Laboratory-Scale Enzymatic/Chemical Syntheses of D- and L-β-Chlorolactic Acid and D- and L-Potassium Glycidate[1]

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Abstract: This paper describes preparations of D- (and L-) chlorolactic acids having enantiomeric excesses greater than 97% by D- (and L-) lactate dehydrogenase catalyzed reduction of chloropyruvic acid with NADH. In syntheses carried out on 0.1–0.5-mol scales, the NADH was regenerated in situ by using glucose 6-phosphate and glucose 6-phosphate dehydrogenase. The enantiomerically enriched chlorolactic acids were converted to the correspondingly enriched potassium salts of glycidic acid (epoxyacrylic acid) by treatment with potassium hydroxide.

Enantiomerically pure epoxides are valuable synthetic intermediates. These compounds are now usually prepared from natural products[2] or by asymmetric epoxidation.[3,4] This paper describes highly enantioselective enzymatic syntheses of D(*S*)- and L(*R*)-β-chlorolactic acids and the conversion of these compounds to the corresponding epoxides (D(*R*)- and L(*S*)-glycidic acid, epoxyacrylic acid) (Figure 1).

Results

L-Lactic dehydrogenase (L-LDH, E.C. 1.1.1.27, rabbit muscle) and D-lactic dehydrogenase (D-LDH, E.C. 1.1.1.28, *Lactobacillus leichmannii*) catalyze the reduction of chloropyruvic acid to L-β-chlorolactic and D-β-chlorolactic acid, respectively. Table I summarizes comparative kinetic parameters for pyruvic acid, chloropyruvic acid, and bromopyruvic acid. The halopyruvic acids are reduced at slightly slower maximum rates than pyruvic acid. The concentrations of halopyruvic acids required to achieved these maximum rates are considerably higher than that of pyruvic acid.

The conditions used to synthesize D- and L-chlorolactic acid reflect the following three constraints. First, the reducing agent in the system (NADH) is too expensive to be used stoichiometrically and must be recycled. The particular recycling system used here (glucose 6-phosphate/glucose 6-phosphate dehydrogenase) was chosen for its convenience for laboratory-scale synthesis;[5] other systems might have worked as well. We note, however, that glucose 6-phosphate dehydrogenase is a very stable enzyme and that it contains no active-site thiol groups which might be inactivated by the halopyruvic acids. Second, the halopyruvic acids (especially bromopyruvic acid) do deactivate both lactic dehydrogenases (presumably by alkylation). To minimize this deactivation, we carried out the reactions by using the less reactive chloropyruvic acid as substrate and maintaining the concentration of this material in the solution at concentrations close to K_m by slow addition. Third, glucose 6-phosphate, 6-phosphogluconate, and especially inorganic phosphate (an impurity in the glucose 6-phosphate starting material) catalyze the decomposition of the reduced form of the nicotinamide cofactor (NADH).[5] To achieve rapid reduction of the chloropyruvic acid, we chose to perform the reaction with most of the nicotinamide cofactor maintained in the reduced form. This strategy protected the enzymes by keeping the concentration of chloropyruvate low but also resulted in relatively short lifetimes and low total turnover numbers for the NAD(H). Table II summarizes the results of reactions carried out on 0.25–0.50-mol scale. The enzymes were used in immobilized form: immobilization facilitates their recovery at the end of the reaction and (for reasons which are not presently clear) improves their stability.[6]

Table I. Michaelis-Menten Constants (K_m) and Relative Rates under Saturating Conditions (v_{max}^{rel}) for Substrates X-O_2CO_2H[a]

	L-LDH		D-LDH	
X	K_m, mM	v_{max}^{rel} [b]	K_m, mM	v_{max}^{rel} [b]
H	0.2	1	2	1
Cl	4	0.4	50	0.8
Br	4	0.5	13	0.4

[a] These parameters, determined with 0.31 mM NADH in 0.6 M Hepes, pH 7.6, at 26 °C, are approximate due to irreversible enzyme inactivation at high halopyruvic acid concentrations. [b] v_{max}^{rel} is the estimated value of v_{max}, relative to v_{max} for pyruvic acid itself.

