L-Lactate Dehydrogenase: Substrate Specificity and Use as a Catalyst in the Synthesis of Homochiral 2-Hydroxy Acids[1]

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Abstract: This paper reports kinetic parameters (K_m, k_{cat}) for reduction of approximately 20 α-keto acids by L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) from 5 sources (porcine heart, rabbit muscle, chicken liver, bovine heart, lobster tail). The L-LDH-catalyzed reduction reaction of four substrates representative of the range of activities observed has been carried out on a preparative scale using rabbit muscle L-LDH, and absolute configurations and values of enantiomeric excess (ee) of the products have been determined: 2-hydroxybutanoic acid, ee >99%, *S*; 2-hydroxypentanoic acid, ee >99%, *S*; cyclopropaneglycolic acid, ee >99%, *S*; 3-phenyllactic acid, ee >99%, *S*. This enzyme-catalyzed reduction provides a practical method for preparing 1–25-g quantities of a range of 2-hydroxy acids with high ee. To illustrate the value of these compounds as chiral synthons, (*S*)-2-hydroxybutanoic acid was converted to (*S*)-1-butene oxide having >98% ee on a 6-g scale.

Transformations based on enzymatic catalysis[2–5] are providing an increasingly valuable component of the methodology of enantioselective synthesis. Some enzymes [for example, porcine pancreatic lipase (EC 3.1.1.3),[6] porcine liver esterase (EC 3.1.1.1),[7] and horse liver alcohol dehydrogenase (EC 1.1.1.1)[8]] used in recent synthetic applications have broad substrate specificity but show variable enantioselectivity; others [for example, fumarase (EC 4.2.1.2),[9] glycerol kinase (EC 2.7.1.30),[10] and glycerol dehydrogenase (EC 1.1.1.6)[11][have high enantioselectivity but narrow substrate specificity. Only a few enzymes [for example, acylase I, (EC 3.5.1.14)][12] simultaneously possess broad substrate specificity and high enantioselectivity. This paper explores the utility in organic synthesis of an enzyme [L-lactate dehydrogenase, L-LDH (EC 1.1.1.27)] that, we believe, belongs in this third category.

L-Lactate dehydrogenase is commercially available and inexpensive (less than $10 per 1000 units). Although this enzyme has an air-sensitive thiol group, it has satisfactory stability if immobilized and protected against autooxidation. L-LDH catalyzes the reduction of pyruvate to L-lactate by NADH in vivo with absolute enantiospecificity.[13] It accepts 2-oxo acids other than pyruvate,[14] but its enantioselectivity in these reactions has not been established. The utility of this enzyme as a catalyst in organic synthesis has not been systematically examined. We have previously used L-LDH in the multigram scale synthesis of L-β-chlorolactic acid having >97% ee.[15] This work convinced us that L-LDH was indeed an efficient catalyst that should be useful in the enantioselective reduction of unnatural 2-oxo acids and encouraged us to examine the substrate specificity and stereospecificity of several commercially available L-lactate dehydrogenases.

The objectives of this work were to explore the range of substrates accepted by L-LDH at a rate useful in organic synthesis, to establish the absolute configuration and enantiomeric excess of representative products, to determine if there are any significant differences in specificity among the enzymes from different sources, and to illustrate practical synthetic procedures using L-lactate dehydrogenase.

Homochiral 2-hydroxy acids are valuable chiral synthons.[16] Procedures currently available for their preparations include chemical methods,[16–19] fermentation,[20] and enzymatic catalysis.[21,31]

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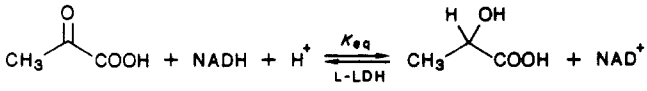
Few of the chemical methods yield products having high values of ee; fermentation methods (almost certainly utilizing lactate dehydrogenase in vivo) can give good values of ee[20] but suffer from low concentrations of product and difficult isolation. Enzymatic methods are relatively straightforward for this application.

L-Lactate dehydrogenase is found in all higher organisms. It is a tetramer with a molecular weight of ca. 140000.[22] The amino acid sequences[23] and crystal structures[24] of several lactate dehydrogenases have been determined, and the mechanism of its catalysis has been partially defined.[25] The tetrameric assembly is composed of subunits whose composition correlates with the tissue in which it is found.[13] The homogeneous isoenzymes from animal heart have H-type subunits (H_4), whereas those from animal muscle have M-type subunits (M_4). The other, heterogeneous, isoenzymes have a mixture of both subunits (H_3M, H_2M_2, HM_3). The isoenzymes have been reported to show significant differences in substrate selectivity and in their sensitivity to substrate or product inhibition.[13] H-type enzymes have higher activity toward a wider range of substrates and are more sensitive to substrate or product inhibition than M-type enzymes.[13] Both types of homogeneous enzymes were tested in this work.

L-Lactate dehydrogenase has several characteristics useful for synthetic application. It has a high specific activity (400–1500 units/mg) and is readily immobilized. The equilibrium constant of the L-LDH-catalyzed reaction of pyruvate greatly favors the reduction;[26] $K_{eq} = 10^{11}$ M^{-1} (eq 1; $K'_{eq} = 10^4$). The supporting



(1)

techniques for the synthetic use of L-LDH, including immobilization[27] and in situ regeneration of NADH,[28] are well developed.

Results

Kinetics Analysis. Five L-lactate dehydrogenases (from rabbit muscle, porcine heart, bovine heart, chicken liver, and lobster tail) were surveyed with several 2-oxo acids in 30 mM phosphate buffer at pH 7.2 and 25 °C in the direction of reduction (Table I). Figure 1 shows representative kinetic data. The relative values of k_{cat} of bovine heart enzyme cited from the studies of others were obtained, with the exception of fluoropyruvate, by using the reactivity of the substrate measured at a single concentration (1.7 or 3.3 mM).

