Microbial Synthesis of Chiral Intermediates for β-3-Receptor Agonists

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ABSTRACT: Chiral intermediates were prepared by biocatalytic processes for the chemical synthesis of β-3-receptor agonists. These include: (i) the microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone **1** to the corresponding (*R*)-alcohol **2** by *Spingomonas paucimobilis* SC 16113. In the biotransformation process, a reaction yield of >85% and an optical purity of 99.5% were obtained for the desired (*R*)-alcohol **2**; (ii) the enzymatic resolution of racemic α-methyl phenylalanine amide, **3,** and α-methyl-4-hydroxyphenylalanine amide, **5,** by amidase from *Mycobacterium neoaurum* ATCC 25795 to prepare the corresponding (*S*)-amino acids **4** and **6**. Reaction yields of 49.9 and 49 M% (theoretical maximum yield 50 M%) and optical purities of 99 and 94% were obtained for the desired (*S*)-amino acids **4** and **6**, respectively; (iii) the asymmetric hydrolysis of methyl-(4-methoxyphenyl)-propanedioic acid, ethyl diester, **7,** to the corresponding (*S*)-monoester **8** by pig liver esterase. A reaction yield of 96 M% and an optical purity of 96% were obtained for (*S*)-monoester **8** when reactions were carried out in a biphasic system containing 10% ethanol at 10°C.
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KEY WORDS: Asymmetric hydrolysis, biocatalysis, chiral intermediates, pig liver esterase, β-3-receptor agonist, resolution using amidase, stereoselective reduction.

Much attention is currently 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 growing awareness of the importance of chirality as the key to the efficacy of many drug products. It is now known that in many cases only one stereoisomer of a drug substance is required for efficacy; the other stereoisomer is either inactive or exhibits considerably reduced acitivity. Pharmaceutical companies are aware that, where the switch from racemate drug substance to enantiomerically pure compound is feasible, new drugs should be homochiral to avoid the possibility of side effects caused by an undesirable stereoisomer. There is the opportunity to double the use of an industrial process by obtaining a separate patent on the use of a single stereoisomer as a more efficient drug. The physical advantages of enantiomers over racemates may confer processing and formulation advantages, as well as lower doses.

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 usually carried out in aqueous solution. This avoids the use of solvent and other environmentally harmful chemicals used in the chemical processes. Furthermore, microbial cells or enzymes derived from biocatalysis can be immobilized and reused for many cycles. Recently, a number of review articles (1–9) have been published on the use of enzymes in organic synthesis.

β-Adrenoceptors have been classified as β1 and β2 (10). Increased heart rate is the primary consequence of β1-receptor stimulation, while bronchodilation and smooth muscle relaxation are mediated by β2-receptor stimulation. Rat adipocyte lipolysis was initially thought to be a β1-mediated process (10). However, recent results indicate that the receptor-mediated lipolysis is neither β1 nor β2, but "atypical" receptors, later called β3-adernergic receptors (12). β3-Adrenergic receptors are found on the cell surfaces of both white and brown adipocytes, and are responsible for lipolysis, thermogenesis, and relaxation of intestinal smooth muscle (13,14). Consequently several research groups are engaged in developing selective β3 agonists for the treatment of gastrointestinal disorders, type II diabetes, and obesity (15–19). We report here an efficient biocatalytic synthesis of chiral intermediates required for the total chemical syntheses of β3 receptor agonists (Scheme 1) such as BRL 37344, BMS-187257, and BMS 210620 (15,16). These include the stereoselective microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone, **1,** to the corresponding (*R*)-alcohol **2** (Scheme 1); the enzymatic resolution of racemic α-methyl phenylalanine amide, **3,** and α-methyl-4-

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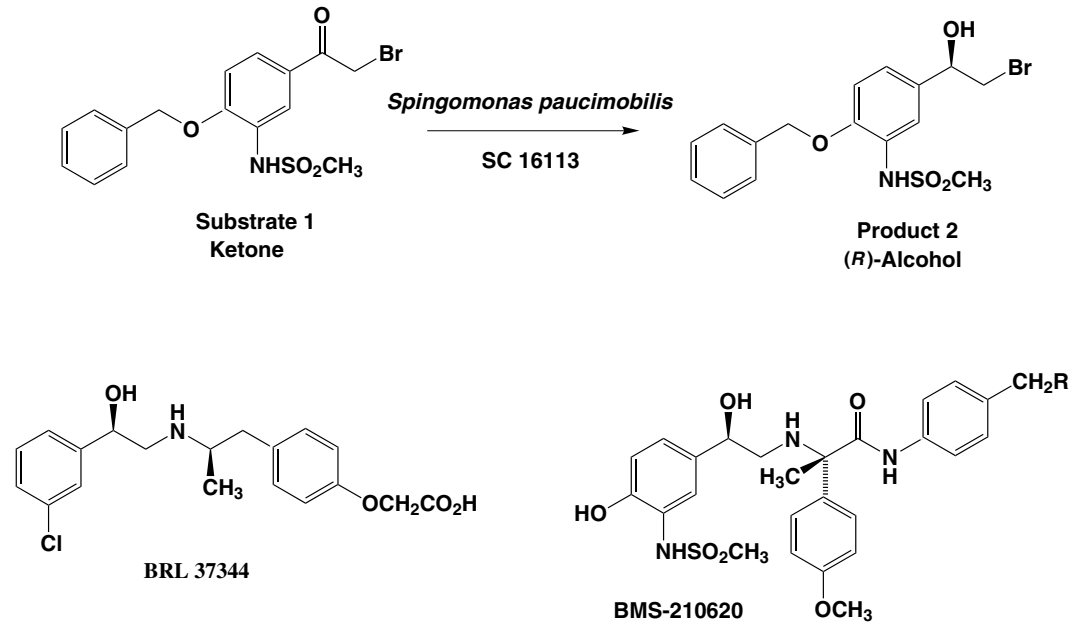


