Enzymatic vs. Fermentative Synthesis: Thermostable Glucose Dehydrogenase Catalyzed Regeneration of NAD(P)H for use in Enzymatic Synthesis[1]

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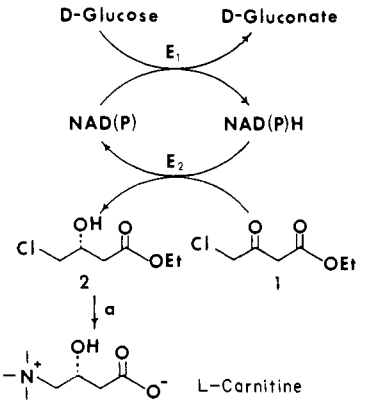
Abstract: Procedures are described for regeneration of reduced nicotinamide cofactors NADH and NADPH from NAD(P) based on glucose and a thermostable glucose dehydrogenase from *Bacillus cereus* immobilized in polyacrylamide gels. The turnover number for NAD in a synthesis of 200 mmol of D-lactate is 40 000. Application of this system to other syntheses is demonstrated with preparations of ethyl (*R*)-4-chloro-3-hydroxybutanoate, (*R*)-2,2,2-trifluoro-1-phenylethanol, ethyl (*S*)-3-hydroxyvalerate, (*S*)-lactaldehyde dimethyl acetal, and (*S*)-3-hydroxybutanal dimethyl acetal. Further investigation of the kinetics regarding the thermoresistance of glucose dehydrogenase in the presence of NaCl has been carried out, and it appears that the enhancement by NaCl of the thermal stability of the enzyme is approximately third order. The immobilized glucose dehydrogenase incubated at 55 °C, pH 7.5, for 7 days is still fully active while many other enzymes are completely inactivated in 1–2 days. Addition of NaCl enhances the thermal stability more significantly than the immobilization does, and a remarkable increase in thermal stability was observed with these two combined factors. The half-life of the immobilized glucose dehydrogenase at 55 °C in a buffer (pH 7.0–7.5) containing 1 M NaCl is more than 30 days compared to 3 min for the free enzyme, corresponding to an overall ~50 000-fold increase in thermal stability.

Asymmetric reduction of carbonyl compounds with microorganisms is increasingly recognized as a valuable approach to organic synthesis.[3-6] Previous studies indicate that baker's yeast has broader substrate specificity than the enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1) from the same species. Many carbonyl derivatives which are not substrates for ADH can be reduced by baker's yeast.[7,8] Sih and co-workers recently have demonstrated that baker's yeast contains at least two oxidoreductases which catalyze the reduction of ethyl 4-chloro-3-oxobutanoate **1** to (*S*)-ethyl-4-chloro-3-hydroxybutanoate (the *S* enantiomer of **2**) of opposite configuration at different rates,[3] and thus the product obtained was partially optically active (55% ee). Modification of the substrate by increasing the size of the ester group has altered the stereochemical course of the yeast reduction and increased the optical yield.[3] We speculate that enzymatic approaches to the synthesis may avoid the problem of competing reactions present in the fermentative processes and give products in higher optical purity using readily available substrates without requiring further modification. Since the ADH-catalyzed reductions require NAD(P)H cofactors which, as well as the enzymes, are very expensive, their regeneration is thus required to make the enzymatic process technologically and economically feasible. Several approaches to the regeneration of NAD(P)H have been developed and applied to large-scale synthesis.[9] Of these, the uses of formate/formate dehydrogenase (FDH),[10] hydrogen/hydrogenase (H_2ase),[11] and glucose-6-phosphate (G-6-P)/G-6-P dehydrogenase (G-6-PDH)[12] have been considered the most practical. These systems, however, still have disadvantages: FDH has relatively low activity (3 ±/mg) and accepts NAD only; H_2ase is extremely sensitive to oxygen; the use of G-6-PDH requires the preparation of its substrate G-6-P, and both G-6-P and its product catalyze the decomposition of NAD(P)H in solution.[12]

Scheme I[a]



[a] E_1, the glucose dehydrogenase from *Bacillus cereus;* E_2, the alcohol dehydrogenase from *Thermoanaerobium brockii* or horse liver; (a) (1) Me_3N, (2) HCl.

