BIOORGANIC CHEMISTRY 18, 116–130 (1990)

Synthesis of L-β-Hydroxyvaline from α-Keto-β-hydroxyisovalerate Using Leucine Dehydrogenase from *Bacillus* Species[1]

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Received September 11, 1989

α-Keto-β-bromoisovaleric acid or its ethyl ester was hydrolyzed with sodium hydroxide to α-keto-β-hydroxyisovalerate and converted *in situ* to L-β-hydroxyvaline by reaction with NADH and NH_3 catalyzed by leucine dehydrogenase from *Bacillus species*. Methyl 2-chloro-3,3-dimethyloxiranecarboxylate and the corresponding isopropyl or 1,1-dimethylethyl esters were prepared by Darzens condensation. These glycidic esters, after hydrolysis by sodium bicarbonate and sodium hydroxide to α-keto-β-hydroxyisovalerate, were also converted to L-β-hydroxyvaline by leucine dehydrogenase. NAD was recycled to NADH with either formate dehydrogenase from *Candida boidinii* or glucose dehydrogenase from *Bacillus megaterium*. Polyethylene glycol–NADH was an effective reductant with formate dehydrogenase and dextran–NAD was effective with glucose dehydrogenase. Reductive amination activity for α-keto-β-hydroxyisovalerate was found in most *Bacillus* strains screened, including *megaterium, subtilis, cereus, pumilus, licheniformis, thuringiensis,* and *brevis*. Highest specific activity was found in *B. sphaericus* ATCC 4525. pH 8.5 was optimum for both glucose dehydrogenase and reductive amination of α-keto-β-hydroxyisovalerate by the *B. sphaericus* enzyme. The apparent K_m for α-keto-β-hydroxyisovalerate was 11.5 mM compared to 1.06 mM for α-ketoisovalerate. The apparent V_{max} with α-keto-β-hydroxyisovalerate was 41% of the value with α-ketoisovalerate, making the enzyme very suitable for the preparation of L-β-hydroxyvaline.

INTRODUCTION

During the past several years syntheses of α-amino acids have been pursued intensely (*1–9*) because of their importance as building blocks for proteins and other compounds of medicinal interest (*10–14*). New methods have been developed for the asymmetric synthesis of β-hydroxy-α-amino acids (*7, 15–25*) because of their utility as starting materials for the total synthesis of monobactam antibiotics. L-β-Hydroxyvaline (**1**) is a key intermediate needed for the synthesis of tigemonam (**2**), a new orally active monobactam developed by Squibb (*26–30*) (Fig.

[1] This work was presented in a poster session of the 46th Annual Meeting of the Society for Industrial Microbiology, Seattle, WA, August 13–18, 1989, Abstract 79.

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0045-2068/90 $3.00

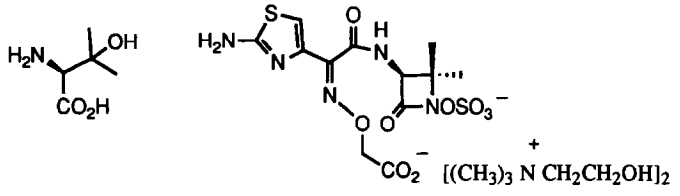

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FIG. 1. Structures of L-β-hydroxyvaline (**1**) and tigemonam (**2**).

1). Procedures for the synthesis and resolution of racemic β-hydroxyvaline (*31–36*) and the asymmetric synthesis of D-β-hydroxyvaline (*37, 38*) have been reported, but the direct synthesis of optically active L-β-hydroxyvaline has not been accomplished (*39*). The present synthesis of tigemonam involves resolution of Z-β-hydroxyvaline by classical chemical methods.

Reductive amination or transamination of α-keto-β-hydroxyisovalerate (**7**) (*40, 41*) by a suitable enzyme could provide an improved route to L-β-hydroxyvaline. Leucine dehydrogenases from *Bacillus sphaericus* (*42*), *B. cereus* (*43*), and *B. megaterium* (*44*) have been used for the synthesis of branched chain amino acids but not for hydroxylated amino acids. Indeed, the *B. sphaericus* enzyme has been reported to be inactive for oxidative deamination of serine and threonine (*45*). This report describes improved synthetic routes to α-keto-β-hydroxyisovalerate (**7**) and the use of leucine dehydrogenase for the synthesis of L-β-hydroxyvaline (**1**) (Scheme 3).

EXPERIMENTAL

Chemical Synthesis and Isolation

α-Keto-β-bromoisovaleric acid **5** was prepared from ethyl-α-ketoisovalerate **3** (*48*) by saponification to acid **4** (*40*) followed by bromination (*41*). Esters **12** and **13** were prepared by conventional methods from the corresponding acid chlorides and alcohols.

Ethyl-α-keto-β-bromoisovalerate **6** was prepared by two methods, a and b.

Method a. A three-necked flask (500 ml) was equipped with a mechanical stirrer, an addition funnel, and argon inlet tube, and a gas outlet tube which was connected (via a Drierite guard tube) to an aqueous KOH trap to capture HBr. Keto ester **3** (*48*) (43.25 g, 0.3 mol) and chloroform (60 ml) were charged into the flask and heated to 50°C in an oil bath. A solution of Br_2 (14.0 ml, 0.27 mol) in chloroform (50 ml) was added dropwise from an addition funnel over 1.5 h. The temperature of the oil bath was maintained near 50°C during addition of the bromine solution. After 20 min, argon was bubbled through the mixture for 0.5 h to remove HBr. The flask was cooled in an ice bath and then the material was slowly poured into a solution of $NaHCO_3$ (16.8 g, 0.2 mol) in water (200 ml) in an

ice bath. No vigorous evolution of CO_2 was observed. The material was transferred to a separatory funnel and the pale yellow organic layer was dried over $MgSO_4$ for 1 h. The solution was filtered and the chloroform was evaporated under vacuum to give an oil. The material was kept under pump vacuum at 35°C for 1 h to furnish 61.6 g (yield 92%) of ester **6**.[3]

Ir ($CHCl_3$): ν (C=O), 1719, 1739 cm^{-1}; ^1H NMR ($CDCl_3$): δ, 1.4 (t, 3H, J = 7 Hz), 2.01 (s, 6H), 4.38 (q, 2H, J = 7 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ, 13.9, 29.6, 60.9, 62.4, 162.3, 190.5 ppm; MS (CI): M + H 223.

