# Asymmetric Reduction of β-Keto Esters with an Enzyme from Bakers' Yeast

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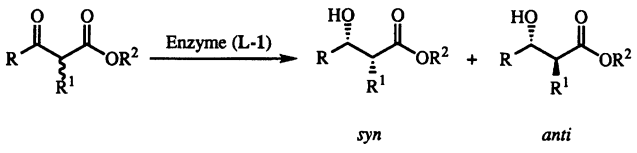
Various β-keto esters have been reduced by one of β-keto ester reductases isolated from bakers' yeast. The corresponding β-hydroxy esters have been obtained in excellent enantiomeric and diastereomeric excesses, respectively. It has also been elucidated that the reductase recognizes the stereochemistry not only at the β-carbon but also at the α-carbon affording one of the four possible diastereoisomers of α-substituted β-hydroxy esters predominantly. The stereoselectivity is excellent and chemical yields are moderate to good.

Biocatalysts have received wide interest in modern organic chemistry in order to synthesize optically active compounds. Bakers' yeast is one of the most popular biocatalysts, because it is available easily, is cheap and does not require special instruments and apparatuses for microbiological studies. Namely, since Deol et al. reported the reduction of various β-keto esters into the corresponding (*S*)-β-hydroxy esters,[1] reduction mediated by bakers' yeast have been investigated extensively. Optically active β-hydroxy esters are important building blocks in the syntheses of biologically active compounds such as pheromones[2—4] and antibiotics[5—7] as well as herbicides and insecticides. However, stereoselectivity exerted by bakers' yeast in the reduction is not necessarily satisfactory for artificial substrates.[8—11] Since a microbe is a bag which contains many enzymes in it,[12] it is conceivable that plural enzymes are responsible to the reduction and some enzymes may reduce a substrate into one enantiomer of the corresponding product, while other enzymes may afford the other enantiomer of the product. When these two groups of enzymes happen to have similar catalytic activity, the product we obtain appears as an almost racemic mixture; a very disappointed result.

Recently, we reported the isolation and characterization of four β-keto ester reductases from bakers' yeast; two of them afford D-β-hydroxy ester and the other two afford the corresponding L-β-hydroxy ester.[13] It has also been reported that certain additives can control stereochemistry of the reduction mediated by bakers' yeast. The additive inhibits specifically one or the other groups of the reductases.[14—16] The inhibition constant, $K_i$, plays a crucial role for the apparent specificity.

One of the four reductases isolated from bakers' yeast, which has been abbreviated as **L-1**, is a unique enzyme in the sense that it is not adsorbed on an ion-exchange resin at pH 7.0 (Toyopearl/DEAE), differently from the other three, on the process of purifying the enzymes. In other words, **L-1**, which affords L-β-hydroxy esters in enantiomerically pure state is an enzyme which is available quite easily from cheap bakers' yeast, and is worth to be studied its potential in organic asymmetric syntheses as a biocatalyst of molecular level.

In this paper, results from the reduction of β-keto esters that have or does not have a substituent at the α-position mediated by a non-adsorbed β-keto ester reductase, **L-1**. **L-1** is a monomeric enzyme with molecular weight of 32 kDa (Eq. 1).[13]




R = H, Me, Et, Pr, $ClCH_2$
$R^1$ = H, Me, Et, Pr, Bu, Hex, Oct, Ph, Allyl, Propargyl
$R^2$ = Me, Et, Pr

(1)

## Results and Discussion

**Substrate Specificity.** First of all, relative activities of **L-1** toward various substrates were determined. **L-1** was added to a solution of the substrate and NADPH. Relative reaction rates were determined by measuring the decrease in the concentration of NADPH with spectrophotometer. Details will be described in Experimental section. Ethyl 4-chloro-3-oxobutanoate was chosen as the standard (activity=100%). Results are summarized in Table 1.

