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Stereochemical Control of Yeast Reductions. 6. Diastereoselectivity of 2-Alkyl-3-oxobutanoate Oxido-reductases.[1]

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ABSTRACT: Two L-oxido-reductases, designated as L-enzyme 1 and L-enzyme 2, purified from bakers' yeast catalyzed the reduction of 2-alkyl-3-oxobutanoates using NADPH as the coenzyme to furnish carbinols of the L-configuration (ee = 0.98). The L-enzyme 2 (MW 36,000) has a more relaxed substrate specificity than L-enzyme 1 (MW 74,000). With the exception of 2-methyl-3-oxobutanoate, L-enzyme 1 and 2 exhibited opposite chiral preferences relative to the C-2 substituent to yield the *anti*(2S,3S)- and the *syn*(2R,3S)-isomer respectively. The degree of diastereoselectivity depends on the C-2 substituent but not on the size of the ester grouping for L-enzyme 1. In contrast, the diastereoselectivity of L-enzyme 2 is not affected by the size of C-2 alkyl substituent and ester groupings. The kinetic parameters of the purified enzymes are consistent with the stereochemical outcome of intact bakers' yeast reductions in that L-enzyme 1 is the dominant enzyme in competitive reductions.

INTRODUCTION

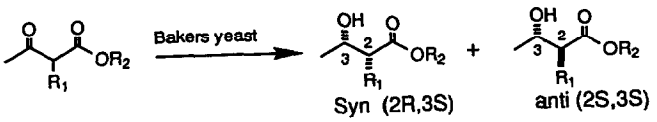
The stereoselective reductions of β-keto esters by bakers' yeast have been used extensively by synthetic chemists for the preparation of optically-active alcohols[2], which are important intermediates for the synthesis of complex natural products. In contrast to acyclic achiral ketonic substrates, bakers' yeast-catalyzed reduction of 2-alkyl-3-oxobutanoate esters generates two chiral centers simultaneously because these substrates undergo facile keto-enol equilibration. Consequently, it should be possible to control the stereochemical course of bakers' yeast reductions to generate one predominant diastereomer out of four possible isomers, if both substrate and product enantiospecificities are met.[3] A survey of the published results from various laboratories reveals that in general bakers' yeast-catalyzed reduction of 2-alkyl-3-oxobutanoate esters are highly enantioselective to furnish the corresponding L-(S)-3-hydroxy esters.[4] However, the diastereoselectivity (*syn/anti* ratio) varies widely depending on the chemical structure of the 2-alkyl substituent and in some cases the size of the alkoxy ester also.[5]

In 1987, our laboratory completed the studies on the β-keto ester reductases from bakers' yeast.[6] Two enzymes, designated as L-enzyme 1 and L-enzyme 2, were isolated, characterized and shown to be the dominant enzymes responsible for the reduction of 2-alkyl-3-oxobutanoate esters. Herein, we report our investigations pertaining to the substrate diastereoselectivity of these purified enzymes and the correlation of these results with those of intact bakers' yeast cells.

RESULTS

Bakers' Yeast-catalyzed Reduction of 2-Alkyl-3-oxobutanoate Esters. Several 2-alkyl-3-oxobutanoate esters were exposed to bakers' yeast and the results are shown in Table 1. As expected, the reduction proceeded with a high degree of enantioselectivity in all cases to give the corresponding L-(S)-3-hydroxybutanoate esters. However, the diastereoselectivity (*syn/anti* ratio) varied widely depending on the 2-alkyl substituent. For the C-2 methyl and propargyl derivatives, the *syn*(2R,3S) isomer was preferentially formed whereas the *anti*(2S,3S) isomer was found to be the dominant product when the C-2 substituent was either allyl or benzyl. Enlarging the size of the ester group appeared to have some effect on diastereoselectivity. For example, with 2-benzyl-3-oxobutanoate esters (**4**, **7**), the *syn/anti* ratio changed from 30:70 to 4:96 when the ethyl ester was changed to the octyl ester.

