Stereochemical Control in Microbial Reduction. XXI. Effect of Organic Solvents on Reduction of α-Keto Esters Mediated by Bakers' Yeast

Kaoru NAKAMURA,* Shin-ichi KONDO, Yasushi KAWAI, and Atsuyoshi OHNO
Institute for Chemical Research, Kyoto University, Uji, Kyoto 611
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Reduction of α-keto esters mediated by bakers' yeast takes place in an organic solvent without immobilization of the microbe. Several factors such as water content, pH of the solution, amount of bakers' yeast, and the property of the organic solvent affect to enantioselectivity of the reduction. In benzene, stereochemistry of the reduction shifts markedly toward preferential production of the (*R*)-α-hydroxy ester. The stereochemical consequence of the present reaction is accounted for by two factors; 1) the inhibition of enzymatic decomposition of the produced (*R*)-α-hydroxy ester in benzene and 2) the enhancement of relative activities of dehydrogenases or reductases in producing the (*R*)-α-hydroxy ester under dilute concentrations of the substrate at the vicinity of enzymes.

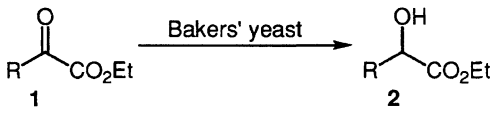
Use of an organic solvent for transesterification mediated by hydrolytic enzymes has widely been developed during the last decade.[1,2] Several advantages have been recognized in using biocatalysts in a non-aqueous solvent, namely, many organic substrates are hardly soluble to water, and highly nucleophilic property of water often causes undesirable side reactions such as hydrolysis and decomposition of the starting materials and products. In addition, since the product is usually soluble to an organic solvent very well, it is separated easily from other materials in the reaction mixture by simple filtration when an organic solvent is employed for the reaction. Nevertheless, little attention has been paid on the use of an organic solvent in enzymatic reactions other than those with hydrolytic enzymes. For organic chemists, one of popular groups of enzymes other than that of hydrolytic enzymes is a group of dehydrogenases. However, in most cases, a dehydrogenase requires the cooperation of an NAD-coenzyme and since the actual reagent for the redox reaction with a dehydrogenase is the coenzyme, stoichiometric amount of the coenzyme is necessary to complete the reaction unless the coenzyme is not reproduced by a composite redox reaction. In a microbial aqueous system, the recycling of the oxidized form of coenzyme is done easily by consumption of glucose. The reproduction of the coenzyme, however, is hardly achieved in an organic solvent because there is no proton source in the system. This is the main reason that organic solvents have been ignored for a wide variety of enzymatic reactions.

In modern organic syntheses, bakers' yeast has often been employed as a convenient and versatile reducing reagent in obtaining chiral alcohols usable for building blocks.[3,4] However only a few reports have concerned about the application of organic medium in the reduction mediated by this microbe.[5—8] The use of an organic solvent as a reaction medium faces another difficulty that the organic solvent may result in serious damage to the cell membrane and releasing the denatured enzymes into the solvent. In order to prevent the cell from the damage, immobilization method has been developed. For example, we reported the reduction of α-keto esters with an immobilized bakers' yeast in hexane, where the stereoselectivity of the reaction was different from that observed in water with unimmobilized one.[5]

Recently, we also found that unimmobilized bakers' yeast survives in an organic solvent affording satisfactory results in the reduction of α-keto esters.[7] The reaction system again changes stereoselectivity from those exerted in aqueous solutions. In this paper, detailed insight of the reaction will be described and the effect of organic solvent on changing the stereoselectivity of the reduction will be discussed.

Results

Bakers' yeast is available in two different states; one is raw (pressed) and the other is dry. Dry bakers' yeast (Oriental Co., Ltd.) was employed because the enantiomeric excess (e.e.) in the product was reproducible better with this state than the other, and the water content in the reaction system was easily controlled by using dry bakers' yeast making it possible to investigate the effect of water on reaction rate and enantioselectivity. The reaction studied in the present research was the reduction of α-keto esters shown in Eq. 1.



1 2

a	R = CH_3	**d**	C_4H_9
b	C_2H_5	**e**	C_5H_{11}
c	C_3H_7	**f**	$(CH_3)_2CH$

(1)

Ethyl 2-oxoheptanoate (**1e**) was employed as the key compound of the survey, because quantitative detection of both the starting material and the product (**2e**) is easy with this ester.