SCHEME 1

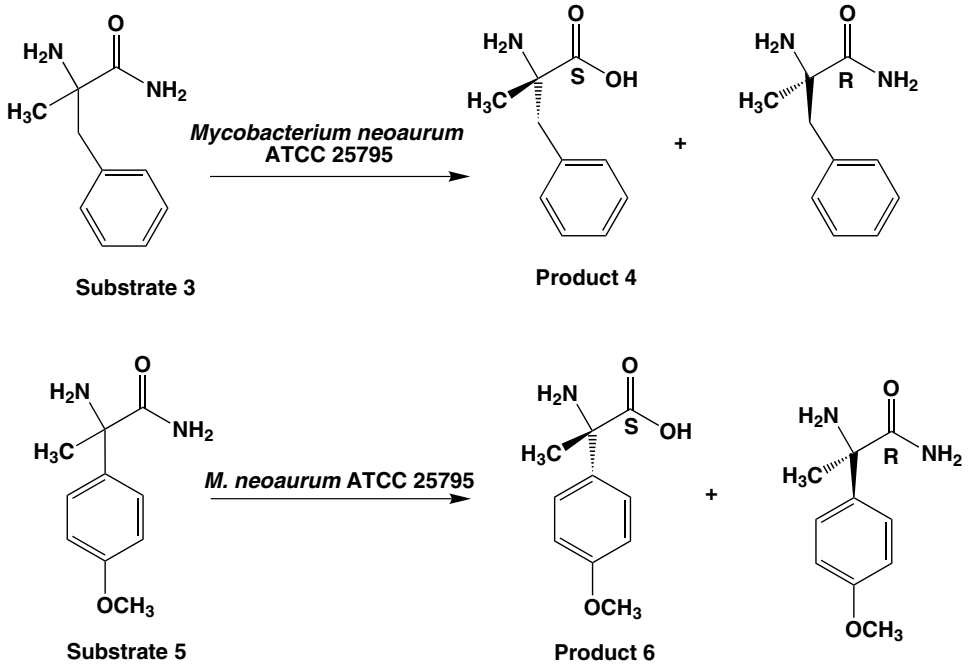
hydroxyphenylalanine amide, **5,** to the corresponding (*S*)-amino acids **4** and **6**, respectively, by an amidase from *Mycobacterium neoaurum* ATCC 25795 (Scheme 2); and the enzymatic asymmetric hydrolysis of methyl-(4-methoxyphenyl)-propanedioic acid, ethyl diester, **7,** to the corresponding (*S*)-monoester **8** by pig liver esterase (PLE) (Scheme 3).

MATERIALS AND METHODS

Materials. Substrates **1, 3, 5,** and **7** and reference standards **2, 4, 6,** and **8** were synthesized by the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ). The physicochemical properties, including spectral characteristics [^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, mass spectra], were in full accord for all these compounds.

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

Growth of microorganisms. For screening purposes, one



SCHEME 2

Diester 7

S-(–)-Monoester 8

SCHEME 3

vial of each culture was used to inoculate 100 mL of medium A containing 1% yeast extract, 1% malt 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 on a rotary shaker. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 25 mM potassium phosphate buffer (pH 6.8), and then used for biotransformation studies.

Biotransformations of compound **1** *by microbial cell suspensions.* Cells of various microorganisms were suspended in 10 mL of 100 mM potassium phosphate buffer, pH 6.0, at 20% (wt/vol, wet cells) cell concentration and supplemented with 10 mg of compound **1** in 0.2 mL of acetonitrile and 250 mg of glucose. The biotransformation of compound **1** was conducted at 28°C, 280 rpm on a rotary shaker. Periodically, samples of 2 mL were taken and extracted with 8 mL of ethyl acetate. After centrifugation, the ethyl acetate phase was collected and filtered through a 0.2 µm LID/X filter (Whatman Inc., Fairfield, NJ). Clarified ethyl acetate filtrate was evaporated under a gentle stream of nitrogen. The oily residue obtained was dissolved in acetonitrile, filtered through a 0.2 µm LID/X filter and analyzed for substrate **1** and product **2** concentrations by high-pressure liquid chromatography (HPLC). The optical purity of compound **2** was determined by chiral HPLC.

TABLE 1
Stereoselective Microbial Reduction of Ketone 1

Microorganisms	Reaction yield[a] of **2** (%)	Optical purity of **2** (%)
Agrobacterium tumefaciens ATCC 15955	7	99
Alcaligenes eutrophus ATCC 17697	15	86
Arthrobacter petroleophagus ATCC 21494	5	99.1
Debaryomyces hansenii ATCC 66354	7	99.2
Mycobacterium sp. ATCC 29676	10	99.5
Rhodococcus rhodochorous ATCC 14347	21	99.9
Hansenula anomala SC 13833	16	96.3
H. anomala SC 16142	18	97
H. saturnus SC 13829	20	99.1
Spingomonas paucimobilis SC 16113	58	99.5

[a]Reaction mixture in 10 mL of cell-suspensions (20% wet cells) contained 10 mg of substrate **1** (dissolved in acetonitrile and supplied at 2% acetonitrile concentration) and 250 mg of glucose. Reactions were carried out at 28°C, 200 rpm on a rotary shaker for 18 h as described in the Materials and Methods section. The reaction yield of **2** and optical purity of **2** were determined by high-performance liquid chromatography as described in the Materials and Methods section. **1**, 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone; **2,** (*R*)-alcohol of **1**.