Method b. Pyridinium hydrobromide perbromide (4.94 g 3.9 mmol) was added to the solution of ethyl-α-ketoisovalerate **3** (*48*) (2.0 g, 13.9 mmol) in CH_2Cl_2 (20 ml). The mixture was refluxed for 1 h in a 50°C oil bath. A vigorous reaction was noticed after dissolution of the reagent. The solution was diluted with CH_2Cl_2 (80 ml) and washed successively with water (2 × 25 ml), 10% $Na_2S_2O_3$ (25 ml), 5% $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$) and the solvent was evaporated under vacuum to give 3.0 g (~97%) of bromo ester **6**. The product was identical (^1H NMR) to the bromo ester made by method a.

2-Chloro-3,3-dimethyloxiranecarboxylic acid, methyl ester **14**. Glycidic ester **14** (bp 50–70° 1.8–1.2 mm, yield 62.3%)[3] was made by the method described for the preparation of ester **16** (reaction temperature −78°C). The product was stored below −20°C. Storage over solid $NaHCO_3$ prevents decomposition to unwanted products; however, an increase in isomerization to the chloro ketone **17** (R = CH_3) was observed.

Ir ($CHCl_3$): ν (C=O), 1750 cm^{-1}; ^1H NMR ($CDCl_3$): δ, 1.4 (s, 3H), 1.63 (s, 3H), 3.9 (s, 3H) ppm [for **17** (R = CH_3): δ, 1.85 (s, 6H), 3.92 (s, 3H) ppm][3]; ^{13}C NMR ($CDCl_3$): δ, 19.9, 53.2, 65.4, 79.7, 164.0 ppm [for **17** (R = CH_3): 29.95, 53.1, 68.7, 166 ppm]; MS (CI): $[M + NH_4]^+$ 182.

2-Chloro-3,3-dimethyloxiranecarboxylic acid, 1-methylethyl ester **15**. Glycidic ester **15** (bp 45–49°C/1 mm, yield 68%)[3] was prepared following, the procedure described for ester **16**.

Ir ($CHCl_3$): ν (C=O) 1738 cm^{-1}; ^1H NMR ($CDCl_3$): δ, 1.27-1.34 (m, 6H), 1.6 (s, 3H), 1.8 (s, 3H): 5.03-5.17 (m, 1H) ppm [for **17** (R = isopropyl, estimated ~15%): 1.8 (s, 6H), 5.1 (m, 1H) ppm]; ^{13}C NMR ($CDCl_3$): δ, 19.7, 21.1, 21.2, 64.9, 70.5, 79.9, 163.8 ppm [for 17 (R = isopropyl): δ, 28.5, 68.3, 162, 192 ppm]; MS (CI): $[M + NH_4]^+$ 210.

2-Chloro-3,3-dimethyloxiranecarboxylic acid, 1-1-dimethyethyl ester **16**. A round-bottomed flask (250 ml) and addition funnel (125 ml) were dried overnight in a 125°C oven. The flask was equipped with a thermocouple, stirring bar, and nitrogen inlet. Acetone (14.7 ml, 0.2 mol) and ester **15** (37.0 g, 0.2 mol) were added to the flask which was chilled in an ice–acetone bath (−10°C). A solution of K-*t*-amylate in toluene (121 ml, 0.22 mol) was added dropwise over 90 min keeping the temperature <2°C. After 60 ml of the K-*t*-amylate had been added, acetone (8 ml,

[3] Partial decomposition was observed during attempted purification of these products. Ester **6** formed the corresponding α-keto-β-hydroxy ester which is the actual intermediate that leads to the desired end product **7**. Chloroglycidic esters **14** and **15** partially isomerize to the corresponding chloro ketone **17** during distillation; **17** also forms the end product **7** during hydrolysis.

0.11 mol) was pipetted into the reaction mixture. After the addition of base was complete, the mixture was stirred for 90 min and quenched with 40% brine (100 ml). The layers were separated and the organic layer was washed with 50% brine (100 ml) and brine (50 ml). All aqueous layers were backwashed with toluene (50 ml). The organic layers were combined and dried ($MgSO_4$). Evaporation of the solvent gave 45.37 g of **16** as a colored oil. The product was distilled through a short path vacuum-jacketed still: bp 54–57°C/1 mm, 36.96 g, yield 90%.

Ir ($CHCl_3$): ν (C=O) 1739 cm^{-1}; ^1H NMR ($CDCl_3$): δ, 1.4 (s, 3H); 1.57 (s, 9H); 1.6 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$): δ, 20, 27.8, 65.2, 80.5, 84.0, 164.0 ppm; MS (CI): $(M + NH_4)^+$ 224.

Anal. Calcd for $C_9H_{15}O_3Cl$: C, 52.30: H, 7.32; Cl, 17.16. Found: C, 52.65; H, 7.38; Cl, 17.19.

Sodium β-hydroxy-α-ketoisovalerate **7**. Chloroglycidic ester **15** (2.0 g, 10.4 mmol) was added to a solution of $NaHCO_3$ (0.87 g, 10.4 mmol) in water (21 ml). The mixture was stirred for 7 h, NaOH (6 N, 2.1 ml, 12.6 mmol) was added, and the mixture was stirred overnight. The pH of the solution was adjusted to 6.4 with dilute HCl and lyophilized. The residue was dissolved in 10 ml H_2O and lyophilized again. The resulting white powder weighed 2.29 g (99%). Sodium β-hydroxy-α-ketoisovalerate **7** was used *in situ* for the enzymatic reduction.

Ir (KBr): ν (C=O) 1707, 1638, 1625 cm^{-1}; ^1H NMR ($CDCl_3$): δ, 1.48 (s 6H), 4.68 (−OH) ppm [for hydrate **9**: δ 1.25 (s, 6H) ppm]; ^{13}C NMR (D_2O): δ, 26.9, 76.5, 173.3, 211 ppm [for hydrate **9**: δ 24.3, 134.3 ppm (weaker absorptions)]; MS (FAB): (M − H) 131; (M + 2 Na-2H) 153.

L-β-Hydroxyvaline **1**. A solution of β-hydroxyvaline (16 ml, ~353 mmol obtained by enzymatic reductive amination) was boiled for 2 min, centrifuged to remove protein, and then chromatographed over Dowex-50 H^+ in a column (3 × 13.5 cm). The material was eluted with H_2O (250 ml) and 1 M NH_4OH (250 ml) and fractions (25 ml each) were monitored by TLC (silica gel, EtOAc : EtOH : AcOH : H_2O, 5 : 2 : 1 : 1; R_f of **1** 0.21); homogeneous fractions were combined and water was evaporated on a rotary evaporator. The residue was dissolved in water (25 ml) and evaporated under vacuum. This process was repeated two more times. The product was redissolved in water (25 ml) and lyophilized to give 0.593 g (54%) of **1** as a light brown solid. The product contained ~0.5 mol H_2O by KF analysis. The sample was dried over P_2O_5 at 40°C under vacuum for 4 h, mp 202°C dec.