Table II. Synthesis of D- and L-β-Chlorolactic Acids and of Potassium D- and L-Glycidate[a]

	L	D
immobilization yields,[b] %		
LDH	27	35
G-6-PDH	14	14
enzyme recoveries,[c] %		
LDH	46	45
G-6-PDH	100	100
reaction time, days	7	3
yields[d]		
Ba-6-P-gluconate·xH_2O	282 g (0.52 mol, 100%)	139 g (0.25 mol, 100%)
β-chlorolactic acid	31.6 g (0.25 mol, 52%)	16.5 g (0.13 mol, 53%)
K glycidate	78%	85%
enantiomeric excess,[e] %		
β-chlorolactic acid	>97	>97
K glycidate	>97	>97
turnover numbers (TTN)[f]		
LDH	5×10^6	2×10^6
G-6-PDH	1×10^8	5×10^7
NAD (H)	394	400

[a] Potassium glycidates were produced from chlorolactic acids on an 8-mmol scale. [b] Immobilization yield = 100 × (immobilized activity/activity in solution before immobilization). [c] These percentages of the starting activities were recovered after a single use of the enzymes in the preparations summarized in this table. [d] Isolated yields. The moles and % reported for Ba-6-P-gluconate are based on enzymatic assays; the isolated samples were ~76% pure with the major impurities being water of hydration and $Ba_3(PO_4)_2$. [e] The same values of ee were obtained by using crude (noncrystallized) β-chlorolactic acid and material which had been isolated by crystallization. [f] Total turnover number (TTN) = mol of product/mol of enzyme (cofactor).

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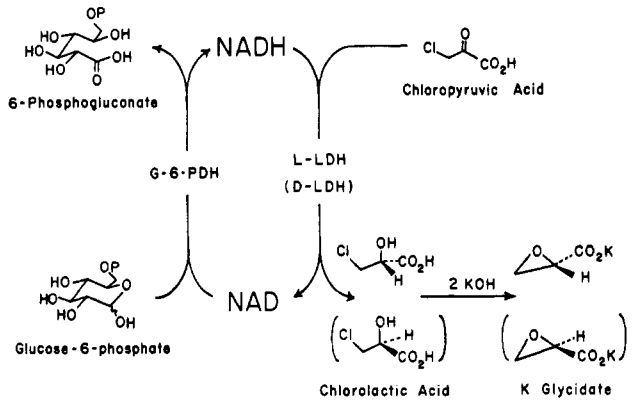

Figure 1. Synthesis of chlorolactic acids and potassium glycidates (G-6-PDH = gluclose 6-phosphate dehydrogenase; LDH = lactate dehydrogenase).

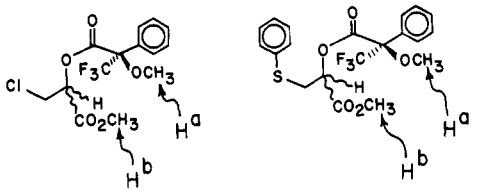

Figure 2. (*R*)-(+)-MTPA derivatives prepared from chlorolactic and glycidic acids.

β-Chlorolactic acid was isolated as the free acid. It is a stable and nonhygroscopic white crystalline solid. Conversion of β-chlorolactic acid to glycidic acid was accomplished by treatment with base and the glycidic acid isolated as its potassium salt.[7] The temperature of the reaction mixture and the amount of potassium hydroxide added (2 equiv) were controlled carefully to prevent undesired hydroxide attack on the epoxide ring (forming glyceric acid).

The enantiomeric purity of the products was determined by examination of the ^{1}H NMR spectra of (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl ((*R*)-(+)-MTPA) derivatives (Figure 2).[8] β-Chlorolactic acid was converted first into its methyl ester. Glycidic acid was treated first with benzenethiol (to open the epoxide ring, presumably with retention of configuration at C_2) and then converted to its methyl ester. The intensities of the resonances due to both the methyl ether protons (H^A, a quartet) and the methyl ester protons (H^B, a singlet) of the diasteriomers produced from racemic chlorolactic and glycidic acids and of the diasteriomers derived from the two enzymatic reactions[9] were compared. By this criterion both the chlorolactic acids and the glycidic acids showed no enantiomeric contamination. The wrong isomer would not have been detected at values of the enantiomeric excess greater than 97%.