Solvent Effect on Enantioselectivity. Various organic solvents were tested to look for the best result on both enantioselectivity and chemical yield. Table 1 summarizes the results from the reduction of **1e**.

The reaction did not take place in a hydrophilic solvent such as ethanol or tetrahydrofuran, probably because the water inside the cell of a microbe flows out into the bulk of solvent and the enzymatic systems of the microbe become out of the order. Among the solvents tested, benzene was chosen for further studies because it gave an excellent result and is easy to be treated. Toluene may be substituted for benzene.

Effect of Water Content. As shown in Fig. 1, while no reaction proceeded in dry benzene, the addition of a drop of water to the system promoted the reaction immediately resulting in the formation of ethyl 2-hydroxyheptanoate (**2e**).

Table 2 indicates that the water content defined by "ml-water/g-bakers' yeast" is important on discussing the variation in e.e. and chemical yield. Relative amount of water against the amount of solvent has no meaning.

Enantiomeric excess and chemical yield from the reduction of **1e** are plotted in Fig. 2 against the water content in the system.

It is recommended to pulverize the dry bakers' yeast as fine as possible in order to increase the contact area between the solid (bakers' yeast) and liquid (bulk solvent) phases. It is known that acidic conditions promote the oxidation of NAD(P)H and basic conditions accelerate the reduction of $NAD(P)^+$.[9] For the present

Table 1. Solvent Effect on the Enantioselectivity in the Reduction of Ethyl 2-Oxoheptanoate by Bakers' Yeast

Solvent[a]	Yield/%[b]	E.e./%[c]	Config.
Cyclohexane	25	55	*R*
Hexane	22	61	*R*
t-Butyl methyl ethr	26	63	*R*
Diisopropyl ether	22	67	*R*
p-Xylene	17	72	*R*
Toluene	25	76	*R*
Mesitylene	19	77	*R*
Benzene	36	77	*R*
t-Butyl aceate	33	77	*R*

a) Dry bakers' yeast (0.2 g ml^{-1} solvent) and distilled water (0.4 ml/g-bakers' yeast) were added to the reaction mixture. b) Chemical yield was determined by GLC analysis. c) Enantiomeric excess was determined by GLC analysis of the corresponding (*R*)-MTPA esters.

Table 2. Effect of Water Contents on the Reduction of Ethyl 2-Oxoheptanoate with Bakers' Yeast in Benzene[a]

Benzene	Water Added	Water/yeast[b]	Yield[c]	E.e.[d]	Config.
ml	ml	ml g^{-1}	%	%	
7	0.28	0.34	14	58	*R*
7	0.56	0.57	36	77	*R*
7	0.65	0.64	27	82	*R*
7	0.84	0.79	24	85	*R*
7	1.12	1.01	18	84	*R*
14	1.12	1.01	11	82	*R*
21	1.12	1.01	7	80	*R*
7	1.68	1.46	5	83	*R*
7	2.24	1.91	3	—[e]	—

a) Dry bakers' yeast, 1.4 g; Substrate, 0.02 M in benzene. b) Amount of water=water added to the system+water originally contained in dry yeast: Net yeast=weighed dry yeast−contained water in it. c) Chemical yields were determined by GLC analysis. d) Enantiomeric excesses were determined by GLC analysis of corresponding (*R*)-MTPA esters. e) Not determined.

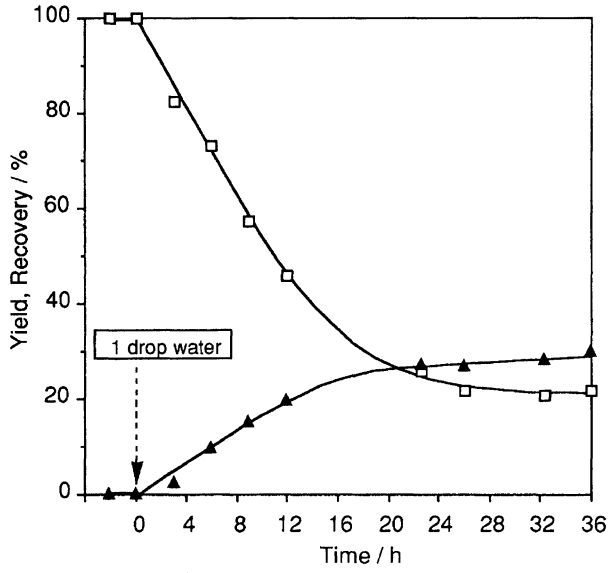


Fig. 1. Reduction of ethyl 2-oxoheptanoate (**1e**, □) to ethyl 2-hydroxyheptanoate (**2e**, ▲) with bakers' yeast in benzene.