We describe here studies of the thermal stability of a mesophilic enzyme glucose dehydrogenase (GDH) from *Bacillus cereus*[13] and the development of combined procedures for use to modify the enzyme to a remarkably stable and active form useful as a practical catalyst in organic synthesis. The enzyme GDH uses glucose as a substrate and accepts either NAD or NADP as a cofactor.[13] It is more stable and more active than the enzyme from other species which have been used in small-scale synthesis.[14]

Results and Discussion

Thermal Stability of Glucose Dehydrogenase. Stability is one of the most important factors which determine the practicality

(1) Supported by the National Science Foundation (Grant CHEM-8318217) and the Robert A. Welch Foundation (Grant A-1004).
(2) NSF Predoctoral fellow.
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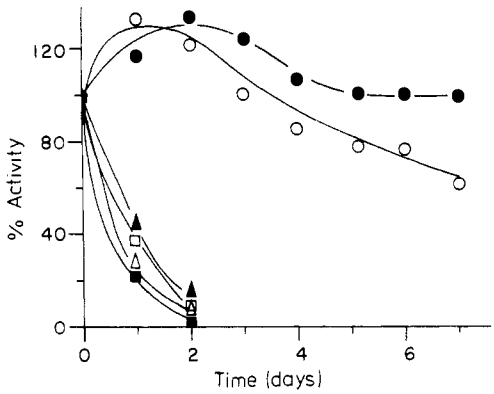

Figure 1. Thermal stability of the glucose dehydrogenase at 55 °C (●) (50 m M triethanolamine buffer–0.5 M NaCl, pH 7.5) compared with L-lactic dehydrogenase from rabbit muscle (▲), horse liver alcohol dehydrogenase (△), thermoanaerobium alcohol dehydrogenase (○), glucose-6-phosphate dehydrogenase from L. *mesenteroides* (□), and formate dehydrogenase from yeast (■). All enzymes were immobilized.

Table I. Half-Life of the Glucose Dehydrogenase at 55 °C in 50 mM Triethanolamine–HCl Buffer Containing Different Concentrations of Salt[a]

salt	pH	half-life (min)[b] soluble enzyme	half-life (min)[b] immobilized enzyme
control[c]	7.0	1.2×10^2	
	7.5	3	6×10^2
NaCl: 0.5 M	7.5	5.6×10	$>5 \times 10^4$
1.0 M	7.5	1.1×10^2	$>5 \times 10^4$
3.0 M	7.5	1.4×10^3	$>5 \times 10^4$
KCl: 0.5 M	7.5	5.2×10	
$MgCl_2$: 0.5 M	7.5	1.4×10	
$CaCl_2$: 0.5 M	7.5	1.1×10	
sodium pyruvate: 0.5 M	7.5	5.3×10	

[a] The enzyme activity was assayed at 25 °C according to the methods described in the Experimental Section. [b] Calculated on the basis of the first-order inactivation of the enzyme. For those values higher than 5×10^4, the numbers were estimated approximately based on the initial rate of inactivation. [c] No salt was added to the buffer solution.

