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# D-Lactate Dehydrogenase

## Substrate Specificity and Use as a Catalyst in the Synthesis of Homochiral 2-Hydroxy Acids

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## ABSTRACT

This note compares the substrate specifity of D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) to that of L-lactate dehydrogenase (L-LDH, EC 1.1.1.27), illustrates three procedures that use D-LDH in synthesis and two methods for recycling NADH, and provides experimental details illustrating the use of D-LDH in organic synthesis.

**Index Entries:** D-lactate dehydrogenase, substrate specificity; D-lactate dehydrogenase, use in synthesis; reduction of 2-oxoacids; 2-hydroxy acids, synthesis; enzymes, immobilized; enzymes, use in synthesis; (*R*)-butene oxide, synthesis.

## INTRODUCTION

L-LDH is a broadly useful enzyme in organic synthesis; it transforms a wide range of 2-oxoacids, such as pyruvate, to *S*-2-hydroxy acids, such as L-lactate [Eq. 1] (*1*). D-LDH converts 2-oxoacids to *R*-2-hydroxy acids [Eq. 2]. We hoped that L-LDH and D-LDH would provide complementary catalysts for organic synthesis. The objective of the work reported in this paper was to establish the potential of D-LDH as a catalyst in organic synthesis, by examining its substrate specificity, stability, and enantioselectivity. D-LDH does indeed reduce a range of 2-oxo acids, but the range is substantially narrower than that of L-LDH. Thus, although D-LDH is a

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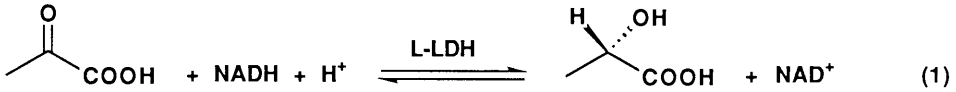
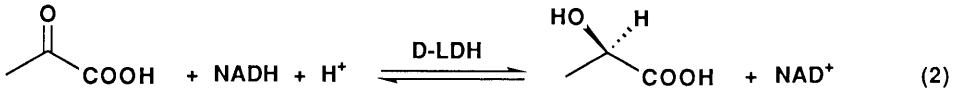
catalyst that is in some sense "enantiomeric" to L-LDH, it is a less useful catalyst than, and is not a true complement to, L-LDH.




(1)




(2)

In vivo, D-LDH catalyzes the NADH-dependent interconversion of pyruvate and D-lactate in anabolic and catabolic pathways. Neither the structure nor mechanism of D-LDH, found in lower organisms, has been studied as extensively as in the case of L-LDH, found in higher organisms (*2–5*). Although the structures of the L- and D-enzymes are thought to be similar, their respective mechanisms of catalysis appear to be different (*5,6*).

Several properties of D-LDH make it appealing for use in synthesis, in addition to its ability to provide the opposite enantiomer obtained from reductions with L-LDH. In vivo, the reduction of pyruvate to lactate is highly favored (*4*). The commercial preparation of D-LDH from *Leuconostoc mesenteroides* was examined in this work because it is inexpensive (~$ 4/1000 U; 1000 U converts ~1 mol of substrate to product per day under assay conditions) and has a high specific activity of 1,000–1,500 U/mg of protein (*7,8*). Like L-LDH, D-LDH has an air-sensitive thiol group, but it is stable if used in an inert atmosphere in the presence of reducing agents such as dithiothreitol to prevent autooxidation. NADH must be recycled, but several good techniques exist for this purpose (for reviews, *see 9* and *10*).

Other methods for the enzyme-catalyzed production of *R*-2-hydroxy acids have been reported using D-LDH (*11*), D-2-hydroxyisocaproate dehydrogenase (*12*), 2-oxocarboxylate reductase (*13*), and L-2-haloacid dehydrogenase (*14*).