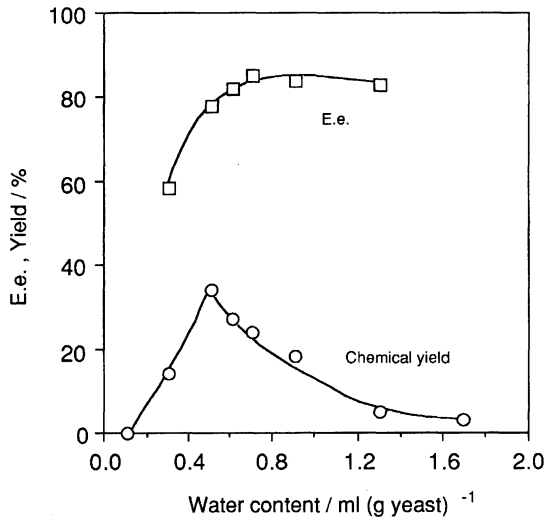


Fig. 2. Effect of water content on the enantioselectivity (□) and chemical yield (○) from the reduction of ethyl 2-oxoheptanoate (**1e**).

research, both oxidation and reduction have to undergo smoothly in order to use the coenzyme repeatedly. Therefore, the effect of acidity of water added to yeast was tested. Although no appreciable improvement on both the enantioselectivity and chemical yield was observed, an acetate buffer of pH 5 (0.1 M, M=mol dm^{-3}) was found to afford reliable results in reproducibility, and this was employed for further reactions.

Reduction of α-Keto Esters. Six α-keto esters, **1a**—**1f**, were subjected to the reduction in benzene and water. Table 3 summarizes the results.

It is recognized that the reduction in benzene shifts the enantioselectivity toward the (*R*)-side in comparison with the reaction in water. Namely, the configurations of the products from the reduction of **1b**—**1e** change from (*S*)-predominance to (*R*)-predominance: inversion of the selectivity.

Enantioselective Decompositions of the Product. As will be described later, there is a possibility that the α-hydroxy esters, **2**, are decomposed in aqueous solutions enantioselectively under the catalysis of enzymes in bakers' yeast. To test the validity of this proposal, enentioselective decompositions of racemic **2e**, as a representative of the α-hydroxy esters, in the presence of bakers' yeast in water, hexane, and benzene were studied quantitatively. Figure 3 demonstrates the time-dependent consumption of **2e** and e.e. observed for **2e** recovered from each solvent system after 24 h.

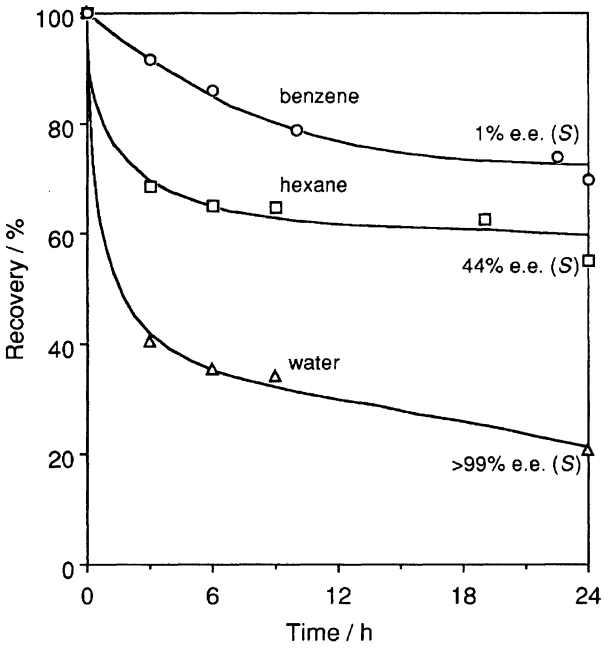


Fig. 3. Decomposition of ethyl 2-hydroxyheptanoate (**2e**) catalyzed by bakers' yeast in benzene (○), hexane (□), and water (△).

