Biocatalytic Synthesis of Chiral Intermediates for Antiviral and Antihypertensive Drugs

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ABSTRACT: The chiral intermediate (1*S*,2*R*) [3-chloro-2-hydroxy-1-(phenylmethyl)propyl] carbamic acid, 1,1-dimethylethyl ester **2a** was prepared for the total synthesis of a human immunodeficiency virus protease inhibitor, BMS-186318. The stereoselective reduction of (1*S*) [3-chloro-2-oxo-1(phenylmethyl)propyl] carbamic acid, 1,1-dimethylethyl ester **1** was carried out using microbial cultures, among which *Streptomyces nodosus* SC 13149 efficiently reduced **1** to **2a**. A reaction yield of 80%, enantiomeric excess (e.e.) of 99.8%, and diastereomeric purity of 99% were obtained for chiral alcohol **2a**. Chiral L-6-hydroxy norleucine **3**, an intermediate in the synthesis of antihypertensive drug, was prepared by reductive amination of 2-keto-6-hydroxyhexanoic acid **4** using beef liver glutamate dehydrogenase. The cofactor NADH required for this reaction was regenerated using glucose dehydrogenase from *Bacillus* sp. A reaction yield of 80% and e.e. of 99.5% were obtained for L-6-hydroxynorleucine **3**. To avoid the lengthy chemical synthesis of the ketoacid, a second route was developed in which racemic 6-hydroxynorleucine [readily available from hydrolysis of 5-(4-hydroxybutyl) hydantoin **5**] was treated with D-amino acid oxidase from *Trigonopsis variabilis* to selectively convert the D-isomer of racemic 6-hydroxynorleucine to 2-keto-6-hydroxyhexanoic acid **4** and L-6-hydroxynorleucine **3**. Subsequently, the 2-keto-6-hydroxyhexanoic acid **4** was converted to L-6-hydroxynorleucine by reductive amination using glutamate dehydrogenase. A reaction yield of 98% and an e.e. of 99.5% were obtained.

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KEY WORDS: Antihypertensive drug, antiviral drug, chiral intermediates, formate dehydrogenase, glutamate dehydrogenase, L-6-hydroxynorleucine, reductive amination, stereoselective reduction.

Increasing understanding of the mechanisms of drug interactions on a molecular level has led to the awareness of the importance of chirality. In many cases, only one enantiomer is efficacious, and the other either is inactive or exhibits considerably reduced activity. Pharmaceutical companies know that, where appropriate, new drugs should be homochiral to avoid possible side effects due to an undesirable stereoisomer.

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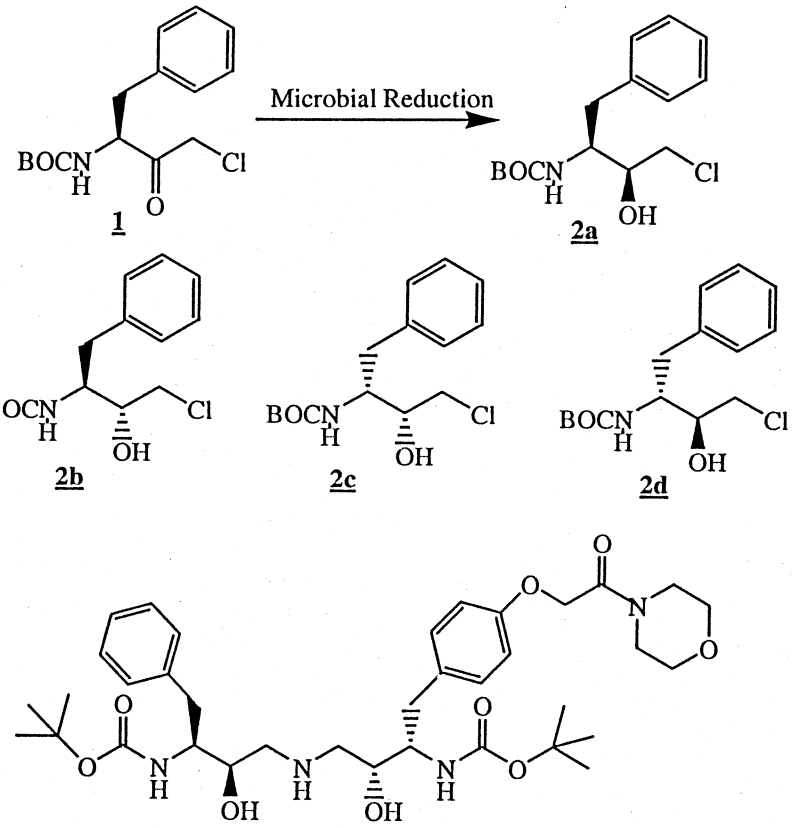
Chiral drug intermediates can be prepared by asymmetric synthesis by either chemical or biocatalytic processes using microbial cells or enzymes derived therefrom. The advantages of microbial- or enzyme-catalyzed reactions over chemical reactions are: (i) they are stereoselective and can be carried out at ambient temperature and atmospheric pressure. This minimizes problems of isomerization, racemization, epimerization, and rearrangement that generally occur during chemical processes. (ii) Biocatalytic processes are generally carried out in aqueous solution, thus avoiding environmentally harmful chemicals, used in the chemical processes, and solvent waste disposal. (iii) Microbial cells or their enzymes can be immobilized and reused many cycles.