Conclusions

The syntheses described here provide practical routes to both enantiomers of chlorolactic and glycidic acid. As described, the procedures should be capable of generating quantities up to several moles. For larger quantities, it would be worthwhile to increase the rates of reactions and the turnover numbers of enzymes and cofactors to improve the economics of the syntheses. The use of larger quantities of immobilized enzymes would be the most direct approach to increased reaction rates.[10] One approach to higher turnover numbers would be to use glucose 6-phosphate containing a lower level of phosphate impurity,[11] or to use an NADH regeneration system which does not involve alkyl phosphates, to minimize acid-catalyzed decomposition of NADH. A second would control the concentration of chloropyruvate at the lowest practical level to minimize the deactivation of the enzymes by alkylation.[12]

An attractive feature of this enzymatic system is that both D- and L-lactate dehydrogenase are commercially available, and both are relatively inexpensive and stable. Thus, both enantiomers of chlorolactic acid and glycidic acid are available. These materials should be useful starting materials for more complex enantioselective syntheses. Direct enzymatic reduction of complex α-keto acids by D- and L-LDH is not practical since the rate of substrate reduction by these enzymes decreases rapidly with increasing substrate chain length.

The major drawbacks of the present system are that the substrate (chloropyruvic acid) is reactive toward the enzyme and that the lifetime of the nicotinamide cofactor is relatively short (turnover numbers for NAD(H) in other systems are 10^3–10^4). Otherwise, the system is straightforward to operate and provides a practical if limited synthetic method to a useful chiral synthon.

Experimental Section

Materials and Methods. Chloropyruvic acid was prepared by the method of Cragoe and Robb[13] and recrystallized from ether/methylene chloride. Bromopyruvic acid was prepared by the metehd of Clark and Kirby.[14] L-Lactic dehydrogenase (rabbit muscle, E.C. 1.1.1.27), D-lactic dehydrogenase (*Lactobacillus leichmannii*, E.C. 1.1.1.28), glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, E.C. 1.1.1.49), β-NAD, and β-NADH were purchased from Sigma. *dl*-β-Chlorolactic acid was purchased from ICN Pharmaceuticals. Glucose 6-phosphate[15] and PAN 500[6] were prepared as described previously. (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid was purchased from Aldrich and converted to its acid chloride by the method of Mosher et al.[8] Water was distilled twice, the second time by using a Corning AG lb glass still. Ether was distilled from a purple solution of sodium and benzophenone dianion. Methanol was distilled from magnesium. Welding-grade argon was used without further purification. ^{1}H NMR spectra of (*R*)-(+)-MPTA derivatives were recorded by using a Bruker (WM 250) 250-MHz spectrometer. The pH of the reaction mixtures was controlled with a Weston Model 7561 pH controller coupled to an LKB 10200 peristaltic pump. Chloropyruvic acid was added to reaction mixtures by using a variable-speed LKB 2120 peristaltic pump.

Assays. The following enzymes and substrates were assayed by using procedures described in Bergmeyer: G-6-PDH[16a] (substituting NAD for NADP); G-6-P;[16b] 6-phosphogluconate;[16c] chloropyruvic and bromopyruvic acid;[16d] D- and L-LDH[16e] (replacing pyruvic acid with 5 mM chloropyruvic acid); NAD[16f] (using G-6-PDH from *Leuconostoc mesenteroides*); NADH[16g] (using pyruvate and L-LDH). Activities are reported in units having dimension of μmoles per minute. Assays for the activities of immobilized enzymes were essentially the same as for soluble enzymes.[6] Aliquots (20–100 μL) of immobilized enzymes (as small particles suspended in buffer) were taken and added to a 3-mL polystyrene cuvette containing 3 mL of buffer and the required substrates. The cuvette was stoppered with Parafilm and shaken for 3–4 s to mix the suspension. The UV absorbance was read at 340 nm for 5 s. This process