Fermentation of Spingomonas paucimobilis *SC 16113. Spingomonas paucimobilis* SC 16113 was grown in a 350-L fermenter. The fermentation process consisted of two inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, frozen vials of *S. paucimobilis* SC 16113 culture were inoculated into 100 mL of medium A. The 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 a 25-L germinator that contained 16 L of medium B (2.2% cerelose hydrate, 1% Hy-yest 412, 0.025% Dow Corning antifoam AF, 0.025% SAG 5639 antifoam). The pH of the medium was adjusted to 6.8 before sterilization. The germinator was run at 500 rpm, 28°C, and 15 liters per minute (Lpm) aeration for 24 h. Fermenters with 550 L of medium B were inoculated with 16 L of germinator-grown inoculum. The fermentations were conducted at 28°C and 185 rpm with 150 Lpm aeration. Samples were taken during the course of the fermentation to measure pH, partial volume of solids, relative viscosity, optical density, and glucose concentration. The off-gas carbon dioxide from the fermenter was monitored continuously with a mass spectral (MS) gas analyzer. The fermenter was harvested after the glucose concentration dropped below 3 g/L. To determine the specific enzyme activity of cells during fermentation, the cells were periodically harvested by centrifugation from 200 mL of culture broth. Cell suspensions (20% wt/vol, wet cells) were prepared in 100 mM potassium phosphate buffer (pH 6.8) and supplemented with 1.0 mg /mL of compound **1** and 25 mg/mL of glucose. The biotransformation of compound **1** was conducted in a 125-mL flask with a reaction volume of 10 mL at 28°C and 200 rpm on a shaker. Periodically, samples were analyzed for the reduction of compound **1** to compound **2** by HPLC.

The specific enzyme activity was expressed as mg of compound **2** produced per h per g of dry cells. The cells were harvested with the aid of a Sharples centrifuge (Alfa-Laval, Springfield, PA) and the wet cell paste was collected and stored at −60°C until further use. About 25 kg of wet cell paste was collected from each fermentation.

Two-stage process: reduction of compound **1** *in a fermenter.* Frozen cells from the above batches were used to conduct the reduction of compound **1** in 5-L and 25-L fermenters

under aerobic or anaerobic (under argon) conditions. Cell suspensions (20% wt/vol, wet cells) in 80 mM potassium phosphate buffer (pH 6.0) were used. Glucose (25 g/L) and compound **1** in *N,N*-dimethylformamide (2 g/L) were supplemented to the cell suspensions, and the reduction was conducted at 37°C and 300 rpm with gentle sparging of argon gas or air at 1 Lpm. The pH was allowed to drop from an intial value of 6.0 to 5.5; thereafter the pH was maintained at 5.5 during the bioreduction process. Periodically, 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC.

Preparative scale biotransformation of **1** was carried out in a 380 L fermenter. Wet cells (40 kg) of *S. paucimobilis* SC 16113 were suspended in 200 L of 80 mM potassium phosphate buffer (pH 6.0). Glucose (25 g/L) and compound **1** in *N,N*-dimethylformamide (2 g/L) were supplemented to the cell suspensions, and the reduction was conducted at 37°C and 300 rpm with gentle sparging of argon gas at 1 Lpm. The pH was allowed to drop from an intial value of 6.0 to 5.5; thereafter the pH was maintained at 5.5 during the bioreduction process. Periodically, 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC. At the end of bioreduction, the aqueous solution (209 L) was extracted with an equal volume of ethyl acetate, and the separated organic layer was concentrated under vacuum at 35°C to 12 L. The ethyl acetate concentrate was further concentrated under vacuum at 35°C to 750 mL, washed three times with 500 mL of aqueous saturated sodium bicarbonate, then washed three times with 500 mL of saturated sodium chloride, and finally washed three times with 500 mL of 0.5 N HCl. The rich ethyl acetate was concentrated under vacuum at 35°C to dryness. The concentrate was suspended in 200 mL of ethanol and heated to 50°C until all solids were dissolved. To the hot solution, 82 mL of 40°C water was added, followed by the addition of 480 mL of *n*-heptane. The solution was stirred for an hour at room temperature and allowed to cool. The crystals formed were filtered, washed with 1 L of water, and dried under vacuum at 45°C to yield 120 g of crude compound **2**. The crude product was further recrystallized to yield 100 g of product **2** in overall 50% yield.

Single-stage fermentation and biotransformation of **1**. *Spingomonas paucimobilis* SC 16113 cultures were grown in 550 L of medium B as described in the two-stage process. At the end of the fermentation stage, cells were microfiltered, diafiltered, and used immediately in the single-stage biotransformation process. The fermentation broth was cooled to about 8°C and transferred to a tank maintained at about 8°C. Cultures were concentrated to 100 L volume by a 40-ft^2 ceramic membrane crossflow microfilter (Millipore Corp., Bedford, MA) at a transmembrane pressure of 45 psi and a recirculation rate of 700 gal/min. The cells were then diafiltered by adding 400 L of 80 mM potassium phosphate buffer (pH 6.0) to the recirculation tank at 3.8 L/h-ft^2. The washed cells were further concentrated, if necessary, to give retentate volume of 100 L, corresponding to about 20% (wt/vol, wet) cell suspensions. The microfiltered and diafiltered cells were used in the biotransformation process. Glucose (25 g/L) and compound **1** in *N,N*-dimethylformamide (2 g/L) were supplemented to the cell suspensions and the reduction was conducted at 37°C and 300 rpm with gentle sparging of argon gas at 1 Lpm. The pH was allowed to drop from an intial pH of 6.0 to 5.5; thereafter the pH was maintained at 5.5 during the bioreduction process. Periodically, 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC.