The use of enzymes in organic synthesis has been reviewed (1–11). This report provides some specific examples of the use of oxidoreductases in stereoselective catalysis and preparation of chiral drug intermediates required for our antiviral and antihypertensive agents (12,13).

An essential step in the life cycle of human immunodeficiency virus (HIV-1) is the proteolytic processing of its precursor proteins. This processing is accomplished by HIV-1 protease, a virally encoded enzyme. Inhibition of HIV-1 protease arrests the replication of HIV *in vitro*. Thus, HIV-1 protease is an attractive target for chemotherapeutic intervention. Recently, Barrish *et al*. (14) reported the discovery of a new class of selective HIV protease inhibitors that incorporate a C_2 symmetric aminodiol core as their key structural feature. Members of this class, and particularly BMS-186318 (Scheme 1), display potent anti-HIV activity in cell culture. In this report, we describe the stereoselective microbial reduction of (1*S*) [3-chloro-2-oxo-1-(phenylmethyl)propyl] carbamic acid, 1,1-dimethylethyl ester **1** to **2a** (Scheme 1). Chiral alcohol **2a** {(1*S*,2*R*)[3-chloro-2-hydroxy-1-(phenylmethy)propyl] carbamic acid, 1,1-dimethyl ethyl ester} is a key intermediate for the total chemical synthesis of BMS-186318.

L-6-Hydroxynorleucine **3** is a chiral intermediate useful for the synthesis of a vasopeptidase inhibitor now in clinical trial and for the synthesis of C-7-substituted azepinones as potential intermediates for other antihypertensive metalloprotease inhibitors (15,16). It has also been used for the synthesis of siderophores, indospicines, and peptide hormone analogs (17–21). Reductive amination of ketoacids using



BOC = butoxycarbonyl

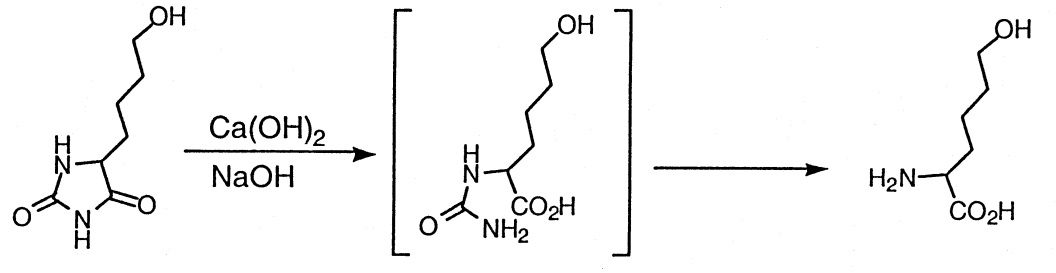
BMS-186318 (Antiviral agent)