of an enzyme as a synthetic catalyst. Enzymes can be inactivated by a number of ways such as denaturation (caused by heat or organic solvents), oxidation, and dissociation. Thermal inactivation is considered the most important mode of inactivation.[15] A study of the thermal stability of several synthetically useful enzymes immobilized in polyacrylamide gels is shown in Figure 1. Although these enzymes are very stable at room temperature, they are quite different in stability at higher temperature. As shown, the immobilized enzyme GDH incubated at 55 °C for 7 days in the presence of NaCl (0.5 M) is still fully active, while other enzymes are completely inactivated in 1–2 days, except the ADH from *Thermoanaerobium brockii* (TADH) which shows a half-life of 7 days under this condition. The reason for the initial increase of activity for GDH and TADH as shown in the figure is not clear. Interestingly, sodium chloride plays an important role in the thermal stability of GDH, but it does not affect other enzymes significantly. The activity of GDH, however, is independent of NaCl; no significant change of the enzyme activity has been observed when assayed in Tris buffer (0.1 M, pH 8.0) containing different concentrations of NaCl (0–3 M). In order to indentify the source of inorganic ions which ensure the thermal resistance of GDH, several inorganic salts have been tested and the results are shown in Table I. It is obvious that monocations such as Na^+ and K^+ are the species which enhance the stability and the enhancement increases with increase of NaCl concentration. The latter characteristic is illustrated in Figure 2 which indicates that

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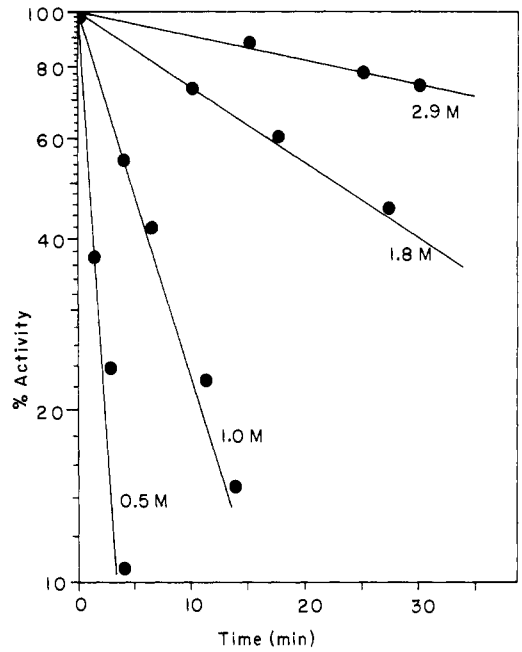


Figure 2. Time course for the thermal inactivation of soluble glucose dehydrogenase at 85 °C with different NaCl concentrations.

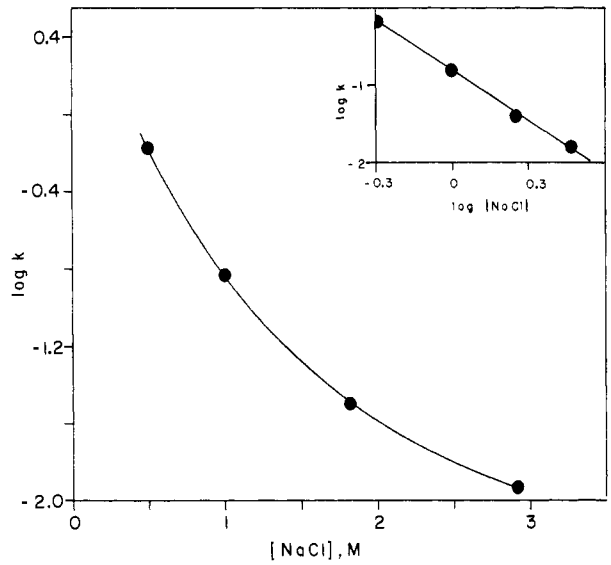


Figure 3. Effect of NaCl concentration on the thermal inactivation of glucose dehydrogenase at 85 °C. The first-order rate constants, k, of thermal inactivation were obtained from Figure 2.

the inactivation of soluble GDH at different concentrations of NaCl essentially follows first-order kinetics at 85 °C. The first-order rate constant, k, of the thermal inactivation must be some function of the concentration of NaCl (eq 1 and 2). Figure