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(9) The chemical shifts ($CDCl_3$) of the resonances of H^A and H^B in the (*R*)-(+)-MTPA derivitives of chlorolactic and glycidic acids were as follows: for D-chlorolactic acid (H^A, δ 3.68, H^B, δ 3.84); for L-chlorolactic acid (H^A, δ 3.63, H^B, δ 3.78); for D-glycidic acid (H^A, δ 3.66, H^B, δ 3.74); for L-glycidic acid (H^A, δ 3.61, H^B, δ 3.71).
(10) The volume of gel containing the immobilized enzymes was less than 10% of the total volume in each of the syntheses; the use of larger quantities of enzyme (and gel) should not be problematic. Careful optimization of the enzyme immobilization yields would also prove advantageous for large-scale syntheses.
(11) Glucose 6-phosphate of higher purity can be prepared by enzymatic phophorylation of glucose using an ATP regeneration system involving phosphoenolpyruvic acid (PEP) and pyruvate kinase. Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.*, in press.
(12) Enzymatic reduction of hydroxypyruvic acid (which is comparable to chloropyruvic acid as a substrate for both D- and L-LDH and is not expected to inactivate these enzymes) followed by two-step conversion to glycidic acid (via the monotosylate) may provide a more economical large-scale route to these epoxides.
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was repeated about 15 times. A linear response was obtained for the change of absorbance with time.

Immobilization of Enzymes.[6] L-LDH, D-LDH, and G-6-PDH were immobilized separately in cross-linked PAN gel with a loading of ca. 2.5 mg protein/g of PAN-500. The reaction mixtures contained enzyme substrates to protect the active sites during the immobilization procedure. The procedure outlined below for D-LDH is representative. Immobilization yields (100 × (activity after immobilization/activity before immobilization)) and substrate concentrations for the other immobilizations were as follows: L-LDH (27%, 8 mM pyruvate, 1.0 mM NADH); G-6-PDH (14%, 6.0 mM G-6-P, 0.6 mM NADP). A commercially available suspension of ca. 20 mg of D-LDH in 4 mL of 3.2 M $(NH_4)_2SO_4$ was centrifuged at 4 °C at 15 000*g* for 10 min. The precipitate was dissolved in 3 mL of 0.3 M Hepes buffer (pH 7.5). This solution was dialyzed against 1 L of a 50 mM Hepes buffer (pH 7.5, deoxygenated with a stream of argon) to decrease the concentration of $(NH_4)_2SO_4$. The resulting solution contained 1160 units of chlorolactic dehydrogenase activity (using 5 mM chloropyruvate, pH 7.6, 25 °C). To PAN 500 (13.0 g) in a 500-mL beaker was added 42 mL of 0.3 M Hepes buffer (pH 7.5, 0.05 M $MgCl_2$) containing 50 mg of sodium pyruvate and 50 mg of NADH. The mixture was stirred vigorously. After 1 min, 650 μL of a dithiothreitol solution (0.50 M) and 5.53 mL of a triethylenetetramine solution (0.50 M) were added. One minute later, the D-LDH-containing solution was added. The mixture gelled after ca. two additional minutes of stirring. The gel was kept at room temperature for 1 h, ca. 200 mL of 0.005 M Hepes buffer (pH 7.5) containing 50 mM ammonium sulfate was added, and the gel was broken into small particles in a Waring blender at low speed for 3 min and then at high speed for 30 s. The gel particles were separated by centrifugation, washed with 200 mL of 50 mM Hepes buffer (pH 7.5), and again separated by centrifugation. The gel particles were suspended in H_2O to produce 20 mL of a suspension containing 405 units of chlorolactic dehydrogenase activity (35% immobilization yield).

