Biocatalytic Synthesis of Some Chiral Drug Intermediates by Oxidoreductases

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ABSTRACT: Chiral intermediates were prepared by biocatalytic processes with oxidoreductases for the chemical synthesis of some pharmaceutical drug candidates. These include: (i) the microbial reduction of 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone (**1**) to *R*-(+)-1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanol (**2**) [*R*-(+)-BMY 14802], an antipsychotic agent; (ii) the reduction of *N*-4-(1-oxo-2-chloroacetyl ethyl) phenyl methane sulfonamide (**3**) to the corresponding chiral alcohol (**4**), an intermediate for D-(+)-*N*-4-{1-hydroxy-2-[(-methylethyl)amino]ethyl}phenyl methanesulfonamide [D-(+) sotalol], a β-blocker with class III antiarrhythmic properties; (iii) biotransformation of *N*ε-carbobenzoxy (CBZ)-L-lysine (**7**) to *N*ε-CBZ-L-oxylysine (**5**), an intermediate needed for synthesis of (*S*)-1-[6-amino-2-{[hydroxy(4-phenyl butyl)phosphinyl]oxy}1-oxohexyl]-L-proline (ceronapril), a new angiotensin converting enzyme inhibitor (**6**) and (iv) enzymatic synthesis of L-β-hydroxyvaline (**9**) from α-keto-β-hydroxyisovalerate (**16**). L-β–Hydroxyvaline (**9**) is a key chiral intermediate needed for the synthesis of *S*-(*Z*)-{[1-(2-amino-4-thiazolyl)-2-{[2,2-dimethyl-4-oxo-1-(sulfooxy)-3-azetidinyl] amino}-2-oxoethylidene]amino}oxyacetic acid (tigemonam) (**10**), an orally active monobactam.
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KEY WORDS: Antihypertensive drug (ceronapril), antipsychotic agent [*R*-(+)-BMY 14802], biocatalysis, β-blocker with class III antiarrhythmic properties (D-sotalol), chiral drug intermediates, monobactam (tigemonam), oxidoreductases.

Recently, much attention has been focused on the interaction of small molecules with biological macromolecules. The search for selective enzyme inhibitors and receptor agonists or antagonists is one of the keys for target-oriented research in the pharmaceutical industry. Increasing understanding of the mechanism of drug interaction on a molecular level has led to increasing awareness of the importance of chirality as the key to efficacy of many drug products. It is now known that often only one stereoisomer of a drug substance is required for efficacy, and the other stereoisomer is either inactive or exhibits considerably reduced activity. Pharmaceutical companies are aware that, where appropriate, new drugs for the clinic should be homochiral to avoid the possibility of side effects due to an undesirable stereoisomer. Where the switch from racemate drug substance to enantiomerically pure compound is feasible, there is the opportunity to double the use of an industrial process. The physical characteristic of enantiomers vs. racemates may confer processing or formulation advantages.

Chiral drug intermediates can be prepared by different routes. One is to obtain them from naturally occurring chiral synthons, mainly produced by fermentation processes. The chiral pool primarily refers to inexpensive, readily available, optically active natural products. A second is to carry out the resolution of racemic compounds. This can be achieved by preferential crystallization of stereoisomers or diastereoisomers and by kinetic resolution of racemic compounds by chemical or biocatalytic methods. Finally, chiral synthons can also be prepared by asymmetric synthesis by either chemical or biocatalytic processes with microbial cells or enzymes derived therefrom. The advantages of microbial or enzyme-catalyzed reactions over chemical reactions are that they are stereoselective and can be carried out at ambient temperature and atmospheric pressure. These minimize problems of isomerization, racemization, epimerization, and rearrangement, which generally occur during chemical processes. Biocatalytic processes are generally carried out in aqueous solution. This avoids the use of environmentally harmful chemicals used in chemical processes and the disposal of solvent waste. Furthermore, microbial cells or enzymes derived therefrom can be immobilized and reused in many cycles.

Recently, a number of review articles (1–9) have been published on the use of enzymes in organic synthesis. This report provides some specific examples of the use of oxidoreductases in stereoselective catalysis and preparation of some chiral drug intermediates required for our antipsychotic, antiarrhythmic (β-blocker), antihypertensive, and antibacterial agents (10–13).

ANTIPSYCHOTIC DRUG (BMY 14802)

Stereoselective microbial reduction of 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone (**1**). During the past few years, much research effort has been di-

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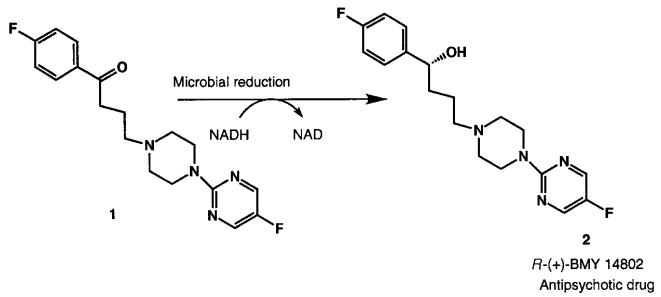


SCHEME 1

rected toward understanding the sigma receptor system in brain and endocrine tissue. This effort has been motivated by the hope that the sigma site may be a target for a new class of antipsychotic drugs (14–17). Characterization of the sigma system helped to clarify the biochemical properties of the distinct haloperidol-sensitive sigma binding site, the pharmacological effects of sigma drugs in several assay systems, and the transmitter properties of a putative endogenous ligand for the sigma site (18–21).

Compound **2**, or *R*-(+)-1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanol [*R*-(+)-BMY 14802], is a sigma ligand, has a high affinity for sigma binding sites, and can selectively inhibit conditioned avoidance and apomorphine-induced stereotype in rats predictive of antipsychotic efficacy. Dopamine cell firing in the substantia nigra, caused by the putative sigma receptors, was inhibited by (+)-3H-3-(3-hydroxyphenyl)-*N*-(1-propyl) piperidine (17,18). In this section, we describe the stereoselective microbial reduction of **1** to yield **2** [*R*-(+)-BMY 14802] (Scheme 1). The reductase that catalyzed the stereoselective reduction of compound **1** to *R*-(+) BMY 14802 (**2**) has been purified to homogeneity.

Materials and Methods

Materials. Standards of compounds **1** and **2** were synthesized by the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ) (22,23).

Microorganisms. Microorganisms (Table 1) were obtained from the American Type Culture Collection (Rockville, MD). Microorganisms were stored at –90°C in vials.

Growth of microorganisms. For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A [glucose (2%), corn steep solids (3.5%), ammonium sulfate (0.5%), soybean oil (0.5%) and calcium carbonate (0.35%), adjusted to pH 6.8 in 1 L of tap water]. Cultures were grown at 28°C and 280 rpm for 48 h. Cultures were harvested by centrifugation at 20,000 × *g* for 15 min, washed with 100 mM potassium phosphate buffer pH 6.9, and used for biotransformation studies.

*Microbial reduction of compound **1**.* Cells of microorganisms were suspended separately in 10 mL of 100 mM potassium phosphate buffer (pH 6.8) at 20% (wt/vol, wet cells) cell concentration and supplemented with 2 mg/mL of compound **1** and 70 mg/mL of glucose. The reaction was conducted at 30°C and 200 rpm for 48 h. Cell suspensions after transformations were extracted with 2 vol of methylene chloride/acetonitrile/isopropanol mixture (60:35:5) After centrifugation, the organic layer was collected and evaporated under a gentle stream of nitrogen. The oily residue obtained was dissolved in methanol, filtered through a 0.2-µm LID/X filter (Whatman, Inc., Fairfield, NJ), and analyzed by gas chromatography (GC).

Growth of Mortierella ramanniana *in a fermentor. Mortierella ramanniana* ATCC 38191 culture was grown in a 380-L fermentor that contained 250 L of medium A. Growth consisted of several inoculum development stages and fermentation.

Inoculum development consisted of F1 and F2 stages. In the F1 stage, frozen vials of *M. ramanniana* ATCC 38191 cultures were inoculated into 100 mL of medium A. Growth was carried out in 500-mL flasks at 28°C and 280 rpm for 48 h. In the F2 stage, 100 mL of F1-stage culture was inoculated into 1.5 L of medium A in a 4-L flask and incubated at 28°C and 180 rpm for 24 h. A germinator with 250 L of medium A was inoculated with 1.5 L of F2-stage inoculum and grown for 24 h at 28°C and 150 rpm with 150 Lpm (liters per min) aeration.

