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Stereoselective Synthesis of Ethyl (2*S*,3*S*)-*Anti*-2-Methyl-3-Hydroxybutanoate Mediated by an Oxidoreductase from *Geotrichum candidum*

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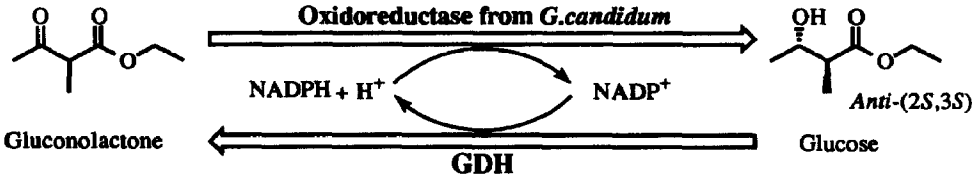
Abstract: *The reduction of ethyl 2-methyl-3-oxobutanoate catalyzed by an oxidoreductase from Geotrichum candidum affords corresponding ethyl (2S,3S)-anti-2-methyl-3-hydroxybutanoate with exclusive diastereo- and enantioselectivity.*

Optically active 2-methyl-3-hydroxybutanoate is highly versatile chiral synthon, because it contains two chiral centers as well as two functional groups that are readily convertible into other functions.[1] It is, therefore, worthwhile to develop a method for stereoselective synthesis of this compound. In a previous paper, we reported that the reduction of alkyl 2-alkyl-3-oxobutanoate by an enzyme obtained from baker's yeast affords the corresponding (2*R*,3*S*)-*syn*-hydroxy ester in an excellent stereoselectivity.[2,3,4] However, the method to prepare other stereoisomer of this ester has not yet been completed.

Now, we report a new method for enzymatic stereoselective synthesis of (2*S*,3*S*)-*anti*-hydroxy esters, the other diastereomer. It is reported that *Geotrichum candidum* is a good reductant of ethyl 2-methyl-3-oxobutanoate to ethyl (2*S*,3*S*)-*anti*-2-methyl-3-hydroxybutanoate, although stereoselectivity is not satisfactory.[5] Therefore, we attempted to isolate an enzyme from *G. candidum* that produces an *anti*-hydroxy ester.



Enzymes were isolated from *G. candidum*[6] (180 g) suspended in 100 ml of base buffer,[7] as reported previously for the isolation of enzymes from baker's yeast.[8] The active fractions[9] were collected through anion exchange column chromatography packed with DEAE-Toyopearl 650 and concentrated to catalyze the reduction of keto esters at 30 °C to furnish the *anti*-hydroxy ester with 95% *d.e.* (diastereomer excess).[10]

For further purification, the enzyme solution containing 20% ammonium sulfate was subjected to column chromatography on Butyl-Toyopearl 650 equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 20% ammonium sulfate. The active fractions were collected and concentrated, and the enzyme solution containing 10% glycerol was stored at –20 °C. All purification steps were run at 4 °C unless otherwise specified.

With the enzyme solution thus obtained, ethyl 2-methyl-3-oxobutanoate was reduced at 30 °C. After usual extraction, the diastereomeric ratio and yield of the reaction were determined on GLC.[11] Enantiomeric purity of the anti product was measured by GLC on a Chiraldex G-TA capillary column.[12,13] The results and conditions are listed in Table 1.

Glucose and glucose dehydrogenase (GDH) were employed to regenerate NADPH from $NADP^+$. It was

confirmed that this enzyme reduces the substrate diastereo- and enantioselectively. However the efficiency from the reaction was found to be unsatisfactory in 50 mM Tris-HCl buffer, and the yields were 66-69%. At the same time, it was observed that pH of the solution decreases as the reaction proceeds. Therefore, the concentration of the buffer was increased to 100 mM in order to keep pH of the solution constant throughout the reaction, and it was found that chemical yield of the hydroxy ester was increased up to 88%. When the reaction was run in 100 mM Bis-Tris-HCl buffer, which has lower pKa than Tris, the chemical yield was improved to 93% without loss of *e.e.*.