Substrate Specificity. The most important observation from Table I is that a variety of compounds are substrates for L-LDH. Because L-LDH is inexpensive, even substrates showing reactivity as low as 0.1% that of pyruvate can be considered for practical-scale (>1 g) reactions using this enzyme. We note that the three enzymes from animal heart and liver—enzymes that are similar in kinetic behavior—generally show higher activity and higher affinity toward the whole range of substrates tested than does the enzyme from rabbit muscle. The enzyme from lobster tail is similar to the enzymes from heart and liver in activity and to the enzyme from rabbit muscle in affinity. With increasing size of the substrate side chain, the value of K_m increases and the values of K_{cat} and k_{cat}/K_m decrease. Glyoxylic acid has a much larger value of K_m than expected based on its size, probably because it exists predominantly as its hydrate in aqueous solution. The range of substrates falls conveniently into three categories on the basis of k_{cat} and K_m (Figure 2). Synthetic reactions are

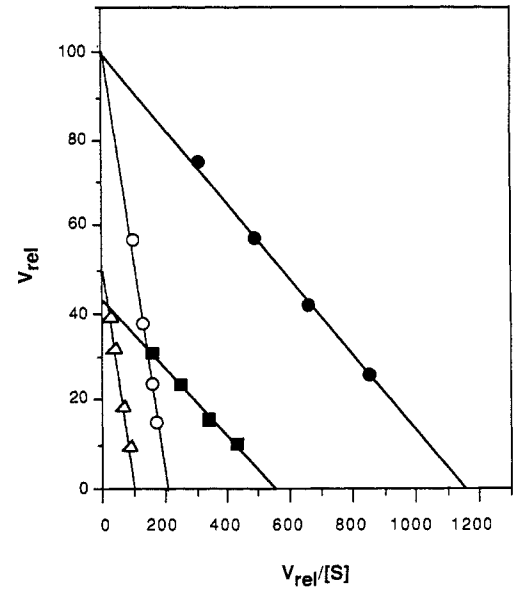
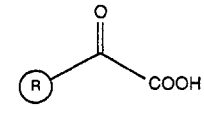


Figure 1. Eadie–Hofstee plots for reduction of several 2-oxo acids by NADH catalyzed by L-lactate dehydrogenase from porcine heart. The compounds were pyruvate (●), 2-oxopentanoate (△), fluoropyruvate (■), and chloropyruvate (○).



R group	Reactivity	k_{cat}
H, CH_3, CH_3CH_2, FCH_2, $ClCH_2$, $BrCH_2$, $HOCH_2$, $HSCH_2$, $HOCH_2CH_2$.	Good	> 10 %
$CH_3CH_2CH_2$, $CH_3CH_2CH_2CH_2$, $(CH_3)_2CH$, CH_3COCH_2, $CH_3SCH_2CH_2$, $C_6H_5CH_2$.	Fair	1-10 %
$(CH_3)_2CH$, $CH_3(CH_2)_4CH_2$, p-$HOC_6H_5CH_2$, Indole-3-CH_2, 2-Furyl.	Poor	< 1 %

Figure 2. Structural characteristics of substrates that are accepted by L-lactate dehydrogenase. Values of k_{cat} are relative to that of pyruvate.

Scheme I. L-LDH-Catalyzed Synthesis of (*S*)-2-Hydroxy Acids

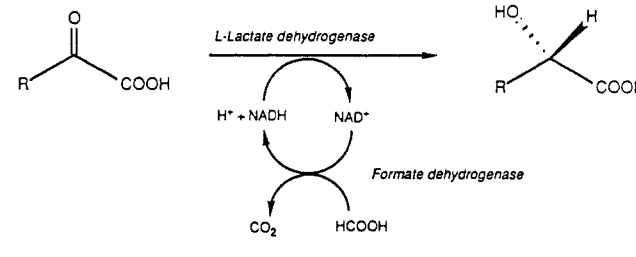


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Table I. Kinetic Parameters (K_m, mM; k_{cat}, min^{-1}; k_{cat}/K_m, M^{-1} min^{-1}) for L-Lactate Dehydrogenases from Different Sources (pH 7.2, 25 °C) (Values Relative to That for Reduction of Pyruvate to L-Lactate)[a]

