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Stereochemical Control in Microbial Reduction. XXIII. Thermal Treatment of Bakers' Yeast for Controlling the Stereoselectivity of Reductions

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The diastereoselectivity in a bakers' yeast reduction of various alkyl 2-alkyl-3-oxoalkanoates can be controlled by a thermal treatment of the yeast in the presence of methyl vinyl ketone. Pretreated bakers' yeast gives the corresponding (2*R*,3*S*)-hydroxy esters selectively. Two β-keto ester reductases, named L-enzyme-1 and L-enzyme-2, are actual reductants of intact bakers' yeast reduction. The thermal stability and inhibition constants of the purified enzymes can dictate the stereochemical outcome of pretreated bakers' yeast reductions.

Optically pure α-branched β-hydroxy esters are utilized as chiral units in the syntheses of various natural products, because they contain two chiral centers as well as two functional groups that are readily convertible into other functions.[1—3] Alkyl 2-alkyl-3-oxoalkanoates are easily enolizable in aqueous solutions, and chiral 2-position racemizes rapidly. Although the chemical reduction of these compounds affords α-branched β-hydroxy esters of four possible stereoisomers, the reduction by bakers' yeast affords the corresponding L-(3*S*)-hydroxy esters with exclusive enantioselectivity. However, the diastereoselectivity, *syn*/*anti* ratio,[4] depends on the structures of the 2-alkyl substituent and alkoxyl group.[5] In order to improve and/or control the stereoselectivity of microbial reduction, various useful methods have been developed. The modification of the substrate is one of them; we have reported that the diastereoselectivity of the bakers' yeast reduction of alkyl 2-alkyl-3-oxobutanoate can be improved by selecting the structure of the ester moiety.[6] The reduction of the neopentyl ester of 2-methyl-3-oxobutanoic acid gives the corresponding *syn*-(2*R*,3*S*)-hydroxy ester, whereas the reduction of *t*-butyl ester yields the corresponding *anti*-(2*S*,3*S*)-hydroxy ester in high diastereomeric excess, respectively. The bakers' yeast reduction of 2-methyl-3-oxoalkanoic acids, instead of esters, affords *anti*-(2*R*,3*R*)-hydroxy acids.[7] The diastereoselectivity is improved in reducing the corresponding dithioester, giving the *syn*-(2*R*,3*S*)-hydroxy ester.[8] Screening of microbes is also a recommended method for obtaining a satisfactory result; reduction by *Geotrichum candidum* and *Rhizopus arrhizus* yields the *anti*-(2*S*,3*S*)-hydroxy esters selectively.[9]

We recently explored a new and valuable technique for the diastereoselective reduction of ethyl 2-alkyl-3-oxobutanoates with bakers' yeast,[10,11] finding that the pretreatment of a microbe at an elevated temperature as well as the addition of methyl vinyl ketone (MVK) into the reaction system improves the stereoselectivity of the reduction, giving the *syn*-(2*R*,3*S*)-hydroxy esters selectively. The method is quite useful because the stereoselectivity can be easily controlled without using screening microbes or modifying the substrate. In this paper, detailed insight into the reaction is described, and the mechanism for this stereochemical control is discussed.

Experimental

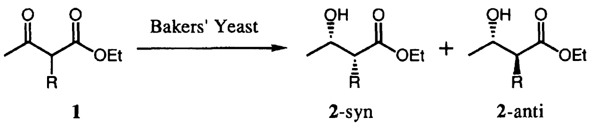
Instruments. IR spectra were recorded on a JASCO FT/IR 5300 spectrophotometer. NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$ solutions with tetramethylsilane (TMS) as an internal reference. Optical rotations were measured with a JASCO DIP-181 digital polarimeter. UV spectra were obtained on a Hitachi U-3210 spectrophotometer. Gas chromatograms were recorded on a Shimadzu GC-14A (PEG-20M Bonded, 25 m) and GC-9A (Chiraldex G-TA, 30 m) gas chromatographs.