Now, we have succeeded in developing a device to obtain (2*R*,3*S*)-*syn*- and (2*S*,3*S*)-*anti*-2-methyl-3-hydroxybutanoate in pure isomers, respectively, out of four possible stereoisomers. We will soon report detailed procedure for the purification of enzyme.

Table 1. Reduction of Ethyl 2-Methyl-3-oxobutanoate[a)]

Enzyme Solution (μl) [b)]	Buffer [c)]	Time (h)	*d.e.*[d)] (%)	*e.e.*[e)] (%)	Yield[f)] (%)
550	50 mM Tris-HCl	24	98	95	66
		48	>99	94	69
500	100 mM Tris-HCl	24	99	95	88
500	100 mM Bis-Tris -HCl [g)]	24	>99	92	93

a) Conditions: 15 mM Substrate (0.5 mmol), NADPH 20 mg, GDH 10 units, Glucose 2 mmol.
b) 500 μl of the solution is equivalent to 1.2 units at 15 mM of substrate concentration. c) pH 7.0
d) For anti stereomer. e) For (2*S*,3*S*)-*anti*-isomer. f) Yield was determined on GLC analysis.
g) Bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane hydrogen chloride buffer; pKa (= 6.46 at 20°C) is lower than Tris. ((trishydroxymethyl)aminomethane).

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References and Notes

1) For a review; Sih, C. J.; Chen, C.-H. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 570-578.
2) Nakamura, K.; Kawai, Y.; Miyai, T.; Honda, S.; Nakajima, N.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 1467-1470.
3) Nakamura, K.; Kawai,Y.; Miyai, T.; Nakajima, N.; Ohno, A. *Tetrahedron Lett.* **1990**, *31*, 1159-1160.
4) For simplicity of expression, we use the terms syn and anti for erythro and threo, respectively.
5) Buisson, D.; Sanner, C.; Larcheveque, M.; Azerad, R. *Tetrahedron Lett.* **1987**, *28*, 3939-3940.
6) *G. candidum* (IFO 4597) was cultivated at 27°C for 2 days in 100 mM potassium phosphate buffer (pH 6.5) containing glycerol (30 g/*l*), yeast ext. (10 g/*l*), and polypeptone (5 g/*l*), then the mould was collected with filtration on paper.
7) 20 mM Tris-HCl buffer (pH 7.0) containing 1.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 3% EtOH.
8) Nakamura, K.; Kawai, Y.; Nakajima, N.; Ohno, A. *J. Org. Chem.* **1991**, *56*, 4778-4783.
9) The reaction mixture was composed of 3.00 ml of 50 mM Tris-HCl buffer (pH 7.0), 50 μl of 6.00 mM NADPH, and 100 μl of enzyme solution. The reaction was started by adding 100 μl of 100 mM ethyl 2-methyl-3-oxobutanoate solution. The activity was assayed by measuring the change in absorbance at 340 nm.
10) Determination of diastereomeric ratio of enzymatic reduction with DEAE- or Butyl-Toyopearl fraction: 1.00 ml of 50 mM Tris-HCl buffer (pH 7.0) containing 45 mU of enzyme, 15 mM ethyl 2-methyl-3-oxobutanoate, and 1.2 equiv. NADPH was incubated for one day at 30°C. After usual work up, the ratio was determined with GLC analysis.
11) Equipped with a capillary PEG 20 M column:*cf.* Nakamura, K.; Miyai, T.; Nagar, A.; Babu, B. R.; Ando, T.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 298-300; The *anti*-isomer has shorter retention time than the corresponding *syn*-isomer.
12) Keto ester was removed by the use of a column chromatography on silica gel with hexane-ethyl acetate (4:1) as an eluent.
13) The absolute configuration of the isomers corresponding to each peak was determined as follows; the retention time of hydroxy ester obtained by yeast reduction (2*R*,3*S* or 2*S*,3*S*) was compared with the hydroxy ester obtained by $NaBH_4$ reduction (containing all four isomers), for which the syn/anti ratio was known by GLC analysis equipped with a capillary PEG 20M column.; *cf.* Nakamura, K.; Miyai, T.; Nagar, A.; Oka, S.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 1179-1187.

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