Biotransformation of compound **1** *in the presence of resin*. XAD-7 resin (50 g) (Amberlite nonionic polymeric adsorbent, 20–60 mesh, polyacrylate, Rohm and Hass, Philadelphia, PA) was washed twice with 200 mL of methanol and then twice with 200 mL of water. The washed resin was suspended in 1 L of 100 mM potassium phosphate buffer (pH 6.0), and 5 g of substrate **1** in dimethylformamide was added to the resin suspension. The substrate was allowed to adsorb onto resin for 18 h at 25°C with continuous mixing. The resin was collected by filtration. Substrate-enriched resin was used in the biotransformation process using microfiltered and diafiltered cells in a 3-L bioreactor. Cell suspensions (1 L) (20% wt/vol, wet cells) were supplemented with compound **1** in *N,N*-dimethylformamide (2 g/L) and glucose (25 g/L), and the reduction was conducted at 37°C and 200 rpm with gentle sparging of argon gas at 0.5 Lpm. The pH was allowed to drop from an intial value of 6.0 to 5.5; thereafter the pH was maintained at 5.5 during bioreduction process. Periodically, 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC. At the end of the biotransformation, the reaction mixture was filtered on a 100 mesh (150 μ) stainless steel screen, and the resin retained by the screen was washed with 1 L of water. The product was then desorbed from the resin with 100 mL of acetonitrile. The acetonitrile solution was then evaporated to dryness under vacuum; the crude product was further purified, crystallized, and recrystallized in an overall 70 M% yield with 90% homogeneity and 99.8% optical purity.

In an alternative process, frozen cells of *S. paucimobilis* SC 16113 were used with resin-adsorbed substrate. Cell suspensions (2 L) (15% wt/vol, wet cells) in 80 mM potassium phosphate buffer (pH 6.0) were supplemented with 200 g of XAD-7 resin containing 20 g of compound **1** and 50 g of glucose. The reduction was conducted at 37°C and 200 rpm with gentle sparging of argon gas at 0.5 Lpm. The pH was maintained at 6.0 during the bioreduction process. Periodically, 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC. At the end of the biotransformation, the reaction mixture was filtered on a 100 mesh (150 μ) stainless steel screen, and the resin retained by the screen was washed with 2 L of water. The product was then desorbed from the resin with 200 mL of acetonitrile. The

acetonitrile solution was then evaporated to dryness under vacuum and the crude product was further purified, crystallized, and recrystallized in an overall 75 M% yield with 91% homogeneity and 99.8% optical purity.

Biotransformation of **1** *by cell extracts of* S. paucimobilis *SC 16113*. *Spingomonas paucimobilis* SC 16113 cells were suspended in 1.3 L of buffer A (50 mM potassium phosphate buffer, pH 6.0, containing 20% glycerol and 1 mM phenylmethylsulfonyl fluoride) at 20% (wt/vol, wet cells) concentration and homogenized to prepare cell suspensions. The cell suspension was disintegrated by two passages through a Microfluidizer (Microfluidics, Newton, MA) at 10,000 psi at 8°C. Disintegrated cells were centrifuged at 20,000 × *g* for 40 min at 4°C; the supernatant solution is referred to as cell extracts. To 1 L of cell extracts, 1 g of substrate **1** in dimethylformamide, 1 g of glucose, 0.1 g of NADP, and 1 kilounit of glucose dehydrogenase were added. The bioreduction was carried out at 30°C, 200 rpm, for 90 min. Periodically 2-mL samples were taken and analyzed for substrate **1** and product **2** concentrations by HPLC. The optical purity of compound **2** was determined by chiral HPLC.

Analytical methods for quantitation and optical purity determination. Analysis of compounds **1** and **2** was carried out using a Hewlett-Packard (HP) 1070 high-performance liquid chromatograph (Palo Alto, CA). A Vydak 5 µm column (25 cm × 4.6 mm, 5 µ) was used at ambient temperature. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid in distilled water) and solvent B (0.1% trifluoroacetic acid in 70% acetonitrile and 30% distilled water). The flow rate was 1 mL/min. The detector wavelength was 211 nm. A gradient elution was carried out for 30 min as follows: 0 min, 50% solvent B; 0–20 min, 100% solvent B; 20–25 min, 100% solvent B; 25–26 min, 50% solvent B. The retention times for the substrate **1** and product **2** were 10.2 min and 8.3 min, respectively. The separation of the two enantiomers of racemic alcohol **2** were achieved by HPLC using a Chiracel OJ-R (46 cm × 15 cm, 5 µ) column (J.T. Baker, Phillipsburgh, PA). The mobile phase consisted of 30% acetonitrile in deionized water. The flow rate was 0.75 mL/min and the detector wavelength was 210 nm. The retention times for the two enantiomers of racemic **2** were 33.1 (*S*-isomer) and 18.9 min (*R*-isomer), respectively.

Growth of M. neoaurum *ATCC 25795*. One vial of *M. neoaurum* ATCC 25795 culture was used to inoculate 100 mL of medium A containing 1% yeast extract, 1% malt extracts, 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 on a rotary shaker. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 100 mM potassium phosphate buffer (pH 7.0), and then used for biotransformation studies.

Fermentation of M. neoaurum *ATCC 25795*. *Mycobacterium neoaurum* ATCC 25795 was grown in 150-L fermenters containing 125 L of medium A. Growth consisted of two inoculum development stages and a fermentation stage. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of culture was inoculated into 100 mL of medium A. The 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. Fermenters containing 125 L of medium A were inoculated with 3 L of F2 stage inoculum and grown at 30°C and 280 rpm with 125 Lpm aeration. During fermentation, cells were periodically harvested by centrifugation from 200 mL of culture broth. Cell suspensions (20% wt/vol, wet cells) were prepared in 100 mM potassium phosphate buffer (pH 7.0) and supplemented with 2.0 mg /mL of compound **3**. The biotransformation was conducted in a 125-mL flask with a reaction volume of 10 mL at 30°C and 200 rpm on a shaker. Periodically samples were removed, and after centrifugation, the separated supernatant solution was collected, filtered through a 0.2 µm LID/X filter, and analyzed by HPLC for substrate **3** and product **4** concentrations. The activity was expressed as mmol of product **4** formed per min per g of dry cells. Cells were harvested at an optimal activity period with the aid of a Cepa centrifuge, and the wet cell pastes were collected and stored at −60°C until further use. Portions of the cells were freeze-dried and stored at 5°C.