SCHEME 1

amino acid dehydrogenases has shown to be a useful method for synthesis of natural and unnatural amino acids (22,23). In this report we also describe the conversion of 2-keto-6-hydroxyhexanoic acid **4** to **3** by reductive amination using beef liver glutamate dehydrogenase and glucose dehydrogenase from *Bacillus* sp. for regeneration of NADH. A second route, which avoids the lengthy chemical synthesis, was developed to prepare the ketoacid by treatment of racemic 6-hydroxy norleucine [readily available from hydrolysis of 5-(4-hydroxybutyl) hydantoin **5**, Scheme 2 (24)] with D-amino acid oxidase from porcine kidney or *Trigonopsis variabilis* and catalase followed by the reductive amination procedure to convert the mixture to **3**.

MATERIALS AND METHODS

Materials. Starting substrate **1** and reference compounds **2a–d** were synthesized in the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ), as described previously (12). The physicochemical properties, including spectral characteristics [^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR,



5-(4-hydroxybutyl) hydantoin
5

Racemic
6-hydroxynorleucine

SCHEME 2

mass spectra], were in full accord for all these compounds. Compound **5** was obtained from Hampshire Chemical Company. Enzymes were purchased from the following sources: D-amino acid oxidase, catalase, glutamate, phenylalanine, and alanine dehydrogenases, Sigma (St. Louis, MO); formate dehydrogenase, Boehringer Mannheim (Indianapolis, IN); glucose dehydrogenase, Amano (Troy, VA). Compound **4** and reference compound **3** were synthesized at the Bristol-Myers Squibb Pharmaceutical Research Institute (13), and physicochemical properties including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra) were in full accord for all these compounds. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM-300 spectrometer (Billerica, MA).

Microorganisms. Microorganisms (Table 1) were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the American Type Culture Collection (Rockville, MD). Microbial cultures were stored at –90°C in vials.

For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A containing 1% malt extract, 1% yeast extract, 2% glucose, and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28°C and 280 rpm for 48 h. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 0.1 M potassium phosphate buffer pH 7.0, and used for reduction studies.

Reduction of **1** *by cell suspensions*. Cells of various microorganisms were suspended separately in 100 mM potassium phosphate buffer (pH 7.0) at 20% (wt/vol, wet cells) cell concentration and supplemented with 1 mg/mL of **1** and 30 mg/mL of glucose. Reduction was conducted at 28°C and 150 rpm. Periodically, 1-mL samples were taken and extracted with 5 mL of *tert*-butylmethylether/toluene (60:40). After centrifugation, the separated organic phase was collected and dried with a nitrogen stream. The oily residue obtained was dissolved in 1 mL of ethanol, filtered through a 0.2 µm LID/X filter (Whatman Inc., Tewksbury, MA), and analyzed by high-performance liquid chromatography (HPLC).

Two-stage process for reduction of **1**. *Streptomyces nodosus* SC 13149 and *Mortierella ramanniana* SC 13850 were grown in a 25-L fermentor containing 15 L of medium A containing 0.025% antifoam. Growth consisted of two inoculum development stages (F1 and F2) and fermentation. In the F1 stage, a frozen vial of each culture was inoculated into 100 mL of medium A contained in a 500-mL flask. Growth was carried out at 28°C and 280 rpm for 48 h on a rotary shaker. In the F2 stage, 10 mL of F1 stage culture was inoculated into 1 L of medium A and incubated at 28°C and 280 rpm for 24 h. Fermentors containing 15 L of medium A were inoculated with 1 L of inoculum of each culture from a F2 stage. Fermentation was conducted at 25°C and 500 rpm with 15 LPM (liters per minute) aeration for 48 h. After 48 h fermentation, cells were collected and stored at –90°C until further use. About 1 kg of wet cell pastes was collected from each fermentation.