Fermentors with 250 L of medium A were inoculated with 30 L of germinator-grown inoculum. Fermentations were con-

TABLE 1
Microbial Reduction of 1 **to** 2 **[BMY 14802][a]**

Microorganisms	Substrate **1** (g/L)	BMY 14802 (g/L)	Optical purity of BMY 14802	
			R-(+)	*S*-(–)
Mortierella ramanniana ATCC 38191	0.02	1.95	98.9	1.1
Pullularia pullulans ATCC 16623	0.04	1.62	1.5	98.5

[a]Microorganisms were grown in a 25-L fermentor for 48 h. Cells were harvested by Cepa centrifuge (New Brunswick Scientific, Edison, NJ) and suspended in 0.1 M phosphate buffer, pH 6.0. Cell suspensions (10% wt/vol, wet cells) were used in the reduction of **1** (2 g/L) at 280 rpm and 28°C for 24 h. The concentrations of **1** and **2** were determined by gas chromatography (GC) and the optical purity of **2** was determined by chiral high-performance liquid chromatography (HPLC). Abbreviations: **1**, 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone; **2**, *R*-(+)-1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanol [*R*-(+)-BMY 14802]; ATCC, American Type Culture Collection (Rockville, MD).

ducted for 40 h at 28°C and 150 rpm with 150 Lpm aeration. To determine the specific activity of cells during fermentation, cells were periodically harvested by centrifugation from 200 mL of culture broth. Cell suspensions (10% wt/vol, wet cells) were prepared in 100 mM potassium phosphate buffer (pH 5.8) and supplemented with 0.5 mg/mL of compound **1** and 70 mg/mL of glucose. The reaction was conducted at 28°C and 280 rpm on a shaker. Periodically, samples were analyzed for the reduction of compound **1** to compound **2** by GC. The specific activity was expressed as micrograms of compound **2** produced per hour per gram of dry cells. After 45 h of fermentation, cells were harvested with the aid of a Sharples centrifuge (Alfa-Laval, Springfield, PA), and the wet cell pastes were collected and stored at −60°C until further use. About 12 kg of wet cell pastes were collected from each fermentation.

Two-stage biotransformation process. Frozen cells from the foregoing batches were used to conduct the reduction of compound **1** in a 5-L BioFlo fermentor (NewBrunswick Scientific, Edison, NJ). Cell suspensions (20% wt/vol, wet cells) in 3 L of 100 mM potassium phosphate buffer (pH 5.8) were used. Compound **1** (6 g) and glucose (200 g) were supplemented to the cell suspensions, and the reduction was conducted at 28°C and 600 rpm with 7 Lpm aeration. Periodically, a 2-mL sample was removed and extracted with 2 vol of methylene chloride/acetonitrile/isopropanol mixture (60:35:5). After centrifugation, the organic layer was collected and dried with a nitrogen stream. The oily residue was dissolved in methanol, filtered through 0.2-μm LID/X filter, and analyzed by GC to determine the percentage conversion of compound **1** to compound **2**. The optical purity of compound **2** was determined by chiral high-pressure liquid chromatography (HPLC).

Single-stage fermentation and biotransformation. The fermentation process consisted of a laboratory sporulation stage and a fermentation stage in which cells of *M. ramanniana* ATCC 38191 were cultivated. Immediately after fermentation, substrate was added to the fermentor, and biotransformation was continued.

In the sporulation stage, frozen vials of *M. ramanniana* ATCC 38191 culture were inoculated into 100 mL of medium A contained in a 500-mL flask. The flask was then incubated at 25°C and 280 rpm for 48 h. One mL of cell suspension from the foregoing flasks was used to inoculate rice flasks to initiate sporulation of the culture at 25°C for 144 h under static conditions. At the end of sporulation time, 100 mL of medium A was added to the flask under aseptic conditions, and the flask was incubated at 25°C and 280 rpm for 18 h. Spore suspension, obtained by the foregoing procedure, was added aseptically to 400 mL sterile water and used to inoculate a 20-L fermentor that contained 15 L of medium A. The fermentor was equipped with pH and dissolved-oxygen probes. The exhaust gas from the fermentor was routed to a mass spectrometer for continuous monitoring of exhaust carbon dioxide and oxygen. Fermentations were conducted at 25°C and 600 rpm with 20 Lpm aeration. During fermentation, samples were periodically taken to check sterility and determine the dry cell weight and glucose concentration. At the end of the fermentation cycle, when residual glucose was depleted, the biotransformation process was initiated by the addition of substrate (2 g/L) and glucose (84 g/L) directly to the fermentor. Subsequently, substrate and product concentrations were monitored. The biotransformation process was considered complete when the residual substrate concentration was less than 7 mg/L. Generally, the biotransformation process was completed in 25 h.

*Isolation of compound **2**.* Fermentation broth (10 L), containing 20 g of compound **2**, was centrifuged to remove cells. The clear supernatant was treated with 5 N NaOH to raise the pH to 8.0 and allowed to stand at room temperature for 1 h. The precipitated solids (70 g) were collected by filtration and dissolved in 500 mL acetone. The acetone solution was filtered and dried over anhydrous sodium sulfate. Acetone was removed by rotary evaporation, followed by drying at 30°C at reduced pressure to recover 14.2 g of white solid compound **2** in overall 70 mol% yield. The GC area percentage purity of the isolated compound **2** was >99%.

Preparation and purification of cell extracts. Mortierella ramanniana ATCC 38191 cells were suspended in buffer A [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5], containing 20% glycerol and 1 mM dithiothreitol (DTT), at a 20% (wt/vol, wet cells) cell concentration. The cell suspensions were disintegrated by sonication for 20 min at 4°C. Disintegrated cells were centrifuged at 20,000 × *g* for 40 min at 4°C, and the supernatant solution was referred to as cell extract.

Cell extract was loaded onto a DEAE cellulose (DE-52 from Whatman Co., Maidstone, England) column (5.0 × 67 cm). The column was washed with 500 mL of buffer A and eluted with a linear gradient of salt (0–0.5 M sodium chloride) in 1 L of buffer A. Fractions that contained reductase activities were pooled and loaded onto a phenyl sepharose column (25 × 15 cm), which was previously equilibrated with buffer A. The column was washed with buffer A and then eluted with buffer A containing 1% Triton × 100. Fractions containing reductase activities were pooled and loaded onto a Pharmacia (Piscataway, NJ) Mono Q column (0.5 × 5 cm) in a Phast (Pharmacia) protein liquid chromatography system. The column was washed with buffer A and subsequently eluted with a salt gradient (0–0.1 M sodium chloride in buffer A).

*Transformation of compound **1** by cell extracts.* Cell extracts, pooled DE-52 fraction, pooled Mono Q fraction, and pooled phenyl sepharose fractions were analyzed for their ability to catalyze the transformation of compound **1** to compound **2**. The reaction mixture contained 0.67 mM NADP, 10 mg/mL glucose, 3.4 units of glucose dehydrogenase, 1 mg/mL of compound **1**, and an appropriate amount of enzyme fraction in 1 mL of buffer A. The reaction was started by the addition of reductase and continued at room temperature on a shaker at 150 rpm. The reaction was quenched by raising the pH of the reaction mixture to 9.5 with 1 N NaOH. The reaction mixture was extracted with 2 mL toluene, and the separated toluene layer was analyzed for compounds **1** and **2** by GC. One unit of activity was defined as the amount of compound **2** formed

(nmol/min). Protein concentration in various fractions was determined by BioRad (Richmond, CA) assays (24).

Polyacrylamide gel electrophoresis. Denaturing gels were run on a Phast system. The purified enzyme was incubated at 90°C for 2 h with 1% sodium dodecyl sulfate (SDS) and 10 mM DTT, then electrophoresed on a 12.5% gel with 0.1% SDS.

Molecular weight (M_r) determination. M_r of the native enzyme was determined by gel filtration on a Superose 12 column (Pharmacia). The column was eluted with 100 mM MES buffer (pH 6.0) that contained 20% glycerol and 1 mM DTT at a flow rate of 1 mL/min. The following standards (Sigma, St. Louis, MO) were used for calibration purpose: sweet potato amylase (200,000) yeast alcohol dehydrogenase (150,000), bovine serum albumin (66,000), bovine erythrocyte carbonic anhydrase (29,000), and horse heart cytochrome (12,384). The subunit M_r was determined by SDS-polyacrylamide gel electrophoresis with low-molecular-weight standards from BioRad.

Analytical methods. Analysis of compounds **1** and **2** was carried out in a Hewlett-Packard (HP) Model 5890 gas chromatograph (Palo Alto, CA) with a flame-ionization detector (FID). An HP ultra-2 column (25 m × 0.32 mm × 0.1 mm film thickness) was used at 230°C injector temperature, 270°C detector temperature, and 230–270°C column temperature (4°C/min) over a 10-min run time. The retention time for compound **1** was 5.2 min and for compound **2** was 5.6 min. Samples were prepared by extracting the reaction mixture with methylene chloride/acetonitrile/isopropanol mixture (60:35:5) and analyzing the separated organic phase directly on GC.

The optical purity of compound **2** was determined by chiral HPLC. A Bakerbond Chiralcel OD column (J.T. Baker, Phillipsburg, PA) was used at 20°C. The mobile phase consisted of hexane/butanol/cyclohexanol (97:2:1). Flow rate was 1 mL/min, and the retention time was 25 min for the *R*-(+) enantiomer and 31 min for *S*-(−) enantiomer.

Results

Among microorganisms evaluated for the reduction of compound **1** to **2**, *M. ramanniana* ATCC 38191 predominantly reduced compound **1** to *R*-(+) compound **2**, and *Pullularia pullulans* ATCC 16623 reduced compound **1** to *S*-(−) compound **2**. Optical purity of >98% was obtained in each reaction (Table 1).