TABLE 1. Diastereoselectivity of bakers' yeast reductions



Compound	Yield (%)	*syn:anti*	$[\alpha]_D^{23}$ in $CHCl_3$	ee
1 R_1=CH_3; R_2=C_2H_5	50	91:9	+6.15° (c=2.42)	0.98 (2R,3S)
2 R_1=$CH_2C{\equiv}CH$; R_2=C_2H_5	75	76:24	+23.13° (c=2.35)	0.98 (2R,3S)
3 R_1=$CH_2CH{=}CH_2$; R_2=C_2H_5	57	23:77	+12.61° (c=1.83)	0.98 (2S,3S)
4 R_1=$CH_2C_6H_5$; R_2=C_2H_5	71	30:70	-9.03 (c=2.90)	0.98 (2S,3S)
5 R_1=CH_3; R_2=C_8H_{17}	64	96:4	+3.30 (c=3.18)	0.98 (2R,3S)
6 R_1=$CH_2CH{=}CH_2$; R_2=C_8H_{17}	37	40:60	+5.10° (c=1.88)	0.98 (2S,3S)
7 R_1=$CH_2C_6H_5$; R_2=C_8H_{17}	11	6:94	-24.31° (c=0.54)	0.98 (2S,3S)

Substrate Specificity of the Purified 2-Alkyl-3-oxo Ester Oxido-reductases. Two enzymes, L-

enzyme 1 (MW 74,000 ± 7,000, previously reported as L-enzyme) and L-enzyme 2 (MW 36,000 ± 3,000) had been purified to homogeneity from the cytosolic fraction of bakers' yeast. Both enzymes were found to catalyze the reduction of a variety of 2-alkyl-3-oxobutanoates in the presence of NADPH but not NADH to yield the corresponding L-(S)-carbinols. The relative activities of these two oxido-reductases towards various keto acids and esters were examined with a view to gaining some insight as to the identity of their natural substrates. Ethyl γ-chloro-3-oxobutanoate was used as a reference standard, because both enzymes exhibited similar relative activity towards this substrate. It is worthy of note that L-enzyme 1 reduced only β-keto esters whereas β-keto acids, α- and γ-keto acids and esters cannot serve as substrates for this enzyme (Table 2). The reaction rates varied widely depending on he structure of the β-keto substrates. The stereoselective reductions generally proceeded much faster for acyclic β-keto esters without α-substituents. On the other hand, L-enzyme 2 appeared to possess a more relaxed substrate specificity. In fact, this enzyme was catalytically-active on all the substrates examined including α-, γ-keto acid and esters albeit at a lower relative rate. Both enzymes exhibited high relative activity towards cyclic β-keto esters.

Stereoselectivity of the Purified Oxido-reductases. The stereoselectivity of the reductions of several 2-alkyl-3-oxobutanoate esters by purified L-enzyme 1 and 2 were examined and the results are shown in Table 3. While both enzymes possess the 3(S) preferred chirality for the reduction of the 3-oxo group in all the substrates examined, L-enzyme 1 and 2 exhibited opposite stereochemical preference for the C-2 alkyl substituent except for 2-methyl-3-oxobutanoates (**1** and **5**). That is, the L-enzyme 2 catalyzed the reduction to yield preferentially the *syn*(2R,3S) isomer whereas L-enzyme 1 gave the *anti*(2S,3S) isomer (Scheme 1). Thus the size of the C-2 substituent has a marked influence on the formation of the *syn/anti* isomers and the reaction rate whereas enlarging the ester group from ethyl to octyl did not appear to have a pronounced effect.

SCHEME 1

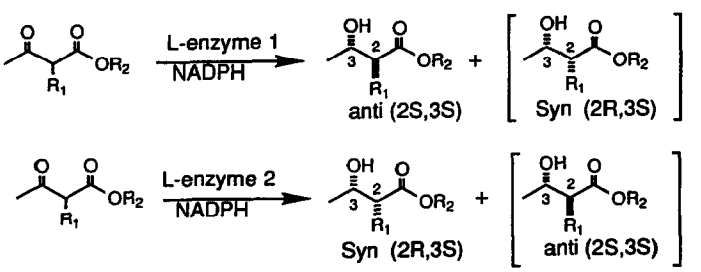
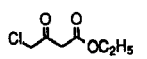
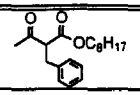
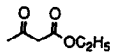
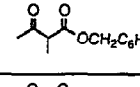
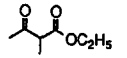
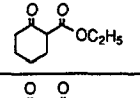
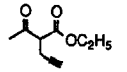
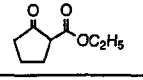
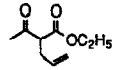
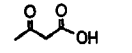
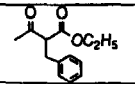
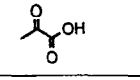
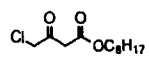
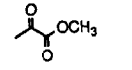
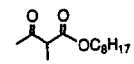
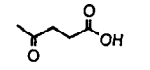
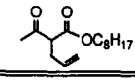
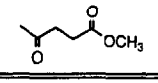