TABLE 1
Stereoselective Microbial Reduction of Ketone 1[a]

Microorganisms	Reaction yield of **2a** (%)	Diastereomeric purity of **2a** (%)	e.e. of **2a** (%)
Streptomyces nodosus SC 13149	56	>99	99.9
Pullularia pullulans SC 13849	47	81	99.9
Candida boidinii SC 13821	39	>99	99.9
Nocardioides albus SC 13910	5	85	99.9
Mortierella ramanniana SC 13850	54	91	99.9
Caldariomyces fumigo SC 13901	51	93	99.9

[a]**1**, (1*S*)[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester; **2a**, (1*S*,2*R*)[3-chloro-2-hydroxy-1-(phenylmethyl)propyl] carbamic acid, 1,1-dimethyl ethyl ester; e.e., enantiomeric excess.

Frozen cells from the above batches were used to conduct the reduction of **1** in a 5-L reactor. Cell suspensions (10% wt/vol, wet cells) in 3 L of 0.1 M potassium phosphate buffer (pH 6.0) were used. Compound **1** (3 g) and glucose (30 g) were added to the reactor, and the reduction was carried out at 28°C and 160 rpm with 1 LPM aeration for 24 h. The pH was maintained between 6.6 and 6.8. Periodically, samples were prepared as described above and analyzed by HPLC to determine the percentage of conversion of **1** to **2a**. The diastereomeric purity and the enantiomeric excess of **2a** were determined by HPLC.

Single-stage process for reduction of ketone **1**. *Streptomyces nodosus* SC 13149 culture was grown in a 25-L fermentor as described above. After 30 h of growth, 15 g of ketone **1** was added to the fermentor, and the biotransformation process was continued for 48 h. The pH was maintained at 6.8 during the biotransformation process. Periodically, samples were prepared as described above and analyzed by HPLC to determine the percentage of conversion of **1** to **2a**. The diastereomeric purity and the enantiomeric excess of **2a** were determined by HPLC.

Isolation of alcohol **2a**. At the end of single-stage bioreduction, 12 L of the reaction mixture was extracted with 24 L of *tert*-butylmethylether/toluene (60:40). The separated organic phase (20 L) was washed with 10 L of 0.1 M sodium chloride, dried over anhydrous sodium sulfate, and evaporated under reduced pressure to obtain 7.5 g of crude product, which was recrystallized from ethyl acetate to obtain 6.5 g (62% overall yield) of white needle crystals of **2a**. The diastereomeric purity and the enatiomeric excess of the isolated chiral alcohol **2a** were >99% and >99.8%, respectively. ^{1}H NMR ($CDCl_3$) δ 1.4 (*s*, 9H), 2.9 (*d*, 2H, *J* = 13 Hz), 3.2 (*s*, 1H), 3.6 (*m*, 2H), 3.85 (*m*, 1H), 4.5 (*s*, 1H), 7.2–7.4 (*m*, 5H). Mass spectrum: *m/z* 302 $(M + H)^+$ (calc. for $C_{15}H_{22}ClNO_2$, 301).

HPLC analysis of compounds **1, 2a–2d**. Analysis of **1** and **2a–2d** were carried out using a Hewlett-Packard (Palo Alto, CA) high-performance liquid chromatograph. A YMC-PACK-ODS-A column (Whatman Inc.; 100 × 4.5 mm, i.d. 5 µm) was used under the following conditions. Mobile phase was 10% methanol (solvent A) and 90% methanol (solvent B) used in a gradient. For the first 25 min the eluant was 100% A; from 25 to 25.2 min the eluant was 25% A and 7% B; from 25.2 to 30 min the eluant was 100% A.

The flow rate was 1 mL/min, and the detection wavelengths were 224, 250, and 280 nm. A diode array detector from Hewlett-Packard was used. The retention times for substrate **1**, compounds **2a**, **2c**, and **2d** were 24, 23.5, and 24.5 min, respectively. The enantiomeric excess of chiral alcohol **2a** was determined by chiral HPLC. A Bakerbond chiralpak AD column (100 × 4.5 mm,i.d. 5 m) was used at ambient temperature; injection volume was 10 µL; mobile phase was 97.5% hexane/1% cyclohexanol/1.5% ethyl acetate mixture; flow rate was 0.8 mL/min; and detection wavelength was 210 nm. The retention times for the compounds **2a**, **2b**, and **2c–2d** were 14, 15.5, and 21.5 min, respectively.