TABLE 2
Reduction of 1 to 2 [BMY 14802] by *Mortierella ramanniana:* Evaluation of Cells During Growth in a 380-L Fermentor[a]

Cell growth time (h)	Concentration of substrate **1** (g/L)	Concentration of **2**, BMY 14802 (g/L)	Optical purity *R*-(+) BMY 14802 (%)
7	0.96	0.42	89.5
19	0.56	1.1	93.3
31	0.04	1.86	99.4
43	0.12	1.8	96.8

[a]During fermentation cells were harvested from 500-mL broth samples and suspended in 100 mM potassium phosphate buffer (pH 5.8). Cell suspensions (20% wt/vol, wet cells) were used to conduct the reduction of **1** (2 mg/mL) at 280 rpm and 20°C for 24 h. Concentrations of **1** and **2** were determined by GC and the optical purity of **2** was determined by chiral HPLC. For abbreviations, see Table 1.

Further research was conducted with *M. ramanniana* ATCC 38191 to convert compound **1** to *R*-(+) compound **2.** Cells of *M. ramanniana* ATCC 38191 were grown in a 280-L fermentor with 250 L of medium. During growth, cells were harvested periodically and used to conduct the reduction of compound **1**. As shown in Table 2, cells harvested after 31 h growth and used in the bioreduction of compound **1** gave a higher reaction yield (93%) and optical purity (>99%) of *R*-(+) compound **2**.

Cells harvested from a 380-L fermentor were evaluated for the reduction of compound **1** in 5- and 15-L fermentors with 3- and 10-L cell suspensions (20% wt/vol, wet cells), respectively. Compound **1** was supplied at 2 g/L concentration. Glucose was supplemented at 70 g/L concentration. After a 24-h reaction period, about 99% yield (99.5% optical purity) of *R*-(+) compound **2** was obtained from both batches. The kinetics of transformation of compound **1** are shown in Table 3. Isolation of *R*-(+) compound **2** from the 10-L fermentation broth was carried out as described in the Materials and Methods section to obtain 14.2 g of product in overall 70 mol% yield and 99% GC and HPLC purity. Isolated *R*-(+) compound **2** gave a melting point of 115°C, specific rotation α $[D]_{25}$ of +26.8 with chloroform as solvent, and optical purity of 99.5% as analyzed by chiral HPLC.

TABLE 3
Reduction of 1 to 2 by Cell Suspensions of *Mortierella ramanniana* ATCC 38191: Preparative 10-L Batch[a]

Reaction time (h)	Concentration of substrate **1** (g/L)	Concentration of **2** [BMY 14802] (g/L)	Conversion (%)	Optical purity of *R*-(+) BMY 14802 (%)
3	1.65	0.31	15	n.d.
6	1.3	0.64	32	n.d.
12	0.71	1.25	62	n.d.
18	0.48	1.61	80	99.4
24	0.04	1.98	99	99.5

[a]Cells of *M. ramanniana* ATCC 38191 were suspended in 10 L of 0. 1 M potassium phosphate buffer (pH 8) at 20% (wt/vol, wet cells) concentration. Glucose (200 g) and **1** (20 g) were supplied, and the reduction was conducted in a 15-L fermentor at 500 rpm, 28°C, 12 Lpm aeration. Abbreviation: n.d., not determined. See Table 1 for other abbreviations.

TABLE 4
Reduction of 1 to [BMY-14802] by Cell Suspensions of *Mortierella ramanianna* ATCC 38191: Effect of Glucose Concentration[a]

Glucose added (g/L)	Concentration of **2** [BMY 14802] (g/L)	Conversion (%)	Optical purity *R*-(+) BMY 14802 (%)
0	0.48	24	
10	1.49	75	99.4
20	1.95	98	99.4
40	1.93	97	99.3

[a]Cells suspensions of *M. ramanniana* ATCC 38191 (8% wt/vol, wet cells) were supplied with **1** (2 mg/mL) and glucose as indicated. Reactions were conducted at pH 5.5, 280 rpm, 28°C, for 20 h. Compounds **1** and **2** were analyzed by GC, and the optical purity of **2** was determined by chiral HPLC. See Tables 1 and 2 for abbreviations.

The effect of pH and temperature on the reduction of compound **1** to *R*-(+) compound **2** by *M. ramanniana* ATCC 38191 was evaluated in a 100-mL reactor. Cell suspensions (20% wt/vol, wet cells) were supplemented with 200 mg compound **1** and 7.5 g glucose, and biotransformation was conducted at 28°C (280 rpm) for 18 h. The optimal pH for the reduction of compound **1** to *R*-(+) compound **2** is 5.5. The optimal temperature for the reduction of compound **1** to *R*-(+) compound **2** is 28°C.

The effect of glucose and substrate concentration on the biotransformation of compound **1** to *R*-(+) compound **2** by cells of *M. ramanniana* ATCC 38191 was evaluated. As shown in Table 4, 20 mg/mL of glucose was enough to obtain a 98% reaction yield. In the absence of added glucose, only 22% reaction yield was obtained. Substrate at 2 and 4 mg/mL concentration gave reaction yields of 100% and 90%, respectively. At higher substrate concentration (6 mg/mL), lower reaction yield (63%) and optical purity (94.5%) were obtained for *R*-(+) compound **2** (Table 5).

A single-stage fermentation/biotransformation process was developed for conversion of compound **1** to compound **2** by cells of *M. ramanniana* ATCC 38191. Cells were grown in a 20-L fermentor with 15 L of medium A as described in the Materials and Methods section. After 40 h of growth in a fermentor, when the residual glucose was depleted (0.1%), the pH of the medium had dropped to 4.5. Upon total consumption of glucose, the pH of the medium started to increase, indicating completion of the fermentation process. When the pH increased to 5.5, compound **1** (2 g/L) and glucose (70 g/L) were added to the fermentor, and the biotransformation process was continued (Fig. 1). The biotransformation process was completed in a 24-h period, with the reaction yield of 99% and the optical purity of 98.9% for *R*-(+) compound **2**. At the end of the biotransformation process, cells were removed by filtration, and product was recovered from the filtrate. Filtrate was adjusted to pH 8.0 and allowed to stand at room temperature for 1 h. The precipitate was filtered and dissolved in isopropyl alcohol (IPA). Product was precipitated as a HCl salt by addition of isopropanolic HCl. Recrystallization from IPA yielded 24 g of *R*-(+) compound **2**. Overall, 80% recovery of product was obtained in 99% HPLC purity and 98.8% optical purity.

Purification of the enzyme that reduces **1** to *R*-(+) compound **2** was carried out from cell extracts of *M. ramanniana* ATCC 38191 (Table 6). Overall, 55-fold purification of enzyme was achieved, which gave a specific activity [nmol *R*-(+) compound **2** formed/min/mg protein] of 268. The purified enzyme has a molecular weight of 29,000 daltons as determined by gel filtration and consists of a single subunit of 29,000 daltons as judged from SDS-polyacrylamide gel electrophoresis. The purified enzyme was inhibited by *p*-hydroxy mercuribenzoate, 1,10-phenanthroline, and 2,2′-bipyridyl at 1 mM concentration of each inhibitor. This indicates the involvement of a sulfhydryl group (and perhaps metals) at the enzyme active site.

ANTIARRHYTHMIC AGENT (β-BLOCKER)

Stereoselective reduction of N-*4-(1-oxo-2-chloroacetyl ethyl) phenyl methane sulfonamide* (**3**). Larsen and Lish (25) reported the biological activity of a series of phenethanolamine-bearing alkyl sulfonamido groups on the benzene ring. Within this series, some compounds possessed adrenergic and antiadrenergic actions, including α-adrenergic receptor block or receptor stimulation, β-adrenergic receptor block or receptor stimulation. D-(+) Sotalol (Scheme 2) is a β-blocker (26). Unlike other β-blockers, it has class III antiarrhythmic properties (27). The β-adrenergic blocking drugs, such as propranolol {(1-[(-methylethyl) amino]-3-(1-naphthalenyloxy)-2-propanol} and sotalol, have been separated chemically into the *dextro* and *levo* rotatory optical isomers, and the activity of the *levo* isomer has been demonstrated to be 50 times that of the corresponding *dextro* isomer (28). In this section, we describe the stereoselective microbial reduction of **3** to the corresponding (+)-**4**, or (+)-*N*-4-(1-hydroxy-2-chloroacetyl ethyl) phenyl methane sulfonamide. The compound (+)-**4** is a key chiral intermediate for the synthesis of D-(+)-sotalol (Scheme 2).

TABLE 5
Reduction of 1 to [BMY-14802] by Cell Suspensions of *Mortierella ramanniana:* Effect of Substrate Concentration[a]

Concentration of substrate **1** (g/L)	Reaction time (h)	Concentration of **2** [BMY 14802] (g/L)	Conversion (%)	Optical purity *R*-(+)-2 (%)
2	24	2	100	99.2
4	24	3.6	90	98.9
6	48	3.8	63	94.5

[a]Cell suspensions of *M. ramanniana* ATCC 38191 (8% wt/vol, wet cells) were supplied with glucose (75 mg/mL) and substrate as indicated. Reactions were conducted at 28°C, 280 rpm, at pH 5.5. Concentrations of **1** and **2** were determined by GC, and the optical purity of **2** was determined by chiral HPLC. See Tables 1 and 2 for abbreviations.