		rabbit muscle			procine heart			bovine heart			chicken liver			lobster tail		
compd	R	K_m	K_{cat}	K_{cat}/K_m	K_m	k_{cat}	K_{cat}/K_m	K_m	k_{cat}	K_{cat}/K_m	K_m	k_{cat}	K_{cat}/K_m	K_m	k_{cat}	K_{cat}/K_m
1	H	18[b]	60[b]	1	3.2	60	2		80[e]		3.5	110	2	14	70	0.7
2	CH_3	0.25	100	100	0.09	100	100	0.05	100	100	0.05	100	100	0.13	100	100
3	CH_3CH_2	7.4	50	2	0.6	50	8	0.08[f]	60[f]	40	0.5	55	6	2.2	55	3
4	$CH_3CH_2CH_2$	5.3	1	0.05	1.9	6	0.3	3.3	6	0.09	1.0	4	0.2	5.2	9	0.2
5	$CH_3(CH_2)_2CH_2$	3.7	1	0.07	1.9	7	0.3	3.0[f]	2[f]	0.03	1.7	7	0.2	4.1	10	0.3
6	$CH_3(CH_2)_3CH_2$		0.03			0.06			0.3[f]			0.06				
7	$(CH_2)_2CH$	9.7	4	0.1	2.5	3	0.1	4.1	3	0.04	1.4	3	0.1	6.0	4	0.09
8	$(CH_3)_2CH$		0.04		3.9	0.3	0.007		0.4[e]			0.4			0.3	
9	$(CH_3)_2CHCH_2$		<0.01		3.6	0.02	0.0005		0.01[f]			0.03				
10	$CH_3CH_2CH(CH_3)$		<0.01		5.2	0.01	0.0002		0.01			0.01				
11	FCH_2	0.89	80	22	0.08	40	45	0.1[g]	30[g]	15	0.6	50	4	0.2	40	30
12	$ClCH_2$	4.0[c]	40[c]	3	0.5	100	20	0.5	80	8	0.7	130	9	2.0	65	4
13	$BrCH_2$	4.0[c]	50[c]	3	2.4	90	3	1.9	250	7	0.5	130	15	1.9	110	8
14	$HOCH_2$	1.0	120	30	0.2	170	80		100[e]		0.2	160	30	1.7	110	8
15	$HSCH_2$	0.6	20	8	0.4	60	15		100[e]		0.3	70	6	0.4	20	7
16	$HOCH_2CH_2$	4.8[b]	25[b]	1	0.7	50	6	1.2	40	2	0.4	30	4	1.3	40	4
17	CH_3COCH_2	3.9	2	0.1	0.8	3	0.3	0.4[f]	10[f]	1	0.8	3	0.2	0.4	2	0.7
18	$CH_3SCH_2CH_2$	5.4	0.3	0.01	3.4	2	0.05		6[f]		4.9	3	0.03	5.7	3	0.07
19	C_6H_5		0			<0.01			0.1[e]			0				
20	$C_6H_5CH_2$	6.5	0.6	0.02	2.2	2	0.08		3[e]		1.7	2	0.06	4.1	8	0.3
21	p-$HOC_6H_5CH_2$		1			1			1			1			2	
22	o-$NO_2C_6H_5CH_2$				0.7[d]	0.01[d]	0.01		0.3							
23	indole-3-CH_2		0.1			0.2			0.1			0.6			0.05	
24	2-furyl		0.1			0.2						0.2				

[a] The specific activities for reduction of pyruvate (units per milligram of enzyme) in 30 mM phosphate buffer (pH 7.2, 25 °C) are 160, 250, 270, 365, and 190 μmol min^{-1} mg^{-1}, respectively, for rabbit muscle, porcine heart, bovine heart, chicken liver, and lobster tail L-lactate dehydrogenase. [b] Lane, R. S.; Dekker, E. E. *Biochemistry* **1969**, *8*, 2958. [c] Hirschbein, B. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1982**, *104*, 4458. [d] Holbrook, J. J.; Stinson, R. A. *Biochem. J.* **1973**, *131*, 739. [e] Meister, A. *J. Biol. Chem.* **1949**, *184*, 117. [f] Meister, A. *J. Biol. Chem.* **1952**, *197*, 309. [g] Eisman, E. H.; Lee, H. A., Jr.; Winer, A. D. *Biochemistry* **1965**, *4*, 606.

Table II. Summary of the L-LDH-Catalyzed Reductions of 2-Oxo Acids to 2-Hydroxy Acids [RCH(OH)COOH]

		conditions								
run no.	products (R)	substrate, mmol	L-LDH, units	FDH, units	NAD, mmol	formate, mmol	time, days	yield, %	AC[a]	ee,[a] %
1	CH_3CH_2 (**25**)	100	400	40	0.50	117	4.8	99	*S*	>99
2	CH_3CH_2 (**25**)	150	*b*	*b*	0.75	170	4.2	96	*S*	>99
3	$CH_3CH_2CH_2$ (**26**)	30	*b*	*b*	0.15	35	3.0	97	*S*	>99
4	$(CH_2)_2CH_2$ (**27**)	30	*b*	*b*	0.20	35	3.7	94	*S*	>99
5	$PhCH_2$ (**28**)	15	*b*	*b*	0.10	19	8.0	96	*S*	>99

[a] AC, absolute configuration; ee, enantiomeric excess. [b] The enzymes recovered from the previous run were reused. The enzymes recovered from the fifth run possessed 60% of the initial activities.

usually run in concentrated (i.e., V_{max}) conditions, and rates are determined by k_{cat}.

Syntheses of (*S*)-2-Hydroxy Acids Using L-LDH. As substrates for illustrative practical-scale syntheses, we selected 2-oxobutanoic acid (**3**), 2-oxopentanoic acid (**4**), cyclopropaneglyoxylic acid (**7**), and phenylpyruvic acid (**20**). Rabbit muscle L-lactate dehydrogenase was chosen as the catalyst, and formate/formate dehydrogenase as the NADH-regenerating system (Scheme I). These 2-oxo acids represent the full range of reactivity reported. The enzymes (L-LDH, FDH) were immobilized in polyacrylamide (PAN) gel. All reactions were carried out by a similar procedure. A 1-equiv aliquot of substrate was allowed to react with 0.005–0.007 equiv of NADH in the presence of a slight excess of formate. The solution was kept in the presence of 1 mM mercaptoethanol, and air was excluded from it. The progress of reaction was followed by observing the amount of HCl solution required to keep the solution between pH 7.4 and 7.6; typical data are summarized in Figure 3. The reaction was stopped when the theoretical amount of HCl solution had been added, the enzyme-containing gels were removed, the aqueous solution was concentrated and acidified, and the products were extracted with ether. The products were almost pure on the basis of their ^1H NMR spectra and did not require further purification. The enzymes recovered from the first run were reused for the next reaction. This recycling was repeated 5 times to prepare 4 different products in 2–25-g quantities. The enzymes recovered after five cycles still possessed more than half of the initial activities. The results from these syntheses are summarized in Table II. The yields ranged from 94 to 99%.