Screening of strains for reductive amination of **4**. Strains inoculated from frozen vials were shaken at 220 rpm at 28°C for 48 h in 50 mL of medium containing (g/L): L-phenylalanine, 10.0; peptone, 10.0; yeast extract, 5.0; K_2HPO_4, 2.0; NaCl, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.2. Cells were harvested by centrifugation, washed with 50 mM potassium phosphate buffer pH 7, and resuspended in 5 mL of the same buffer containing 1 mM dithiothreitol. Cells were sonicated for 2 min, then centrifuged for 20 min at 101,000 × *g*. Extracts were assayed spectrophotometrically for reductive amination. The assay for amino acid dehydrogenase contained, in a volume of 1.0 mL: 0.75 M NH_4OH adjusted to pH 8.75 with HCl, 0.4 mM NADH, 25 mg/mL compound **4**, and extract. Absorbance change per minute at 340 nm was used to calculate the activity of the enzyme.

HPLC analysis of 6-hydroxynorleucine. Analysis of enantiomeric purity and of the amount of 6-hydroxynorleucine was performed with a Chiralpak WH (Diacel Chemical Industries, Ltd., Easton, PA) 25 × 0.46 cm column; the mobile phase was 0.3 mM $CuSO_4$; flow rate was 1 mL/min; temperature was 40°C; and detection was at 230 nm.

Enzymatic preparation of **3** *from racemic 6-hydroxynorleucine. (i) D-Amino acid oxidase from porcine kidney*. Racemic 6-hydroxynorleucine (0.5 g) in 20 mL of 50 mM potassium phosphate buffer (pH 7) was treated with porcine kidney D-amino acid oxidase (49 U, 350 mg) and beef liver catalase (182 U, 8 mg) to give a mixture of ketoacid and 0.236 g compound **3** with 97% e.e. The enzymes were added in increments, and the reaction took 10 d to complete. The reductive amination was then carried out as described above. 6-Hydroxynorleucine (0.484 g) was produced (97% yield) with >99% e.e.

(ii) D-Amino oxidase from T. variabilis. *Trigonopsis variabilis* ATCC10679 was grown in a 250-L fermentor at 28°C on medium containing (g/L): KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2$, 0.5; H_3BO_3, 0.1; $(NH_4)_2MoO_4$, 0.04; $MnSO_4 \cdot 4H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$; 0.04; $CuSO_4 \cdot 5H_2O$, 0.045; $FeSO_4 \cdot 7H_2O$, 0.025; DL-methionine, 3; biotin, 0.02; thiamine, 0.1; cysteine, 0.72; cerelose hydrate, 22. Cell paste (129 g) was harvested 50 h after inoculation and stored frozen. Racemic 6-hydroxynorleucine (0.514 g) in 70 mL of 50 mM potassium phosphate buffer, pH 7, was shaken with 14 g washed *T. variabilis* ATCC 10679 cells for 21 h at 28°C and 200 rpm to give **3** with e.e. > 99%. Cells were removed by centrifugation, and the supernatant was treated with glutamate dehydrogenase, glucose dehydrogenase, NH_3, glucose, and NAD as described above. Compound **3** (0.469 g, 91% yield) was obtained with >99% e.e. After the completion of the reaction, **3** was isolated as described above. The isolated yield was 0.394 g (76.6%), and the e.e. was 99%. The ^1H NMR spectrum agreed with the structure.