Biotransformations of compound **3** *by microbial cell suspensions*. The cells of *M. neoaurum* ATCC 25795 were suspended in 100 mM potassium phosphate buffer, pH 7.0, at 10% (wt/vol, wet cells) cell concentration and supplemented with 1 mg/mL of compound **3.** Freeze-dried cells of *M. neoaurum* ATCC 25795 were suspended in 100 mM potassium phosphate buffer, pH 7.0, at 1% cell concentration and supplemented with 1 mg/mL of compound **3.** The biotransformation was conducted at 30°C, 280 rpm on a rotary shaker. Periodically, samples of 1 mL were taken and after centrifugation, the supernatant solution was collected, filtered through a 0.2 µm LID/X filter, and analyzed for substrate **3** and product **4** concentrations. The biotransformation of substrate **5** was carried out in a similar manner.

Samples (2 mL) from the reaction mixture were analyzed for substrate and product concentration with a Hewlett-Packard 1070 high-performance liquid chromatograph. A Phenomenex Cyanopropyl column (150 × 4.6 mm, 5 µ) was used. The mobile phase consisted of 5% isopropanol in hexane. The flow rate was 230 nm. The optical purity of products **4** and **6** was determined by chiral HPLC. The separation of the two enantiomers of products **4** and **6** was achieved by a Chiralcel WH column (250 × 4.6 mm, 5 µ). The mobile phase was 10 mM $CuSO_4$. The flow rate was 1 mL/min, and the detector wavelength was 230 nm. The retention times for the two enantiomers of **4** were 8.0 and 16.7 min, respectively. The retention times for the two enantiomers of **6** were 12.9 and 18.9 min, respectively.

Preparation of cell extracts of M. neoaurum *ATCC 25795*. Cultures of *M. neoaurum* ATCC 25795 were grown in a 150-L fermenter as described earliar. Cell suspensions (10% wt/vol, wet cells) in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 10% glycerol (buffer

B) were disintegrated by Microfluidizer M-110F (Microfluidics, Inc.) at 10,000 psi at 4°C. The lysates were centrifuged at 20,000 × g for 30 minutes at 4°C, and the clear supernatant collected was referred to as cell extracts.

Purification of amidase. Cell extracts of *M. neoaurum* ATCC 25795 (200 mL) were loaded on a DEAE (diethylaminoethyl) cellulose column (5 × 50 cm) previously equilibrated with buffer B. The column was washed with buffer B and eluted with 2 L of buffer B containing sodium chloride in a linear gradient of 0–0.5 M. Fractions containing amidase activity were pooled and used for biotransformation of substrates **4** and **6**.

Biotransformations of **3** *and* **5** *by cell extracts*. Cell extracts from *M. neoaurum* ATCC 25795 were evaluated for the biotransformations of compounds **3** and **5**. The reaction mixture contained 40 mg of substrate in 20 mL of cell extracts. The reactions were conducted at 30°C, 200 rpm on a shaker. Concentrations of substrates **3** and **5** and products **4** and **6** were determined by HPLC. The optical purities of products **4** and **6** were determined by chiral HPLC as described above.

Hydrolysis of **7**. The enzymatic asymmetric hydrolysis of **7** was carried out in 25 mM potassium phosphate buffer at pH 7.2, at 10°C. The reaction mixture contained 18 mL of buffer and 2 mL of organic solvent (as indicated) containing 200 mg of diester **7** and 500 units of enzyme PLE (Sigma Chemicals, St. Louis, MO). The pH of the reaction was maintained at 7.2 by addition of 5 N NaOH. Periodically, samples of 1 mL were taken and extracted with 4 mL of ethyl acetate. After centrifugation, the separated organic layer was filtered through a 0.2 µm LID/X filter (Whatman Inc.) . A portion of the clarified ethyl acetate filtrate was dried under a stream of nitrogen. The resulting material was dissolved in mobile phase and analyzed by HPLC for optical purity of monoester **8**. The analysis of diester **7** and monoester **8** were carried out by gas chromatography (GC) assay. An HP-1 capillary column (12 m × 0.2 mm × 0.33 mm) was used with the oven at 110°C and the injector and detector at 250°C. The retention times for the diester **7** and monoester **8** were 19.05 and 9.5 min, respectively. The separation of two enantiomers of monoester **8** was achieved by chiral HPLC with a Chiralpak AD (Diacel Chemical Co., Easton, PA) column (0.46 × 25 cm). Mobile phase was 10% isopropanol, 10% cyclohexanol, and 0.1% formic acid; retention times of two enantiomers of racemic **8** were 9.04 and 11.2 min, respectively.

Semipreparative-scale hydrolysis of diester **7**. A semipreparative-scale asymmetric hydrolysis of diester **7** was carried out in a biphasic system using 10% ethanol as a cosolvent. The reaction mixture contained 270 mL of 25 mM potassium phosphate buffer (pH 7.2) and 30 mL of ethanol containing 3 g of diester **7** and 22,000 units of crude PLE (Amano International Enzyme Co., Troy, VA). The reaction was carried out at 10°C, 125 rpm agitation and pH 7.2 for 11 h. The pH was maintained at 7.2 with 5 N NaOH. At the end of reaction, the reaction mixture was centrifuged and filtrate was passed through an ultrafiltration device using a 10,000 MW cut-off membrane to remove most of the protein. The membrane was washed with 30 mL of 25 mM potassium phosphate buffer, pH 7.2. Combined permeate and wash (380 mL) were acidified to pH 2.2 with 6 N HCl and 10.5 grams of SP 207 resin was added to adsorb monoester product **8** onto resin at ambient temperature. Product containing resin was collected by filtration, and desorption of product **8** from the resin was carried out using 215 mL of methanol in the first extraction and 105 mL of methanol in the second extraction. Combined methanol extracts (300 mL) were evaporated under reduced pressure at 40°C to obtain 2.6 g of pale yellow oily residue. The monoester **8** was isolated in 86.3 M% overall yield. The optical purity of isolated *S*-(−)-monoester **8** was 96.9%. The ^{1}H NMR and MS of the isolated product were consistent with monoester **8,** and the specific rotation of monoester $[\alpha]_D$ was −14.4 (c = 1.1 in methanol).