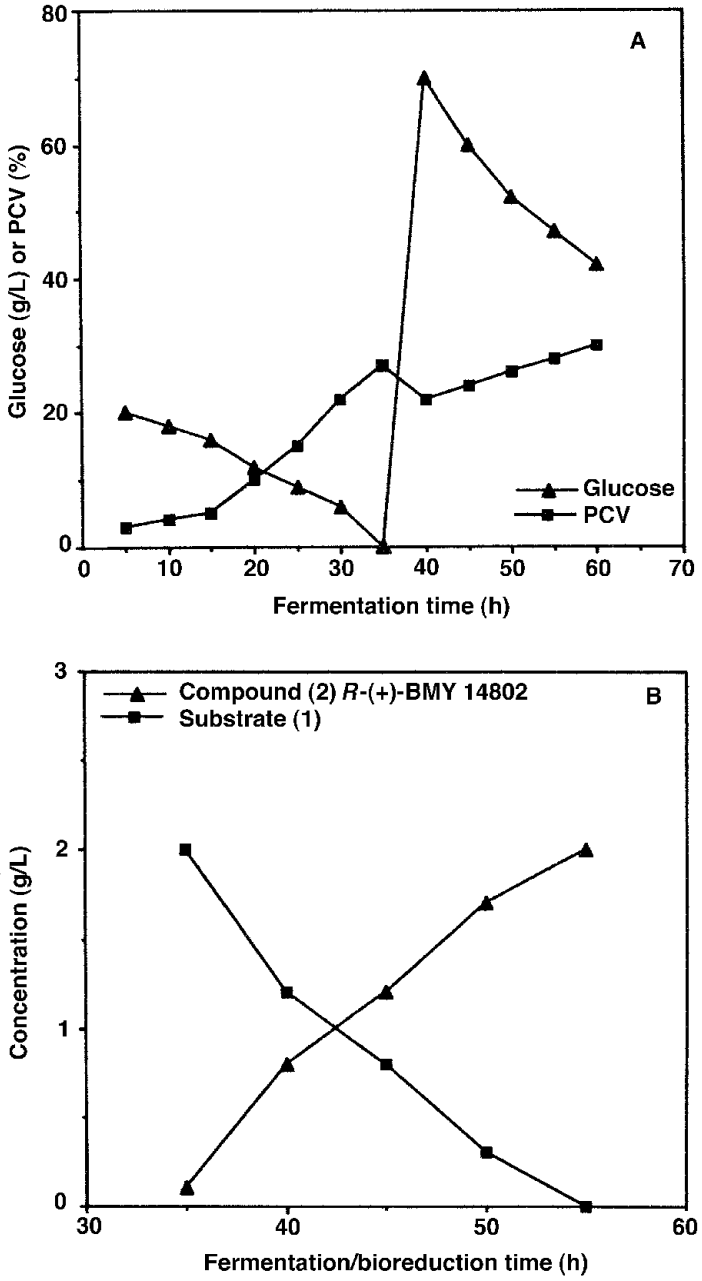


FIG. 1. Growth of *Mortierella ramanniana* in a 15-L fermentor during single-stage fermentation/biotransformation. (A) Residual glucose and packed cell volume (PCV); (B) substrate **1** and compound **2**. Abbreviations: **1**, 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone; **2**, *R*-(+)-1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanol [*R*-(+)-BMY 14802].

Materials and Methods

Microorganisms. Microorganisms (Table 7) were obtained from our culture collection in the Microbiology Department of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the American Type Culture Collection. Microorganisms were stored at –90°C in vials.

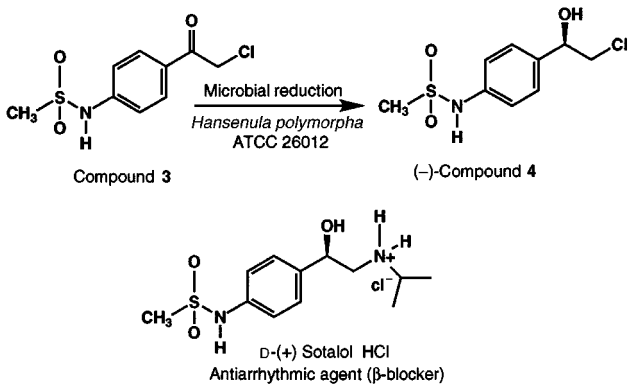
Growth of microorganisms. For screening purposes, one vial of each culture was used to inoculate 100 mL of medium B, which contained 2% glucose, 1% malt extract, 1% yeast extract, and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 25°C, 280 rpm for 48 h. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 10 mM potassium phosphate buffer pH 6.8, and used for biotransformation studies.

*Transformation of compound **3** by microbial cell suspensions.* Cells of various microorganisms were suspended in 10 mL of 10 mM potassium phosphate buffer pH 6.8 at 20% (wt/vol, wet cells) cell concentration and supplemented with 20 mg compound **3** and 750 mg glucose. Transformation of

TABLE 6
Purification of BMY 14802 Reductase from *Mortierella ramanniana* ATCC 38191[a]

Step	Volume (mL)	Total units (nmol/min)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Cell extracts	800	5104	1040	4.9	100	1
DE-52 pooled fraction	173	3962	38	105	77.6	21.4
Phenyl sepharose chromatography	15	1700	6.5	261	33	53
Mono-Q column chromatography	6	1494	6	268	29	55

[a]Reaction mixture for enzyme assay in 1 mL of 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 5.5) contained 0.67 mM NADP, 10 mg glucose, 4 units of glucose dehydrogenase, and 1 mg of **1**. Reactions were conducted at 25%C, 100 rpm. Concentrations of **1** and **2** were determined by GC. See Tables 1 and 2 for abbreviations. For additional details on methods, see the Materials and Methods section.



SCHEME 2

compound **3** was conducted at 28°C, 200 rpm on a rotary shaker. Periodically, 2-mL samples were taken and extracted with 4 mL ethyl acetate. After centrifugation, the ethyl acetate phase was collected and filtered through a 0.2-µm LID/X filter. Clarified ethyl acetate filtrate (0.6 mL) was analyzed by GC to determine the concentrations of compounds **3** and **4**. The remaining ethyl acetate filtrate (3 mL) was dried under a stream of nitrogen. The oily residue obtained was dissolved in a mobile phase (hexane/*n*-butanol, 65:35) and analyzed by chiral HPLC to determine the optical purity of compound **4**.

Analytical methods. The reduction of compound **3** to compound **4** in the reaction mixture samples was monitored by GC with FID. A Hewlett-Packard fused-silica capillary column (cross-linked methyl silicone, 15 m long, 0.33-µm film thickness, 0.32 mm i.d.) was used at 250°C detector temperature and 190°C injection temperature. The initial oven temperature was 195°C, and the final temperature was 205°C. Temperature was increased at 0.5°C/min. Helium was used as the carrier gas at 25 mL/min. The retention time for substrate **3** was 10.2 min, and for the corresponding reduced product **4** it was 9.6 min.

The optical purity of product **4** was determined by chiral HPLC. A Bakerbond Chiralcel OB column was used at ambient temperature. Injection volume was 20 µL, mobile phase was 65% hexane with 35% *n*-butanol, flow rate was 0.5 mL/min, and detector wavelength was 230 nm. The retention time was 16.32 min for the *R*-(+) enantiomer and 13.63 min for the *S*-(–) enantiomer.

Gas chromatography–mass spectrometry (GC–MS) analysis. A GC–MS method was developed to obtain MS data on compound **4**. A Hewlett-Packard ultra 2 column (25 mm, 0.2 mm i.d., 0.33 µm) was used at 260°C injector temperature and 260°C detector temperature. The initial oven temperature was 280°C. Temperature was increased at 10°C/min, starting at 5 min.

Nuclear magnetic resonance (NMR) analysis. The H NMR

TABLE 7
Microbial Reduction of *N*-4-(1-oxo-2-chloroacetyl ethyl) Phenyl Methane Sulfonamide 3[a]

Microorganisms	Reaction time (h)	Conversion to alcohol **4** (%)	Optical purity of alcohol **4** (%)
Pichia methanolica ATCC 58403	40	80	26
Pullularia pullulans ATCC 16623	68	73	75
Hansenula polymorpha ATCC 26012	22	95	99
Trichoderma polysporium SC 14962	22	86	77
Rhodococcus sp. ATCC 29675	22	53	91
Rhodococcus sp. ATCC 21243	22	64	97.5
Nocardia salmonicolor SC 6310	22	45	99

[a]Cell suspensions (20% wt/vol of wet cells) of each microbial culture in 10 mL of 10 mM phosphate buffer were supplemented with 20 mg of substrate and 750 mg glucose. Bioreduction of **3** was carried out at 28°C and 200 rpm on a rotary shaker. The concentrations of substrate **3** and product **4** (the corresponding alcohol to **3**) were determined by GC, and the optical purity of **4** was determined by chiral HPLC with a Chiralcel OB column. Abbreviation: **4**, (+)-*N*-4-(1-hydroxy-2-chloroacetyl ethyl) phenyl methane sulfonamide.

spectra were recorded at ambient temperature on a Brucker (Karlsruhe, Germany) AP-300 MHZ spectrometer with deuterochloroform as solvent and 3% tetramethylsilane as an internal standard.

Growth of Hansenula polymorpha *ATCC 26012 in a fermentor. Hansenula polymorpha* ATCC 26012 was grown in a 380-L fermentor with 250 L of medium B containing 0.25% antifoam. Growth consisted of inoculum development stages and fermentation.