Materials. Organic reagents and solvents were purchased from Nacalai Tesque, Inc. and Aldrich Chemical Co. Dry bakers' yeast was purchased from Oriental Yeast Co. and stored in a refrigerator. Ethyl 2-alkyl-3-oxobutanoates and ethyl 2-alkyl-3-oxopropanoates were prepared as reported previously.[6,12] The detailed procedure for purifying the enzymes was described in a previous paper.[13]

Enzyme Assay. A 50 µL aliquot of an enzyme solution was added to 3.00 mL of a solution of 0.10 M (1 M=1 mol dm^{-3}) potassium phosphate buffer (pH 7.0) that also contained ethyl 4-chloro-3-oxobutanoate (0.98 mM) and NADPH (0.09 mM). The enzyme activity was determined spectrophotometrically at 30 °C by following the decrease in the absorbance of NADPH at 340 nm.

General Procedure for the Reduction of Various α-Alkyl β-Keto Esters with Pretreated Bakers' Yeast. In general, a suspension of 20 g of dry bakers' yeast in 60 mL of water was preincubated for 30 min at 50 °C in the presence of an appropriate concentration of MVK, and cooled to 30 °C; then, 1 mmol of a substrate was added to this reduction system. The mixture was magnetically stirred for 24 h at 30 °C. Hyflo Super-Cel and ethyl acetate were added, and the mixture was filtered. The precipitates were washed with ethyl acetate. The combined washings and filtrate were washed with water and brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/ethyl acetate (5/1) used as an eluent, giving the corresponding hydroxy ester. The chem-

Table 1. Effect of MVK and Thermal Treatment on the Diastereoselectivity of Bakers' Yeast Reduction[a)]



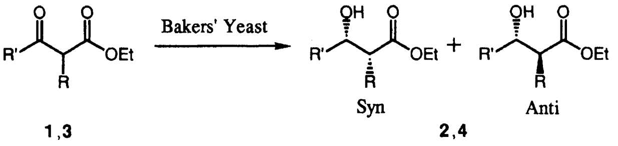
	Substrate R	*syn/anti* Ratio (Yield/%)[b)] None	MVK[c)]	Heat[d)]	MVK[e)] heat
1a	Me	87/13(75)	96/ 4(72)	93/ 7(69)	97/ 3(68)
1b	Et	66/34(80)	86/14(76)	80/20(72)	92/ 8(57)
1c	Pr	74/26(70)	85/15(68)	92/ 8(51)	93/ 7(50)
1d	Allyl	30/70(71)	84/16(66)	65/35(65)	96/ 4(68)
1e	Propargyl	66/34(80)	91/ 9(73)	86/14(79)	94/ 6(71)

a) Dry Bakers' Yeast, 20g; Water, 60 mL; Substrate, 1 mmol. b) Isolated yield. c) Preincubated for 30 min in the presence of 83 mM of MVK. d) Preincubated for 30 min at 50 °C. e) Preincubated for 30 min at 50 °C in the presence of 60 mM of MVK.

ical yields are listed in Tables 1 and 2. The enantiomeric and diastereomeric excesses (e.e. and d.e.) in the product were determined by the same procedures as those reported in previous papers.[6,14,15)]

Results and Discussion

Two different states of bakers' yeast are available. One is raw, and the other is dry. Dry bakers' yeast was employed for the present research due to its higher reproducibility in selectivity of the reduction. Ethyl 2-alkyl-3-oxobutanoate was employed as substrates for the microbial reduction shown in Eq. 1.



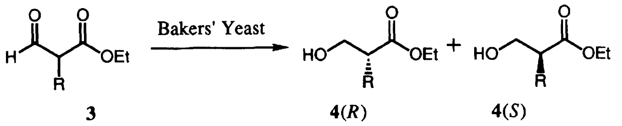
	R	R'		R	R'
1a, 2a	Me	Me	**3a, 4a**	H	Me
1b, 2b	Me	Et	**3b, 4b**	H	Et
1c, 2c	Me	Pr	**3c, 4c**	H	Pr
1d, 2d	Me	Allyl	**3d, 4d**	H	Allyl
1e, 2e	Me	Propargyl	**3f, 4f**	H	Bu

(1)