Ir (KBr): ν (C=O) 1611 cm^{-1}; ^1H NMR (D_2O): δ, 1.15 (s, 3H), 1.38 (s, 3H), 3.5 (s, 1H) ppm; ^{13}C NMR (D_2O): δ, 24.3, 28.3, 64.4, 70.8, 173.1 ppm; $[\alpha]_D$ + 3° (c = 3.5, H_2O); + 11.9° (c = 1.3, 6 N HCl).[4]

Anal. Calcd for $C_5H_{11}NO_3 \cdot 0.13\ H_2O$: C, 44.32; H, 8.38; N, 10.34; H_2O, 1.73. Found: C, 44.67; H, 8.28; N, 10.17; H_2O, 1.89 (KF).

[4] Compound **1** was crystallized from isopropyl alcohol and water to give a colorless crystalline solid. Specific rotation and mp were unchanged. The literature value for specific rotation (Ref. (*31*)) is +13.5° (c = 2, 5 N HCl) and +6° (c = 4, H_2O). Specific rotation of the HCl salt of **1** (SQ 30,519) obtained via resolution of the *N-t*-butoxycarbonyl derivative (Dr. J. L. Moniot and Mr. J. Heikes, Squibb work) was found to be +15° (c = 1, 6 N HCl) and +6.4° (c = 1, H_2O).

Bacterial Strains and Growth Conditions

Bacillus strains were obtained from the American Type Culture Collection and the Squibb collection and are listed in Table 1. For screening purposes, 1 ml of each culture was used to inoculate 100 ml of medium containing per liter: tryptone (17 g), soytone (3 g), NaCl (5 g), glucose (2.5 g), yeast extract (1 g), and K_2HPO_4

TABLE 1

Synthesis of L-β-Hydroxyvaline by *Bacillus* Strains

Strain	No.	Specific activity[a] (units/mg protein)	L-β-Hydroxyvaline (mM)	
			A[b]	B[c]
B. subtilis	SC 13794[d]	0.1289	38.0	73.2
B. subtilis	SC 10253	0.0343	11.0	69.8
B. subtilis	SC 8548	0.1124	10.3	20.0
B. megaterium	ATCC 39118	0.1232	66.4	74.5
B. megaterium	SC 3593[d]	0.2402	16.2	66.0
B. megaterium	SC 6394[d]	0.3123	61.0	66.4
B. megaterium	SC 6423[d]	0.2706	10.3	79.5
B. megaterium	SC 6446[d]	0.2476	13.8	46.9
B. megaterium	SC 3781	0.1544	18.9	16.3
B. megaterium	SC 3782	0.1156	19.3	60.5
B. stearothermophilus	ATCC 12980[e]	0.0000	0.0[e]	0.0[e]
B. stearothermophilus	ATCC 7953[e]	0.0000	0.0[e]	0.0[e]
B. sphaericus	ATCC 4525	0.8705	6.5	57.7
B. sphaericus	SC 3574	0.5695	0.0	37.2
B. cereus	ATCC 14579	0.4326	8.7	65.3
B. cereus	SC 12147	0.4971	21.3	61.3
B. cereus	SC 10856	0.1892	68.4	76.1
B. pumilus	SC 11128	0.0802	2.5	62.7
B. pumilus	SC 8513	0.2079	2.5	68.4
B. licheniformis	SC 12148	0.1039	40.5	73.7
B. licheniformis	SC 11075	0.0613	7.7	58.9
B. circulans	SC 12999	0.0000	0.0	7.2
B. circulans	SC 10275	0.0074	0.0	6.6
B. polymyxa	SC 1522	0.0000	5.9	0.0
B. thuringiensis	SC 2928	0.5259	2.8	73.6
B. brevis	SC 3812	0.2760	0.0	34.3
B. coagulans	SC 9261	0.0194	0.0	48.3
B. alvei	SC 9230	0.0000	0.0	0.0

[a] Assay contained 10 mM α-keto-β-hydroxyisovalerate, 0.3 mM NADH, 0.75 M NH_4Cl–NH_4OH, pH 9.5.

[b] After incubation with 1 M NH_4Cl, 1 M glucose, 100 mM α-keto-β-hydroxyisovalerate, and 2 mM NAD.

[c] After incubation with 1 M ammonium formate, 100 mM α-keto-β-hydroxyisovalerate, 40 units/ml formate dehydrogenase, and 2 mM NAD.

[d] L-β-Hydroxyvaline was assayed after 68 h.

[e] Cells were grown and incubations were carried out at 55°C.

(2.5 g). The medium was adjusted to pH 7 with HCl. After 16 h of growth at 30°C, cells were collected by centrifugation, washed with 0.1 M potassium phosphate, pH 7, and resuspended in 5 ml of this buffer. Bacteria were disrupted by sonication and 0.2-ml samples were evaluated in two 1-ml reaction systems for synthesis of L-β-hydroxyvaline:

A. 1 M NH_4Cl, 1 M glucose, 2 mM NAD, and 0.1 M α-keto-β-hydroxyisovalerate.

B. 1 M ammonium formate, 2 mM NAD, 0.1 M α-keto-β-hydroxyisovalerate, and 40 units/ml formate dehydrogenase from *Candida boidinii*.

Reactions were run at 30°C for 48 h except as noted in Table 1 and initial pH was 8.2.

For preparative purposes, *B. sphaericus* ATCC 4525 was grown at 30°C in a 250-liter fermentor to the end of log phase on medium containing per liter: yeast extract (20 g), glucose (10 g), and K_2HPO_4 (2 g). The medium was adjusted to pH 7. Bacteria in 10 mM potassium phosphate buffer, pH 7, containing 0.01% mercaptoethanol were disrupted by sonication; the sonicate was partially purified by heating for 20 min at 60°C (*46*) followed by centrifugation for 10 min at 28,000*g*. The supernatant was stored at −20°C and used as a source of leucine dehydrogenase.