Derivatives of 3-oxobutanoate react relatively fast and the reactivity increases with the increase in length of the alcohol moiety. Thus, in the series of 3-oxo-

Table 1. Substrate Specificity $RCOCH(R^1)COOR^2$ of **L-1**

| R | $R^1$ | $R^2$ | Activity(%) | R | $R^1$ | $R^2$ | Activity(%) |
|---|---|---|---|---|---|---|---|
| $ClCH_2$ | H | Et | 100 | Me | Et | Et | 6.5 |
| Me | H | Me | 5.8 | Me | Pr | Et | 12.6 |
| Et | H | Me | 0 | Me | Allyl | Et | 14.7 |
| Pr | H | Me | 0 | Me | Propargyl | Et | 18.3 |
| Ph | H | Me | 0 | H | Me | Et | 89.4 |
| Me | H | Et | 13.5 | H | Et | Et | 86.6 |
| Et | H | Et | 8.4 | H | Pr | Et | 81.0 |
| Pr | H | Et | 0 | H | Allyl | Et | 92.7 |
| Me | H | Pr | 16.0 | H | Bu | Et | 19.1 |
| Et | H | Pr | 0 | H | Hex | Et | 10.8 |
| Pr | H | Pr | 0 | H | Oct | Et | 5.1 |
| Me | Me | Et | 13.0 | H | Ph | Et | 13.6 |

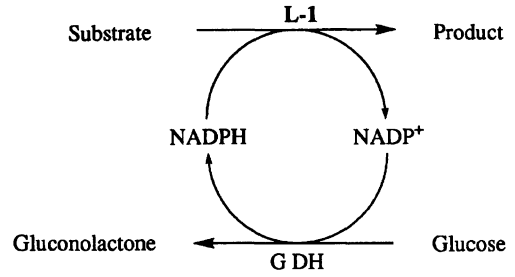
butanoate, the propyl ester reacts fastest and the ethyl ester follows it: the reactivity of methyl ester is only one third of the propyl ester. In the series of 3-oxopentanoate, the corresponding ethyl ester only is reactive. Thus, only one additional methyl group at 4-position of the substrate decreases the enzymatic activity strongly. Since the bulkiness of ethyl group is not much different from chloromethyl group, the high reactivity of ethyl 4-chloro-3-oxobutanoate might come from the induction effect of the chloro substituent. Polar interaction in the acid side of the pocket of the enzyme thus plays an important role on the reactivity.

It is interesting to note that increase in the molecular size reduces the reactivity. Total nine atoms in the chain length of a substrate seems too long to be trapped in the pocket of this enzyme. Thus, size of the pocket of **L-1** might be small. At the same time, the observation that the methyl esters, which might be small substrates in principle, are not satisfactory substrate in contrast to the expectation, suggests that the pocket requires the best fit, probably in order to set the reacting position at the correct site. Thus, we obtained an insight into a suitable substrate for this particular enzyme.

When the substrate has an alkyl substituent at the α-position, the reactivity is much greater than those without the substituent. In addition, it has been found that aldehydic substrate reacts faster than the corresponding keto esters. Similar tendency was observed by Yamada et al.[18] with an NADPH-dependent aldehyde reductase from *Sporobolomyces salmonicolor*, which reduces 4-chloro-3-oxobutanoates to the corresponding L-hydroxy esters.

Of all these 2-alkyl substrates, 2-allyl and 2-propargyl derivatives react faster than those substituted by simple alkyl group such as methyl and ethyl derivatives. As seen in the reaction with the substrate having 2-allyl or 2-propargyl group, the presence of an olefinic bond at the α-position of carbon chain increases the reactivity of **L-1**. Polar group in 2-alkyl moiety may fit well with the active site of the enzyme.

**Reduction of Alkyl 3-Oxoalkanoates.** Based on the results mentioned above, the enzymatic reduction of β-keto esters was attempted in a preparative scale. When a microbe is subjected to a reaction as a catalyst, the chemical system to regenerate a coenzyme (NADH or NADPH) is equipped in it already. However, when an enzyme is employed as a catalyst for a large scale reaction, it is necessary to construct another chemical cycle to regenerate the consumed coenzyme. The recycling system should not denature the reductase and, at the same time, should proceed preferably faster than the reduction process. In addition, the chemical yield of the regenerated NADH or NADPH should be as close to 100% as possible in order to maintain catalytic efficiency of the reductase at a high level. Consequently, we decided to employ an enzymatic system, instead of artificial chemical process, to regenerate the coenzyme, and found that glucose dehydrogenase from *Bacillus megaterium* (GDH)/Glucose couple is suitable for this purpose (Eq. 2). It should be noted that **L-1** is an NADPH-dependent enzyme.