## METHODS

The preparation of substrates, kinetic measurements, immobilization of enzymes, the determination of enantiomeric excess (ee) of 2-hydroxy acids according to the method of Mosher (*15*), and general experimental procedures are described elsewhere (*1*). When enzymes were confined within dialysis membranes (the MEEC technique, *16*), the following procedure was followed: a section of dialysis tubing (SpectraPor 2, 12-14,000 MW cut off, 16 mm flat width, Spectrum Medical Industries or VWR) was rinsed with distilled water and a knot was tied at one end. The enzymes

Table 1
Relative Rates of Reduction ($k_{cat}$)
of 2-Oxoacids(RCOCOOH) with D-LDH and with L-LDH.
The Values are Relative to that for Reduction of Pyruvate to Lactate

| Compound | R | D-LDH *Leuconostoc mesenteroides* | L-LDH[a] rabbit muscle (Isozyme M) | L-LDH[a] bovine heart (Isozyme H) |
|---|---|---|---|---|
| **1** | H | 36 | 18 | 60 |
| **2** | $CH_3$ | 100[b] | 100 | 100 |
| **3** | $CH_3CH_2$ | 15 | 50 | 50 |
| **4** | $CH_3CH_2CH_2$ | <0.01[c] | 1 | 6 |
| **5** | $CH_3(CH_2)_2CH_2$ | <0.01[c] | 1 | 7 |
| **6** | $CH_3(CH_2)_3CH_2$ | <0.01 | 0.03 | 0.06 |
| **7** | $(CH_2)_2CH$ | <0.01 | 4 | 3 |
| **8** | $(CH_3)_2CH$ | <0.01[c] | 0.04 | 0.3 |
| **9** | $(CH_3)_2CHCH_2$ | <0.01[c] | <0.01 | 0.02 |
| **10** | $CH_3CH_2CH(CH_3)$ | <0.01[c] | <0.01 | 0.01 |
| **11** | $ClCH_2$ | 80[d] | 40[c] | 100 |
| **12** | $BrCH_2$ | 40[d] | 40[c] | 90 |
| **13** | $HOCH_2$ | 44 | 120 | 170 |
| **14** | $HOOCH_2CH_2$ | <0.01 | | |
| **15** | $CH_3COCH_2$ | 0.4 | 2 | 3 |
| **16** | $C_6H_5CH_2$ | 2 | 0.6 | 2 |
| **17** | *p*-$HOC_6H_5CH_2$ | 1 | 1 | 1 |

[a] From ref. *1*.
[b] This value corresponds to 7.3 mmol/min/mg of D-LDH (*L. mesenteroides*).
[c] These substrates exhibit no activity with D-LDH from *Lactobacillus confusus* (*11*).
[d] From *ref. 24*.

were dissolved in an aliquot of the reaction mixture and transfered to the tubing using an Eppendorf pipet with a plastic tip. The other end of the tubing was then tied shut, taking care to exclude as much air as possible.

Experimental details for synthetic procedures are provided below.

## RESULTS AND DISCUSSION

### Substrate Specificity

The substrate specificity of D-LDH from *Leuconostoc mesenteroides* was examined (Table 1) according to the method previously described (*1*). We report values for $k_{cat}$ ($V_{max}$) for the reduction of 2-oxo acids to the corresponding 2-hydroxy acids because synthetic reactions are usually performed at concentrations of substrates that saturate the enzyme. Values of $k_{cat}$ are compared to the values previously determined for the H and M isozymes of L-LDH.

Table 2
D-LDH-Catalyzed Reductions of 2-Oxobutyric Acid (**3**) and Phenyl Pyruvate (**16**)

| Reaction | Compound reduced | Scale, mmol | Yield | Preparation of D-LDH[a] | NADH recycling method[b] |
|---|---|---|---|---|---|
| 1 | **3** | 150 | 90% | immobilized | Formate/FDH |
| 2 | **3** | 150 | 95% | immobilized | Formate/FDH |
| 3 | **3** | 7 | 91% | MEEC | Formate/FDH |
| 4 | **16** | 5 | 98% | MEEC | Formate/FDH |
| 5 | **16** | 50 | 72% | soluble | Glucose/GlcDH |

[a]MEEC = membrane-enclosed enzymatic catalysis.
[b]FDH = formate dehydrogenase; GlcDH = glucose dehydrogenase.