TABLE 2. Substrate specificity of the purified α-substituted β-keto ester reductases.[a]

Substrate[b]	L-enzyme 1 (% activity)	L-enzyme 2 (% activity)	Substrate[b]	L-enzyme 1 (% activity)	L-enzyme 2 (% activity)
	100	100		0.54	2.6
	11	15		0.16	67
	2.6	33		15	55
	1.1	53		5.2	32
	9.0	23		0	2.0
	0.5	3.9		0	1.5
	106	161		0	3.0
	2.1	22		0	0.3
	0.9	6.3		0	1.8

[a]The enzyme activity was measured according to the method described in the experimental section using 19 units of the purified enzyme.

[b]All the substrate concentrations were 5 mM except the octyl α-alkylacetoacetates (**5a-7a**) where 1 mM was used to avoid the turbidity which could complicate the spectrophotometric measurement.

TABLE 3. Diastereoselectivity of the purified L-enzyme 1 and L-enzyme 2

Substrate	L-enzyme 1			L-enzyme 2		
	% c	*syn:anti*	ee	% c	*syn:anti*	ee
1	46	90:10	0.98	26	99:1	0.98
2	25	10:90	0.98	45	99:1	0.98
3	89	1:99	0.98	27	99:1	0.98
4	<1	19:81	0.98	4.5	90:10	0.98
5	5	91:9	0.98	19	99:1	0.98
6	1.5	1:99	0.98	7.5	99:1	0.98
7	<1	20:80	0.98	3	99:1	0.98

The kinetic parameter, K_m (Michaelis constant) and k_{cat} (turnover number) of the purified oxido-reductases on 2-methyl, 2-allyl and 2-propargyl-3-oxobutanoate ethyl esters were measured in order to gain an insight into the diastereoselectivity of bakers' yeast reductions of these compounds (Table 4). In general, the K_m values are higher for substrates bearing a 2-alkyl substituent. For example, the K_m values of L-enzyme 1 and 2 for ethyl-γ-chloroacetate are $1x10^{-3}$M and $1.25x10^{-3}$M respectively. As the size of the 2-alkyl substituent is increased, an increase in the value of K_m is noted. The second order rate constants (k_{cat}/K_m) for all three substrates were higher for L-enzyme 1 than for L-enzyme 2 (Table 4).

Reduction of 2-Alkyl-3-oxobutanoates by (R)-3-Hydroxybutanoate Oxidoreductases. The reduction of a series of β-keto esters by two commercial (R)-3-hydroxybutanoate oxidoreductases were investigated and the results are shown in Table 5. As can be seen, the enzyme from *Rhodo-pseudomonas spheroides* had a very narrow substrate specificity and was inert towards substrates possessing an α-substituent. In contrast the enzyme from *Pseudomonas lemoignes* exhibited a broader substrate specificity and was active on straight chain and branched β-keto esters to yield D-carbinols of high optical purity (ee >0.98). Moreover, the enzyme preferentially formed the *syn*(2S,3R) isomer on all the β-keto esters tested. The *syn/anti* ratio varied from 86:14 for 2-benzyl-3-oxobutanoate (**4**) to 99:1 for both 2-allyl- and 2-propargyl-3-oxobutanoate (**2**) (Table 6).

TABLE 4. The kinetic parameters of the purified enzymes.[a]

Substrate[b]	L-enzyme 1			L-enzyme 2			$(k_{cat}/K)_1/(k_{cat}/K)_2$
	K (mM)	k_{cat} (sec^{-1})	k_{cat}/K ($mM^{-1}sec^{-1}$)	K (mM)	k_{cat} (sec^{-1})	k_{cat}/K ($mM^{-1}sec^{-1}$)	
1	3.61 ± 0.19	14.83 ± 0.37	4.11 ± 0.13	6.75 ± 0.75	11.04 ± 0.59	1.64 ± 0.10	2.51 ± 0.23
2	10.01 ± 1.09	24.14 ± 1.64	2.41 ± 0.11	19.55 ± 4.12	40.01 ± 5.27	2.05 ± 0.17	1.18 ± 0.28
3	6.30 ± 1.25	67.14 ± 7.73	10.66 ± 0.97	11.86 ± 4.97	20.67 ± 5.48	1.74 ± 0.28	6.13 ± 1.25

[a]The kinetic constants were determined by using .5 μg L-enzyme 1 or 0.28 μg L-enzyme 2 at various concentrations of 2-alkyl-3-oxobutanoates (**1-3**) (dissolved in 10 μl ethanol) in the presence of .24 mM NADPH at pH 6.5 at 23°C. The data were analyzed by the Lineweaver-Burk method using nonlinear regression analysis and the standard deviations are shown.