RESULTS

Microbial reduction of **1**. Microorganisms were screened for the transformation of ketone **1** to chiral alcohol **2**. As shown in Table 1, among the cultures evaluated, *Hansenula anomola* SC 13833, *H. anomola* SC 16142, *Rhodococcus rhodochrous* ATCC 14347, and *Spingomonas paucimobilis* SC 16113 gave the desired alcohol **2** in >96% optical purity and >15% reaction yield. *Spingomonas paucimobilis* SC 16113 catalyzed the efficient conversion of ketone **1** to the desired chiral alcohol **2** in 58% reaction yield and >99.5% optical purity.

Since substrate **1** is insoluble in water, the effect of solvents on dissolved substrate **1** and its supply in the biotransformation reaction mixture were evaluated. Dimethylformamide at 2–5% concentrations was found to be the best cosolvent to supply the substrate in the biotransformation process (Table 2).

The fermentation of *S. paucimobilis* SC 16113 culture was carried out in a 750-L fermenter as described in the Materials and Methods section. From each fermentation batch, about 60 kg of wet cell paste was collected. Cells harvested from the fermenter were used to conduct the biotransformation in 1-, 10-, and 210-L preparative batches under aerobic or anaer-

TABLE 2
Effect of Cosolvent in Microbial Reduction of Ketone 1

Cosolvent (%)	Reaction yield[a] of **2** (%)	Optical purity of **2** (%)
Dimethylformamide (10)	80	99
Dimethylformamide (5)	84	99.1
Dimethylformamide (2)	85	99.4
Dimethylsulfoxide (10)	73	98
Dimethylsulfoxide (5)	73	98.2
Dimethylsulfoxide (2)	80	98.2
Acetonitrile (2)	59	99.2

[a]Reaction mixture in 10 mL of cell suspensions (20% wet cells) contained 10 mg of substrate **1** (dissolved in solvent as indicated and supplied at solvent concentration as indicated) and 250 mg of glucose. Reactions were carried out at 28°C, 200 rpm on a rotary shaker for 18 h as described in the Materials and Methods section. The reaction yield of **2** and optical purity of **2** were determined by high-performance liquid chromatography (HPLC) as described in the Materials and Methods section. See Table 1 for abbreviations.

TABLE 3
Semipreparative Batches for Microbial Reduction of Ketone 1[a]

Condition	Batch size (L)	Reaction time (h)	Substrate input (g/L)	Reaction yield of **2** (%)	Optical purity of **2** (%)
Aerobic	1	4	1	85.3	98.5
Aerobic[b]	2	4	1	87	98.6
Aerobic	1	3	1	87.9	98.5
Anaerobic[b]	1	3	1	89.7	98.7
Anaerobic	1	6	2	87	99.4
Anaerobic	2	3	1	90	98.5
Anaerobic	10	3	1	95	99.4
Anaerobic	200	2	1	84	99.5

[a]Cells were suspended in 80 mM potassium phosphate buffer (pH 6.0) at 20% (wt/vol, wet cells) concentration. Cell suspensions were supplemented with substrate **1** (in dimethylformamide) and glucose (25 g/L). Reactions were carried out at 37°C, 300 rpm under anaerobic conditions. The reaction yield and optical purity of **2** were determined by HPLC as in the Materials and Methods section. See Tables 1 and 2 for abbreviations.
[b]Microfiltered and diafiltered cells were used.

obic conditions. The cells were suspended in 80 mM potassium phosphate buffer (pH 6.0) to 20% (wt/vol, wet cells) concentration. Compound **1** (1–2 g/L) and glucose (25 g/L) were added to the fermenter, and the reduction reaction was carried out at 37°C. Results are as shown in Table 3. In some batches, the microfiltered and diafiltered cells were used directly in the bioreduction process. In all batches of biotransformation, reaction yields of >85% and optical purities of >98% were obtained.

The isolation of compound **2** from the 200-L preparative batch was carried out as described in the Materials and Methods section to obtain 100 g of product **2**. The isolated **2** gave a homogeneity index of 83% and an optical purity of 99.5% as analyzed by chiral HPLC. The MS and NMR data of isolated compound **2** and the standard compound **2** were virtually identical.

In an alternative process, frozen cells of *S. paucimobilis* SC 16113 were used with resin-adsorbed substrate at 5 and 10 g/L substrate concentrations as described in the Materials and Methods section. Results are as shown in Table 4. In this process, an average reaction yield of 85% and an optical purity of >99% were obtained for product **2**. At the end of the biotransformation, the reaction mixture was filtered on a 100 mesh (150 μ) stainless steel screen, and the resin retained by the screen was washed with 2 L water. The product was then desorbed from the resin and crystallized in an overall 75 M% yield with 91% homogeneity and 99.8% optical purity.

The reduction of compound **1** to compound **2** was also carried out using cell extracts of *S. paucimobilis* SC 16113. Glucose dehydrogenase was used to regenerate the cofactor NADPH required for the reduction. After 90 min reaction time, 80% conversion of ketone **1** to chiral alcohol **2** was obtained. About 0.6 g of chiral alcohol **2** was isolated from the reaction mixture in 90% chemical purity. The isolated compound **2** gave 99.4% optical purity.