Flask inoculum development was carried out in F1 and F2 stages. In the F1 stage, frozen vials of *H. polymorpha* cultures were inoculated into 100 mL of medium B in 500-mL flasks and incubated at 25°C, 280 rpm for 48 h. In the F2 stage, 100 mL of F1-stage cultures was inoculated into 1.5 L of medium B in 4-L flasks and incubated at 25°C, 180 rpm for 24 h. A seed fermentor with medium B was inoculated with the entire contents of a single F2-stage inoculum. Growth in a seed fermentor was conducted at 28°C, 10 psig, 200 Lpm aeration, 120 rpm agitation for 12 h.

Fermentors with 250 L of medium B were inoculated with 13 L of seed fermentor-grown inoculum. Fermentations were conducted at 25°C, 150 rpm agitation, 150 Lpm aeration. Upon depletion of carbon source (generally 24 h), cells were harvested with the aid of a Sharples centrifuge. Wet cell pastes were collected and stored at −60°C until further use. About 8 kg of wet cell pastes was collected from each fermentation.

*Transformation of **3** by cell suspensions of* H. polymorpha. Transformation of **3** to **4** was conducted in a fermentor in 3-L batches at 25°C, 200 rpm. Compound **3** (4 mg/mL), glucose (75 mg/mL), and cells (20%, wet cells) were used. During bioreduction, the substrate and product concentrations were determined by in-process GC assays, and the optical purity of **4** was determined by chiral HPLC.

*Isolation of compound **4**.* Typically, 3 L fermentation broth with 11 g of **4** was extracted twice with equal volumes of ethyl acetate. The organic phase was separated and dried over anhydrous sodium sulfate. The ethyl acetate extract was concentrated under reduced pressure at 40°C. The oily liquid obtained was dissolved in mobile phase that consisted of 25% methanol in deionized water and purified on a preparative SepTech HPLC (Wakefield, RI). The column size was 2 × 10 inch and was packed with C18 (25 µ) material purchased from SepTech. The mobile phase was 25% methanol in deionized water. The detection wavelength was 230 nm, and the flow rate was 100 mL/min. The fraction containing compound **4** was recovered, and methanol was removed under reduced pressure. The water was removed by freeze-drying. About 8.2 g of compound **4**, with 97% GC purity and >99% optical purity, was obtained.

Preparation of cell extract. Hansenula polymorpha ATCC 26012 was suspended in buffer B (50 mM potassium phosphate buffer, pH 6.8 with 1 mM DTT) at a 20% (wt/vol, wet cells) cell concentration. Cell suspensions were disintegrated by three passages through a Microfluidizer (Newton, MA) at 13,000 psi pressure at 4°C. Disintegrated cells were centrifuged at 20,000 × *g* for 40 min at 4°C. The supernatant solution obtained after centrifugation is referred to as cell extract.

*Reduction of compound **3** by cell extract.* One liter of cell extract was supplemented with 2.5 g of compound **3**, formate dehydrogenase (500 units), 0.7 mM NAD^+, and 25 g sodium formate. The reaction was carried out in a pH-stat at pH 6.8, 150 rpm agitation, and 28° C. Periodically, samples were taken and analyzed for reaction yield and optical purity of compound **4** as described earlier. After complete conversion of **3** to **4**, the reaction mixture was extracted with 2 vol ethyl acetate, and the product was isolated by preparative HPLC as described earlier. About 2.1 g of compound **4**, with 98% purity and by GC 99.4% optical purity, was isolated.

Results

Microorganisms were screened for the transformation of compound **3** to compound **4**. As shown in Table 7, among cultures evaluated, *Rhodococcus* spp. ATCC 29675 and ATCC 21243, *Nocardia salmonicolor* SC 6310, and *H. polymorpha* ATCC 26012 gave desired product **4** in >90% optical purity. *Hansenula polymorpha* ATCC 26012 catalyzed the efficient conversion of compound **3** to compound **4** in 95% reaction yield and >99% optical purity.

Growth of *H. polymorpha* ATCC 26012 culture was carried out in a 380-L fermentor as described in the Materials and Methods section. Cells harvested from the fermentor were used to conduct transformation in a 3-L preparative batch. Cells were suspended in 3 L of 10 mM potassium phosphate buffer pH 7.0 (wt/vol, wet cells) at a cell concentration of 20%. Compound **3** (12 g) and glucose (225 g) were added to the fermentor, and the reduction reaction was carried out at 25°C, 200 rpm, pH 7. About 95% conversion of compound **3** to compound **4** was obtained in 20-h reaction period (Table 8). The isolation of compound **4** from the reaction mixture was carried out by preparative HPLC as described in the Materials and Methods section to obtain 8.2 g of product. The isolated **4** gave a specific rotation $[\alpha]_D$ Na of +20 and optical purity of >99% as analyzed by chiral HPLC. The mass spectra and H NMR spectra of isolated compound **4** and standard compound **4** were virtually identical. Reduction of **3** to **4** was also carried out with cell extracts of *H. polymorpha* ATCC 26012. Formate dehydrogenase was used to regenerate cofactor NADH, required for the reduction reaction. After 18 h reaction time, 95% conversion of **3** to **4** was obtained. About 2.1 g of **4** was isolated from the reaction mixture. The isolated **4** gave 98% purity by GC area and 99.4% optical purity.

ANTIHYPERTENSIVE DRUG

Biotransformation of N*ε-carbobenzoxy (CBC)-L-lysine* (**7**) *to* N*ε-CBZ-L-oxylysine* (**5**). As requirements for optical purity of pharmaceuticals become more stringent, enzymic methods for production of chiral synthons are receiving increased attention. Compound **5** is an intermediate needed for synthesis of a new angiotensin-converting enzyme inhibitor with the trivial name ceronapril (29), or **6**: (*S*)-1-[6-amino-2-{[hydroxy(4-phenylbutyl)phosphinyl]oxy}1-oxohexyl]-L-proline], which is being

TABLE 8
Preparative Scale Reduction of 3 by *Hansenula polymorph*[a]

Reaction time (h)	Yield of alcohol **4** (g/L)	Optical purity of alcohol 4 (%)
4	1.1	n.d.
8	1.8	n.d.
12	2.4	n.d.
16	3.2	99.4
20	3.8	99.5

[a]Bioreduction of **3** to **4** (the corresponding chiral alcohol to **3**) was carried out in a 5-L fermentor. Cell suspension (3 L) was supplemented with 12 g of substrate and 100 g of glucose. Reaction was conducted at 28°C, 200 rpm. Substrate **3** and product **4** concentrations were determined by GC, and the optical purity of **4** was determined by chiral HPLC. See Table 7 for abbreviations.

developed for treatment of hypertension (Scheme 3). The precursor of this intermediate, L-oxylysine, has also been used to prepare an analog of the antibiotic butirosin (30). Compound **5** has been prepared from L-lysine by diazotization with sodium nitrite and sulfuric acid, followed by treatment of the product L-oxylysine with benzyl chloroformate (29) As an alternative approach, we have explored the enzymic oxidation of L-lysine or **7** to the keto acid **8**, followed by reduction with a dehydrogenase of appropriate enantioselectivity.

Materials and Methods

Materials. L-2-Hydroxyisocaproate (L-HIC)dehydrogenase was a generous gift from Dr. H. Schütte, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. Commercial sources were: lysyl oxidase, Yamasa Shoyu Co., Ltd. (Choshi, Chiba, Japan); L-amino acid oxidase Type 1 from *Crotalus adamanteus*, Sigma Chemical Company; polyethyleneglycol (PEG)-2000-NADH, Braunschweiger Biotechnologie (Braunschweiger, Germany).

Enzyme assays. L-Amino acid oxidase was monitored by coupling the H_2O_2 evolved in the reaction to the oxidation of *o*-dianisidine catalyzed by horseradish peroxidase (31). The reaction solution contained in 1 mL: 50 mM potassium phosphate pH 7.4, 1 mM L-lysine or lysine derivative, 0.2 mM *o*-dianisidine, and 10 µg (2.17 units) horseradish peroxidase. The reaction was started by the addition of L-amino acid oxidase, and the increase in absorbance at 460 nm was monitored (absorbance = 11.3 $mM^{-1}cm^{-1}$). All continuous spectrophotometric assays were performed at 25°C.

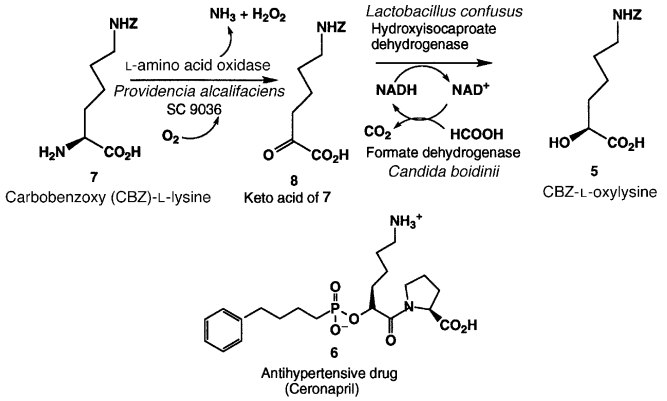
The reaction solution for L-HIC dehydrogenase, coupled to L-amino acid oxidase, contained, in 1 mL: 0.1 M potassium phosphate pH 7.4, 1 mM lysine or lysine derivative, 2000 units bovine liver catalase, and 1.7 units L-HIC dehydrogenase. Reactions were started by addition of L-amino acid oxidase, and the absorbance decrease at 340 nm was monitored.