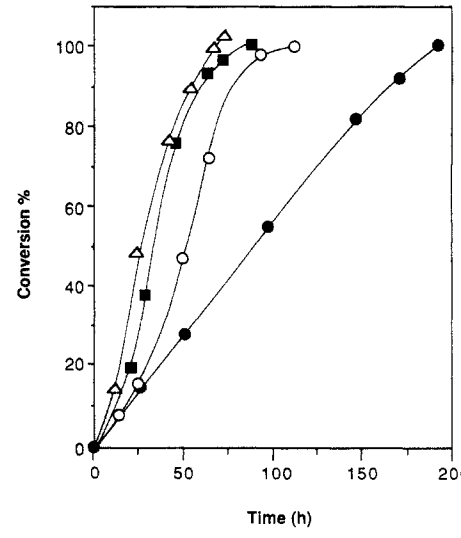


Figure 3. Reaction progression for the L-LDH-catalyzed reductions of 2-oxo acids (RCOCOOH) to (*S*)-2-hydroxy acids (RCHOHCOOH): (○) R = CH_3CH_2, run 1; (△) R = $CH_3CH_2CH_2$, run 3; (■) $(CH_2)_2CH$, run 4; (●) R = $C_6H_5CH_2$, run 5.

Determination of Absolute Configuration and Enantiomeric Purity. We assigned the absolute configuration of the enzymatically reduced products (**25–28**) on the basis of the analysis of ^1H NMR spectra of the (*R*)-(+)-MTPA derivatives.[29,30] We

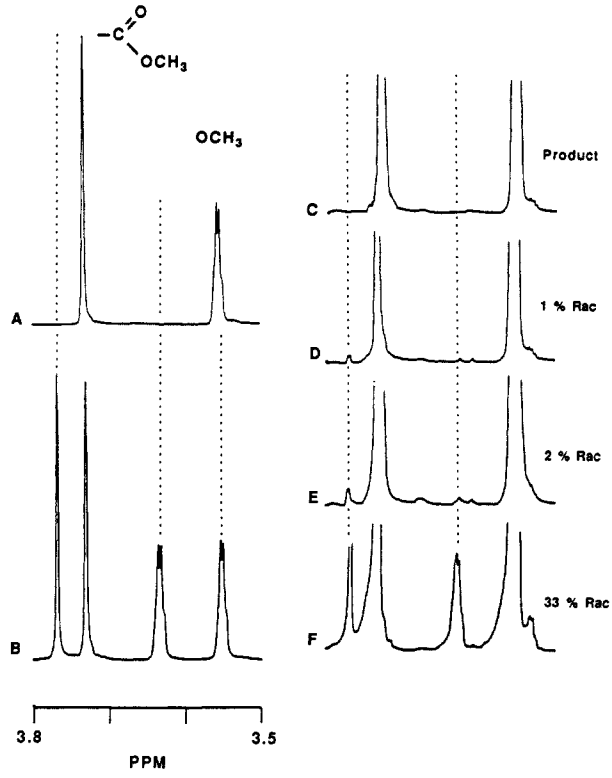


Figure 4. ^{1}H NMR spectra (300 MHz) at 3.2–4.0 ppm of the ester methyl and methoxy of the (*R*)-(+)-MTPA derivatives: (A) enzymically reduced (*S*)-2-hydroxybutanoic acid; (B) (*R,S*)-2-hydroxybutanoic acid; (C) base line detail of A; (D) C, with 1% B; (E) C, with 2% B; (F) C, with 33% B.

correlated the ^{1}H NMR chemical shifts for one to three characteristic protons of each MTPA derivative with its configuration based on the correlation scheme described by Yamaguchi.[29] These correlations suggest that all of the enzymatically reduced products have an *S* configuration (Table II), as expected from the specificity of L-lactate dehydrogenase for (*S*)-2-lactate.

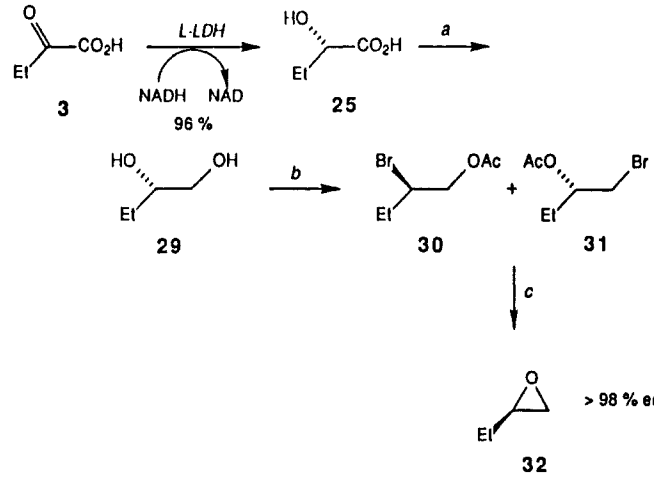
We determined the values of ee by carefully adding 1–33% of the MTPA ester of the racemic 2-hydroxy acid to the MTPA ester of the enzymatically reduced product (Figure 4). With careful calibration, we were able to detect the impurity of the minor enantiomer in quantities as low as 0.5%.

Synthesis of (*S*)-1-Butene Oxide. As an illustration of the potential utility of the 2-hydroxy acids as chiral synthons, we synthesized (*S*)-1-butene oxide (**32**) chemically in three steps from (*S*)-2-hydroxybutanoic acid (Scheme II). The overall yield was 55% based on 2-oxobutanoic acid. The optical purity of the epoxide was determined by ^{1}H NMR spectroscopy in the presence of Eu(hfc)$_3$ (Figure 5). With careful calibration, we could detect 1% enantiomeric impurity. We conclude that **32** has >98% ee.

Discussion

Range of Substrates Useful in Organic Synthesis. Among the kinetic parameters, the relative value of k_{cat} provides the best basis for the determination of substrate utility in organic synthesis. The value of k_{cat} limits the fastest possible rate of reaction with a substrate showing low activity; provided that K_m is not too large, it thus limits the practical scale of the reaction. The range of values of k_{cat} observed in this work is approximately 10^4. On the basis of experience with the compounds in Table II, we found that it is practical to carry out multigram (1–5-g) syntheses in convenient laboratory-scale reactions using substrates having reactivities as low as 0.1–1% that of pyruvate (Figure 2). We believe that this scale can be increased to more than 10 g without difficulty by extending the reaction time or reusing the recovered enzymes. We conclude that the substrates having reactivity >10% that of pyruvate can be used for >50-g-scale preparations; the substrates having reactivity between 1 and 10% that of pyruvate can be reduced on >10-g scale; the substrates having reactivity 0.1–1% that of pyruvate can be reduced without major difficulty on a multigram scale.