Discussion

The Role of Water. Water content plays a crucial role in the reduction mediated by bakers' yeast in an organic solvent as demonstrated in Fig. 2. The fact that relative amount of water against bakers' yeast, but not against the bulk solvent, is important in affording satisfactory results clearly indicates that the water added to the system interacts with the bakers' yeast or enzymes in it. A rapid increase in the chemical yield can be seen as the water content increases up to 0.6 ml-water/g-bakers' yeast, then the yield decreases gradually as the water content increases further. Thus, about 70—100% water in weight against bakers' yeast affords the best result. After the ratio exceeds 1.9, almost no reaction proceeds practically. This is the reason that raw bakers' yeast, which contains large amount of water, does not afford satisfactory result in chemical yield as well as its reproducibility.

Table 3. Reductions of α-Keto Esters by Bakers' Yeast in Water and Wet Benzene[a)]

Substrate		Water		Benzene[b)]	
	R	Yield/%	E.e./%	Yeld/%	E.e./%
a	CH_3	79	93 (*S*)	56	73 (*S*)
b	C_2H_5	44	78 (*S*)	39	13 (*R*)
c	C_3H_7	23	76 (*S*)	44	63 (*R*)
d	C_4H_9	5	99 (*S*)	54	86 (*R*)
e	C_5H_{11}	9	92 (*S*)	26	86 (*R*)
f	$(CH_3)_2CH$	63	19 (*R*)	49	90 (*R*)

a) Dry bakers' yeast, 4 g; Substrate, 0.4 mmol; Benzene, 20 ml. b) The solvent contains 3.2 ml of potassium phosphate buffer (pH 5).

The necessity of small amount of water has also been reported in transesterification of alcohols catalyzed by a lipase,[10—15] although the amount of water required for transesterification with a lipase is much less than that for the reduction with bakers' yeast. The major reason for this difference may be accounted for by the fact that bakers' yeast is a microbe, a living thing, and a lipase is an enzyme, a material; in order to keep a microbe survive (or, at least, to keep the enzyme system operating), much water might be required than to keep the conformation of a protein. Usually, the oxidation and reduction of the coenzyme is catalyzed by different enzymes, and the reduced form of this coenzyme is produced by the pentose-phosphate pathway.[16] Without a recycling system(s) of NAD(P)H, the true reagent for the reduction, it is natural that no detectable reduction takes place.

One of the reasons that the presence of large amount (1.5 ml g^{-1} in the ratio or more) of water inhibits the reduction stems from the decrease in the surface area of bakers' yeast; the well-pulverized bakers' yeast begins to form aggregates again at higher water contents. The other candidate for the reason is the cooperation of autolytic enzymes in the presence of large amount of water. Autolysis of wet cells by adding an organic solvent is well-known and this technique has been applied for the extraction of enzymes from the cell of a

microbe.[17]

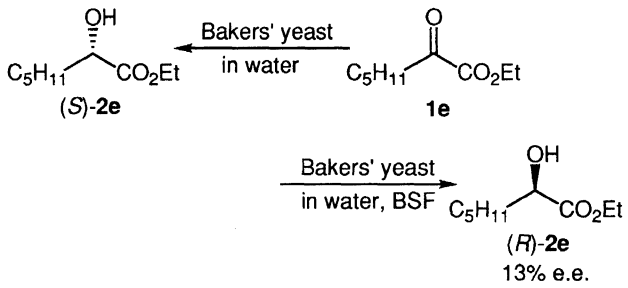
All reductions in organic solvents so far studied tend to afford the (*R*)-predominant products. The same tendency has been observed in the reduction in hexane mediated with raw bakers' yeast immobilized by polyurethane,[5] although the effect in immobilized system is small. Details will be discussed in the following section.