RESULTS

Enzymatic synthesis of compound **2a**. About 100 microorganisms were screened for the stereoselective reduction of **1** to **2a**. The reaction yields, diastereomeric purity, and e.e. of **2a** obtained with the best six cultures were as shown in the Table 1. Lower diastereomeric purities (<70%) and reaction yields (<20%) were obtained with other cultures. The reaction yield and diastereomeric selectivity were dependent upon the microorganism used during the reduction of **1**. *Streptomyces nodosus* SC 13149, *Candida boidinni* SC 13821, *M. ramanniana* SC 13850, and *Caldariomyces fumago* SC 13901 gave >39% reaction yields, >91% diastereomeric purities, and 99.9% e.e. of product **2a.**

Further research was conducted using *S. nodosus* SC 13149 and *M. ramanniana* SC 13850 to convert ketone **1** to the corresponding chiral alcohol **2a**. Cells were grown in a 25-L fermentor for 48 h, collected, and suspended in 100 mM potassium phosphate buffer pH 6.8; and the resulting cell suspensions were used to carry out the two-stage process for biotransformation of **1** as described in the Materials and Methods section. After 24 h biotransformation, a reaction yield of 67%, e.e. of 99.9%, and diastereomeric purity of >99% were obtained for chiral alcohol **2a** using cells of *S. nodosus* SC

TABLE 2
Stereoselective Microbial Reduction of Ketone 1: Two-Stage Process[a]

Microorganism	Reaction time (h)	Compound **2a** (g/L)	Yield (%)	Diastereomeric purity of **2a** (%)	e.e. of **2a** (%)
Streptomyces nodosus SC13149	12	0.56	56		
	24	0.67	67	>99	99.9
Mortierella ramanniana SC13850	12	0.22	22		
	24	0.54	54	91	99.9

[a]For abbreviations see Table 1.

TABLE 3
Stereoselective Microbial Reduction of Ketone 1: Single-Stage Process Using *Streptomyces nodosus* SC 13149[a]

Reaction time (h)	Compound **2a** (g/L)	Yield **2a** (%)	Diastereomeric purity of **2a** (%)	e.e. of **2a** (%)
24	0.56	56		
48	0.80	80	>99	99.9

[a]For abbreviations see Table 1.

TABLE 4
2-Keto-6-hydroxyhexanoic Acid Dehydrogenase in Microbial Strains

Microbe	Strain	U/mL[a]
Bacillus licheniformis	SC12772	0.0099
Bacillus licheniformis	SC12148	0.0368
Rhodococcus sp.	SC13810	0.0177
Sporosarcina ureae	ATCC 6473	0.0103
Sporosarcina ureae	ATCC 13888	0.0613
Thermoactinomyces intermedius	ATCC 33205	0.2161

[a]Cells from a 50-mL culture were assayed as described in the Materials and Methods section, except that *Thermoactinomyces intermedius* was grown at 53°C and assayed at 50°C. U, units.

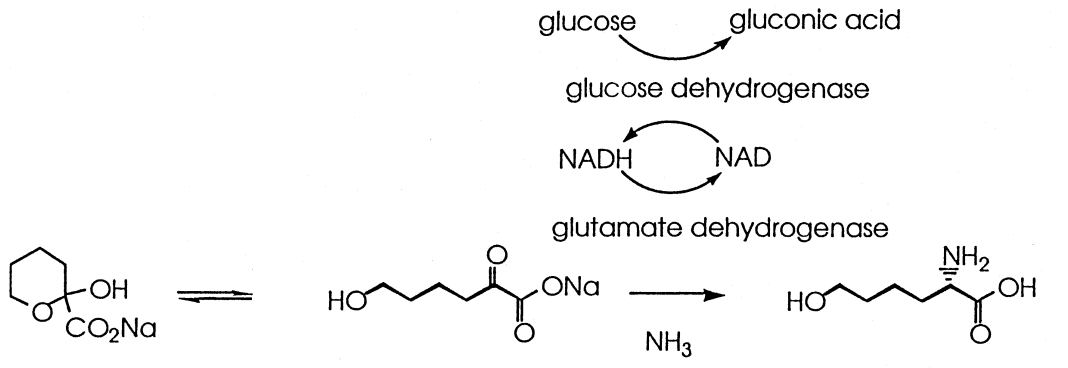
13149. *Mortierella ramanniana* SC 13850 gave a reaction yield of 54%, e.e. of 99.9% and diastereomeric purity of 90% for chiral alcohol **2a** (Table 2).