Enzyme Assays and Reactions

Activity of leucine dehydrogenase was determined in a system that contained in 1.0 ml: α-keto-β-hydroxyisovalerate or α-ketoisovalerate, 0.75 M NH_4Cl–NH_4OH buffer, 0.3 mM NADH, and 3 to 10 μl of cell extract. Absorbance decrease at 340 nm was monitored. All components except the keto acid were added and a blank value for NADH oxidation was measured before the reaction was started by the addition of keto acid. Further details are given in legends to the tables and figures.

The glucose dehydrogenase assay used for screening strains contained in 1.0 ml: 0.5 M glucose, 3 mM NAD, 0.1 M Tris chloride, pH 8, and 10 μl of sonicated cell extract. For determining optimum pH, 0.75 M NH_4Cl–NH_4OH buffer was substituted for Tris. The reaction was started by addition of enzyme, and absorbance increase at 340 nm was monitored.

Protein was determined by the dye-binding method of Bradford (*47*) using bovine serum albumin as standard.

Preparative reactions with glucose dehydrogenase and leucine dehydrogenase were run in an initial volume of 16 ml at pH 8.5 and 30°C. pH was maintained by addition of 3 M NH_4OH with a Brinkmann pH stat. Reactions contained 0.1 to 0.5 M α-keto-β-hydroxyisovalerate, 1 M glucose, 0.2 M NH_4Cl, 0.5 mM NAD, 0.01% mercaptoethanol, 44 units leucine dehydrogenase from *B. sphaericus* ATCC 4525, and 29 units glucose dehydrogenase from *B. megaterium*.

HPLC Analysis

Samples were diluted with water and heated in a boiling water bath for 2 min to stop the reaction and precipitate proteins. L-β-Hydroxyvaline was assayed with a

Hewlett–Packard 1090 HPLC equipped with a diode array detector. A Bakerbond Chiralpak WH 25 × 0.46-cm column was used. Injection volume was 20 μl, mobile phase was 0.3 mM $CuSO_4$, flow rate was 1.5 ml/min, temperature was 45°C, and detection wavelength was 230 nm. The standard was racemic β-hydroxyvaline.

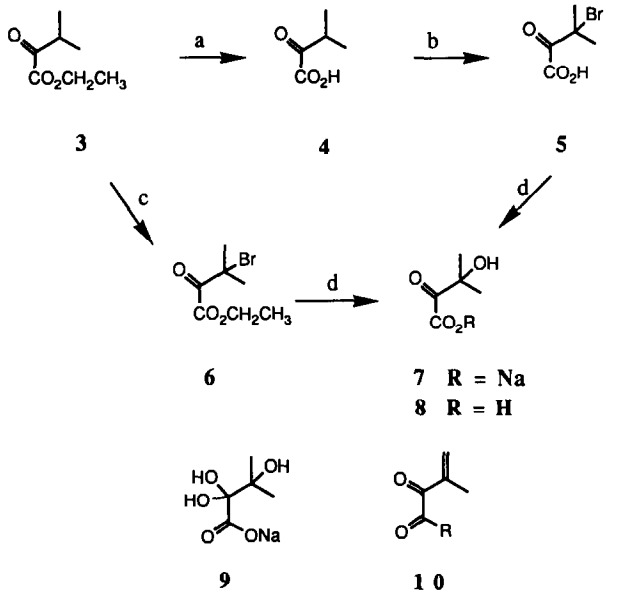
Enzymes and Cofactors

Polyethylene glycol–N^6-(2-aminoethyl)-NADH (PEG–NADH) and formate dehydrogenase from *C. boidinii* were gifts from H. Schutte (Gesellschaft fur Biotechnologische Forschung mbH, Federal Republic of Germany). Dextran–NAD (attached through C8 to dextran D4133 with a six-carbon spacer) and leucine dehydrogenase from *Bacillus* species were from Sigma. Glucose dehydrogenase from *Bacillus megaterium* was purchased from Amano International Enzyme Co.

RESULTS

Synthetic Routes to α-Keto-β-hydroxyisovalerate (7)

α-Keto-β-hydroxyisovalerate **7** was prepared by three methods.
Method a: Via α-keto acid **4** (*Scheme 1*). We have recently reported (*48*) an efficient method for the synthesis of α-keto ester **3**. Saponification of ester **3** (aq NaOH, EtOH) to keto acid **4** followed by bromination (Br_2, 50°C) produced the



a. NaOH, H_2O, EtOH b. Br_2 c. Br_2, CH_2Cl_2 or PyHBr•Br_2
d. NaOH, H_2O

SCHEME 1. Syntheses of α-keto-β-hydroxyisovalerate (7) via ethyl-α-ketoisovalerate (3).

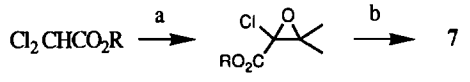
bromoketo acid **5** (*40, 41*). Hydrolysis of **5** with 2 eq of aq NaOH formed α-keto-β-hydroxyisovalerate (**7**). The ^1H NMR and ^{13}C NMR analyses showed the presence of 15–20% of the hydrate **9**. Acidification of the hydrolysis reaction mixture with HCl formed acid **8**. Rebasification with aq NaOH gave material identical (^1H NMR) to the starting material. The solution of salt **7** was used for the *in situ* enzymatic reduction.

Method b: Via β-bromo-α-keto ester **6** (*Scheme 1*). Bromination of ester **3** with Br_2 or PyHBr · Br_2 gave bromo ester **6** in ~90 and 97% yields, respectively. Ester **6** was converted to salt **7** by treatment with aq NaOH in the manner described in method a. This synthesis is a step shorter than method a. Formation of the possible by-product **10** (see method c) was not observed during preparation of salt **7** by methods a and b.

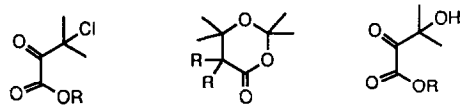
Method c: Via α-chloroglycidic esters **14–16** (*Scheme 2*). Darzens condensation (*49*) of acetone with methyl dichloroacetate **11** (K-*t*-amylate, toluene) formed the chloroglycidic ester **14** in 62% yield. Product **14** contained ~13% chloro ketone **17** (R = CH_3). Reaction at low temperature (−78°C) showed the formation of the bis-acetone condensation side-product **18** (R = Cl, OH). Under the hydrolysis conditions both the chloroglycidic ester **14** and the chloro ketone **17** (R = CH_3) form the desired product **7**.