Enantioselective Decompositions of the Product in Water. In a previous paper from our laboratory, it was reported that the reduction of **1e** by bakers' yeast immobilized by polyurethane affords (*R*)-**2e** in hexane, whereas the antipode is obtained from the reduction in water.[5] So far water is employed as the solvent, immobilization of the microbe exerted no difference in enantioselectivity. It was proposed, therefore, that the stereochemical shift stems from the enantioselective decomposition of (*R*)-**2e** under aqueous conditions.[18] Although the reduction itself affords the (*R*)-predominant product, the preferential decomposition of the (*R*)-enantiomer remains the (*S*)-predominant ester as the final and isolable product from an aqueous system.[5]

Figure 3 suggests that **2e** disappears faster in water than it does in organic solvents, and 80% of the starting material has already been consumed in water after 24 h. The e.e. of the remained starting material is the highest in water among the systems studied. There is no doubt that enantioselective decomposition of **2e** takes place in an aqueous system. On the other hand, practically no such decomposition of **2e** proceeds in benzene and the starting material remains as the racemic mixture of the enantiomers. It is conceived that the consumption of **2e** in benzene is due to uncatalyzed hydrolysis of the ester. The difference in the amount of unreacted **1e** between different solvents, therefore, is attributed to the amount of enantioselective decompositions catalyzed by the microbe. Thus, the e.e. in the product observed in the benzene system seems to afford accurate enantioselectivity of the reduction exerted by bakers' yeast. The decomposition is prohibited in certain extent in hexane, but not completely.

However, the enantioselective decomposition alone cannot explain the whole shift of the selectivity. The stereochemistry associated with the reduction of **1f** also changes on changing the solvent from water to benzene. Here, it has been confirmed that the product, **2f**, which has a branched-chain substituent is not decomposed enantioselectively in water[6] in spite that a large shift to the (*R*)-predominance is observed in benzene (Table 2). Another example can be seen in the reduction of **1a**. The shift of enantioselectivity toward the (*R*)-side is also seen in the reduction of this ester in benzene. It has again been confirmed that ethyl lactate (**2a**) is not decomposed enantioselectively in aqueous solutions.[5]

Recently, Ushio et al. reported[19] that enantioselective decomposition of **2e** catalyzed by bakers' yeast is inhibited completely by the addition of 1-butanesulfonyl fluoride (BSF) to the reaction system. The inhibitor has also been applied to the present reduction system in water, and has been found to afford (*R*)-**2e** in 23% chemical yield and 13% e.e. in contrast to the production of (*S*)-predominant product under normal aqueous conditions (Eq. 2).



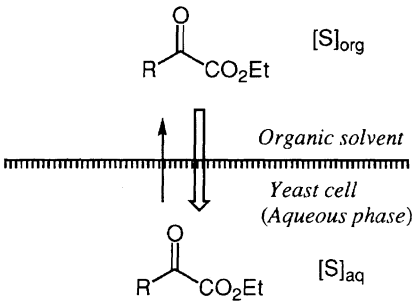
(2)

The enantioselectivity of the reduction is apparently shifted from the (*S*)-preferency to the (*R*)-preferency on addition of BSF, but the shift is too small to regard the enantioselective decompositions of the product as the whole factor to change the selectivity on changing the reaction medium from water to organic solvent.

Thus, it has been confirmed that there is a factor(s) other than enantioselective decomposition of the product in producing the (*R*)-predominant product in the reduction in an organic solvents.

Effect of Substrate Concentration. In a water/organic solvent biphase medium, a substrate is partitioned between two phases. Thus the concentration of the substrate in the water phase, where bakers' yeast exists, is different from that in a uniphasic water solution. Consequently, the effective concentration of a substrate at the vicinity of dehydrogenases that exist inside the cell of a microbe differs from the stoichiometric one in the bulk of organic solvent as schematically illustrated in Scheme 1.

Since K_m and V_{max} of a substrate are different for different enzymes, respectively, the change in the concentration of a substrate should affect the kinetics with each enzyme, and the enantioselectivity may change as the consequence of the change in relative reactivity



Scheme 1.

(V_{max}/K_m) of each enzyme.

The concentrations of **1e** and **1f** in the aqueous phase of the biphasic media calculated from the partition coefficients for these substrates are <0.1 mM and 0.64 mM with benzene, and 0.17 and 5.8 mM with hexane under usual conditions employed for the reduction. The values are much less than the stoichiometric one (20 mM; see Experimental). This reduction of the concentration seems to be another candidate for the factor to change the enantioselectivity of the reduction. Accordingly, the effect of the dilution of **1f** in water was tested in detail. It should be noted that resulting **2f** does not undergo catalytic enantioselective decomposition in water. Not only in aqueous system but also in organic system, the decrease in concentration of the substrate contributes to increase e.e., which again proves the effect of diluted concentration of a substrate on improving the enantio selectivity. The results are plotted in Fig. 4.