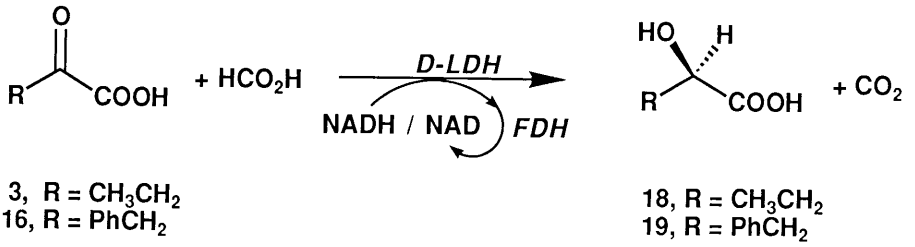
D-LDH accepts fewer substrates than do the H and M isozymes of L-LDH. In particular, alkyl chains longer than ethyl (e.g., **5–10**) are not tolerated. Surprisingly, 2,4-dioxoacids (e.g., **15**) are poor substrates ($k_{cat} < 1$). Simple aromatic compounds (**16** and **17**) are fair substrates ($10 > k_{cat} > 1$). Several compounds with electronegative groups attached to the methyl group of pyruvate are good substrates (e.g., **11–13**), but react more slowly in reactions catalyzed by D-LDH than by L-LDH ($k_{cat} > 10$).

## (*R*)-2-Hydroxy Acids

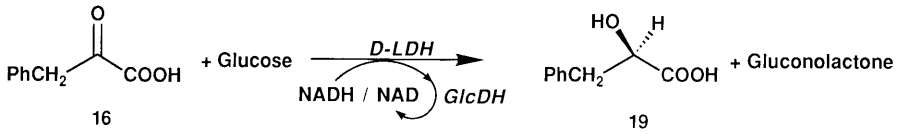
As representative examples, we reduced **3** (a good substrate) and **16** (a fair substrate) in reactions catalyzed by D-LDH. Pure products were obtained merely by acidification of the reaction mixture and extraction into ether. The resulting (*R*)-2-hydroxy acids, 2-hydroxybutyric acid **18** and phenyllactic acid **19**, each had an ee of >98%. In the context of these reactions, we compared three methods of manipulating the enzyme and two methods of recycling NADH (Table 2); these comparisons are discussed below.

## Manipulation of D-LDH

We used D-LDH immobilized in polyacrylamide (PAN) gel (*17*) (Table 2, reactions 1 and 2), enclosed within a dialysis membrane (the MEEC technique, reactions 3 and 4), and in soluble form (reaction 5). Although immobilization of L-LDH in PAN gel was straightforward (*1,17*), immobilization yields for D-LDH were consistently poor (3–10%). Use of the MEEC technique was operationally much easier and avoided the loss of enzyme activity during the immobilization procedure. The rate of reaction using MEEC was approximately one-half the rate when immobilized enzyme was used. In the case of D-LDH, the MEEC technique is preferable to immobilization on PAN, although the MEEC technique was not tried on a large scale where transport may significantly limit the rate of reaction. The use of soluble enzyme required no effort, but the catalyst was not recovered (*18*).




Scheme 1. D-LDH-catalyzed reductions using formate/formate dehydrogenase to recycle NADH.



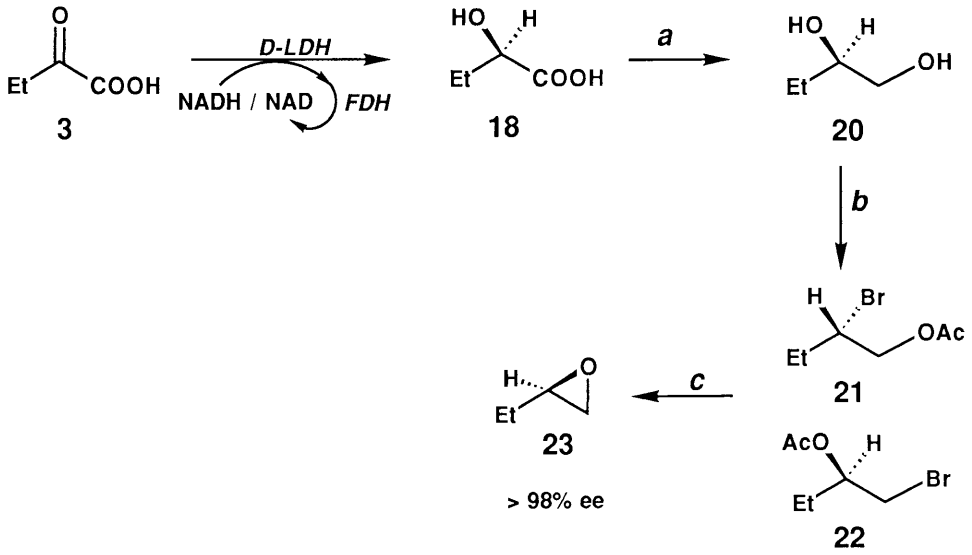

Scheme 2. D-LDH-catalyzed reduction of phenyl pyruvate using glucose/glucose dehydrogenase to recycle NADH.