Condensation of isopropyl dichloroacetate **12** and *t*-butyl dichloroacetate **13** with acetone (K-*t*-amylate, 0°C) formed chloroglycidic esters **15** in 68% (83% at −78°C) and **16** in 90% yields, respectively. Use of K-*t*-amylate as a base in Darzens condensation produced the α-chloroglycidic esters in reproducible high yields. The most hindered *t*-butyl ester **13** gave minimal side-products even when the reaction was conducted at 0°C.

Hydrolysis of esters **14–16**, first with aqueous $NaHCO_3$ (1.1 eq, the corresponding β-hydroxy esters **19** are formed predominantly at this stage) and then with NaOH (1 eq), produced salt **7**. Formation of the by-product **10** (R = ONa) varied from 1 to 3% (estimated by 270 MHz ^1H NMR).



11 R = CH_3 **14** R = CH_3
12 R = $CH(CH_3)_2$ **15** R = $CH(CH_3)_2$
13 R = $C(CH_3)_3$ **16** R = $C(CH_3)_3$



17 **18** **19**

a. acetone, K-t-amylate, toluene b. aq $NaHCO_3$, NaOH

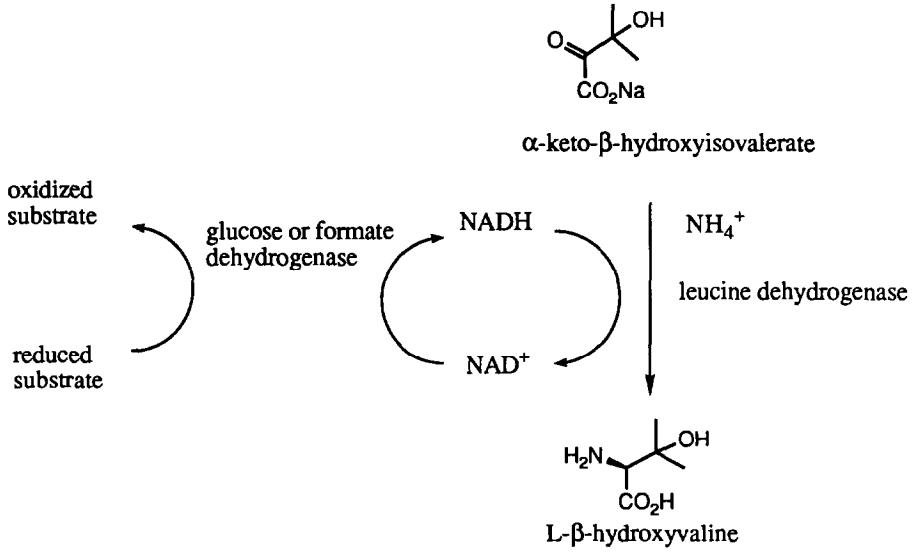
SCHEME 2. Synthesis of α-keto-β-hydroxyisovalerate (**7**) via α-chloroglycidic esters.

Rearrangements of α-haloglycidic esters to α-keto-β-halo esters have been reported in the literature (*50–55*). We found that the thermolysis of neat **15** (100°C, 15 min) yielded a mixture of **17** (R = $CH(CH_3)_2$, 85%) and **10** (R = $OCH(CH_3)_2$, 15%). However, the rearrangement of **15** in hot toluene (95°C) furnished chloro ketone **17** (R = $CH(CH_3)_2$) in 87% yield. Hydrolysis of this material with aqueous $NaHCO_3$, followed by NaOH, formed salt **7** which contained ~1% of olefin **10**.

The facile conversion of chloroglycidic esters to α-keto-β-hydroxyisovalerate **7** is new. The improved syntheses of chloroglycidic esters **14–16** from readily available starting materials provide a viable alternative for commercial production of the precursor salt **7**.

Reductive Amination of α-Keto-β-hydroxyisovalerate by Leucine Dehydrogenase Coupled to Formate Dehydrogenase

Enzymatic reductive amination was carried out as outlined in Scheme 3. A solution containing 0.5 M α-keto-β-hydroxyisovalerate from ester **6**, 2 M ammonium formate, 2 mM NAD, 1.5 mg (50 units) leucine dehydrogenase from *Bacillus* species, and 40 mg (80 units) formate dehydrogenase from *C. boidinii* in 16 ml, pH 8, was incubated 41 h at 30°C. HPLC analysis of the reaction solution indicated a 71% conversion of the ester to L-β-hydroxyvaline. L-β-Hydroxyvaline was isolated from the reaction as described under Experimental. HPLC analysis of the recovered material showed that leucine dehydrogenase produced exclusively the L-isomer of β-hydroxyvaline (Fig. 2). α-Keto-β-hydroxyisovalerate from bromo acid **5** was also converted to L-β-hydroxyvaline in 82% yield by a similar procedure.



SCHEME 3. Enzymatic synthesis of L-β-hydroxyvaline.