As expected, the enantioselectivity is shifted toward the (*R*)-side as the concentration of **1f** decreases. Namely, the increase in the enantioselectivity at lower concentrations is appreciable. It is interesting to note that the partition coefficient of hexane is 34 times larger than that of benzene and the latter solvent affords better result in enantioselectivity than the former by keeping the substrate concentration in the aqueous phase very low (Table 1). Being judged from the result obtained here, a dehydrogenase(s) which reduces an α-keto ester to the corresponding (*R*)-α-hydroxy ester (*R*-enzyme) may have smaller K_m than the dehydrogenase(s) which affords the (*S*)-product (*S*-enzyme). We cannot discuss on the difference in V_{max} of these enzymes at present.

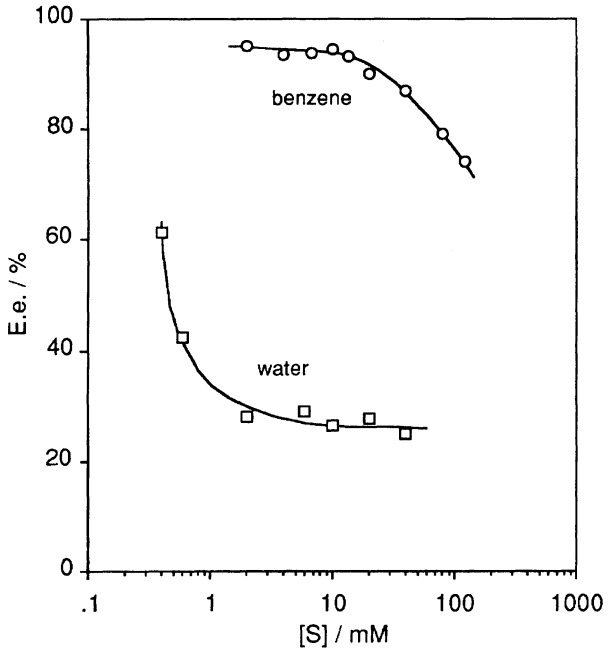


Fig. 4. Effect of substrate concentration on the enantioselectivity of the reduction of ethyl 3-methyl-2-oxobutanoate (**1f**) in benzene (○) and water (□).

It has, thus, been elucidated that benzene containing small amount of water affords satisfactory results in producing (*R*)-α-hydroxy ester by the reduction of α-keto ester mediated by dry bakers' yeast. The bakers' yeast is not necessary to be immobilized when certain amount of water is contained in the system. The change in enantioselectivity stems from two reasons; one of which is the prevention of catalyzed enantioselective decompositions of (*R*)-α-hydroxy ester produced by the reduction, and the other is the acceleration of *R*-enzymes relative to *S*-enzymes under concentration-controlled conditions.

Experimental

Instruments. ^{1}H NMR spectra were recorded on a Varian VXR-200 spectrometer in $CDCl_3$. Gas–liquid chromatograms were recorded on a Shimadzu GC-14A gas–liquid chromatograph. Thermogravimetric analysis of bakers' yeast was recorded on a Shimadzu TGA-50 thermogravimetric analyzer.

Materials. Ethyl pyruvate (**1a**) and ethyl lactate (**2a**) were purchased from Nacalai Tesque, Inc. 2-Oxobutanoic acid and 1-butanesulfonyl chloride were purchased from Tokyo Kasei Co. Ethyl 3-methyl-2-oxobutnoate (**1f**) and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid ((*R*)-(+)-MTPA) were obtained from Aldrich Chemical Co. Dry bakers' yeast was purchased from Oriental Yeast Co. and stored in a refrigerator.

Ethyl 2-oxobutanoate (**1b**), ethyl 2-oxopentanoate (**1c**), ethyl 2-oxohexanoate (**1d**), and ethyl 2-oxoheptanoate (**1e**) were prepared according to the literature procedures.[5]

1-Butanesulfonyl Fluoride. 1-Butanesulfonyl fluoride was synthesized by the same procedure as described in the literature.[20] ^{1}H NMR spectrum and other physical constants as well as elemental analyses gave satisfactory results.