(2)

The results from the reduction of β-keto esters that have no substituent at the α-position ($R^1$=H) are listed in Table 2, where it is recognized that the enantioselectivity exerted by **L-1** is perfect; the reduction with **L-1** gives the corresponding L-hydroxy esters for every reactive substrates. These results are in contrast with the reduction mediated by bakers' yeast, which usually gives a mixture of enantiomers.

It is interesting to note that the future of reduction with **L-1** is uniquely different from that with bakers' yeast. A long ester such as 3-oxohexanoate is reduced by bakers' yeast in an excellent stereoselectivity, whereas **L-1** cannot catalyze the reduction of this ester. The observation suggests that one of D-enzymes in bakers' yeast prefers rather large substrate more than L-enzymes. At present, we have no rational proposal for unreactivity of methyl 3-oxopentanoate in the reduction with **L-1**. However, the fact that the enantioselectivity of the reduction with bakers' yeast is quite low, most part of this keto ester might be reduced by a D-enzyme. On the other hand, the keto ester which has a polar group in the acid moiety seems to be a preferable substrate of **L-1**.

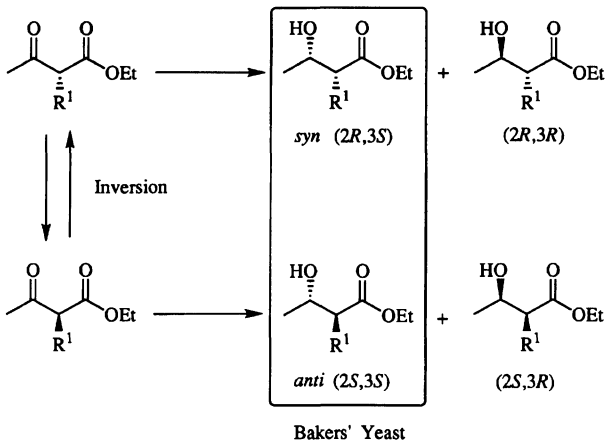
**Reduction of Ethyl 2-Alkyl-3-oxoalkanoates.** When a β-hydroxy ester has a substituent at the α-position, there exists diastereoisomerism in this compound. We made attempts to synthesize only one of these four possible diastereoisomers of β-hydroxy esters, and obtained partly successful results by the reduction of the corresponding α-substituted β-keto esters mediated by bakers' yeast. For example, with an appropriate structure of the alkoxy moiety, an alkyl 2-methyl-3-oxobutanoate affords the (2*R*,3*S*)-*syn*- or (2*S*,3*S*)-*anti*-isomers of the corresponding alkyl 2-methyl-3-hydroxybutanoate in 97 : 3 ratio depending on the reaction conditions.[19] No 3*R*-isomers were detected. Substitution of the α-methyl group by an allyl group, on the other hand, reverses the isomer ratio to 3 : 97.[19] The inhibitor technique described in the introductory remarks (vide supra) improves or changes the ratio. Unfortunately, however, no satisfactory results have been obtained for substrates that have an α-substituent other

Table 2. Reduction of β-Keto Esters $RCOCH(R^1)COOR^2$ with Bakers' Yeast or **L-1**

| R | $R^1$ | $R^2$ | Bakers' yeast | | | **L-1**[a] | | |
|---|---|---|---|---|---|---|---|---|
| | | | Yield(%) | e.e.(%) | Config. | Yield(%) | e.e.(%) | Config. |
| Me | H | Me | — | — | | 13 | >99 | *S*(L) |
| Et | H | Me | 46 | 12[b] | *R*(D) | 0 | — | |
| Me | H | Et | 66 | 77[b] | *S*(L) | 44 | >99 | *S*(L) |
| Et | H | Et | 61 | 4[b] | *R*(D) | 30 | >99 | *S*(L) |
| Pr | H | Et | 50 | 90[c] | *R*(D) | 0 | — | |
| $ClCH_2$ | H | Et | 62 | 43[b] | *S*(D) | 99 | >99 | *R*(L) |
| Me | H | Pr | — | — | | 18 | >99 | |

a) Substrate; 0.7 mmol: Buffer; 0.1 M Tris-HCl, pH 7, 70 ml: Temp; 30 °C: Time; 48 h.
b) Ref. 15. c) Ref. 17.

