 *April* 1995; *accepted* 23 *May* 1995)