A single-stage fermentation–biotransformation process was developed for conversion of ketone **1** to chiral alcohol **2a** with cells of *S. nodosus* SC 13149 as described in the Materials and Methods section. A reaction yield of 80%, diasteromeric purity of >99%, and an e.e. of 99.8% were achieved (Table 3) . From a 12-L reaction mixture, 6.5 g of chiral alcohol **2a** was isolated as white needle crystals in overall 62% yield. The diastereomeric purity and the e.e. of the isolated chiral alcohol were >99% and >99.8%, respectively.

Enzymatic synthesis of **3**. A variety of ketoacids can be converted to L-amino acids by treatment with a suitable amino acid dehydrogenase. Initial screening using HPLC analysis, with formate dehydrogenase for regeneration of NADH, showed that phenylalanine dehydrogenase from *Sporosarcina* sp. and beef liver glutamate dehydrogenase converted 0.1 M compound **4**, sodium salt mixture, completely to **3**. Additional screening of amino acid dehydrogenases with spectrophotometric enzyme assays using **4**, sodium salt as substrate showed that glutamate dehydrogenases from *Candida utilis* and *Proteus* sp. were active using NADPH but not NADH. Leucine dehydrogenase partially purified from *B. sphaericus* ATCC 4525 (25) and alanine dehydrogenase from *B. subtilis* were not active. Of 132 cultures screened, an extract from *Thermoactinomyces intermedius* ATCC 33205 was the most active (Table 4). This strain has been shown to be a source of thermostable phenylalanine dehydrogenase (26) as well as leucine dehydrogenase (27).

Beef liver glutamate dehydrogenase was used for preparative reactions at 10% total substrate concentration. As depicted in Scheme 3, compound **4**, sodium salt, in equilibrium with 2-hydroxytetrahydropyran-2-carboxylic acid, sodium salt, is converted to **3**, and the reaction requires ammonia and NADH. NAD produced during the reaction was recycled to NADH by the oxidation of glucose to gluconic acid using glucose dehydrogenase from *B. megaterium*. The optimal pH for glutamate dehydrogenase with this substrate was determined to be about 8.76. We previously reported that glucose dehydrogenase had a broad pH optimum centered at about 8.5 (27). The kinetics of the reaction are shown in Figure 1. Reaction was complete in about 3 h with reaction yields of 89–92%, and e.e. was >99%.

Chemical synthesis and isolation of **4** required several steps. In a second, more convenient process (Scheme 4), the ketoacid was prepared by treatment of racemic 6-hydroxynorleucine (produced by hydrolysis of **5**) hydantoin] with D-amino acid oxidase and catalase. After the e.e. of the remaining **3** increased to >99%, the reductive amination procedure was used to convert the mixture containing **4** and **3** entirely to **3** with yields of 91 to 97% and e.e. of >99%.