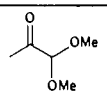
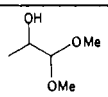
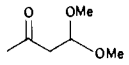
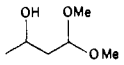
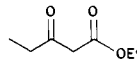
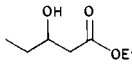
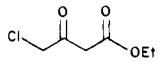
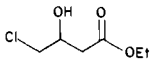
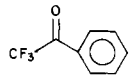
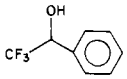
$$k = x[\mathrm{NaCl}]^y \quad (1)$$

$$\log k = \log x + y \log [\mathrm{NaCl}] \quad (2)$$

$$k = 0.15/[\mathrm{NaCl}]^{3.3} \quad (3)$$

3 indicates the relation between the concentration of NaCl and log k (k values were calculated from the half-life obtained from Figure 2 with the equation $k = 0.693/\text{half-life}$). A replot of log k vs. log [NaCl] gives a linear line shown in Figure 3 (insert). From the slope (y) and intersection (log x) of the line, the relation between k and NaCl is thus obtained and shown in eq 3. It appears that the enhancement effect of NaCl is about third order. Although we carried out experiments regarding the stability of enzymes without removal of oxygen, we should mention that no significant change was observed in the absence of oxygen. It is

Table II. Fermentative vs. Enzymatic Reductions of Carbonyl Compounds[a]

substrate	product	compd no.	% ee (configuration)[b]			
			Baker's yeast	YADH	HLADH	TADH
		3	c	100 (S)	100 (S)	51 (R)
		4	c	100 (S)	89 (S)	91 (S)
		5	40 (S)[d]	e	84 (S)	88 (S)
		2	55 (S)[f]	g	98 (R)	90 (R)
		6	44 (R)[h]	i	i	94 (R)

[a] Enzymatic reactions were carried out on 10-mmol scales. Specific activities are in the range of 1–10 U/mg unless otherwise mentioned. [b] Determined by converting the carbinol to (R)-(+)-MPTA ester and analyzed by 90-MHz ^{1}H NMR: Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* 1973, *95*, 512. [c] Data are not available. [d] See ref 2. [e] Poor substrate (the specific activity is <0.01 U/mg). [f] See ref 2. [g] The enzyme was inactivated immediatedly after adding the substrate. [h] Bucciarrelli, M.; Forni, A.; Moretti, I.; Torres, G. *Chem. Commun.* 1978, 456. [i] Not a substrate.

clear that immobilization and addition of NaCl enhance the thermal stability of GDH and the latter affects the enzyme more significantly. The half-life of soluble GDH at pH 7.5 in the absence of NaCl is 3 min, compared to 10 h for the immobilized enzyme under the same conditions. When NaCl was added to the suspension of immobilized GDH, a remarkable enhancement of thermal stability was observed. The half-life of immobilized GDH at 55 °C in a triethanolamine buffer (50 mM, pH 7.5) containing 1 M of NaCl is more than 30 days compared to 3 min for the free enzyme (Table I), corresponding to an overall ~ 50 000-fold increase in thermal stability.

So far, it is not clear why NaCl increases the thermal stability of GDH. A substantial amount of work has been devoted to studying the effects of salts on protein stability;[16] the results indicate that inorganic ions may bind to the charged species of proteins or reduce the solubility of the hydrophobic region of proteins by increasing the ionic strength of the solution. Both effects will make proteins more compact than they would be and thus prevent thermal denaturation and intermolecular aggregation. The thermal stability of GDH is also sensitive to solution pH. The optimal region for the enzyme to be thermally stable is 6.5, and a decrease in thermal stability was observed at pH lower or higher than 6.5. Apparently, proton dissociation and association of some functional groups in the enzyme must be involed in the mechanism. Further investigation of the mechanisms of proton transfer affecting thermal stability of the enzyme will be carried out.