Scheme II. Synthesis of (*S*)-1-Butene Oxide[a]



[a] Conditions: (a) BH_3–THF, 0 °C, 20 h (91%); (b) 30% HBr–AcOH, −15 to +25 °C, 3 h (90%); (c) $C_5H_{11}OK$, $C_5H_{11}OH$, room temperature (71%).

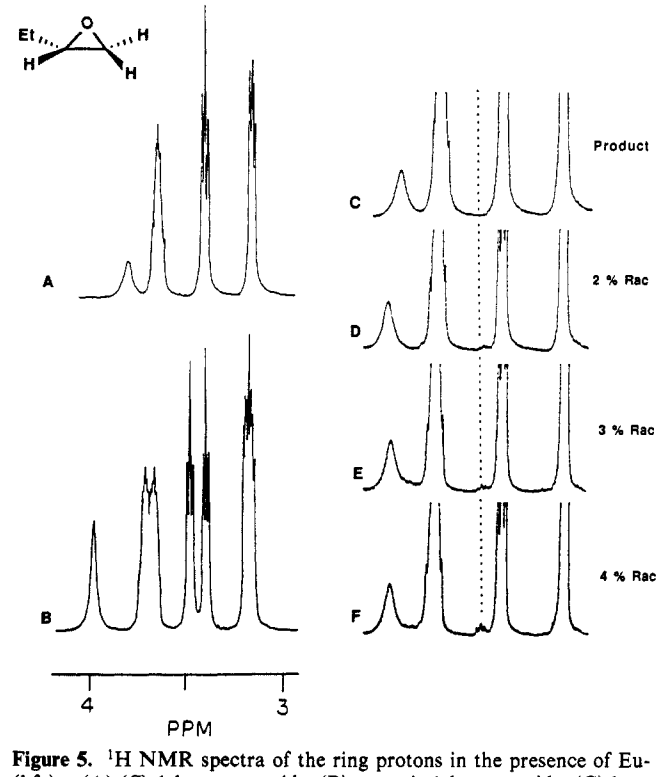


Figure 5. ^{1}H NMR spectra of the ring protons in the presence of Eu(hfc)$_3$: (A) (*S*)-1-butene epoxide; (B) racemic 1-butene oxide; (C) base line detail of A; (D) A with 2% B; (E) A with 3% B; (F) A with 4% B. Each NMR sample was prepared by mixing 60 μL of Eu(hfc)$_3$ (100 mg/mL $CDCl_3$), 0.5 mL of $CDCl_3$, and 10 μL of epoxide.

Enantioselectivity. Although L-lactate dehydrogenase generates only L-lactate in the direction of reduction, the enantioselectivity of L-LDH toward other substrates had not been established. Only one unnatural substrate, 3-chloropyruvate, has been shown to be reduced in an L-LDH-catalyzed reaction with high enantiose-

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lectivity (>97% ee).[15] On the basis of the results described in Table II, we conclude that L-LDH is highly enantioselective for substrates other than pyruvate. We believe that L-LDH will prove highly enantioselective toward the whole range of unnatural substrates listed in Table I.

Synthetic Procedures. The practicality of operation of L-LDH-catalyzed reductions is determined by the stability and cost of enzymes, the efficiency of the NADH-regenerating system, the stability and yield of products, the ease with which products can be isolated, the simplicity of analytical methods used to follow the progress of reaction, and related matters. The enzymes used as catalysts in this work are rabbit muscle L-lactate dehydrogenase and yeast formate dehydrogenase. All are readily immobilized in polyacrylamide gel (PAN), and L-LDH can also be used enclosed in a membrane (membrane-enclosed enzymatic catalysis,[31] MEEC). The MEEC technique is more convenient than covalent immobilization and is especially useful when a large quantity of enzyme is required. We believe that the MEEC technique will be the method of choice for syntheses using relatively unreactive substrates and requiring large amounts of L-LDH. PAN-immobilized L-LDH will be the better choice for large-scale syntheses using highly reactive substrates and a small amount of enzymes and requiring recycling of the enzymes.

The progress of a reaction carried out using L-LDH can be conveniently followed titrimetrically by observing the amount of HCl solution added to keep the pH of the reaction mixture neutral. The enzymatically reduced products are stable under the reaction conditions and can be isolated in almost pure form and in excellent yield by simple extraction with ether. Formate dehydrogenase is expensive, but the cost can be reduced by recycling the recovered enzymes. The reactant and product (CO_2) used in the NADH-regenerating system do not complicate the workup of the reaction. We believe that synthetic procedures using L-LDH are straightforward, practical, and economical.