L-β-Chlorolactic Acid. A 2.5-L aqueous solution containing dithiothreitol (3.6 mM), EDTA (1.4 mM), $MgCl_2$ (7.0 mM), and glucose 6-phosphate (0.51 mol, 0.20 M) was adjusted to pH 7.6 with KOH. The solution was transferred to a 5-L three-necked flask equipped with a magnetic stirrer and a pH electrode. The solution was degassed with argon and NAD (0.53 mmol) and an aqueous suspension of PAN-immobilized L-LDH (2300 units) and G-6-PDH (278 units) were added. The reaction was carried out under an argon atmosphere at ambient temperature (ca. 20 °C), and the pH was maintained at pH 7.4–8.0 by the pH stat controlled addition of 1.0 M KOH solution. An aqueous solution of chloropyruvic acid (1.0 M, maintained at 5 °C) was added dropwise at an average rate of 3 mL h^{-1} by using a peristaltic pump. Several times during the course of the reaction additional NAD was added to maintain a 0.05–0.20 mM concentration of NAD(H) in the reaction mixture. A total of 0.49 mol of chloropyruvic acid and 1.27 mmol of NAD were added over the course of the reaction. After 172 h the PAN was allowed to settle and the solution decanted. The PAN was washed with 0.05 M Hepes buffer (pH 7.5), separated by centrifugation, and resuspended in fresh buffer. The resulting suspension (500 mL) contained 100% of the original G-6-PDH activity and 46% of the original L-LDH activity. To the decanted reaction solution was added 1 mol of $BaCl_2$, followed by 2 L of ethanol. The white precipitate which formed was filtered, washed with 50% aqueous ethanol, and dried under vacuum, yielding 282 g of barium 6-phosphogluconate·xH_2O (0.52 mol by enzymatic assay, 100% yield). The solution was made acidic with HCl, saturated with NaCl, and extracted in several fractions into a total of 5 L of ethyl acetate. The ethyl acetate solution was dried with Na_2SO_4 and filtered. An aliquot (40 mL) was saved for determination of optical purity. The remaining solution was concentrated by rotary evaporation and then dried under vacuum, yielding 55 g (0.44 mol, 91%) of crude product. The chlorolactic acid was recrystallized from 1:9 toluene/benzene yielding 31.6 g of white needles: mp 88–89 °C, $[\alpha]^{25}_D$ 4.14 ± 0.08° (*c* 9.05 g/100 mL, H_2O) (0.25 mol, 52% yield); ^1H NMR (acetone-d_6) δ 9.0–11.0 (b, variable and exchangeable with D_2O, 2, OH), 4.55 (m, 1, methine), 3.85 (d, 2, methylene); IR (Nujol) 3450 (OH), 2300–3600 (OH), 1718 cm^{-1} (carbonyl).

Anal. Calcd for $C_3H_5ClO_3$: C, 28.94; H, 4.05; Cl, 28.47. Found: C, 28.94; H, 4.01; Cl, 28.45.

D-β-Chlorolactic Acid. The procedure for the enzymatic synthesis of D-β-chlorolactic acid was essentially the same as that for L-β-chlorolactic acid, with the following exceptions. The reaction mixture initially contained 0.25 mol of glucose 6-phosphate, 405 units of PAN-immobilized D-LDH (with 5 mM chloropyruvic acid), and 386 units of PAN-immobilized G-6-PDH. The reaction was complete after 3 days, with a total of 0.25 mol of chloropyruvic acid and 0.625 mmol of NAD having been added. The D-LDH retained 45% and the G-6-PDH retained 100% of their initial enzymatic activities. The yield of barium 6-phosphogluconate was quantitative, while D-β-chlorolactic acid was obtained in a 66% crude yield (20.4 g, 0.164 mol) and a 53% yield (14.0 g, 0.132 mol) after recrystallization: mp 88–89 °C, $[\alpha]^{25}_D$ 3.97 ± 0.08° (*c* 9.05 g/100 mL, H_2O).

Methyl β-Chlorolactates. To 0.5 g of *dl*-, D-, or L-β-chlorolactic acid in a round-bottomed flask equipped with a reflux condenser and under an argon atmosphere was added 20 mL of dry methanol followed by 0.5 mL of acetyl chloride. The reaction mixture was heated at reflux for 1.5 h and then concentrated by rotary evaporation affording a yellow oil: ^1H NMR ($CDCl_3$) δ 4.55 (t, 1, methine), 3.85 (s, 3, methyl), 3.85 (d, 2, methylene), 3.7–4.7 (variable, OH); IR (neat) 3500 (OH), 2980 (C–H), 1740 cm^{-1} (carbonyl).