than methyl and allyl groups. Furthermore, simple methyl or ethyl esters do not afford satisfactory results for the substrates with any α-substituents we studied. The alkoxy moiety has to have a bulky alkyl group such as *t*-butyl or neopentyl group.[19] Thus, it is necessary to look for a suitable catalyst/conditions versatile for obtaining diastereomerically as well as enantiomerically pure α-substituted β-hydroxy esters ($R^1 \neq H$) which are again useful building blocks in the syntheses of biologically active compounds.[20—22] A group of these keto esters is particularly interesting as the substrates, because enolization makes their (2*S*)- and (2*R*)-enantiomers in rapid equilibrium under the reduction conditions. Consequently, these starting materials stay as a racemic mixture throughout the reduction (Eq. 3), and it is not necessary to quench the reduction on its half-way in order to obtain enantiomerically pure product. This is a point largely superior to kinetic resolution of an acid by hydrolysis of its corresponding ester mediated by a lipase.[23,24]




(3)

Since it is known that two L-enzymes from bakers' yeast (**L-1** and **L-2**) are the sole enzymes responsible to the reduction of α-substituted β-keto esters,[25] and that **L-1** results in excellent stereoselectivity, the reduction was tried for a variety of α-substituted β-keto esters under the catalysis of **L-1** with the GDH/Glucose system as the NADPH-recycling device. The results are listed in Table 3. No need to say that all the reductions mediated by **L-1** exert excellent stereoselectivities to afford the corresponding (2*R*,3*S*)-isomers solely in moderate to good chemical yields.

**Reduction of Ethyl 2-Formylalkanoates.** Another interesting example for the application of **L-1** can be seen in the reduction of α-substituted β-formyl esters (R=H). Although there appears no chirality at the reaction center, the β-position of the product, upon the reduction of esters in this group, the vicinal α-position remains to be a chirality center. Therefore, the product has an enantioisomerism. Reduction of a series of these esters mediated by bakers' yeast was reported by Züger et al. about a decade ago.[26] However, the reported results were miserable. Previously, we reported that the reduction of α-substituted β-formyl esters has been controlled by modifying the ester substituent of the formyl esters.[27] Thus the reduction of the 2,2-dimethylpropyl ester afforded the corresponding (*R*)-hydroxy ester selectively although the enantioselectivity did not exceed 90%.

Contrary, to the results from bakers' yeast, the enantioselectivity exerted by **L-1** is perfect for all the esters subjected to the reduction as listed in Table 4. On the other hand, the chemical yield tends to decrease as the α-alkyl substituent becomes longer. This may be a limitation of an enzymatic reaction and will be a point to be revised in future in order to take the reduction into a versatile device in general organic syntheses.

**Absolute Configurations of Ethyl 2-Alkyl-3-hydroxypropionates.** Absolute configurations of ethyl 2-alkyl-3-hydroxypropionates discussed herein are not known except for that of ethyl 2-ethyl-3-hydroxypropionate. In order to establish the stereochemistry, we studied the absolute configurations of these hydroxy esters.

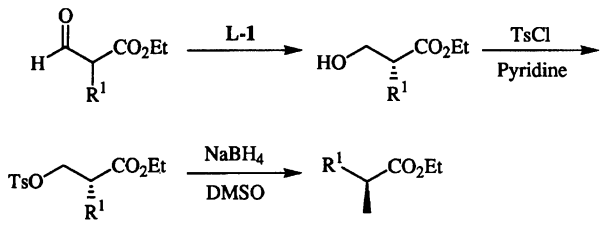
All ethyl 2-alkyl-3-hydroxypropionates obtained by the reduction of ethyl 2-formylalkanoates by means of the enzyme **L-1**, except for ethyl 2-ethyl-3-hydroxypropionate, exert dextro rotation. The hydroxyl groups in 2-propyl- and 2-butyl-3-hydroxypropionates were converted into the corresponding tosylates. The optical rotations of the tosylates are again positive for both.