The catalase assay contained, in 1.0 mL: 0.1 M potassium phosphate pH 7.4, and 0.06% H_2O_2. The absorbance decrease after addition of enzyme was monitored at 240 nm (absorbance = 0.0436 mM^{-1} cm^{-1}). Protein was determined by the dye-binding method of Bradford (24) with bovine serum albumin as standard.

Analytical methods. HPLC analyses of compound **7** transformations were performed with a Hewlett-Packard Hypersil C_{18} 20 × 4.6 cm column, with 5 µm particle size. Column temperature was 40°C, mobile phase was 37% methanol and 63% water with 0.05% H_3PO_4, flow rate was 1 mL/min, detection wavelength was 215 nm, and injection volume was 5 µL. Retention times were 9.8 min for carbobenzoxy-6-amino-2-oxohexanoic acid (**8**), 13.7 min for **5**, and 23.3 min for **7**, with the keto acid (**8**) peak skewed toward higher retention times. Samples were boiled for 2 min, centrifuged, and filtered before HPLC analysis.

Optical purity of **5** was determined by derivatization and separation of diastereomers by GC (32).

Microbial growth conditions. Providencia alcalifaciens SC 9036 was obtained from the Squibb culture collection and is ATCC strain 13159. It was grown on medium C, described by Szwajcer *et al.* (33), which contained 1% peptone, 0.2% casein hydrolysate, 0.2% yeast extract and 0.6% NaCl at pH 7.2 to 7.4. Growth was at 37°C, 100 rpm in shake flasks. A 200-mL overnight culture was used to inoculate a 15-L tank that contained the same medium at 37°C, stirred at 200 rpm, and aerated at 20 Lpm. After 11 h, cells were harvested by centrifugation, washed with 50 mM potassium phosphate buffer pH 7.4, and stored frozen at −18° until used for biotransformation.



SCHEME 3

Enzyme localization. Cells (1.4 g wet weight) were collected from a 17-h shake flask culture and sonicated in 15 mL 50 mM potassium phosphate pH 7.4. Debris was removed by centrifugation for 10 min at 12,000 × *g*. The extract supernatant was centrifuged for 1 h at 101,000 × *g* to give a supernatant and pellet fraction. The pellet was resuspended in 2 mL 50 mM potassium phosphate, pH 7.4. Oxidation of **7** was measured by incubating 5 mM of **7** with 0.6 mL of extract or with 101,000 × *g* pellet or supernatant fractions in 1.5 mL of 0.1 M potassium phosphate pH 7.4 and 3000 units catalase for 16 h. The amount of **8** produced by 1 mg of *C. adamanteus* venom L-amino acid oxidase under these conditions was used as a standard for 100% conversion in the HPLC assay.

Results

The enzymatic approach to synthesis of compound **5** is shown in Scheme 3. *Crotalus adamanteus* (rattlesnake) venom L-amino acid oxidase has been reported to oxidize compound **7** to the corresponding keto acid **8** (34), and L-HIC dehydrogenase from *Lactobacillus confusus* converts the keto acid **8** to compound **5** (Robison, R.S., M.G. Doremus, and L.J. Szarka, unpublished results). Lysyl 2-oxidase from *Trichoderma viride* was tested as a microbial alternative to the snake venom enzyme. Substrate specificity studies (Table 9) showed that the activity of the enzyme is greatly reduced with ε-substituted lysine derivatives. Activity with *N*ε-formyl-, acetyl-, trifluoroacetyl-, *t*-butoxycarbonyl (BOC)-, or **7** was less than 2% of the activity with lysine. There was no activity with L-oxylysine. On the other hand, the snake venom oxidase was active with the ε-substituted lysine derivatives but had little activity with L-lysine. The methyl and ethyl esters of lysine were substrates for the *T. viride* oxidase but were not utilized by the snake venom enzyme.

The product from lysine oxidation by *T. viride* lysyl oxidase was not a substrate for L-HIC dehydrogenase (Table 9) or for several other dehydrogenases that were screened at pH 7.4 or 9. With snake venom L-amino acid oxidase, *N*ε-formyl-, acetyl-, CBZ-, or trifluoroacetyl-L-lysine were good substrates when the oxidation was coupled to L-HIC dehydrogenase, but *N*ε-*t*-BOC-L-lysine was not an effective substrate. The product of lysine oxidation by the *T. viride* enzyme has been shown to cyclize to a Schiff base (35) and this may interfere with utilization by L-HIC dehydrogenase.

Alternatively, although L-HIC dehydrogenase shows rather broad specificity (36) it may require a substituent on the ε-amino of lysine for activity.

*Synthesis of compound **5**.* HPLC analysis showed that 0.3 units/mL *T. viride* lysyl oxidase, coupled to 0.8 units/mL L-HIC dehydrogenase, was able to convert 1 mg/mL compound **7** to 1 mg/mL compound **5** after 48 h in a reaction mixture that also contained 1 mM NAD, 0.2 M sodium formate, 0.7 units/mL formate dehydrogenase, and 1250 units/mL catalase in 0.1 M potassium phosphate at pH 8. In an effort to find a more active **7** oxidase activity, several microbial strains known to possess L-amino acid oxidase were screened. Four strains of *Proteus* and *Providencia* were found to oxidize compound **7** to the keto acid **8**. *Providencia alcalifaciens* SC 9036 gave the highest conversion to keto acid **8** and was selected for further study.

Compound **7** was converted to **5** in 95% yield by the following procedure. CBZ-L-lysine **7** (5.6 g) was added to 1 L of a solution that contained 0.1 M potassium phosphate pH 7.4 and 10 g of *P. alcalifaciens* SC 9036 cells, and the mixture was incubated at 200 rpm for 27 h at 30°C. Compound **7** is nearly insoluble at pH 7.4 but went into solution as the reaction proceeded. After 24 h, HPLC analysis indicated complete conversion to the keto acid **8**. After cells were removed by centrifugation, 0.2 M sodium formate, 1 mM NAD, 32 units formate dehydrogenase, and 66 units L-HIC dehydrogenase were added, and the solution was incubated for 64 h at 28°C. HPLC analysis indicated conversion to 18.9 mM compound **5**. The isolated product (m.p. 74–75°C, standard m.p. 76°C) had 98.4% HPLC homogeneity and 98.5% optical purity. ^{1}H and ^{13}C NMR spectra were identical with those from the chemically produced compound: Infrared (IR) (KBr): ν (O-H) 3335 cm^{-1}; ^{1}H NMR (CD_3OD): δ, 1.37–1.85 (*m*, 6H), 3.03–3.18 (*t*, 2H), 4.03–4.13 (*q*, 1H), 4.75–5.00 (*bm*, HOD), 5.00–5.03 (*s*, 2H), 7.20–7.40 (*m*, 5H) ppm; ^{13}C NMR (CD_3OD): δ, 23.7, 30.9, 35.3, 41.9, 67.6, 71.6, 129.0, 129.2, 129.7, 138.7, 159.2, 178.1 ppm.

TABLE 9
Substrate Specificities of L-Amino Acid Oxidase and L-2-Hydroxyisocaproate (HIC) Dehydrogenase

Enzyme	Substrate	Specific Activity (units/mg oxidase)
Lysyl oxidase	L-Lysine	1.663
Trichoderma viride	L-Oxylysine	0
	*N*ε-Acetyl-L-lysine	0.003
	*N*ε-Formyl-L-lysine	0.033
	*N*ε-BOC-L-lysine[a]	0
	*N*ε-Trifluoacetyl-L-lysine	0.027
	*N*ε-CBZ-L-lysine[a]	0.003
	*N*ε-CBZ-L-lysine methyl ester	0
	L-Lysine methyl ester	0.847
	L-Lysine ethyl ester	1.083
L-Amino acid oxidase	L-Lysine	0
Crotalus adamanteus	L-Oxylysine	0.001
	*N*ε-Acetyl-L-lysine	0.405
	*N*ε-Formyl-L-lysine	0.338
	*N*ε-BOC-L-lysine	0.254
	*N*ε-Trifluoroacetyl-L-lysine	0.406
	*N*ε-CBZ-L-lysine	0.405
	*N*ε-CBZ-L-lysine methyl ester	0.006
	L-Lysine methyl ester	0.001
	L-Lysine ethyl ester	0
T. viride lysyl oxidase	L-Lysine	0
coupled to	L-Lysine methyl ester	0
HIC dehydrogenase	L-Lysine ethyl ester	0
C. adamanteus	*N*ε-Acetyl-L-lysine	0.062
L-amino acid oxidase	*N*ε-Formyl-L-lysine	0.12
coupled to	*N*ε-BOC-L-lysine	0
HIC dehydrogenase	*N*ε-Trifluoroacetyl-L-lysine	0.028
	*N*ε-CBZ-L-lysine	0.12

[a]Abbreviations: BOC, *t*-butoxycarbonyl-; CBZ, carbobenzoxy- .

TABLE 10
Localization of *N*ε-Carbobenzoxy-L-lysine (7) Oxidase and Catalase Activities in *Providencia alcalifaciens*

Fraction	Catalase (units/mg)	Oxidase[a] (% conversion)	Protein (mg/mL)
Cell extract	1328	97.6	4.3
10,100 × *g* pellet	318	96.8	5.1
101,000 × *g* supernatant	2197	0	2.4

[a]Percentage conversion of 5 mM compound **7** to the keto acid carbobenzoxy-6-amino-2-oxohexanoic acid (**8**), measured by HPLC as described in the Materials and Methods section. See Table 1 for abbreviations.