Enzymatic resolution of **3** *and* **5** *to the corresponding (*S*)-amino acids* **4** *and* **6**. The cells (10% wt/vol, wet cells) of *M. neoaurum* ATCC 25795 were evaluated for biotransformation of compound **3** to compound **4**. The reaction was completed in 75 min with a reaction yield of 48 M% (theoretical max. 50%) and an optical purity of 95% for the desired product **4**. Freeze-dried cells of *M. neoaurum* ATCC 25795 were suspended in 100 mM potassium phosphate buffer (pH 7.0) at 1% concentration, and cell suspensions were used for the biotransformation of compound **3**. The reaction was completed in 60 min with a reaction yield of 49.5 M% (theoretical max. 50%) and an optical purity of 99% for the desired product **4**. The kinetics of reaction are shown in Figure 1. Biotransformation of compound **3** was also carried out using a purified amidase. A reaction yield of 49 M% and an optical purity of 99.9% were obtained for the desired product **4** after 60 min reaction time.

Freeze-dried cells of *M. neoaurum* ATCC 25795 and partially purified amidase were used for the biotransformation of compound **5**. A reaction yield of 49 M% and an optical purity of 78% were obtained for the desired product **6** using freeze-

TABLE 4
Semipreparative Batches for Microbial Reduction of Ketone 1 in the Presence of Resin XAD-7[a]

Batch size (L)	Reaction time (h)	Substrate input (g/L)	Product **2** on resin (g/L)	Product **2** in aqueous (g/L)	Reaction yield of **2** (%)	Optical purity of **2** (%)
1	73	5	4	0.12	82	98.5
2	93	10	7.8	0.29	81	98.6

[a]Cells were suspended in 80 mM potassium phosphate buffer (pH 6.0) at 20% (wt/vol, wet cells) concentration. Cell suspensions were supplemented with substrate **1** (on XAD-7 resin; Rohm and Haas, Philadelphia, PA) and glucose (25 g/L). Reactions were carried out at 37°C, 300 rpm under anaerobic conditions. The reaction yield and optical purity of **2** were determined by HPLC as in the Materials and Methods section. See Tables 1 and 2 for abbreviations.

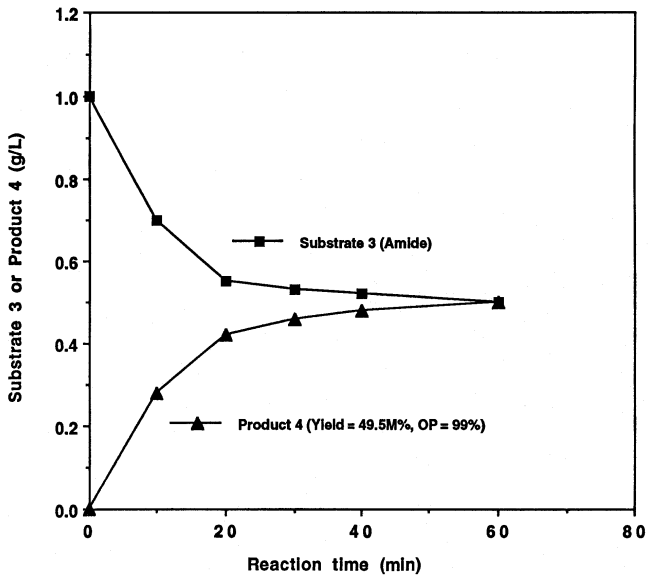


FIG. 1. Resolution of racemic α-methyl phenylalanine amide **3** by *Mycobacterium neoaurum* ATCC 25795. **3,** racemic α-methyl phenylalanine amide; **4,** (*S*)-amino acid of **3**; OP, optical purity.

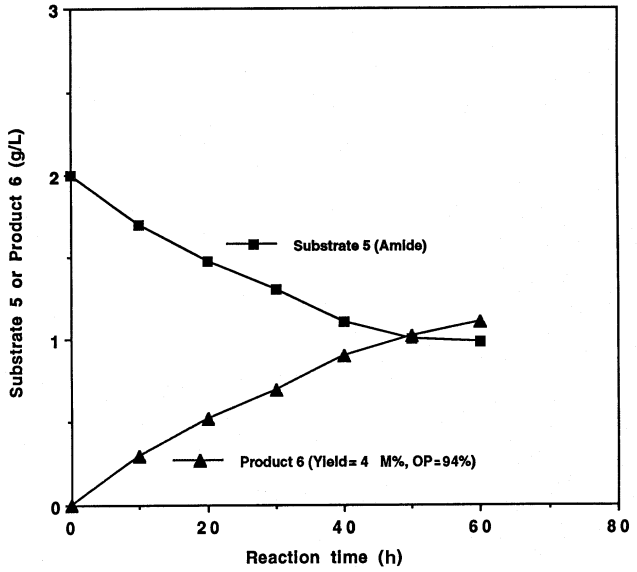


FIG. 2. Resolution of racemic α-methyl-4-hydroxyphenylalanine amide, **5,** by *M. neoaurum* ATCC 25795. **6,** (*S*)-amino acid of **5**; for other abbreviations see Figure 1.