Ethyl 2-ethyl-3-oxobutanoate (**1b**) and ethyl 2-allyl-3-oxobutanoate (**1d**) were employed as typical compounds for the investigation, because the former is converted into the corresponding *syn*-(2*R*,3*S*)-hydroxy ester by reduction, while the latter is converted into the *anti*-(2*S*,3*S*)-product in low diastereoselectivity, respectively.[6)]

Effect of MVK on Diastereoselectivity. We reported in a previous paper that a certain oxidoreductase in bakers' yeast is inhibited selectively by methyl vinyl ketone (MVK).[13)] Therefore, MVK was chosen again as a selective inhibitor of the present bakers' yeast reduction. The bakers' yeast was preincubated for 1 h in the presence of an appropriate concentration of methyl vinyl ketone; a substrate was then added to this preincubated system. The diastereomeric ratio in the product was determined by a GLC analysis.[16)] In Fig. 1, the diastereoselectivity in the product is shown as a function of the MVK concentration. Apparently, the diastereoselectivity in the *syn*-product increases with an increase in the MVK concentration; the best result was obtained with a concentration of 83 mM for both substrates.

Table 2. Reduction of Various Ethyl 2-Alkyl-3-oxopropanoate with Pretreated Bakers' Yeast[a)]



	Substrate R	e.e./%, (Configuration), Yield/% None	MVK[b)] heat
3a	Me	45 (*R*) 58	>99 (*R*) 50
3b	Et	68 (*S*) 60	>99 (*R*) 40
3c	Pr	69 (*S*) 69	>99 (*R*) 75
3d	Allyl	21 (*S*) 61	98 (*R*) 29
3f	Bu	25 (*S*) 63	>99 (*R*) 55

a) Dry Bakers' Yeast, 20 g; Water, 60 mL; Substrate, 1 mmol. b) Preincubated for 30 min at 50 °C in the presence of 60 mM of MVK.

Effect of the Temperature on the Diastereoselectivity. We reported in a previous paper that the thermal stability of each enzyme is different.[13)] It would therefore be expected that a thermal treatment of bakers' yeast distorts the relative activity levels of the enzymes operating for the reduction, and alters the stereochemical outcome. Since bakers' yeast heated at above 60 °C exerts no reducing activity at all, a suitable pretreatment temperature is 50 °C. The bakers' yeast was preincubated for an appropriate time interval

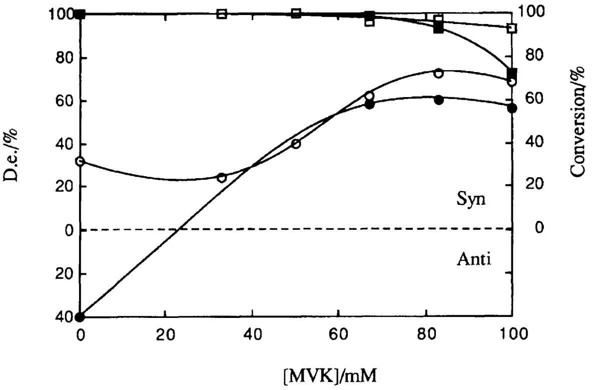


Fig. 1. Effect of MVK on diastereoselectivity (circles) and reactivity (squares) of the reduction of ethyl 2-ethyl-3-oxobutanoate (**1b**, open symbols) and ethyl 2-allyl-3-oxobutanoate (**1d**, closed symbols) by bakers' yeast.

at 50 °C, and cooled to 30 °C; a substrate was then added to this preincubated system. The diastereoselectivity in the product thus obtained is plotted in Fig. 2 as a function of the period for the thermal treatment at 50 °C. Figure 2 indicates that the diastereoselectivity of the reduction is meaningfully affected by the period of the thermal treatment. Although a prolonged treatment increases the *syn*-selectivity, a treatment longer than 30 min causes a decrease in both the selectivity and reactivity.

Since, both the addition of MVK and a thermal treatment improve the *syn*-selectivity, a combination of these two techniques affords the most favorable result. Indeed, as shown in Fig. 3, the diastereoselectivities of thermally treated bakers' yeast are drastically improved up to a satisfactory level by the addition of MVK. The best result was obtained at a concentration of 60 mM. These particular conditions were thus employed for further reactions.