## Recycling of NADH

Two systems were used to recycle NADH: one based on formate/formate dehydrogenase (EC 1.2.1.1.) (Scheme 1), and one based on glucose/glucose dehydrogenase (EC 1.1.1.47) (Scheme 2). The system based on FDH displayed the following advantages: FDH is more stable that GlcDH; and the product of the regeneration system, $CO_2$, does not complicate the workup. GlcDH has the merits of lower cost and higher specific activity compared to FDH. In general, recycling systems operate on natural substrates so that large amounts of enzyme are not needed; this consideration reduces the benefit of lower cost and higher specific activity. From this work, we conclude that the formate/FDH recycling system is preferable to the glucose/GlcDH recycling system.

## Use of D-LDH in Synthesis

Homochiral 2-hydroxy acids in general are useful chiral synthons (*1*). To illustrate the use of D-LDH in organic synthesis, we prepared 5 g of (R)-(+)-butene oxide (**23**) in > 98% ee from 2-oxobutyric acid **3** (Scheme 3) following the method of Golding and Ellis (*19*). The (unoptimized) overall yield for the four-step synthesis was 48%. The optical purity of **21** prepared according to Scheme 1 is due entirely to the enzyme-catalyzed step. We have prepared both enantiomers of **23** by several enzymatic methods and conclude that the methods based on D- and L-LDH are preferable (*20*). Using the same chemical steps, we transformed *R*-propylene




Scheme 3. Synthesis of (*R*)-1-Butene Oxide. [a]$BH_3$·THF, 0–25°C, 24 h (81%). [b]31% HBr-AcOH, 0 to 25°C, 1 h (82%). [c]$C_5H_{11}OK$, $C_5H_{11}OH$, 0°C, 1 h (81%).

glycol (obtained by fermentation of glucose) into *R*-methyl oxirane (*21*). The combination of D- and L-LDH was also used to prepare D- and L-glycidate (*22*).

## CONCLUSION

D-LDH is a moderately useful enzyme for organic synthesis. In comparison with L-LDH, D-LDH has a narrower substrate specificity and is less amenable to immobilization in PAN gel. We note several operational details: the MEEC technique is a useful alternative to immobilization in PAN gel; the best regeneration system for NADH uses formate/FDH; and D-LDH provides (*R*)-2-hydroxy acids with high (>98%) ee and these products can readily be used to further synthetic transformations.

## EXPERIMENTAL SECTION

### (*R*)-2-Hydroxybutanoic Acid (*18*)

*Method A*

A 1-L, three-necked round-bottomed flask was equipped with a magnetic stirring bar, three septa, a pH electrode connected to a pH controller, an inlet and outlet via a bubbler for argon, and a polypropylene tube for the pH stat-controlled addition of HCl. The reaction vessel was charged with 2-oxobutyric acid **3** (15.4 g, 150 mmol), sodium formate