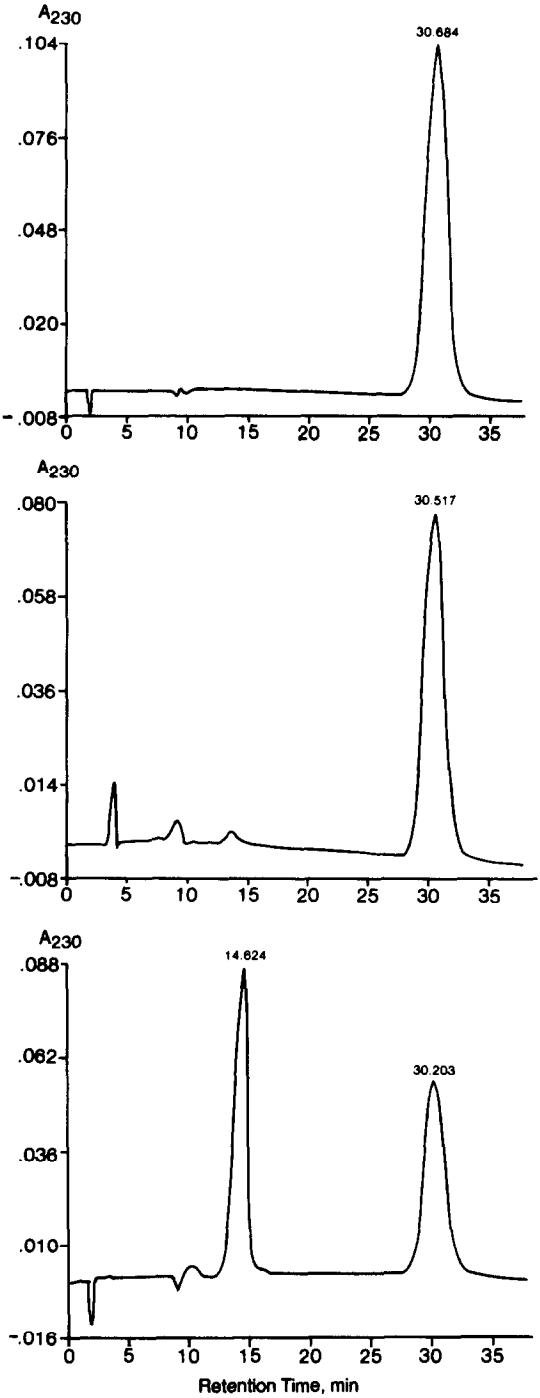


FIG. 2. HPLC analysis on Chiralpak WH of 1.33 mg/ml solutions of enzymatically synthesized L-β-hydroxyvaline (top), chemically synthesized L-β-hydroxyvaline (middle), and D, L-β-hydroxyvaline (bottom).

Distribution of α-Keto-β-hydroxyisovalerate Amination Activity in Bacillus Strains

Leucine dehydrogenase activity is found mainly in *Bacillus* strains (*45*). Screening of *Bacillus* strains was carried out to find strains with high specific activity for reductive amination of α-keto-β-hydroxyisovalerate and to identify any strains able to regenerate NADH for the reaction. Of 28 strains screened (Table 1), *B. sphaericus* ATCC 4525 had the highest specific activity. *B. megaterium* ATCC 39118 has been selected for high levels of glucose dehydrogenase (*44*) and was able to produce L-β-hydroxyvaline when glucose, NAD, NH_4Cl, and α-keto-β-hydroxyisovalerate were added to cell extracts. All other strains had less than 6% of the glucose dehydrogenase activity of this strain (data not shown), but extracts of a few of the strains were also able to produce L-β-hydroxyvaline when provided with glucose, NAD, and NH_4Cl. *B. cereus* ATCC 14579 was the only strain able to produce significant amounts of L-β-hydroxyvaline when intact cells were provided with glucose and α-keto acid, giving a maximum conversion of about 50%.

Reductive Amination by Leucine Dehydrogenase from B. sphaericus ATCC 4525 Coupled to Glucose Dehydrogenase

pH 8.5 was optimum for both glucose dehydrogenase from *B. megaterium* and reductive amination of α-keto-β-hydroxyisovalerate by the *B. sphaericus* enzyme (Fig. 3). Rates of L-β-hydroxyvaline formation in a coupled system containing both enzymes were similar with 0.5, 1, or 2 mM NAD (data not shown). When reactions were carried out in a pH stat as described under Experimental, solutions of **7**, prepared from 0.5 M **6**, 0.25 M **14**, 0.25 M **15**, and 0.25 M **16**, were converted to L-β-hydroxyvaline in yields of 75, 100, 71, and 83%, respectively.

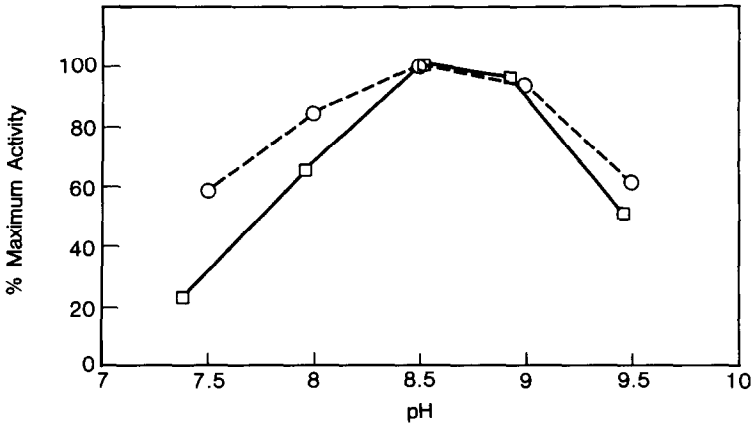


FIG. 3. Effect of pH on reductive amination and glucose dehydrogenase activities. (□), Amination, 0.75 M NH_4Cl–NH_4OH, 0.3 mM NADH, 10 mM α-keto-β-hydroxyisovalerate from **6** and *B. sphaericus* extract; (○), glucose dehydrogenase, 0.75 M NH_4Cl–NH_4OH, 0.5 M glucose, 3 mM NAD, and glucose dehydrogenase from *B. megaterium*.

TABLE 2

Effect of Pyridine Nucleotide Derivatives on Synthesis of L-β-Hydroxyvaline

Pyridine nucleotide	L-β-Hydroxyvaline (mM)	
	Formate dehydrogenase[a]	Glucose dehydrogenase[b]
1 mM PEG–NADH	82.1	10.1
1 mM Dextran–NAD	34.1	79.9
1 mM NAD	81.8	77.3

[a] Incubation for 22 h at 30°C with 1 M ammonium formate, pH 8.5, 100 mM α-keto-β-hydroxyisovalerate, 0.01% mercaptoethanol, 5 units/ml formate dehydrogenase, and 6 units/ml leucine dehydrogenase from *B. sphaericus*.

[b] Incubation for 22 h at 30°C with 1 M glucose, 1 M ammonium chloride, pH 8.5, 100 mM α-keto-β-hydroxyisovalerate, 0.01% mercaptoethanol, 5 units/ml glucose dehydrogenase, and 6 units/ml leucine dehydrogenase from *B. sphaericus*.

Kinetic Parameters

Using the *B. sphaericus* heat-treated extract with 0.75 M NH_4Cl–NH_4OH, pH 8.5, and 0.3 mM NADH, the apparent K_m for α-keto-β-hydroxyisovalerate (prepared from **6**) was 11.5 mM compared to a value of 1.06 mM for α-ketoisovalerate. The apparent V_{max} with α-keto-β-hydroxyisovalerate was 41% of the value with α-ketoisovalerate.

Effect of High Molecular Weight NAD Derivatives

When reduced pyridine nucleotide was regenerated with formate dehydrogenase, PEG–NADH was as effective as NAD and dextran–NAD was less effective for synthesis of L-β-hydroxyvaline by leucine dehydrogenase (Table 2). Conversely, with glucose dehydrogenase to reduce pyridine nucleotide, PEG–NADH was relatively ineffective for synthesis of L-β-hydroxyvaline, but dextran–NAD was as effective as NAD.