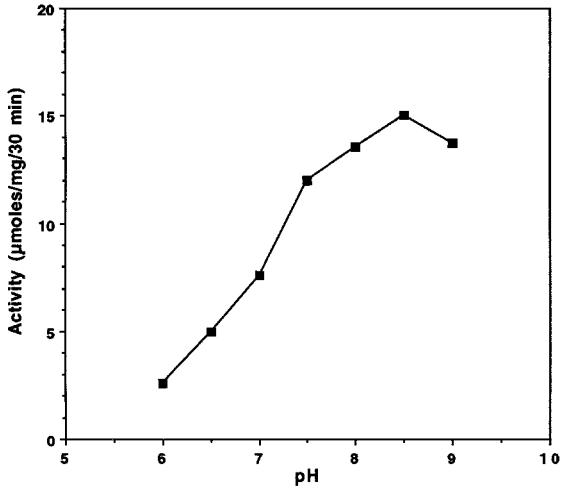


FIG. 2. Effect of pH on biotransformations. Oxidation of *N*ε-carbobenzoxy (CBZ)-L-lysine (**7**): 100 mM potassium phosphate buffer, 1 mM **7** and 5 mg (wet weight)/mL *Providencia alcalifaciens* were incubated at 28°C and 200 rpm for 30 min. A high-pressure liquid chromatography assay was used to determine activity.

L-Amino acid oxidase has been associated with a particulate fraction of the cell in several *Proteus* species (37,38). Cells, extracts, or the particulate fraction from *Providencia alcalifaciens* were effective in oxidizing **7** to the keto acid **8**, but the 101,000 × *g* supernatant had no activity (Table 10). No **7** oxidase activity was found in the 101,000 × *g* pellet when H_2O_2 production was measured. When conversion to the keto acid was measured by HPLC, **7** oxidase was found in the particulate fraction and was absent from the supernatant (Table 10). High catalase activity was found in both the pellet and supernatant fractions, with higher specific activity in the supernatant. The high level of catalase accounts for the inability to measure **7** oxidation by H_2O_2 production.

The pH optimum for oxidation of **7** by *P. alcalifaciens* cells, coupled to L-HIC dehydrogenase, was about 8.8 (Fig. 2). The optimal pH for reduction of the keto acid **8** by L-HIC dehydrogenase, with NADH, was about 6.6 (Fig. 3). At pH 7.4 and 0.2 mM NADH, the apparent K_m for **8** was 10.4 mM, and the Lineweaver-Burk plot was linear. With 0.2 mM PEG-NADH, the apparent K_m for the keto acid was 2.2 mM and the apparent V_{max} was 17% of the value with NADH. At pH 7.4 and 4 mM compound **8**, apparent K_m for NADH was 12 mM, and there was substrate inhibition by concentrations of NADH above 0.2 mM. Under the same conditions, the apparent K_m for PEG-NADH was 50 mM, and there was substrate inhibition above 0.4 mM. Apparent V_{max} under these conditions with PEG-NADH was 71% of the value with NADH. Schütte *et al.* (36) have reported that NADH concentrations greater than 0.24 mM inhibit L-HIC dehydrogenase.

ANTIINFECTIVE DRUG (TIGEMONAM)

Enzymatic synthesis L-β-hydroxyvaline (**9**) *from sodium α-keto-β-hydroxyisovalerate* (**16**). During the past several years, syntheses of α-amino acids have been pursued intensively because of their importance as building blocks of compounds of medicinal interest (39–42). New methods have been developed for the asymmetric synthesis of β-hydroxy-α-amino acids (43–46) because of their utility as starting materials for the total synthesis of monobactam antibiotics. Compound **9** is a key chiral intermediate needed for the synthesis of tigemonam, or *S*-[*Z*]-{[1-(2-amino-4-thiazolyl)-2-{[2,2-dimethyl-4-oxo-1-(sulfooxy)-3-azetidinyl]amino}-2-oxoethylidene]amino}oxyacetic acid (**10**) (Scheme 4), a new orally active monobactam (47–49).

Procedures for the synthesis and resolution of racemic β-hydroxyvaline (50–53) and the asymmetric synthesis of D-β-hydroxyvaline have been reported (54), but the direct synthesis of **9** has not been accomplished. In this section, we describe the direct synthesis of **9** by the reductive amination of **16** with leucine dehydrogenase from *Bacillus sphaericus* ATCC 4525. Leucine dehydrogenases from *B. sphaericus* (55), *B. cereus* (56), and *B. megaterium* (57) have been used for the synthesis of branched-chain amino acids but not for hydroxylated amino acids. The *B. sphaericus* enzyme has

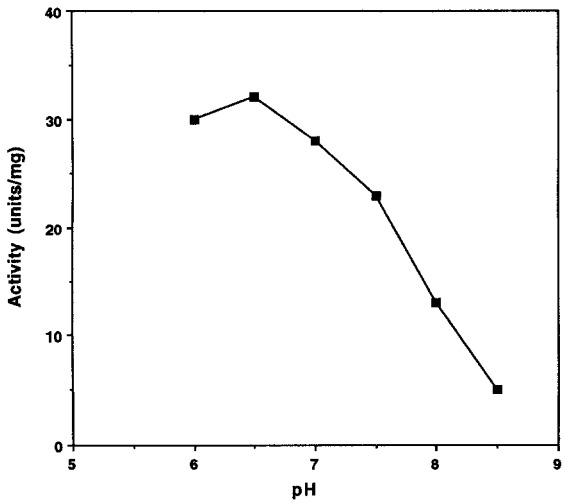
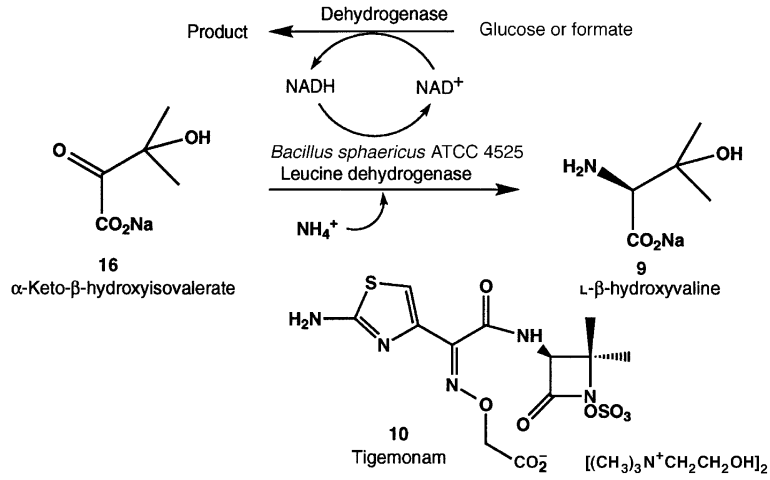


FIG. 3. Reduction of carbobenzoxy (CBZ)-6-amino-2-oxohexanoic acid (**8**) by L-hydroxyisocaproate (HIC) dehydrogenase. Reaction mixture in 1 mL contained 100 mM potassium phosphate buffer, 2 mM Compound **8**, 0.3 mM NADH, and 1 μg L-HIC dehydrogenase.



SCHEME 4

been reported to be inactive for oxidative deamination of serine and threonine (58).

Materials and Methods

Materials. α-Keto-β-bromoisovalerate (**11**), ethyl α-keto-β-bromoisovalerate (**12**), 2-chloro-3,3-dimethyloxiranecarboxylic acid methyl ester (**13**) (glycidic ester **13**), 2-chloro-3,3-dimethyloxiranecarboxylic acid-1-methylethyl ester (**14**) (glycidic ester **14**), 2-chloro-3,3-dimethyloxiranecarboxylic acid-1,1-dimethylethyl ester (**15**) (glycidic ester **15**), **16,** and **9** were synthesized by the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute as described previously (12). Polyethylene glycol-N^6-(2-aminoethyl)-NADH (PEG-NADH) and formate dehydrogenase were gifts from H. Schütte (Gesellschaft für Biotechnologische Forschung mbH). Dextran NAD (attached through C8 to dextran D4133 with a six-carbon spacer) and leucine dehydrogenase from *Bacillus* sp. were purchased from Sigma Chemicals. Glucose dehydrogenase from *B. megaterium* was purchased from Amano International Enzyme Co. (Troy, VA).

Microorganisms. Microorganisms (Table 11) were obtained from our culture collection in the Microbiology Department of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the American Type Culture Collection. Microorganisms were stored at –90°C in vials.

Growth of microorganisms. For screening purposes, one vial of each culture was used to inoculate 100 mL of medium D, which contained 0.25% glucose, 0.3% soytone, 1.7% tryptone, 0.5% NaCl, 0.1% yeast extract, and 0.25% K_2HPO_4 adjusted to pH 7.0 before sterilization. Cultures were grown at 30°C, 280 rpm for 16 h. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 100 mM potassium phosphate buffer pH 7.0, and resuspended in 5 mL of the same buffer. Cultures were disrupted by sonication, and 0.2-mL samples were evaluated in two 1-mL reaction systems for synthesis of **9**: (i) 1 M NH_4Cl, 1 M glucose, 2 mM NAD, and 0.1 M compound **16**. (ii) 1 M ammonium formate, 2 mM NAD, 0.1 M compound **16**, and 40 units/mL formate dehydrogenase from *Candida boidinii*. Reactions were carried out at 30°C for 48 h at an initial pH of 8.2.