Measurement of Water Content in Dry Bakers' Yeast. Pulverized dry bakers' yeast (7.50 mg) was subjected to thermogravimetric analyzer and was heated at 130 °C for 30 min. The decrease in weight was 10.56%. The water content in bakers' yeast thus measured was taken into consideration to calculate total amount of water in the reaction mixture.

General Procedure for the Reduction in Water. Reduction of Ethyl 2-Oxoheptanoate (1e). As a typical example, 172.2 mg (1 mmol) of ethyl 2-oxoheptanoate was added to a suspension of 5 g of pulverized dry bakers' yeast in 50 ml of distilled water, and the whole mixture was stirred at 30 °C until no starting material was detected on gas chromatography (PEG 20 M bonded, 25 m, 130 °C). Then Hiflo Super-Cel (10 g) and dichloromethane were added to the mixture, and the resulted suspension was filtered over celite. The filtrate was extracted three times with each 50 ml of dichloromethane. The combined organic layer was washed with brine, dried over sodium sulfate, and the solvent was evaporated under reduced pressure. Chemical yield of the product was determined by GLC analysis (PEG 20M bonded, 25 m, 130 °C) with pentadecane as an internal standard. Residual oil was subjected to preparative gas chromatography (PEG 20M, 1.5 m, 140 °C) giving 10.5 mg (6% yield) of **2e**. Enantiomeric purity of the product

was measured as will be described below.

General Procedure for the Reduction in an Organic Solvent. Four milliliters of an acetic acid/sodium acetate buffer solution (0.1 M, pH 5.0) was added to a suspension composed of 50 ml of benzene, 172.2 mg (1 mmol) of **1e**, and 10 g of pulverized dry bakers' yeast, and the whole suspension was stirred for 24 h, then was filtered. The solvent in the filtrate was evaporated under reduced pressure. Chemical yield of the product was determined by GLC analysis (PEG 20M bonded, 25 m, 130 °C) with pentadecane as an internal standard. The residual oil was worked up in the same manner as described above giving 27.2 mg (16% yield) of **2e**. Enantiomeric purity of the product was measured as will be described below. Results from other organic solvents are also summarized in Table 1. Table 2 lists the results from variation in relative amount of materials in the reaction system.

Reduction of Ethyl 3-methyl-2-oxobutanoate (1f) in a Larger Scale. To a suspension of pulverized dry bakers' yeast (139 g) in 700 ml of benzene containing 2.00 g (14 mmol) of **1f**, 83.2 ml of 0.1 M, acetic acid–sodium acetate buffer (pH 5) was added. The whole suspension was shaken for 24 h at 30 °C, then filtered. The filtrate was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The residual oil was distilled at 72 °C under the pressure of 20 mmHg (1 mmHg=133.322 Pa) to give 1.12 g (55% yield) of **2f**. The enantiomeric purity of the product was determined to be 90% by the method which will be described below.

Determination of Enantiomeric Purity of the Product. The α-hydroxy esters were converted into the corresponding (*R*)-(+)-α-methyl-α-(trifluoromethyl)phenylacetic acid (MTPA) esters by the Mosher's methods.[21] The diastereomeric excess in the ester was determined on GLC (PEG 20M bonded, 25 m, 150—180 °C). The absolute configurations were determined by comparing retention times on GLC of the MTPA esters with those of the corresponding authentic samples.[5]

Partition Coefficients for 1f and 1e between an Organic Solvent and Water. A solution composed of 10 ml of benzene, 28.8 mg (20.0 mM) of **1f**, and 12.6 mg (5.93 mM) of pentadecane was prepared. To 10.0 ml of distilled water, 2.0 ml of the benzene solution was added and the mixture was shaken at 30 °C for 1 h. The concentration of **1f** in the benzene phase was determined to be 17.2±0.2 mM by GLC analysis (PEG 20M, 1.5 m, 95 °C) with pentadecane as an internal standard. Consequently, the partition coefficient of **1f** between benzene and water at 30 °C was calculated to be 31.0±2.0. The partition of **1f** between hexane and water was measured similarly, and the coefficient was calculated to be 3.39±0.09 at 30 °C.

Partition of **1e** between 2.0 ml benzene and 30.0 ml water was measured similarly. However, the solubility of **1e** to benzene is so high that the partition coefficient could not be calculated accurately. The value was estimated to be larger than 200. Partition coefficient of **1e** between hexane and water was calculated to be 117±13 after the same experiment described above.

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