DISCUSSION

Leucine dehydrogenase from *B. sphaericus* proved to be very suitable for synthesis of L-β-hydroxyvaline. When the reaction is run with 0.25 to 0.5 M α-keto acid, the apparent K_m of 11.5 mM is sufficiently low and the apparent V_{max} is 41% of the value for α-ketoisovalerate (reported to be the best substrate for reductive amination (*45*)). Although $E^{\circ\prime}$ for reductive amination of α-keto-β-hydroxyisovalerate has not been determined, other amino acid dehydrogenase reactions have $E^{\circ\prime}$ of −0.13 to −0.14 V (*56*). Using the glucose dehydrogenase ($E^{\circ\prime}$ = −0.45 V) or

formate dehydrogenase ($E^{\circ\prime} = -0.42$ V) reactions (*56*) to regenerate NADH provides a large $\Delta E^{\circ\prime}$ to drive the reaction to completion.

Other enzymatic approaches to the synthesis of L-β-hydroxyvaline were not successful. Serine hydroxymethyltransferase from *Escherichia coli* catalyzed cleavage of L-β-hydroxyvaline to glycine (and presumably acetone), but the reaction did not proceed in the synthetic direction.[5] A branched chain amino acid transaminase would probably also be suitable for this transformation, although the equilibrium for the reaction would not be as favorable as in the coupled reductive amination. *Corynebacterium glutamicum* ATCC 13032, which has a branched chain transaminase coupled to glutamate dehydrogenase (*57*), was able to produce leucine or valine from the corresponding α-keto acids, but converted α-keto-β-hydroxyisovalerate to a product which appeared to be valine.[5]

Leucine can be produced in membrane reactors employing PEG–NADH as a cofactor retainable by an ultrafiltration membrane, formate dehydrogenase to regenerate the cofactor, and leucine dehydrogenase (*42*). PEG–NADH and formate dehydrogenase were also suitable for L-β-hydroxyvaline production. However, PEG–NADH was relatively ineffective with glucose dehydrogenase as has been reported (*58*). Dextran–NAD was effective with glucose dehydrogenase and was less effective with formate dehydrogenase. Either PEG–NADH with formate dehydrogenase or dextran–NAD with glucose dehydrogenase could be employed for production of L-β-hydroxyvaline in a membrane reactor.

ACKNOWLEDGMENTS

We thank Dr. C. M. Cimarusti for helpful suggestions, our colleagues in Analytical R & D for their help, and Dr. D. Walker, Ms. J. Manello, Dr. M. Loots, and Ms. P. Minicuci for literature searches.

REFERENCES

1. WILLIAMS, R. M. (1989) *in* Synthesis of Optically Active α-Amino Acids (Baldwin, J. E., and Magnus, P. D., Eds.), Vol. 7, Pergamon, Oxford/New York.
2. O'DONNELL, M. J., BENNETT, D. W., AND WU, S. (1989) *J. Amer. Chem. Soc.* **111,** 2353–2355.
3. STORK, G., LEONG, A. Y. W., AND TOUZIN, A. M. (1976) *J. Org. Chem.* **41,** 3491–3493.
4. COREY, E. J., SACHDEV, H. S., GOUGOUTAS, J. Z., AND SAENGER, W. (1970) *J. Amer. Chem. Soc.* **92,** 2488–2501.
5. EVANS, D. A., ELLMAN, J. A., AND DOROW, R. L. (1987) *Tetrahedron Lett.* **28,** 1123–1126.
6. YAOZHONG, J., CHANGYOU, Z., AND HURI, P. (1989) *Synth. Commun.* **19,** 881–888.
7. BOLD, G., DUTHALER, R. O., AND RIEDIKER, M. (1989) *Angew. Chem. Int. Ed. Engl.* **28,** 497–498.
8. O'DONNELL, M. J. (1988) *Tetrahedron* **44,** 5253–5614.
9. SCHMIDT, U., RESPONDEK M., LIEBERKNECHT, A., WERNER, J., AND FISCHER, P. (1989) *Synthesis,* 256–261, and literature cited in Ref. (*1–9*).
10. IMADA, A., KITANO K., KINTAKA, K., MUORI, M., AND ASAI, M. (1981) *Nature (London)* **289,** 590–591.

[5] R. L. Hanson, unpublished results.