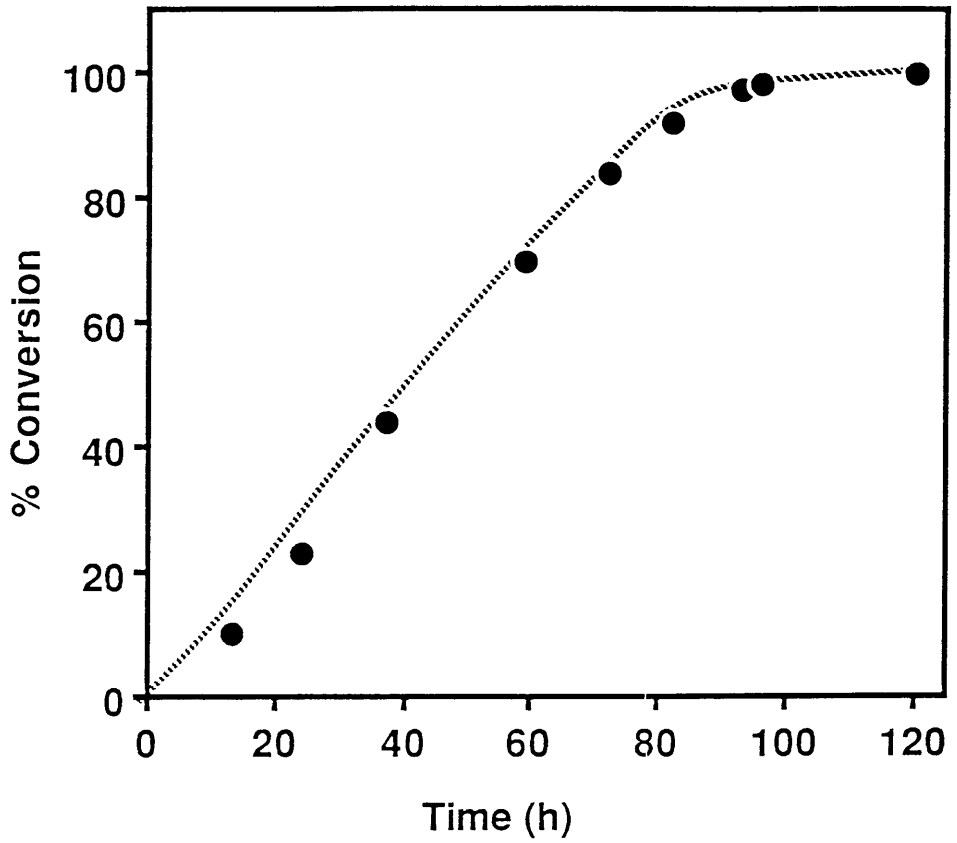



Fig. 1. Reaction progress for the D-LDH-catalyzed reduction of **3** to **18** for reaction 1. The percent conversion is based on the consumption of 2.56 *M* HCl during the reaction.

(11.65 g, 170 mmol), 2-mercaptoethanol (0.05 mL, 0.75 mmol), and Tris-HCl (0.45 g, 4 mmol) in 300 mL of $H_2O$. The pH of the solution was adjusted to pH 7.5 and the solution was degassed for 1 h. A catalytic amount of NAD was added (0.5 g, 0.75 mmol) followed by D-LDH (150 U) and FDH (46 U) immobilized in 100 mL of PAN-1000 gel. During the course of the reaction, the vessel was kept under a positive pressure of argon and the pH was kept at pH $7.5 \pm 0.1$ by the pH stat-controlled addition of 2.56 *M* HCl from a buret via a peristaltic pump. The progress of the reaction was followed by monitoring the consumption of HCl (Fig. 1). After 5 d, the reaction was complete. The enzyme-containing gels were removed by centrifugation, washed twice with 50-mL portions of degassed $H_2O$, and stored at 4°C in 50 m*M* Tris buffer (containing 3 m*M* dithiothreitol). The combined aqueous portions were concentrated to ~70 mL, acidified to pH 2 with conc. HCl, and extracted 4 times with 250-mL portions of ether. The combined ethereal extracts were dried ($MgSO_4$) and evaporated to provide a clear oil that solidified to a white, crystalline solid after

further drying. The solid was dried at 0.5 torr for 12 h to provide 13.9 g (134 mmol, 89%) of **18:** mp 53–55°C (decomp.); $[\alpha]_D^{20} - 5.6°$ (*c* 3.71, $CHCl_3$); $^1H$ NMR ($CDCl_3$) $\delta$ 0.99 (t, $J = 7.4$ Hz, 3 H), 1.75 (m, 1 H), 1.87 (m, 1 H), 4.24 (dd, J = 6.8 and 4.5 Hz, 1 H), 6.72 (br, exchangeable with $D_2O$, 2 OH); IR (Nujol) 3600–2500 (br, OH), 1730 (C=O) $cm^{-1}$. The ee of the *O*-(+)-MTPA derivative of the methyl ester was >98%. The reaction was repeated with the recovered gels to provide 14 g of **18** in 95% yield. (In the second reaction, the aqueous layer was extracted an additional 4 times with 250-mL portions of ether.)