Preparation of leucine dehydrogenase. Bacillus sphaericus ATCC 4525 was grown in a 380-L fermentor with 250 L of medium E, which contained 2% yeast extract, 1% glucose, 0.2% K_2HPO_4, and 0.25% antifoam, adjusted to pH 7.0. Growth consisted of inoculum development stages and fermentation.

Flask inoculum development was carried out in F1 and F2 stages. In the F1 stage, frozen vials of *B. sphaericus* ATCC 4525 cultures were inoculated into 100 mL of medium E, contained in 500-mL flasks, and incubated at 30°C, 280 rpm for 16 h. In the F2 stage, 100 mL of F1-stage cultures were inoculated into 1.5 L of medium E in a 4-L flask and incubated at 30°C, 180 rpm for 24 h.

Fermentors with 250 L of medium E were inoculated with 6 L of inoculum from F2 stage. Fermentations were conducted at 30°C, 150 rpm agitation, 150 Lpm aeration. Upon depletion of carbon source (generally 24 h), cells were harvested with the aid of a Sharples centrifuge. Wet cell pastes were collected and stored at –60°C until further use. Cells were suspended in 10 mM potassium phosphate buffer (pH 7.0), containing 0.01% mercaptoethanol, and disrupted by sonication. The sonicate was partially purified by heating for 20 min at 60°C, followed by centrifugation at 28,000 × *g* for 10 min. The supernatant was stored at –20°C as source of leucine dehydrogenase.

Enzyme assays. Leucine dehydrogenase activity was monitored by decrease in absorbance at 340 nm. The reaction mixture in 1 mL contained **16** or α-ketoisovalerate, 0.75 M NH_4Cl–NH_4OH buffer, 0.3 mM NADH, and 3–10 μL cell extract. All components except keto acid were added, and a

TABLE 11
Synthesis of L-β-Hydroxyvaline (9) by *Bacillus* Strains

Strain	Specific activity[a] (units/mg protein)	L-β-Hydroxyvaline (mM) Experiment 1[b]	Experiment 2[c]
B. subtilis SC 13794	0.1289[d]	38	73.2
B. subtilis SC 10253	0.0343	11	69.8
B. megaterium ATCC 39118	0.1232[d]	66.4	74.5
B. megaterium SC 6394	0.3123[d]	61	66.4
B. sphaericus ATCC 4525	0.8705	6.5	57.7
B. cereus SC 14579	0.1892	68.4	76.1
B. pumilis SC 8513	0.2079	2.5	68.4
B. licheniformis SC 12148	0.1039	40.5	73.7
B. thuringiensis SC 2928	0.5259	2.8	73.6
B. brevis SC 3812	0.276	0	34.3
B. coagulans SC 9261	0.0194	0	48.3

[a]Assay contained 10 mM α-keto-β-hydroxyisovalerate (**16**), 0.3 mM NADH, 0.75 mM NH_4Cl–NH_4OH, pH 9.5.
[b]After incubation with 1 M NH_4Cl, 1 M glucose, 100 mM compound **16**, and 2 mM NAD.
[c]After incubation with 1 M ammonium formate, 100 mM compound **16**, 40 units/mL formate dehydrogenase, and 2 mM NAD.
[d]Compound **9** was assayed after 68 h of reaction.

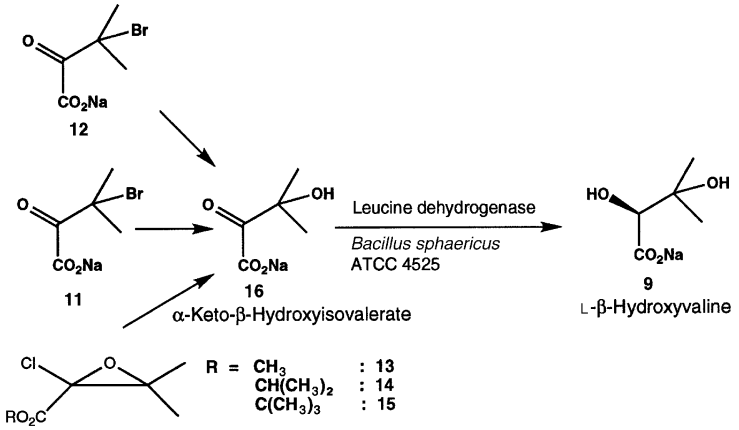
blank value for NADH oxidation was measured before the reaction was started by the addition of keto acid. Further details are given in the legends to the Tables and Figures.

Glucose dehydrogenase activity was monitored by the increase in absorbance at 340 nm. The reaction mixture in 1 mL contained 0.5 M glucose, 0.1 M Tris chloride buffer pH 8.0, 3 mM NAD, and 10 µL cell extract. For determining optimal pH, 0.75 M NH_4Cl–NH_4OH buffer was substituted for Tris chloride. The reaction was started by addition of enzyme. Protein was determined by the dye-binding method of Bradford (24), with bovine serum albumin as standard.

*Preparative synthesis of **9***. 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 compound **16**, 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 to precipitate proteins. Compound **9** 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 mL, 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.

A solution of **9** (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^+ (Dow Chemical, Midland, MI) 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 thin-layer chromatography (silica gel; EtOAc/EtOH/AcOH/H_2O, 5:2:1:1; R_f of **9** = 0.21). Homogeneous fractions were combined, and



SCHEME 5

TABLE 12
Effect of Pyridine Nucleotide Derivatives on Synthesis of L-β-Hydroxyvaline (9)

	L-β-Hydroxyvaline (mM)	
Pyridine nucleotide	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]Reaction was carried out for 22 h at 30°C with 1 M ammonium formate, pH 8.5, 100 mM compound **16**, 0.01% mercaptoethanol, 5 units/mL formate dehydrogenase, and 6 units/mL leucine dehydrogenase from *Bacillus sphaericus*.
[b]Reaction was carried out for 22 h at 30°C with 1 M glucose, 1 M ammonium chloride, pH 8.5, 100 mM compound **16**, 0.01% mercaptoethanol, 5 units/mL glucose dehydrogenase, and 6 units/mL leucine dehydrogenase from *B. sphaericus*. See Table 11 for abbreviation.

water was evaporated under vacuum. This process was repeated two more times. The product was dissolved in water (25 mL) and lyophilized to give 0.593 g (54% yield) of **9** as a light brown solid. The sample was dried over P_2O_5 at 40°C under vacuum for 4 h. M.p., 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° (1.3, 6 N HCl). Anal. calc'd. 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 (Karl Fischer determination).

Results

Distribution of **16** amination activity in *Bacillus* strains was examined. Leucine dehydrogenase activity is mainly found in *Bacillus* strains (56). Screening of *Bacillus* strains was carried out to find a strain with high specific activity for reductive amination of **16** and to identify any strains able to regenerate NADH for the reaction. Of various strains screened (Table 11), *B. sphaericus* ATCC 4525 has the highest specific activity. *Bacillus megaterium* ATCC 39118 has been selected for high levels of glucose dehydrogenase (57) and was able to produce **9** when glucose, NAD, NH_4Cl, and **16** 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 **9** when provided with glucose, NAD, NH_4Cl, and **16**. *Bacillus cereus* ATCC 14579 was the only strain able to produce significant amounts of **9** when intact cells were provided with glucose and **16**, and gave a maximum conversion of about 50%.

Enzymatic reductive amination of **16** by leucine dehydrogenase, coupled to formate dehydrogenase, was carried out as outlined in Scheme 4. A solution of 0.5 M compound **16** from compound **12**, 2 M ammonium formate, 2 mM NAD, 1.5 mg (50 units) leucine dehydrogenase from *Bacillus* sp., and 40 mg (80 units) formate dehydrogenase from *C. boidinii* in 16 mL of buffer (pH 8) was incubated at 30°C for 41 h. HPLC analysis of the reaction mixture indicated a 71% conversion of the ester **12** to compound **9**. Compound **9** was isolated from the reaction as described in the Materials and Methods section. HPLC analysis of the recovered material showed that leucine dehydrogenase produced exclusively the L-isomer of β-hydroxyvaline. Compound **16** from compound **11** was also converted to compound **9** in 82% reaction yield by a similar procedure.

Reductive amination of compound **16** by leucine dehydrogenase, coupled to glucose dehydrogenase, was carried out. Both enzymes had optimal pH for activity of 8.5. Rates of **9** 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 in the Materials and Methods section, solutions of **16**, prepared from 0.5 M **12**, 0.25 M **13**, 0.25 M **14**, and 0.25 M **15** (Scheme 5) were converted to **9** in yields of 75, 100, 71, and 83%, respectively.

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 **9** by leucine dehydrogenase (Table 12). Conversely, with glucose dehydrogenase to reduce pyridine nucleotide, PEG-NADH was relatively ineffective for synthesis of **9**, but dextran-NAD was as effective as NAD.

The apparent K_m for **16** (prepared from **12**) was 11.5 mM, compared to a value of 1.06 mM for α-ketoisovalerate, when using heat-treated extracts of *B. sphaericus* ATCC 4525 as source of leucine dehydrogenase. The apparent V_{max} with **16** was 41% of the value with α-ketoisovalerate.

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