Experimental Section

General Procedures. L-Lactate dehydrogenases (porcine heart, bovine heart, chicken liver, rabbit muscle, lobster tail; EC 1.1.1.27) were obtained as crystalline suspensions in ammonium sulfate solution or as lyophilized powders from Sigma. Formate dehydrogenase (yeast; 1.2.1.2) was obtained in lyophilized form from Boeringer Mannheim. The enzymes in lyophilized form were used as received and those in ammonium sulfate solution centrifuged before use. All enzymes used for kinetic measurements were dissolved in 30 mM phosphate buffer solution. Enzymes used as catalysts in synthesis were immobilized in PAN-1000 as described elsewhere.[27] Biochemicals were obtained from Sigma and used as received. (*R*)-(+)-MTPA-Cl [from (*R*)-(+)-methoxy(trifluoromethyl)phenylacetic acid],[30] chloropyruvic acid,[15] cyclopropaneglyoxylic acid potassium salt,[32] and 4-hydroxy-2-oxobutanoic acid[33] were prepared as described. 2,4-Dioxovaleric acid in aqueous solution was prepared by hydrolysis of its ethyl ester (Aldrich). 3-Methyl-2-oxopentanoic acid, 4-methyl-2-oxopentanoic acid sodium salt, (*p*-hydroxyphenyl)pyruvic acid, α-oxo-2-furanacetic acid, and 3-indolepyruvic acid were obtained from Aldrich. 3-Mercaptopyruvic acid was obtained from Fluka. Other 2-oxo acids were obtained from Sigma. Chemicals were reagent grade and used without further purification unless otherwise indicated. Water was distilled twice, the second time from glass. Chemical shifts in the ^1H NMR spectra were referenced to TMS or solvent peaks and those in the ^{13}C NMR spectra to DSS or solvent peaks. IR spectra were recorded from thin films or Nujol mulls. Optical rotations were measured on a Perkin-Elmer 242 polarimeter. Centrifugations were performed in a Sorvall RC-5B refrigerated superspeed centrifuge or an Eppendorf Model 5414 centrifuge. Control of pH was accomplished with an LKB peristaltic pump and Chemtrix pH controller. Boiling points were uncorrected.

Kinetic Measurements. The activities of L-lactate dehydrogenases were measured in the direction of reduction in the presence of NADH in 30 mM phosphate buffer (pH 7.2) at 25 °C by following the decrease in UV absorbance at 340 nm. The enzymes, NADH, and substrates were dissolved in 30 mM phosphate buffer (pH 7.2) before use. The assay solution contained, at pH 7.2, 30 mM phosphate, 0.2 mM NADH, various concentration of substrates, and various amount of enzymes. The concentration of substrates increased from 0.2 to 8 K_m, and the amount of enzymes was increased by a factor of 1000 in going from pyruvate to poor substrates. The kinetic parameters were obtained from Eadie–Hofstee plots. The activities of formate dehydrogenases were measured in the direction of oxidation at pH 7.5 and 25 °C by following the increase in UV absorbance at 340 nm. The assay solution contained 80 mM Tris, 150 mM formate, 0.7 mM NAD, and about 0.02 units/mL of enzyme. The immobilized enzymes were assayed under the same conditions as for the soluble enzymes.

L-LDH-Catalyzed Synthesis of (*S*)-2-Hydroxy Acids. We performed five reactions with recycled enzymes to prepare four different products on a 15–150-mmol scale.

(*S*)-2-Hydroxybutanoic Acid (25). Run 1. A 300-mL aqueous solution containing 2-oxobutyric acid (**3**; 101 mmol), sodium formate (117 mmol), mercaptoethanol (0.5 mmol), and Tris (2.5 mmol) was transferred to a 1-L three-necked flask equipped with a magnetic stirring bar, three septa, and a Fisher pencil combination electrode connected to a Chemtrix pH controller. The solution was adjusted to pH 7.5 with concentrated KOH solution. NAD (0.5 mmol) and a 100-mL aqueous suspension of immobilized L-LDH (400 units) and FDH (40 units) were introduced into the flask. A bubbler and a nitrogen supply were attached with needles through septa to the flask. An HCl solution (2.56 N) placed in a 50-mL buret was connected (by a silicon tube through a septum to the flask) via a peristaltic pump, which was remotely controlled by the pH controller. The flask was checked for a tight seal, and then nitrogen was bubbled through the solution for 30 min to degas it. The solution was maintained under positive nitrogen pressure. The solution was kept at pH 7.4–7.6 by the controlled addition of 2.56 N HCl. The reaction was followed by observing the amount of HCl solution added. The reaction was stopped when 39.5 mL of 2.56 N HCl had been added (4.8 days). The enzyme-containing gel particles were isolated by centrifugation, washed twice with degassed distilled water, resuspended in cold degassed water containing 2 mM mercaptoethanol, and assayed by the UV method. The combined washes (400 mL) were acidified to pH 2.0 with 6 N HCl and extracted continuously with diethyl ether for 4 days. The ethereal phase was dried over anhydrous $MgSO_4$ and concentrated by rotary evaporation to give an oily residue that solidified after further drying in vacuo to yield a white solid. The solid was dried at 0.5 Torr for 10 h to yield 10.4 g (100 mmol, 99%) of 2-hydroxybutanoic acid: mp 54.5–55.5 °C dec (lit.[34] mp 55–55.5 °C); $[\alpha]^{23}_D$ +7.15° (*c* 8.13, $CHCl_3$); IR (Nujol) ν_{max} 3500–2650 (br), 1733(s), 1000(m), 1130(w), 1060(w), 790(w), 730(w) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.72 (br, variable, and exchangeable with D_2O, 2 OH), 4.24 (dd, 1 H), 1.88 (m, 1 H), 1.75 (m, 1 H), 1.00 (t, J = 7.42 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 179.4, 71.4, 27.3, 8.9; MS, *m/e* 105.054 92 (calcd for $C_4H_8O_3$, 105.055 16).

Run 2. The second run was performed on a 150-mmol scale with the enzymes recovered from the first run. The starting materials included 150 mmol of 2-oxobutanoic acid, 170 mmol of sodium formate, 0.75 mmol of NAD, 0.75 mmol of mercaptoethanol, and 3.75 mmol of Tris. The overall procedure, except for the workup, was followed as described in run 1. The reaction was stopped when 59.8 mL of 2.56 N HCl had been added (4.2 days). The mother liquor obtained after the removal of the enzyme-containing gel particles was concentrated by rotary evaporation in vacuo to 80 mL, acidified to pH 2 with 6 N HCl, and extracted 4 times with 170-mL portions of ether. The ethereal layer was dried over anhydrous $MgSO_4$ and evaporated to give the crude white product. Further drying for 24 h in vacuo provided almost pure white solids (15.02 g, 144 mmol, 96%): mp and spectroscopic data were indistinguishable from those described in run 1.