Methyl β-Chlorolactate (*R*)-(+)-MTPA Derivatives. To D-, L-, or *dl*-methyl β-chlorolactate (0.042 g, 0.30 mmol) in a 5-cm test tube was added (*R*)-α-methyl-α-(trifluoromethyl)phenylacetyl chloride (0.152 g, 0.60 mmol) followed by 10 drops of CCl_4 and 10 drops of pyridine. After 8 h the contents of the tube were transferred to a separatory funnel and diluted with 30 mL of ether. The ethereal solution was washed three times each with 10-mL portions of 0.1 N HCl, saturated Na_2CO_3 solution, and water. The ethereal solution was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation. The resulting oil was purified by preparative TLC on silica gel by using 1:1 CH_2Cl_2/pentane as solvent.

Potassium Glycidates.[7] A 10-mL round-bottom flask containing a solution of 1.05 g of KOH in anhydrous methanol was equilibrated in an ice bath. To this solution was added dropwise a solution of 1.00 g (8.03 mmol) of *dl*-, D-, or L-chlorolactic acid in 2 mL of anhydrous methanol at such a rate that the temperature did not exceed 10 °C (~15 min). The reaction mixture was stirred an additional hour at room temperature and kept overnight at 5 °C. Potassium chloride was removed by filtration and washed with cold methanol. To the combined filtrate and wash solution was added dry ether to precipitate the potassium glycidate completely. The product was dried under reduced pressure: *dl*-potassium glycidate (0.89 g, 88% yield); L-potassium glycidate (0.79 g, 78% yield); D-potassium glycidate (0.85 g, 85% yield). ^1H NMR (D_2O; ppm from DSS): 3.35 (d of d, 1), 2.65–3.02 (m, 2).

Methyl β-(Thiophenoxy)lactate (*R*)-(+)-MTPA Derivatives. To a solution of thiophenol (0.192 g, 1.75 mmol) in 3 mL of dry methanol was added *dl*-, D-, or L-potassium glycidate (0.200 g, 1.59 mmol). The reaction mixture was stirred at room temperature for 48 h, the methanol removed by rotary evaporation, and the resulting solid filtered and washed with benzene. The solid was dissolved in H_2O and the solution filtered, made acidic with HCl, and extracted with ether. The ether layer was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation, yielding a white solid. To a solution (ca 5 mL) of this solid in dry methanol in a round-bottomed flask equipped with a reflux condenser was added 0.20 mL of acetyl chloride. The mixture was heated at reflux for 2 h and concentrated by rotary evaporation. The resulting oil was dissolved in ether, and the solution was passed through a short column of alumina and concentrated by rotary evaporation. To a portion of the resulting oil (0.064 g, 0.30 mmol) and (*R*)-(+)-MPTACl (0.60 mmol, 0.152 g) in a 5-cm test tube were added 10 drops of CCl_4 and 10 drops of pyridine. After 8 h the contents of the tube were transferred to a separatory funnel and diluted with 30 mL of ether. The ethereal solution was washed three times each with 10-mL portions of 0.1 M HCl, saturated Na_2CO_3, and water. The ethereal solution was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation. The resulting oil was purified by preparative TLC on silica gel using 1:1 CH_2Cl_2/pentane as solvent.

Registry No. D-β-Chlorolactic acid, 82079-44-5; L-β-chlorolactic acid, 61505-41-7; D-potassium glycidate, 82044-23-3; L-potassium glydicate, 82079-45-6; pyruvic acid, 127-17-3; chloropyruvic acid, 3681-17-2; bromopyruvic acid, 1113-59-3; methyl β-chlorolactate MTPA, 82044-24-4; methyl β-(thiophenoxy)lactate MTPA, 82044-25-5; methyl β-chlorolactate, 32777-04-1; D-LDH, 9028-36-8; L-LDH, 9001-60-9; G-6-PDH, 9001-40-5.