*Method B*

A 100-mL graduated cylinder with a stirring bar was charged with **3** (750 mg, 7 mmol), sodium formate (544 mg, 8 mmol), Tris-HCl (40 mg, 5 m*M*), and dithiothreitol (0.5 mL of an 0.5 *M* solution, 5 m*M*) in 50 mL of $H_2O$. The pH of the solution was lowered to near pH 8 with 10 *M* NaOH and then adjusted to pH 7.5 with 1 *M* NaOH. The cylinder was stoppered with a septum through which protruded a pH probe, an inlet and outlet for argon, and a polypropylene tube for the pH stat-controlled addition of HCl. The solution was degassed for 1 h with argon and then a catalytic amount of NADH (20 mg, 0.6 mmol) was added. A 2-mL aliquot of the reaction mixture was removed via syringe and added to a polypropylene tube containing D-LDH (0.42 mL of a suspension in 3.2 *M* $(NH_4)_2SO_4$, 5000 U) and FDH (50 mg, 45 U). The solution was transfered into a section of dialysis tubing and the enzyme-containing bag was placed in the reaction mixture. During the course of the reaction, the pH of the solution was kept at pH 7.2–7.5 by pH stat-controlled addition of 1.13 *N* HCl and argon was gently bubbled through the solution to remove $CO_2$ as it formed. Within 3 d, the reaction was complete (96% completion within 2 d). The enzyme solution was placed in a new bag and stored for 1 d at 4°C in 50 mL of 5 m*M* Tris buffer (pH 7.5, 5 m*M* dithiothreitol) before being used in the reduction of phenyl pyruvate **16** (*see below*). Acidification of the reaction mixture and extraction of the product into ether as described in Method A provided 663 mg (6.4 mmol, 91%) of pure **18** as a white crystalline solid whose spectral data matched that of the material produced in Method A. The ee of the *O*-(+)-MTPA derivative of the methyl ester was >98%.

## (*R*)-Phenyllactic Acid (*19*)

*Method A*

The reduction of phenyl pyruvate by the MEEC method used the enzymes recovered from the reduction of **3** (Method B, *above*). The reaction vessel and reaction conditions were also the same. The reaction solution contained sodium phenylpyruvate **16** (1.04 g, 5 mmol), sodium formate (410 mg, 6 mmol), NADH (30 mg), dithiothreitol (5 m*M*), Tris-HCl (40 mg, 5 m*M*) in 50 mL of $H_2O$. The reaction was complete within 3 d. The enzyme-containing bag was removed and stirred for 6 h in 50 mL

of 50 m*M* Tris buffer (pH 7.5, 5 m*M* dithiothreitol). Evaporation at reduced pressure of the combined reaction mixture and dialysate provide a white solid that was sparingly soluble in 25 mL of $H_2O$. This suspension was extracted 3 times with 50-mL portions of ether. The combined ethereal extracts were dried ($MgSO_4$) and concentrated in vacuum to provide 0.997 mg (5 mmol, 98%) of a white, crystalline solid whose spectral data were in agreement with those of material produced using L-LDH (*1*). The ee of the *O*-(+)-MTPA derivative of the methyl ester was >98%.

*Method B*

A solution of sodium phenylpyruvate **16** (10.2 g, 50 mmol) and glucose (9.01 g, 50 mmol) in 750 mL of 0.1 *M* Hepes buffer (pH 7.0, 2.5 m*M* dithiothreitol, 1.5 m*M* EDTA) was placed in a 3-L, three-necked round-bottomed flask with a stirring bar, a pH electrode, and an inlet and outlet for argon. The reaction mixture was degassed and a catalytic amount of NAD (148 mg, 0.212 mmol) was added followed by soluble D-LDH (700 U) and GlcDH (10 U) in 40 mL of PAN-450 gel. During the course of the reaction, pH-stat controlled addition of 2.0 *M* NaOH maintained the pH of the solution near pH 7.0. After 3 d, the reaction was complete and the enzyme-containing gel was removed by centrifugation; recovered GlcDH activity was negligible. Addition of conc. HCl adjusted the pH of the solution to pH 3 and the solution was continuously extracted with ether during a period of 2 d. Removal of ether by rotary evaporation at reduced pressure provided a white solid that was recrystalized in 135 mL of $CHCl_3$ to provide 6.0 g of **19** (36 mmol, 72%).