SCHEME 3

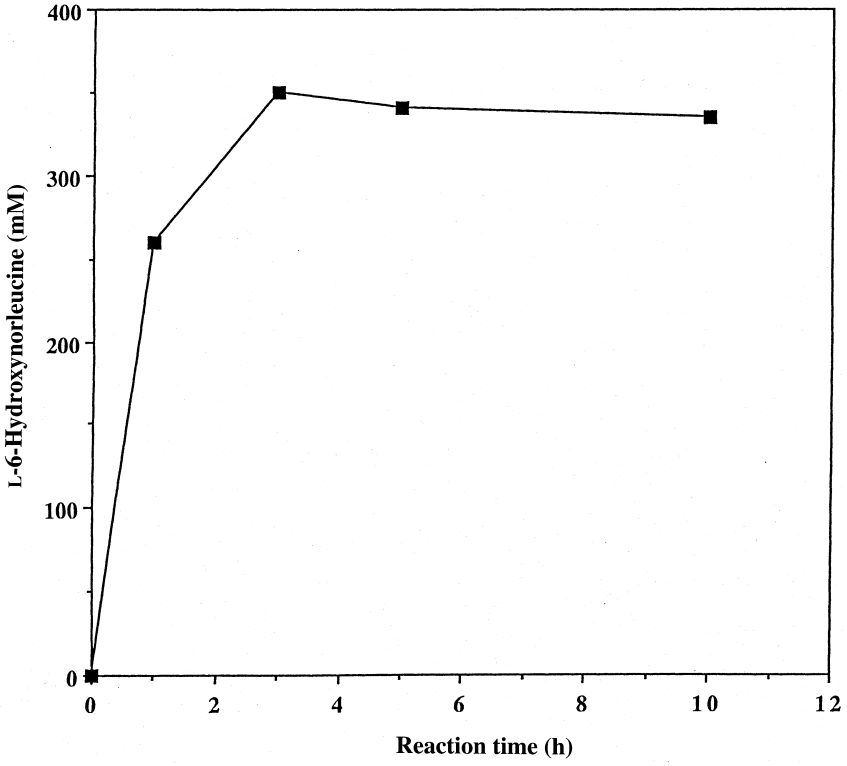
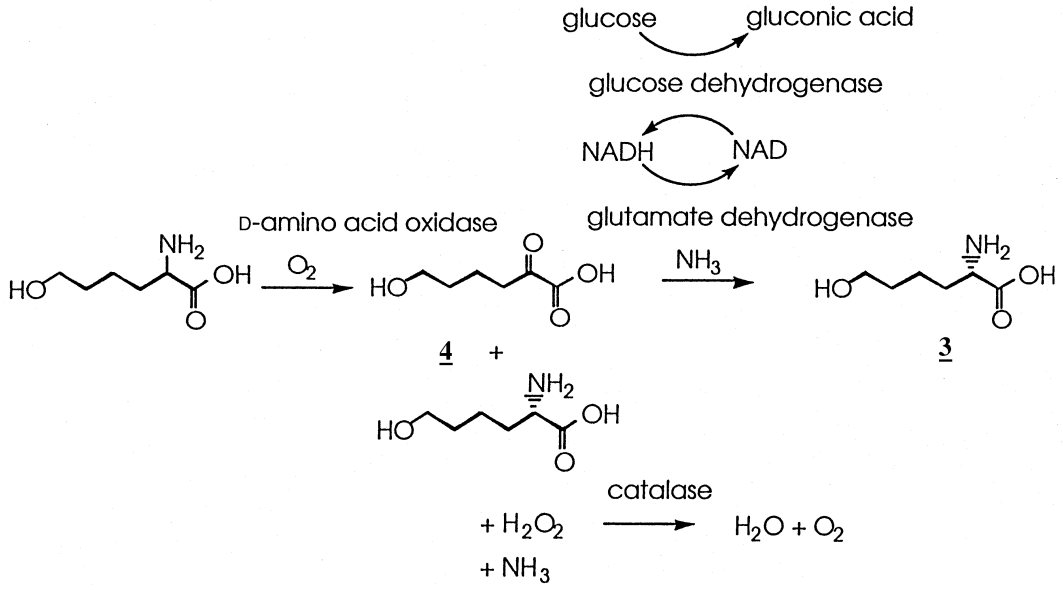


FIG. 1. Biotransformation of 2-keto-6-hydroxyhexanoic acid to L-6-hydroxynorleucine using beef liver glutamate dehydrogenase. NADH was recycled using glucose dehydrogenase. At 3 h, the yield was 92% and the enantiomeric excess was >99%.

Sigma porcine kidney D-amino acid oxidase and beef liver catalase or *T. variabilis* whole cells (source of oxidase and catalase) were used successfully for this transformation.

Compound **3** was readily prepared from the corresponding ketoacid by reductive amination using beef liver glutamate dehydrogenase. Phenylalanine dehydrogenase from *Sporosarcina* sp. or *Th. intermedius* extract was also effective. Either formate dehydrogenase or glucose dehydrogenase was useful for NADH regeneration. Preparation of the ketoacid required several steps, but a shorter route was found in which racemic 6-hydroxynorleucine was pre-pared by hydrolysis of **5**. Conversion of the D-enantiomer to the ketoacid using D-amino acid oxidase followed by



SCHEME 4

in situ reductive amination gave nearly complete conversion to **3**.

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