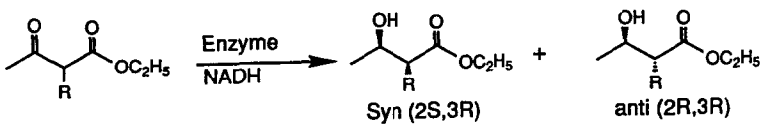
[b]The kinetic constants of ethyl 2-benzyl-3-oxobutanoate (**4**) and the octyl esters were not measured due to the difficulty in preparing a homogeneous solution at high concentrations.

TABLE 5. The relative rates of reduction of β-keto esters by two (R)-3-hydroxybutanoate oxido reductases

Substrate	*Pseudomonas lemoignei*		*Rhodopseudomonas spheroides*	
	ΔA_{340}/min	% activity	ΔA_{340}/min	% activity
Ethyl-γ-chloro-3-oxobutanoate	0.035	100	0.003	100
1	0.015	43	0.0015	50
2	0.018	51	0	0
3	0.042	120	0	0
4	0.012	34	0	0

The assay mixture contained: 1 ml of 0.1 M sodium phosphate (pH 6.5), 0.3 mM NADH, 5 mM β-keto ester (dissolved in 10 μl dimethylformamide) and 1 unit of the indicated enzyme. The decrease of absorbance at 340 nm was monitored continuously.

TABLE 6. Stereoselectivity of (R)-3-hydroxybutanoate oxidoreductase from *Pseudomonas lemoignei*



Substrate	% Conversion	*syn*(2S,3R): *anti*(2R,3R)	$ee_{(2S,3R)}$
1	98	89:11	0.98
2	99	99:1	0.98
3	99	99:1	0.98
4	96	86:14	0.98

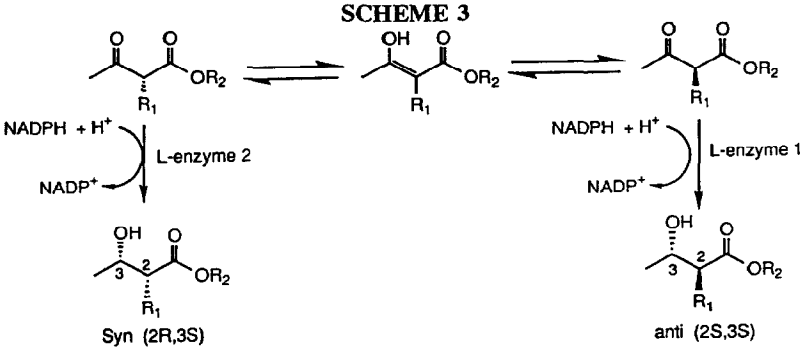
DISCUSSION

Two β-keto ester oxido-reductases were purified to homogeneity from bakers' yeast. L-enzyme 1 (MW 74,000 ± 7,000) appears to be a dimeric protein which could be converted into two identical subunits (MW 42,000 ± 5,000) by treatment with SDS. This enzyme was inhibited by

p-chloromercuribenzoaate and heavy metal ions such as Cu^{2+} and Hg^{2+}. In contrast, L-enzyme 2 consists of a single polypeptide (MW 36,000 ± 3,000) and does not require sulfhydryl reducing agents to retain its enzyme activity.

Both enzymes utilize NADPH as the coenzyme and reduced a variety of β-keto esters (Table 2). However, L-enzyme 1 is much less active towards β-keto esters possessing an α-substituent. In contrast L-enzyme 2 has a more relaxed substrate specificity and was capable of reducing α- and γ-keto acids and esters as well. Both enzymes delivered the hydride of the NADPH molecule from the Re face of the β-keto esters to yield (S)-carbinols. However, the diastereoselectivity of the two enzymes differed in that L-enzyme 2 preferentially formed the *syn*(2R,3S) isomer whereas L-enzyme 1 favored the formation of the *anti*(2S,3S) isomer except when the C-2 alkyl substituent is small such as in ethyl-2-methyl-3-oxobutanoate (**1**). The high overall stereoselectivity exhibited by these enzymes may be rationalized by Scheme 3.