Table 3. Reduction of Ethyl 2-Alkyl-3-oxobutanoates $RCOCH(R^1)COOR^2$ with Bakers' Yeast or **L-1**

| R | $R^1$ | $R^2$ | Bakers' yeast[a] | | **L-1**[b] | |
|---|---|---|---|---|---|---|
| | | | Yield(%) | *syn* : *anti* | Yield(%) | *syn* : *anti* |
| Me | Me | Et | 59 | 87 : 13 | 54 | >99 : 1 |
| Me | Et | Et | 80 | 74 : 26 | 31 | >99 : 1 |
| Me | Pr | Et | 70 | 74 : 26 | 54 | >99 : 1 |
| Me | Allyl | Et | 59 | 35 : 65 | 60 | >99 : 1 |
| Me | Propargyl | Et | 80 | 68 : 32 | 72 | >99 : 1 |

a) Ref. 19. b) Substrate; 0.7 mmol: Buffer; 0.1 M Tris-HCl, pH 7, 70 ml: Temp; 30 °C: Time; 48 h.

The tosylates were subjected to the reduction with sodium borohydride in DMSO (Eq. 4). The resulted ethyl 2-methylalkanoates exert positive optical rotations revealing that the absolute configurations of these esters are *R*.[28,29] Thus, ethyl 2-propyl- and 2-butyl-3-hydroxypropionates obtained by the reduction with **L-1** were determined to be the (*R*)-isomers. The optical rotation of ethyl 2-ethyl-3-hydroxypropionate ($[\alpha]_D^{25}$ −2.53 ($c$=3.95, MeOH)) was compared with that reported in the literature[30] and ethyl 2-phenyl-3-hydroxypropionate was converted into its 3-acetoxy derivative and optical rotation of this derivative ($[\alpha]_D^{25}$ +52.55 ($c$= 1.50, EtOH)) was compared with that reported in the literature.[31] Thus, absolute configurations of both 2-ethyl- and 2-phenyl-3-hydroxypropionates have ben determined to be *R*. The absolute configurations of other 3-hydroxypropionates (R′=Allyl, Hex, Oct) obtained by means of **L-1** were postulated to be *R* by comparing their retention times on HPLC analysis of their MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) esters. Consequently, it has been concluded that the reduction mediated by the **L-1** of ethyl 2-formylalkanoates affords the (*R*)-enantiomer in all the cases.




(4)

## Experimental

**Instruments.** $^1$H NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. Gas chromatography was recorded on a Yanaco G-2800 (PEG-20M, 1.5 m), Shimadzu GC-14A (PEG-20M bonded, 25 m) and GC-9A (Chiraldex G-TA, 20 m) gas chromatographs. HPLC was performed with a Hitachi 655 Liquid chromatography with an IRICA 852-III spectrophotometer using a Cosmosil 5SL column.

Table 4. Reduction of Ethyl 2-Formylalkanoates $RCOCH(R^1)COOR^2$ with **L-1**

| R | $R^1$ | $R^2$ | Yield(%)[b] | e.e.(%)[c] | Config. |
|---|---|---|---|---|---|
| H | Et | Et | 69 | >99 | *R* |
| H | Pr | Et | 66 | >99 | *R* |
| H | Allyl | Et | 66 | >99 | *R* |
| H | Bu | Et | 24 | >99 | *R* |
| H | Hex | Et | 14 | >99 | *R* |
| H | Oct | Et | 4 | >99 | *R* |
| H | Ph | Et | 20 | >99 | *R* |

a) Substrate; 0.7 mmol: Buffer; 0.1 M Tris-HCl, pH 7, 70 ml: Temp; 30 °C: Time; 24 h. b) Isolated yield. c) Determined by HPLC analysis of the (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) ester.