(*S*)-2-Hydroxypentanoic Acid (26). Run 3. The synthesis of **26** was performed on a 30-mmol scale using the procedure for run 2. The starting materials comprised 30 mmol of sodium 2-oxopentanoate (**4**), 35 mmol of sodium formate, 0.2 mmol of NAD, 1.5 mmol of Tris, 0.3 mmol of mercaptoethanol, and 1.14 N HCl for titration. The reaction was stopped when 26.7 mL of 1.14 N HCl had been added (3 days). The products obtained as white solids weighed 3.27 g (28 mmol, 94%): mp 57.8–58.5 °C (lit.[34] mp 34 °C for racemic material); ^1H NMR ($CDCl_3$) δ 6.92 (br s, 2 OH), 4.26 (q, J = 4 Hz, 1 H), 1.78 and 1.68 (md, 2 H), 1.46 (m, 2 H), 0.93 (t, J = 7 Hz, 3 H); MS, *m/e* 119.070 75 (calcd for $C_5H_{10}O_3$, 119.070 81).

(*S*)-Cyclopropaneglycolic Acid (27). Run 4. The synthesis of **27** was performed on a 30-mmol scale using the procedure for run 3 except that potassium cyclopropaneglyoxylate **7** was the starting material. The reaction was stopped when 26.4 mL of 1.14 N HCl had been added (3.7 days). The products obtained as white solids weighed 3.35 g (29 mmol, 97%): mp 97–98.5 °C; ^1H NMR ($CDCl_3$) δ 5.6 (br s, 2, 2 OH), 3.81

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Table III. ^{1}H NMR Chemical Shifts (ppm) for Three Characteristic Protons from (*R*)-(+)-MTPA Derivatives [RCH(O-MTPA)COOCH$_3$] of 2-Hydroxy Acids

acids	AC[a]	CH_3OOC	CH_3O	CH_3CH_2
$CH_3CH_2CH(OH)COOH$	*R*	3.77	3.63	0.87
	S	3.73	3.55	0.99
$CH_3CH_2CH_2CH(OH)COOH$	*R*	3.77	3.63	0.84
	S	3.73	3.55	0.92
$(CH_2)_2CHCH(OH)COOH$	*S*	3.75	3.57	
$C_6H_5CH_2CH(OH)COOH$	*R*	3.75	3.54	
	S	3.75	3.31	

[a] AC, absolute configuration.

(d, J = 7 Hz, 1 H), 1.17 (m, 1 H), 0.57 and 0.46 (md, 4 H); ^{13}C NMR (CD_3COCD_3) δ 175.8, 72.6, 15.3, 2.0, 1.9; MS, *m/e* 117.054 78 (calcd for $C_5H_5O_3$, 117.055 16).

(*S*)-3-Phenyllactic Acid (28). Run 5. The synthesis of **28** was performed on a 15-mmol scale using the procedure for run 2. The starting materials comprised 15 mmol of phenylpyruvic acid, 19 mmol of sodium formate, 0.1 mmol of NAD, 1.5 mmol of Tris, and 0.3 mmol of mercaptoethanol. The reaction was stopped when 13.3 mL of 1.14 N HCl had been added (8 days). The products obtained as white solids weighed 2.43 g (14.6 mmol, 96%): mp 124-125 °C (lit.[34] mp 124-125 °C); ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 5 H), 5.23 (br s, 2 OH), 4.4 (dd, 1 H), 3.18 (dd, 1 H), 2.97 (dd, 1 H); MS, *m/e* 167.071 37 (calcd for $C_9H_{10}O_3$, 167.0781).

(*R*)-(+)-MTPA Derivatives of 2-Hydroxy Acids. The MTPA derivatives were synthesized as described elsewhere.[30] The 2-hydroxy acids were methylated with diazomethane in ether to the corresponding methyl esters, which were treated with (*S*)-(+)-MTPA-Cl in CCl_4-benzene in the presence of 3-(dimethylamino)propylamine to obtain the corresponding MTPA derivatives. ^{1}H NMR chemical shifts for some characteristic protons are summarized in Table III.

Determination of Enantiomeric Excess by ^{1}H NMR Spectroscopy. (*S*)-2-Hydroxy Acids. For calibration, five samples were prepared by mixing 0.5 mL of $CDCl_3$ and 15 μL of one of the following: (1) the MTPA derivative of **25**; (2) the MTPA derivative of racemic **25**; (3) **1**, with 1% of **2**; (4) **1**, with 2% of **2**; (5) **1**, with 33% of **2**. The minor enantiomer could be detected at 0.5% of the major enantiomer. The enantiomeric excess was determined on the basis of the difference in chemical shift for methoxy protons.

(*S*)-1-Butene Oxide. For calibration, five samples were prepared by mixing 60 μL of Eu(hfc)$_3$ (100 mg/mL $CDCl_3$), 0.5 ml of $CDCl_3$, and 10 μL of one of the following: (1) **32** only; (2) racemic **32** only; (3) **1**, with 2% of **2**; (3) **1**, with 4% of **2**; (4) **1**, with 6% of **2**. One percent of the minor enantiomer could be detected.