SCHEME 3



We envisage that the keto form of the 2-alkyl-3-oxobutanoates are in rapid equilibrium with each other via the enol intermediate. The L-enzyme 1 binds preferentially to the 2(S)-alkyl-3-oxobutanoate whereas the L-enzyme 2 prefers the 2(R)-alkyl-3-oxobutanoate. The hydride of NADPH is then delivered from the Re face by the enzymes to furnish the respective carbinols.

Several oxidoreductases in bakers' yeast have been isolated. Furuchi et al.[7] reported the isolation of an oxidoreductase that catalyzed the reduction of benzyl-2-methyl-3-oxobutanoate to give L-carbinols with a *syn*(2R,3S)/*anti*(2S,3S) ratio of 8/1. However, this enzyme appears to be different from L-enzyme 2, for it was reported to be inert towards ethyl-2-methyl-3-oxobutanoate (**1**). On the other hand, our L-enzyme 2[6] appears to be identical to L-enzyme 1 reported by Nakamura et al.[8]; both enzymes have MW around 32,000. In terms of substrate specificity, our L-enzyme 1 (MW 74,000) resembles that of a minor enzyme L-enzyme 2 (MW 32,000) described by Nakamura et al. but the MW of these enzymes appear to be different. It is likely that our L-enzyme 1 is a dimeric

protein which was easily dissociated into its monomer by treatment with SDS. This difference could be attributed to the different sources of the bakers' yeast (Red Star vs. Oriental yeast).

The purified (R)-3-hydroxybutanoate oxidoreductase from *Pseudomonas lemoignei* has a broad substrate specificity and reduces a variety of 2-alkyl-3-oxobutanoates to furnish the *syn*(2S,3R) diastereomer using NADH as the hydride donor. This enzyme is used clinically for the determination of ketone bodies in urine.[9]

The rates of metabolism of unnatural substrates by microorganisms are determined by many factors including the permeability of the compound, the location and the relative concentration of the enzymes. Since the ratio of the concentration of L-enzyme 1 and 2 in Red Star bakers' yeast was estimated to be approximately 1:1, the ratio of the k_{cat}/K_m value of the two L-enzymes could reasonably account for the stereochemical outcome of the yeast reduction of 2-methyl- and 2-allyl-3-oxobutanoates. That is, the L-enzyme 1 has the higher k_{cat}/K_m value for both substrates. Consequently, it has a more pronounced influence on the diastereopreference of intact yeast reductions of these two substrates. On the other hand, the k_{cat}/K_m values are about the same for 2-propargyl-3-oxobutanoate and other factors mentioned above become more dominant. Nevertheless, our studies of the purified L-oxido-reductases from bakers' yeast enabled us to gain an insight into the diastereoselectivity of these enzymes which until now have not been closely examined.

EXPERIMENTAL

Materials. Red Star bakers' yeast was the product of Universal Food Corp., Milwaukee, Wisconsin. α-NADH (80-90%), NADP (Sigma grade, 98-100%), enzymatically reduced NADPH (type III, 98-99%), glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), (+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid [(+)-MTPA], and (R)-3-hydroxybutanoate NAD oxido reductase were purchased from Sigma. Ethyl 2-methyl-3-oxobutanoate and organic solvents were from Aldrich.

Bakers' Yeast Reductions of 2-Alkyl-3-oxobutanoates. The incubations were carried out in a flask containing 10 ml of tap water, 0.5 ml of an ethanolic solution of β-keto ester (50 mg), and 1 g Red Star bakers' yeast. After 20 hr (1-4) or 40 hr (5-7) incubation on a rotary shaker (2" stroke, 250 rpm), the products were recovered by extraction with ethyl acetate three times, dried over sodium sulfate and then evaporated to dryness under reduced pressure.

Enzymatic Reductions of 2-Alkyl-2-oxobutanoates. The incubation mixture contained: 5-10 μg of the purified enzyme, 0.2 ml of an ethanolic solution of the β-keto ester (20 mg), 1.3 equiva-

lents of glucose-6-phosphate, 10 units of glucose-6-phosphate dehydrogenase, 0.2 mM NADPH in a total volume of 10 ml of 0.1 M sodium phosphate buffer, pH 6.5. The mixture was incubated on a rotary shaker (2" stroke, 1000 rpm) at 23°C for 24 hr. The carbinolic products were worked up as described for the yeast reductions.