**Materials.** Organic reagents and solvents were purchased from Nacalai Tesque Co., Wako Pure Chemical Ind., Ltd., and Aldrich Chemical Co. Solvents and commercially available materials were used without purification unless otherwise indicated. Ether, benzene, and pyridine were refluxed on sodium hydride or calcium hydride and distilled before the use. The detailed procedure for purification of the enzyme was described in the previous paper.[13] Alkyl 3-oxoalkanoates, ethyl 2-alkyl-3-oxobutanoates, and ethyl 2-formylalkanoates were prepared according to the method described in the previous papers,[19,32,26] respectively. All the compounds employed for the present reactions afforded satisfactory results in $^1$H NMR and IR spectroscopies.

**Enzyme Assay.** A 50 μl of the solution of **L-1** was added to 3.00 ml of a solution of 0.10 M potassium phosphate buffer (pH 7.0) that also contained a substrate (2 mM, 1 M=1 mol dm$^{-3}$) and NADPH (0.09 mM). The reaction rate was determined spectrophotometrically at 30 °C by following the decrease in absorbance of NADPH at 340 nm. One unit of enzyme activity is the amount of enzyme which oxidizes 1 μmol of NADPH per minute at 30 °C under these conditions.

**General Procedure for the Reduction of Various β-Keto Esters with L-1.** A concentrated solution of **L-1** (5 units), NADPH (10 mg), GDH (20 units), glucose (500 mg), a substrate (0.7 mmol), and a buffer solution (0.1 M Tris-HCl, pH 7.0, 70 ml) were placed in a 100 ml flask. The reaction vessel was shielded from light with an aluminum foil. The reaction mixture was shaken for 24 or 48 h, depending on the reactivity of the substrate,at 30 °C. Then, Hiflo-Super-Cell and ethyl acetate were added

to the mixture and resulted suspension was filtered. The organic portion was concentrated under reduced pressure and the residue was subjected to column chromatography on silica gel with hexane/ethyl acetate (9/1) as an eluent to give the corresponding hydroxy ester. The $^1$H NMR spectra of the products were the same with those of the products obtained by the reduction with sodium borohydride and were assignable to the expected structure. Chemical yield and enantiomeric/diastereomeric excess (e.e./d.e.) in each product are listed in Tables 2, 3, and 4. The e.e./d.e. and *syn*/*anti* ratio in the product were determined by the same procedures as those reported in the previous papers.[11,19,27]

**Reduction of Ethyl (+)- 2- Alkyl- 3- hydroxypropionate to the Corresponding 2-Methylalkanoate.** The conversion of ethyl 2-alkyl-3-hydroxypropionate into ethyl 2-methylalkanoate followed the method reported in the reference.[33] Tosylates were prepared by a standard procedure. Ethyl (+)-2-alkyl-3-hydroxypropionate (8.43 mmol) and tosyl chloride (10.5 mmol) were added to pyridine (30 ml) at 0 °C successively and the mixture was stirred overnight. The mixture was neutralized with 2 M HCl and resulted solution was extracted with ethyl acetate. The extract was dried over anhydrous magnesium sulfate and the organic solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/ethyl acetate (5/1) to give the corresponding tosylate in 49—65% yield.

Ethyl 2-tosyloxymethylpentanoate: $[\alpha]_D^{25}$ +0.16 ($c$=4.32, ether).

Ethyl 2-tosyloxymethylhexanoate: $[\alpha]_D^{25}$ +1.09 ($c$=5.25, ether).

The tosylate (5.1 mmol), $NaBH_4$ (10.0 mmol), and DMSO (25 ml) were placed in a 50 ml flask. The mixture was stirred for 3 h at 85 °C. Then, the reaction mixture was neutralized with 2 M HCl and the resulted solution was extracted with ethyl acetate. The extract was dried over anhydrous magnesium sulfate and the organic solvent was evaporated under reduced pressure. The product was obtained by distillation of the residue with a Kugelrohr apparatus.

Ethyl 2-methylpentanoate: $[\alpha]_D^{25}$ +3.96 ($c$=1.03, ether); lit,[28] *R*-form: $[\alpha]_D^{25}$ −7.93 ($c$=13.60, ether).

Ethyl 2-methylhexanoate: $[\alpha]_D^{25}$ +20.0 ($c$=4.60, ether); lit,[28] *S*-form: $[\alpha]_D^{25}$ +7.91 ($c$=4.296, ether).

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