(*S*)-1-Butene Oxide (32). An oven-dried 300-mL three-necked round-bottomed flask fitted with a silicon stopper, magnetic stirring bar, dropping funnel, and reflux condenser was cooled to room temperature under nitrogen. Compound **25** (15 g, 144 mmol), from run 2, was dissolved in dry THF (80 mL) and transferred into the flask with a long cannula under nitrogen. The flask then was cooled to 0 °C in an ice-ethanol bath. The borane solution in THF (1 M, 260 mL) was placed in the dropping funnel and added dropwise over 2 h. The mixture was allowed to stir under nitrogen at room temperature for 20 h. A water-THF mixture (1:1, 60 mL) was slowly added to hydrolyze the unreacted hydrides. The heterogeneous mixture was stirred until no more gas evolution occurred and poured into cold, saturated, aqueous Na_2CO_3 solution (100 mL). The organic layer was separated and the aqueous layer extracted 4 times with 100-mL portions of THF. The combined organic phase was dried over anhydrous $MgSO_4$ and evaporated. The residue was distilled at reduced pressure (9 Torr) to obtain diol **29** (11.7 g, 91%): bp 94-96 °C [lit.[35] bp 90-95 °C (6 Torr)]; $[\alpha]^{23}_D$ -15.35° (*c* 2.6, EtOH)[lit.[35] $[\alpha]^{22}_D$ -12.87° (*c* 2.6, EtOH)]; IR (neat) ν_{max} 3330 (s), 2960 (s), 2920 (s), 2870 (s), 1460 (s), 1120 (s), 1050 (s), 980 (s), 910 (m), 855 (m) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.61 (m, 2 H), 3.41 (m, 1 H), 2.74 (br s, 2 OH), 1.44 (m, 2 H), 0.93 (t, J = 7.5 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 73.4, 65.8, 26.0, 9.7.

A three-necked, 100-mL, round-bottomed flask fitted with a magnetic stirring bar, dropping funnel, and reflux condenser was charged with the diol **29** (11.5 g, 128 mmol). The flask was cooled to -15 °C in an ice-ethanol bath. A solution of 30% HBr-AcOH (97 mL, 345 mmol) was added from the dropping funnel at -15 °C over 30 min. The yellow homogeneous mixture was stirred at -15 °C for 1 h and then at room temperature for 1.5 h. The solution was poured into ice-water (200 mL) and neutralized immediately with solid Na_2CO_3 (92 g). A yellow oil separated to the bottom. The heterogeneous mixture was extracted once with a 200-mL portion and twice with 100-mL portions of ether. The combined ethereal phase was washed twice with aqueous $NaHCO_3$ solution and once with brine, dried over anhydrous $MgSO_4$, and evaporated. The yellow, oily residue was distilled at reduced pressure (9 Torr) to obtain colorless product **31** (22.3 g, 116 mmol, 91%). [The ^{1}H NMR spectrum indicated that the product contained 7% 1-acetoxy-2-bromobutane (**30**)]: bp 68-70 °C [lit.[35] bp 85-86 °C (25 Torr)]; $[\alpha]^{23}_D$ -23.16° (*c* 4.14, ether) [lit.[35] $[\alpha]^{23}_D$ -21.2° (*c* 3.54, ether)]; IR (neat) ν_{max} 2970 (s), 2920 (m), 2880 (m), 1740 (s), 1460 (m), 1430 (m), 1370 (s), 1230 (s), 1170 (m), 1020 (s), 960 (m) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.91 (q, J = 5.9 Hz, 1 H), 3.45 (ddd, 2 H), 2.07 (s, 3 H), 1.69 (m, 2 H), 0.90 (t, J = 7.45 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 170.3, 73.5, 33.5, 25.5, 20.8, 9.2.

To a three-necked, 250-mL, round-bottomed flask equipped with a magnetic sitrring bar, pressure-equalizing dropping funnel, and 10-cm Vigreux column connected to an efficiently cooled condenser and receiver was added compound **31** (22.1 g, 115 mmol) in dry 1-pentanol (20 mL) followed by the slow addition of potassium pentylate (1.18 N, 97.1 mL, 115 mmol) from the dropping funnel with stirring at room temperature over 30 min. A white precipitate of potassium bromide formed. After the addition was complete, the flask was warmed in an oil bath at ca. 140-150 °C to attain distillation. Colorless fractions in the range of 59-62 °C were collected to give **32** (5.86 g, 81.2 mmol, 71%) (to distill off only the desired product and ensure >90% yield, the Vigreux column should be cooled frequently with a hair dryer during distillation): bp 59-62 °C (lit.[35] bp 62-63 °C); $[\alpha]^{23}_D$ -11.2° (*c* 4.9, dioxane) [lit.[36] $[\alpha]^{16}_D$ -12.25° (*c* 6, dioxane)]; ^{1}H NMR ($CDCl_3$) δ 2.87 (m, 1 H), 2.72 (dd, 1 H), 2.45 (dd, 1 H), 1.53 (dq, 2 H), 0.98 (t, J = 7.50 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 53.10, 46.4, 25.3, 9.41.

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Acidities of Anilines in Dimethyl Sulfoxide Solution

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Abstract: The equilibrium acidities of aniline and 26 of its derivatives have been measured in Me_2SO solution by an overlapping indicator method. The pK_a's cover a range of 14.8 units, from 2,4-dinitroaniline (pK_a = 15.9) to aniline (pK_a = 30.7). A comparison with values obtained by the H_- method for anilines, and also for fluorene carbon acids, is made and discussed. The pK_a values for anilines in Me_2SO are about 10 units higher than the ion pair pK_a's reported in liquid ammonia due in part to the superior ability of NH_3 to solvate the proton. A Hammett plot for 5 meta points and hydrogen gave ρ = 5.67. Points for 4-PhS, 4-CF_3, 4-$MeSO_2$, 4-$PhSO_2$, 4-MeCO, 4-PhCO, 4-CN, 4-F_3CSO_2, and 4-NO_2 deviated significantly from this line. The deviations are the result in part of enhanced solvation of the substituents resulting from direct conjugation with the anilide ion. These substituent solvation assisted (SSAR) effects contribute significantly to the need for σ^- constants in Hammett correlations. A pK_a = 56 in Me_2SO is estimated for CH_4 by extrapolation; similar extrapolations place the pK_a of $PhCH_3$ at 43 and that of NH_3 at 41.

The first acidity measurement of aniline appears to be that of McEwen using a method developed by Conant and Wheland. He assigned an ion pair pK_a of 27 to aniline in benzene, relative to MeOH in benzene, which was arbitrarily given an ion pair pK_a

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