11. SYKES, R. B., CIMARUSTI, C. M., BONNER, D. P., BUSH, K., FLOYD, D. M., GEORGOPADAKOU, N. H., KOSTER, W. H., LIU, W. C., PARKER, W. L., PRINCIPLE, P. A., RATHNUM, M. L., SLUSARCHYK, W. A., TREJO, W. H., AND WELLS, J. S. (1981) *Nature (London)* **291,** 489–491.
12. CIMARUSTI, C. M. (1986) *Gazz. Chim. Ital.* **116,** 169.
13. KOSTER, W. H., AND BONNER, D. P. (1987) Frontiers of Antiobiotic Research, Academic Press, San Diego.
14. MILLER, M. J. (1986) *Acc. Chem. Res.* **19,** 49–56.
15. EVANS, D. A., SJOGREN, E. B., WEBER, A. E., AND CONN, R. E. (1987) *Tetrahedron Lett.* **28,** 39–42.
16. ROEMMELE, R. C., AND RAPOPORT, J. (1989) *J. Org. Chem.* **54,** 1866–1875, and cited references.
17. ITO, Y., SAWAMURA M., SHIRAKAWA, E., HAYASHIZAKI, K., AND HAYASHI, T. (1988) *Tetrahedron* **44,** 5253–5262.
18. GUANTI, G., BANFI, L., AND NARISANO, E. (1988) *Tetrahedron* **44,** 5553–5562.
19. HIRAMA, M., HIOKI, H., AND ITO, S. (1988) *Tetrahedron Lett.* **29,** 3125–3128.
20. GRAUERT, M., AND SCHOLLKOPF, U. (1985) *Liebigs Ann. Chem.,* 1817–1824.
21. SCHOLLKOPF, U., NOZULAK, J., AND GRAUERT, M. (1985) *Synthesis,* 55–56.
22. KUROKAWA, N., AND OHFUNE, Y. (1986) *J. Amer. Chem. Soc.* **108,** 6041–6043.
23. ITO, Y., SAWAMURA, M., AND HAYASHI, T. (1986) *J. Amer. Chem. Soc.* **108,** 6405–6406.
24. EVANS, D. A., AND WEBER, A. E. (1986) *J. Amer. Chem. Soc.* **108,** 6757–6761.
25. SAITO, S., BUNYA, N., INABA, M., MORIWAKE, T., AND TORII, S. (1985) *Tetrahedron Lett.* **26,** 5309–5312.
26. GORDON, E. M., ONDETTI, M. A., PLUSCEC, J., CIMARUSTI, C. M., BONNER, D. P., AND SYKES, R. B. (1982) *J. Amer. Chem. Soc.* **104,** 6053–6060.
27. PARKER, W. L., COHEN, E. M., AND KOSTER, W. H. (1988) U.S. Patent 4751220; *Chem. Abst.* **109,** 116074a (1988).
28. KOSTER, W. H., SLUSARCHYK, W. A., DEJNEKA, T., KRONENTHAL, D. R., PERRI, M. G., PILKIEWICZ, F. G., ROUTH, F. L., SUNDEEN, J. E., WEAVER, E. R., AND ZAHLER, R. (1985) 25th Intersci. Conf. Antimicrobial Agents and Chemotherapy, Abstract 368, September 1985.
29. SLUSARCHYK, W. A., DEJNEKA, T., GOUGOUTAS, J. Z., KOSTER, W. H., KRONENTHAL, D. R., MALLEY, M. F., PERRI, M. G., ROUTH, F. L., SUNDEEN, J. E., WEAVER, E. R., ZAHLER, R., GODFREY, J. D., MUELLER, R. H., AND VON LANGEN, D. J. (1986) *Tetrahedron Lett.* **27,** 2789–2792.
30. YOSHIDA, C., HORI, T., MOMONOI, K., NAGUMO, K., NAKANO, J. KITANI, T., FUKUOKA, Y., AND SAIKAWA, I. (1985). *J. Antibiot.* **38,** 1536–1549.
31. EDWARDS, G. W., AND MINTHORN, M. L. JR. (1968) *Canad. J. Biochem.* **46,** 1227–1230.
32. BERSE, C., AND BESSETTE, P. (1971) *Canad. J. Chem.* **49,** 2610–2611, and cited references.
33. For absolute configuration of β-hydroxyvaline refer: BEYERMAN, H. C., MAAT, L., RIJKE, D., AND VISSER, J. P. (1967) *Recueil* **86,** 1057–1060.
34. SHANZER, A., SOMEKH, L., AND BUTINA, D. (1979) *J. Org. Chem.* **44,** 3967–3969.
35. GODFREY, J. D., MUELLER, R. H., AND VAN LANGEN, D. J. (1986) *Tetrahedron Lett.* **27,** 2793–2796.
36. MIX, H. (1962) *Z. Physiol. Chem.* **327,** 41–48.
37. SCHOLLKOPF, U., NOZULAC, J., AND GROTH, U. (1982) *Synthesis,* 868–870.
38. BELOKON, Y. N., BULYEHEV, A. G., VITT, S. V., STRUCHKOV, Y. T., BATSANOV, A. S., TIMOFEEVA, T. V., TSYRYAPKIN, V. A., RYZHOV, M. G., LYSOVA, L. A., BAKHMUTOV, V. I., AND BELIKOV, V. M., (1985) *J. Amer. Chem. Soc.* **107,** 4252–4259.
39. L-β-hydroxyvaline **1** has been obtained by the degradation of the antibiotic YA-56: OHASHI, Y., ABE, H., AND ITO, Y. (1973) *Agric. Biol. Chem.* **37,** 2283–2287.
40. RADHAKRISHNAN, A. N., WAGNER, R. P., AND SNELL, E. E. (1960). *J. Biol. Chem.* **235,** 2322–2331.
41. WAGNER, R. P., AND ARMSTRONG, F. B. (1970) *in* Methods in Enzymology (Tabor, H., and Tabor, C. W., Eds.), Vol. 17, Part A, pp. 765–770, Academic Press, New York.
42. WICHMANN, R., WANDREY, C., BUCKMANN, A. F., AND KULA, M. (1981) *Biotechnol. Bioeng.* **23,** 2789–2802.

43. SCHUTTE, H., HUMMEL, W., TSAI, H., AND KULA, M. (1985) *Appl. Microbiol Biotechnol.* **22,** 306–317.
44. MONOT, F., BENOIT, Y., LEMAL, J., HONORAT, A., AND BALLERINI, D. (1987) *in* Proceedings, 4th European Congress on Biotechnology 1987 (Neijssel, O. M., van der Meer, R. R., and Luyben, K. C. A. M., Eds.), Vol. 2, pp. 42–45, Elsevier, Amsterdam.
45. OHSHIMA, T., MISONO, H., AND SODA, K. (1978) *J. Biol. Chem.* **253,** 5719–5725.
46. HUMMEL, W., SCHUTTE, H., AND KULA, M. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* **12,** 22–27.
47. BRADFORD, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
48. SINGH, J., KISSICK, T. P., AND MUELLER, R. H. (1989) *Org. Prep. Proced. Int.* **21**(4), 501–504.
49. VILLIERAS, J., CASTRO, B., AND FERRACUTTI, N. (1970) *Bull. Soc. Chim.* **4,** 1450–1455, and references therein.
50. COUTROT, P., AND LEGRIS, C. (1975) *Synthesis,* 118–120.
51. MCDONALD, R. N., AND SCHWAB, P. A. (1964) *J. Org. Chem.* **29,** 2459–2460.
52. VILLIERAS, J., AND FERRACUTTI, N. (1970) *Bull. Soc. Chim.* **7,** 2699–2701.
53. KIRRMANN, A., AND NOURI-BIMORGHI, R. (1968) *Bull. Soc. Chim.* **8,** 3213–3220.
54. TSUBOI, S., FURUTANI, H., AND TAKEDA, A. (1987) *Synthesis,* 292–293.
55. TSUBOI, S., FURUTANI, H., TAKEDA, A., KAWAZOE, K., AND SATO, S. (1987) *Bull. Chem. Soc. Japan* **60,** 2475–2479.
56. WALSH, C. (1979) Enzymatic Reaction Mechanisms, p. 313, Freeman, New York.
57. GROEGER, U., AND SAHM, H. (1984) *in* Proceedings, 3rd European Congress on Biotechnology 1984, Vol. 1, pp. I-85–I-90, Verlag Chemie, Weinheim.
58. OKUDA, K., URABE, I., AND OKADA, H. (1985) *Eur. J. Biochem.* **151,** 33–38.