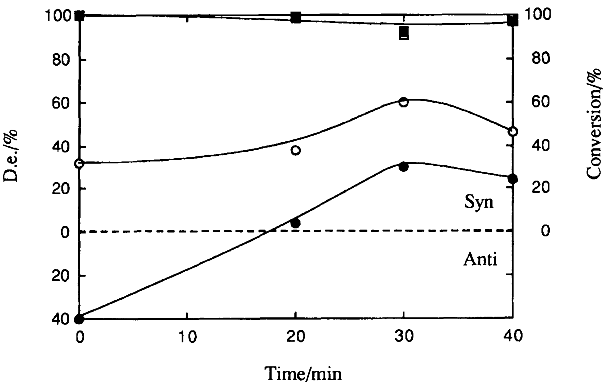


Fig. 2. Effect of thermal treatment of bakers' yeast on the diastereoselectivity (circles) and reactivity (squares) of the reduction of ethyl 2-ethyl-3-oxobutanoate (**1b**, open symbols) and ethyl 2-allyl-3-oxobutanoate (**1d**, closed symbols).

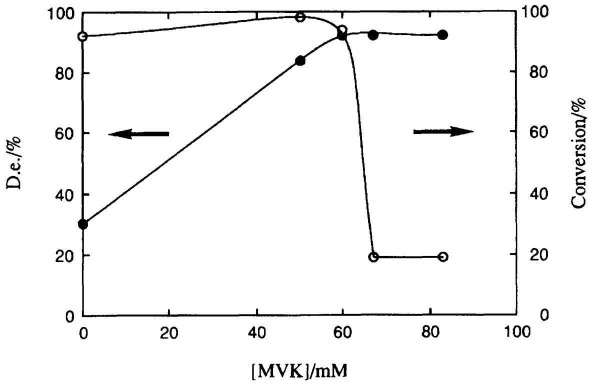


Fig. 3. Effect of MVK on diastereoselectivity (closed circles) and reactivity (open circles) of thermally treated bakers' yeast in the reduction of ethyl 2-allyl-3-oxobutanoate (**1d**).

Reduction of α-Alkyl β-Keto Esters. Various ethyl 2-alkyl-3-oxobutanoates, **1a**—**e**, were subjected to reduction under the reaction conditions described above. The results are summarized in Table 1. The enantiomeric excess was more than 99% in every case. It is obvious that the combination method is quite useful for obtaining the *syn*-(2*R*,3*S*)-hydroxy ester. Namely, the method reverses the diastereoselectivity of the reduction of **1d**.

α-Formyl esters **3** are analogs of the corresponding keto esters **1**, and are reduced by bakers' yeast, resulting in hydroxy esters **4** which have only one chiral center at the α-position, or the reacting position does not create a chirality center. The reduction of these formyl esters by bakers' yeast was reported by Züger et al., although the results are unsatisfactory.[12] Various ethyl 2-alkyl-3-oxopropanoates, **3a**—**f**, were subjected to reduction under the same reaction conditions as described above; the results are summarized in Table 2. The enantioselectivity exerted by this method is perfect for all of the esters subjected to the reduction. Namely, the method reverses the configurations of the products from the reductions of **3b**—**f**. The method is also useful for obtaining the (2*R*)-hydroxy esters.

Enzymatic Study for Stereochemical Control. We recently succeeded in analysing the diastereoselectivity in the bakers' yeast reduction of α-alkyl-β-keto esters quantitatively at the enzyme level.[17] It was elucidated that two enzymes, L-enzyme-1 and L-enzyme-2, are responsible for the reduction by bakers' yeast, and that the stereoselectivities of these competing enzymes are quite different. The former affords the *syn*-(2*R*,3*S*)-hydroxy esters with perfect stereoselectivity for all of the substrates subjected, whereas the selectivity of the latter enzyme depends on the structure of the substrate, resulting in a mixture of *syn*-(2*R*,3*S*)- and *anti*-(2*S*,3*S*)-hydroxy esters.