Cofactor Regeneration. To illustrate the practicality of the glucose/GDH system for NAD(P)H regeneration and to compare the system with others developed before,[9] a synthesis of 200 mmol D-lactic acid was carried out at room temperature. The reaction was complete in a period of a 6-day operation, and the product D-lactic acid was ioslated in 90% yield. At the conclusion of the reaction, both enzymes are still fully active. The turnover numbers are the following: NAD, 40 000; GDH, 10^7; D-lactic dehydrogenase (LDH), 10^8. Since the concentration of NAD (0.01 mM) used in the reaction is about 0.1 K_m of that for LDH and GDH, the reaction is not operating at the maximum rate. We note that the rate can be increased by increasing the concentration of either NAD or enzymes. The cofactor regeneration illustrated has two advantages. First, the high stability of both enzymes and the high turnover number of NAD certainly will reduce the operating cost. The price of NAD, for example, is reduced from $1000/mol to 2.6¢/mol. Second, this high turnover of NAD regeneration indicates that the enzymatic reduction of NAD to NADH in each cycle is almost completely stereoselective, i.e., the hydride is transfered to position 4 of the nicotinamide ring with almost 100% accuracy.

Synthesis. The cofactor regeneration system developed here has been applied to other enzymatic syntheses with NAD-dependent ADH from horse liver (HLADH) or baker's yeast (YADH) and NADP-dependent TADH as catalysts, and the results are summarized in Table II. The product of each of the synthetic reactions was simply isolated by extraction and the yield for each reaction was about 90% except that for compound **2** which gave 72% yield. In most cases the enzymes recovered at the conclusion of reactions are still active (80–90%) and can be reused. In an attempt to prepare compound **2** from **1** with YADH catalysis, the immobilized YADH was inactivated immediately after adding compound **1** and no desired product was isolated. As shown in the table, the enantiomeric purity of each enzymatic product is much higher than that obtained by fermentation. In some cases yeast will reduce the carbonyl compounds which are not substrates for YADH. Trifluoroacetophenone, for example, is not a substrate for YADH but can be reduced by yeast. These results confirm the study reported by Sih and co-workers that yeast contains more than one oxidoreductase. The compounds prepared here are useful either as synthons or as a chiral solvent (compound **5**) for the determination of optical purity by NMR techniques.[18] Compound **3** can be easily converted to L-lactaldehyde for use in the synthesis of L-fucose[19] and L-rhamnose[20] with aldolase/isomerase catalysts. Both unusual L sugars are important components of a number of glycoprotein determinants.[21] The high optical purity of compound **2**, especially prepared from HLADH catalysis, allows preparation of enantiomerically pure L-carnitine which is a valuable pharmaceutical.[3] D-Carnitine is a competitive inhibitor of the L-enantiomer and causes physiological side effects. Although chemical methods for the synthesis of carnitine are available, they produce a racemic mixture.[22] Biochemical routes of L-carnitune

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have been reported before,[23] but they have the drawbacks that the enzymes used are not readily available and the procedures are rather complex, and that enzyme substrates need to be modified in order to enhance the enantioselectivity.[3] A current interesting method for the preparation of L-carnitine is based on kinetic resolution: hydrolysis of *O*-acyl-DL-carnitine with cholinesterase.[24] The attractive feature of this method is that the reaction does not need a cofactor, although the process requires a separation of carnitine from *O*-acylcarnitine and, in principle, yields a maximum of 50% of product based on racemic starting material.

Despite the high optical purity of compounds prepared here by enzymatic methods, we should mention that asymmetric reduction of carbonyl compounds using chiral metal hydrides or chiral transition-metal catalysts is highly developed and is an area of current intense activity.[25] The method is quite flexible and a broad range of carbonyl compounds can be reduced asymmetrically. It is, however, not quite successful in reductions of aliphatic ketones (such as those described in this work) with respect to stereoselectivity, particularly if both groups attached to the carbonyl center are similar in size or contain functional groups which either interfere with the stereospecific coordination of metals or cause side reactions.