## (*R*)-1-Butene Oxide (*23*)

A dry, 300-mL three-necked round-bottomed flask equipped with a dropping funnel, reflux condensor, and magnetic stirring bar was charged with **18** (13.9 g, 134 mmol) in 30 mL of dry THF. The flask was cooled in an ice bath and 240 mL of borane-THF (1.0 *M* solution in THF, 240 mmol) was added dropwise during the course of 2 h. The ice bath was removed and the mixture was stirred for an additional 22 h. Excess hydride was cautiously quenched with 25 mL of $H_2O$. A white precipitate formed and was separated and redissolved in 100 mL of $H_2O$. The organic layer was separated and the combined aqueous layers were saturated with potassium carbonate and extracted 3 times with 100-mL portions of ether. The combined organic phases were dried ($MgSO_4$), concentrated, and distilled at reduced pressure (30 mm, 122–125°C) to provide 9.7 g of **22** (108 mmol, 81%) whose analytical data agreed with literature values (*20,21*): $[\alpha]_D^{21}$ + 12.6° (*c* 3.23, EtOH); $^1$H NMR ($CDCl_3$) $\delta$ 0.90 (t, $J$=7.5 Hz, 3 H, $CH_3$), 1.41 (m, 2 H, $CH_3CH_2$), 3.37 (m, 1 H, CH), 3.58 (m, 2 H, $CH_2O$), 3.67 (br s, 2 H, 2 X OH); IR (neat) 3350 (br OH), 1045 (C–O) cm$^{-1}$.

Conversion of diol **20** to acetoxybromopropanes **21** and **22** proceeded as follows. A dry, 250-mL three-necked round-bottomed flask equipped

with a dropping funnel, reflux condensor, and magnetic stirring bar was charged with **20** (9.7 g, 108 mmol) and the flask was cooled in an ice bath. A solution of 31% HBr-AcOH (73 mL, 339 mmol) was added dropwise during the course of 5 min. The ice bath was removed and the reaction mixture was stirred at room temperature for 45 min. The mixture was then poured into water (200 mL) in a 2-L beaker (in an ice bath) and immediately neutralized with solid $Na_2CO_3$ (~90 g). The solution was extracted 3 times with 150-mL portions of ether and the combined ethereal layers were dried ($MgSO_4$) and evaporated. The residual oil was distilled at reduced pressure (21 mm, 87–91°C) to provide 17.3 g of **22** (89 mmol, 82%; $^1$H NMR spectroscopy indicated that the product contained approximately 7% of 1-acetoxy-2-bromobutane **21**) as a clear liquid whose analytical data agreed with literature values (*20,21,23*): $[\alpha]_D^{21}$ +17.8° (*c* 2.73, EtOH); $^1$H NMR ($CDCl_3$) δ 0.90 (t, *J* = 7.4 Hz, 3 H, $CH_3CH_2$), 1.69 (m, 2 H, $CH_3CH_2$), 2.07 (s, 3 H, $CH_3CO$), 3.45 (m, 2 H, $CH_2O$), 4.91 (m, 1 H, CH); IR (neat) 1735 (C=O) cm$^{-1}$.

To obtain oxirane **23,** a 100-mL round-bottomed flask equipped with a magnetic stirring bar and a pressure-equalizing dropping funnel capped with a drying tube was charged with the mixture of **21** and **22** from the preceding step (17.3 g, 89 mmol) and cooled in an ice bath. Freshly prepared potassium pentylate (70.5 mL, 1.175 *M*) was added dropwise during the course of 1 h. (A white precipitate of potassium bromide immediately formed.) The ice bath was removed and the reaction mixture was stirred at room temperature for an additional 1 h. The flask was then fitted with a 10-cm Vigreaux column, with a condensor cooled to −10°C, and with receiving flasks cooled in a $CO_2$/EtOH bath. An oil bath, preheated to 130°C, was placed under the reaction flask and 5.2 g of **23** (72 mmol, 81%) was collected by careful, slow distillation. Analytical data agreed with literature values (*21*): bp 59–62°C; $[\alpha]_D^{21}$ +14.8°C (*c* 1.18, ether); $^1$H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.5 Hz, 3 H, $CH_3$), 1.54 (m, 2 H, $CH_3CH_2$), 2.44 (m, 1 H, $CH_2O$), 2.70 (m, 1 H, $CH_2O$), 2.85 (m, 2 H, CH). The enantiomeric excess of **23,** measured by $^1$H NMR spectroscopy (*21*) in the presence of Eu(hfc), was >98%.

## ACKNOWLEDGMENTS

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