In a previous paper, we reported that MVK inhibits L-enzyme-1 noncompetitively with 50.6 mM of K_i and inhibits L-enzyme-2 irreversibly with 6.38 mM of K_i.[13] This results suggest that L-enzyme-1 retains half of its

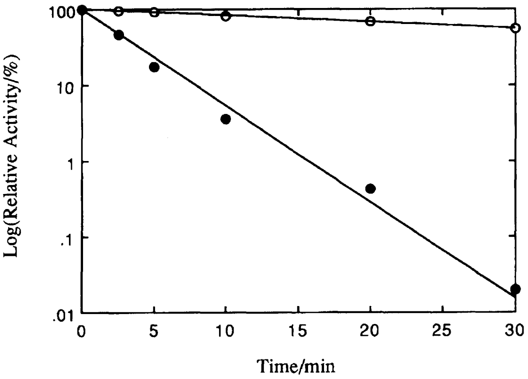


Fig. 4. Thermal stability of L-enzyme-1 (open circles) and L-enzyme-2 (closed circles) at 50 °C.

original activity, whereas L-enzyme-2 retains only less than 10% of its activity in the presence of 60 mM of MVK.

The thermal stability of each enzyme at 50 °C is shown in Fig. 4, which reveals that the half-lives of L-enzyme-1 and L-enzyme-2 are 35 and 1.8 min, respectively. Thus, L-enzyme-2 is much more unstable than L-enzyme-1.

Thus, there remains no doubt that L-enzyme-2 in bakers' yeast is completely denatured after preincubation at 50 °C for 30 min and exposed to 60 mM of MVK remaining L-enzyme-1 as the solely responsible catalyst for reduction under the reaction conditions. Consequently, the (2*R*,3*S*)- and the (2*R*)-products are afforded stereoselectively in the reductions of ethyl 2-alkyl-3-oxobutanoates (**2**) and ethyl 2-alkyl-3-oxopropanoates (**4**), respectively.

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References

1) K. Mori, *Tetrahedron*, **45**, 3233 (1989).

2) T. Oishi and T. Nakata, *Acc. Chem. Res.*, **17**, 338 (1984).

3) S. Masamune and W. Choy, *Aldrichimica*, **15**, 47 (1982).

4) For simplicity of expression, we use the terms *syn* and *anti* for *erythro* and *threo*, respectively.

5) G. Fráter, U. Müller, and W. Günther, *Tetrahedron*, **40**, 1269 (1984).

6) K. Nakamura, T. Miyai, A. Nagar, S. Oka, and A. Ohno, *Bull. Chem. Soc. Jpn.*, **62**, 1179 (1989).

7) H. Watabu, M. Ohkubo, H. Matsubara, T. Sakai, S. Tsuboi, and M. Utaka, *Chem. Lett.*, **1989**, 2183.

8) T. Itoh, Y. Yonekawa, T. Sato, and T. Fujisawa, *Tetrahedron Lett.*, **27**, 5405 (1986).

9) D. Buisson, C. Sanner, M. Larcheveque, and R. Azerad, *Tetrahedron Lett.*, **28**, 3939 (1987).

10) K. Nakamura, Y. Kawai, T. Miyai, and A. Ohno, *Tetrahedron Lett.*, **31**, 3631 (1990).

11) K. Nakamura, Y. Kawai, and A. Ohno, *Tetrahedron Lett.*, **32**, 2927 (1991).

12) M. F. Züger, F. Giovannini, and D. Seebach, *Angew. Chem., Int. Ed. Engl.*, **22**, 1012 (1983).

13) K. Nakamura, Y. Kawai, N. Nakajima, and A. Ohno, *J. Org. Chem.*, **56**, 4778 (1991).

14) K. Nakamura, T. Miyai, K. Ushio, S. Oka, and A. Ohno, *Bull. Chem. Soc. Jpn.*, **61**, 2089 (1988).

15) Y. Kawai, M. Tsujimoto, S. Kondo, K. Takanobe, K. Nakamura, and A. Ohno, *Bull. Chem. Soc. Jpn.*, **67**, 524 (1994).

16) K. Nakamura, T. Miyai, A. Nagar, B. R. Babu, T. Ando, and A. Ohno, *Bull. Chem. Soc. Jpn.*, **63**, 298 (1990).

17) K. Nakamura, Y. Kawai, T. Miyai, S. Honda, N. Nakajima, and A. Ohno, *Bull. Chem. Soc. Jpn.*, **64**, 1467 (1991).