Determination of the Extent of Conversion and the *syn/anti* Ratios. The conversions and *syn/anti* ratios of the yeast and enzymatic reduction were measured using a 3 ft Carbowax 20 M (Alltech) column except for the 2-methyl 3-oxo esters (**1** and **5**), in which a 6 ft column was used. The flow rate of N_2 gas was 30 ml/min; the temperatures of the injector and the detector were 250°C and 270°C, respectively. The temperature of the column, the keto-substrate retention time, and the carbinol-products (*anti/syn* retention times were as follows: **1**, 120°C, 6 min 51 sec, 10 min 36 sec, 10 min 49 sec; **5**, 165°C, 13 min 52 sec, 19 min 34 sec, 20 min 44 sec; **3**, 105°C, 2 min 28 sec, 3 min 49 sec, 5 min 28 sec; **6**, 165°C, 7 min 28 sec, 11 min 34 sec, 13 min 56 sec; **2**, 130°C, 3 min 15 sec, 5 min 30 sec, 6 min 54 sec; **4**, 175°C, 5 min 24 sec, 6 min 56 sec, 8 min 15 sec; **7**, 215°C, 9 min 12 sec, 14 min 22 sec, 16 min 34 sec. The assignment of the *syn* and *anti* diastereomers with respect to the sequence of elution was in accordance with the reported values.[9] The % conversions and *syn/anti* ratios were determined by using an integrator.

Determination of Enantiomeric Excess (ee). After the conversion and *syn/anti* ratio have been analyzed, the crude residue was applied onto a silica gel column (1.2 x 20 cm) and eluted with ethyl acetate/hexane, 1:7 for the ethyl ester and 1:10 for the octyl ester. The purified carbinolic product was converted into the corresponding (+)-MTPA esters by reacting with 2 drops of (+)-MTPA-Cl and one drop of pyridine with stirring at 23°C. After the reaction was complete, the resulting (+)-MTPA ester was extracted with diethyl ether three times. The ethereal layer was washed with 0.1 M HCl twice followed by water until neutral. HPLC analysis of the resulting (+)-MTPA ester was conducted using an Alltech μPorasil column (10 μ, 30 cm x 4.6 cm I.D.). The (+)-MTPA esters of the $NaBH_4$ reduced α-alkyl-β-hydroxybutyrates were used to find the suitable solvent systems for the separations: hexane/ether, 10:1 for the ethyl esters at a flow rate of 1.0 ml/min and 30:1 for the octyl esters at a flow rate of 1.5 ml/min. The absolute configuration of each peak was determined by comparing the HPLC profiles of the (+)-MTPA esters of the compounds obtained by yeast reduction (2R,3S and 2S,3S), to those derivatives derived from *Pseudomonas lemoignei* β-hydroxybutyrate dehydrogenase reduction (2R,3R and 2S,3R) and $NaBH_4$ reduction (contained all four isomers) with known *syn/anti* ratios determined by GC analysis. The elution order was *syn*(2S,3R), *syn*(2R,3S), *anti*(2R,3R), and *anti*(2S,3S) for all the (+)-MTPA esters

of the various 2-alkyl-3-hydroxybutyrates.

Enzyme Assay. The enzyme reaction was followed spectrophotometrically via the decrease in absorbance at 340 nm using a Gilford spectrophotometer at 23°C. A 1 ml cuvette with a 1 cm light path was employed. The assay system contained: 0.2 mM NADPH, suitable quantity of the purified enzyme, and 10 μl of an ethanolic solution of 0.5 M β-keto ester. One unit of this enzyme activity is defined as the amount of enzyme which oxidized 1 nmol of NADPH per minute at 23°C under the assay condition (ϵ of NADPH at 340 nm = 6220 $M^{-1}cm^{-1}$).

Stereoselective Reductions of 2-Alkyl-3-oxobutanoates by (R)-3-Hydroxybutanoate Oxidoreductase of *P. lemoignei*. The incubation mixture contained 10 ml of 0.1 M sodium phosphate (pH 6.5), 0.5 ml of an ethanolic solution of ethyl α-alkylacetoacetate (40 mg), 25 units of oxidoreductase (*Pseudomonas lemoignei*), 2 mM NAD^+, 1 equivalent of glucose-6-phosphate, 20 units of glucose-6-phosphate dehydrogenase. After 48 hr stirring at room temperature, the product was recovered by extraction with ethyl acetate three times. The conversion and *syn/anti* ratio of the crude extract was determined by GC analysis. The ee value of the ethyl 2-alkyl-3-hydroxybutyrate was measured by HPLC analysis of the corresponding (+)-MTPA ester (Table 6).

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