Conclusion

In summary, this work demonstrates the practicality of enzymatic synthesis requiring NAD(P)H regeneration in which glucose is the ultimate reducing agent. By selecting an appropriate ADH as a catalyst, several useful compounds can be prepared in high optical purity. The cofactor regeneration system used here has several advantages. First, the enzyme GDH is more stable than any other enzymes used in nicotinamide cofactor regeneration; it can be operated at high temperature, providing the coupled enzyme is also thermostable (e.g., TADH).[15] Second, the enzyme GDH accepts both NAD and NADP, and the reduction of NAD(P) is essentially irreversible by hydrolysis of the initially formed glucono-δ-lactone to gluconate. Third, glucose is inexpensive and innocuous to most enzymes and NAD(P)H. Moreover, many enzymes in solution containing glucose are more stable than those in solution without glucose. Fourth, the enzyme GDH is commercially available, inexpensive, and easy to manipulate and immobilize. The only disadvantage of this system is that the gluconate produced may complicate the workup procedure. Although the separations by extraction reported here are straightforward, they are more complex than those required with FDH and H_2ase. The simplicity of the system, however, makes it very convenient and practical for use in large-scale synthesis.

Experimental Section

Materials and Methods. Formate dehydrogenase was from Boehringer. Other enzymes and biochemicals were from Sigma. Organic solvents used were reagent grade. ^{1}H NMR was determined with an EM390 instrument (90 MHz). Enzyme assays were carried out at room temperature (25 °C) following Bergmeyer procedures[26] with a Beckman DU-6 UV/vis spectrophotometer. Enzymes were immobilized in polyacrylamide gels (PAN) according to the procedures described previously.[27] TLC was developed with $CHCl_3$:ether = 8:2 v/v with a silica gel plate coated on plastic film (from E. Merck), and the compound was detected with anisaldehyde reagent.[28]

Study of Enzyme Stability. Enzymes (either immobilized or soluble) were incubated in an oil bath at different temperatures in a buffer containing different concentrations of salts. Aliquots were withdrawn periodically for determination of activity at 25 °C. For immobilized enzymes, the incubated mixture was stirred when the sample was taken. For kinetic studies of the NaCl effect on the thermal stability of soluble GDH, the enzyme (2 mg) was dissolved in 1 mL of imidazole buffer (50 mM, pH 6.5) containing different concentrations of NaCl. The mixture in a rubber-stoppered test tube was heated in an oil bath with the temperature controlled at 85 ± 1 °C (this condition was simply chosen in order to facilitate the kinetic study). Ten microliters of the sample was withdrawn periodically and added to a Tris buffer (0.1 M, pH 8.0, 1 mL, 25 °C) containing 60 mM of glucose and 2 mM of NAD. The GDH activity was measured at 25 °C by recording the change of absorbance at 340 nm.

Test of Cofactor Regeneration. A synthesis of D-lactic acid was carried out. The reaction was performed under argon at 25 °C in a 500-mL solution containing sodium pyruvate (0.4 M), NAD (0.01 mM), glucose (0.41 M), dithiothreitol (1 mM), and immobilized GDH (540 U in 7 mL of gel) and D-lactic dehydrogenase (510 U in 5 mL of gel). The reaction pH was automatically controlled at 8.0 by adding a 2 N NaOH solution. The reaction was complete in 6 days as shown by enzymatic determination of pyruvate.[26] The enzyme-containing gels were separated and D-lactic acid was extracted into ether as described previously[9,10] in 90% yield based on pyruvate. Both enzymes recovered were still fully active at the conclusion of the reaction. The turnover number for NAD was 40 000.

Determination of Optical Purity. The optical purity of compounds **2**, **4**, **5**, and **6** was determined by converting them to (*R*)-(+)-MPTA ester[29] and measuring by ^{1}H NMR (90 MHz). For compound **3**, it was hydrolyzed with Dowex 50 (H^+ form) to lactaldehyde (see below) and aliquotes of the solution were oxidized to lactic acid with aldehyde dehydrogenase as catalyst and NAD as cofactor. The concentrations of D- and L-lactic acid were then determined enzymatically with D- and L-LDH, respectively.[26]

Enzymatic Synthesis. Compounds **3**–**6** were prepared on 10-mmol scale. A reaction mixture (80 mL) containing NaCl (0.3 M), dithiothreitol (2 mM), Tris (5 mM), NAD(P) (0.1 mM), substrate (10 mmol), and GDH (60 U, 1 mL of gel) and ADH (20 U, 3–4 mL of gel) both immobilized in PAN separately was stirred under argon at room temperature with pH automatically controlled at 7.5 by adding 0.1 N NaOH solution through a peristaltic pump. The reaction was monitored by measuring the concentration of substrate enzymatically with HLADH. In general, the reaction was complete within 10 h. After removal of the PAN-immobilized enzymes by decantation, the solution was extracted thrice with 50-mL portions of ether. The combined ether was dried over Na_2SO_4 and evaporated to an oily residue which showed[9] a single spot in TLC. The R_f values for each compound are the following: 0.52 (**3**), 0.58 (**4**), 0.66 (**5**), 0.88 (**6**). For the preparation of compound **2**, the addition of substrate was controlled so that no accumulation of the substrate would occur (because it tends to inactivate the enzyme). In this particular preparation, the substrate was divided into ten portions and one proton was added every hour. The proton chemical shifts of the (*R*)-(+)-MPTA esters are as follows: Compound **2** (*R* enantiomer) δ 1.20 (t, CH_3 of Et); 2.71 (d, CH_2–CO); 3.65 (s, OCH_3); 3.81 (d, $ClCH_2$); 4.12 (q, CH_2 of Et); 5.60 (m, carbinol H); 7.48 (m, C_6H_5); for *S* enantiomer δ 2.80 (d, CH_2CO); 3.60 (s, OCH_3). Compound **4** (*S* enantiomer) δ 1.32 (d, CH_3); 1.85 (m, CH_2); 3.25 (d, OCH_3); 3.28 (d, OCH_3); 3.55 (s, OCH_3); 4.20 (t, CH of $CH(OCH_3)_2$); 5.20 (h, Carbinol H); 7.45 (m, C_6H_5); for *R* enantiomer δ 4.40 (t, CH of CH–$(OCH_3)_2$). Compound **5** (*S* enantiomer) δ 0.90 (t, CH_3 of CH_2–CH_3); 1.20 (t, CH_3 of OCH_2–CH_3); 1.75 (q, CH_2 of CH_2–CH_3); 2.65 (m, CH_2 of CH_2–COOEt); 3.55 (s, OCH_3); 4.10 (m, CH_2 of OCH_2CH_3); 5.40 (h, carbinol H); 7.40 (m, C_6H_5); for *R* enantiomer δ 3.52 (s, OCH_3). Compound **6** (*R* enantiomer) δ 3.539 (s, OCH_3); 6.30 (q, carbinol H); 7.40 (m, C_6H_5); for *S* enantiomer δ 3.45 (s, OCH_3).

Synthesis of L-Lactaldehyde from 3. A mixture containing compound **3** (1 g), water (20 mL), and Dowex 50 (H^+) (1 g) in a flask was heated with stirring in a boiling water bath for 30 min. After filtration, the resin was washed with 10 mL of cold water. The combined filtrate was adjusted to pH 3.5 with 0.5 N NaOH solution and stored in a refrigerator. Enzymatic determination of the lactaldehyde obtained with HLADH indicated that 97% of lactaldehyde was obtained on the basis of **3**. Determination of the optical purity of lactaldehyde with aldehyde dehydrogenase and LDH as described above indicated that the lactaldehyde obtained had the L-configuration with 100% ee.

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