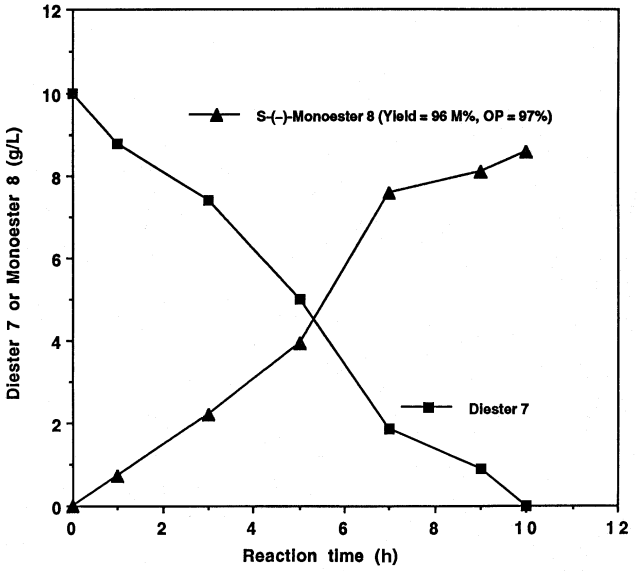


FIG. 3. Asymmetric hydrolysis of methyl-(4-methoxyphenyl)-propanedioic acid ethyl ester, **7,** by pig liver esterase. **8,** (*S*)-monoester of **7**. For other abbreviation see Figure 1.

dried cells. The reaction was completed in 50 h. By using partially purified amidase, a reaction yield of 49 M%, and an optical purity of 94% were obtained for desired product **6** after 70 h reaction time. The kinetics of reaction are shown in Figure 2.

Asymmetric hydrolysis of **7**. Various organic solvents were tested for the PLE-catalyzed asymmetric hydrolysis of diester **7** in a biphasic system. The results (Table 5) indicate that the reaction yields and optical purities of monoester **8** depended upon the solvent used in asymmetric hydrolysis. Tetrahydrofuran, methylisobutyl ketone, and hexane inhibited PLE. Lower reaction yields (28–56 M%) and lower optical purities (59–72%) were obtained using *t*-butylmethyl ether, dimethylformamide, and dimethylsulfoxide as cosolvents. Higher optical purities (>91%) were obtained using methanol, ethanol, and toluene as cosolvents. Ethanol gave the highest reaction yield (96.7%) and optical purity (96%) for monoester **8**.

The effects of temperature and pH were evaluated for the PLE-catalyzed hydrolysis of diester **7** in a biphasic system using ethanol as a cosolvent. It was observed that the optical purity of the desired monoester **8** increased with decreasing temperature from 25 to 10°C. The optimal pH for asymmetric hydrolysis of diester **7** in a biphasic system using ethanol as a cosolvent is 7.2 at 10°C (Table 6).

A semipreparative scale asymmetric hydrolysis of diester **7** was carried out in a biphasic system using 10% ethanol as a cosolvent. Substrate (3 g) was used in a 300-mL reaction mixture. The reaction was carried out at 10°C, 125 rpm agitation, and at pH 7.2 for 11 h. Kinetics of the reaction are as shown in Figure 3. A reaction yield of 96 M% and an optical purity of 96.9% were obtained. From the reac-

TABLE 5
Effect of Solvent on Asymmetric Hydrolysis of Methyl-(4-methoxyphenyl)-propanedioic Acid, Ethyl Diester, 7[a]

Solvent	Reaction time (h)	Diester **7** (mg/mL)	Monoester **8** (mg/mL)	Yield of monoester **8** (M%)	Optical purity of monoester **8** (%)
Methanol	22	0	0.65	37	92
Ethanol	22	0	1.7	96.7	96
Acetonitrile	22	0	0.5	28.2	59.3
Dimethylformamide	22	0	0.85	48.3	68.5
Dimethylsulfoxide	22	0.61	1	56.9	72
Acetone	22	0	1.44	81.9	65.1
Methylethylketone	48	0	1.36	77.3	82.1
Methylisobutylketone	64	2.01	0	0	
t-Butylmethylether	22	0.76	0.8	46	64.4
Tetrahydrofuran	48	2	0	0	
Toluene	22	0.18	0.59	33.6	91
Hexane	64	2.05	0	0	

[a]**8,** (*S*)-monoester of **7**.

TABLE 6
Effect of Temperature and pH on Asymmetric Hydrolysis of 7[a]

Temperature (°C)	pH	Diester **7** (mg/mL)	Monoester **8** (mg/mL)	Yield of monoester **8** (M%)	Optical purity of monoester **8** (%)
25	7	0	1.3	80	85
25	7.2	0	1.41	91	85
25	8	0	1.45	93	82
15	7.2	0	1.35	80.3	90.5
10	6.8	0	1.38	80.5	80.5
10	7.2	0	1.54	95	96.3
10	8	0	1.58	96	94

[a]For abbreviations see Table 5.

tion mixture, 2.6 g of monoester **8** was isolated in 86.3 M% overall yield. The optical purity of isolated *S*-(−)-monoester **8** was 96.9%. The ^{1}H NMR and MS of the isolated product were consistent with monoester **8** and the specific rotation of monoester $[\alpha]_D$ was −14.4 (c = 1.1 in methanol).

DISCUSSION

The asymmetric reduction of carbonyl compounds by baker's yeast has been reviewed (3,20–22). The synthetically useful alcohol dehydrogenases from yeast (23), horse liver (2), and secondary alcohol dehydrogenase (24,25) usually reduce carbonyl compounds to give an (*S*)-alcohol. Recently, Bradshaw *et al.* have demonstrated the use of alcohol dehydrogenase from *Lactobacillus kefir* (26) and *Pseudomonas* sp. (27) as catalysts for synthesis of chiral aromatic, cyclic, and aliphatic alcohols from their corresponding ketones.

In our continuing effort to prepare chiral synthons for drug development, we have demonstrated the microbial reduction of *N*-(4-1-oxo-2-chloroacetyl ethyl) phenyl methane sulfonamide to the corresponding *R*-(+)-alcohol (28), an intermediate for the synthesis of D-(+)-sotalol (β-blocker). We have prepared *R*-(+)-BMY-14802, an effective antipsychotic drug, by the stereoselective microbial reduction of 1-(4-fluorophenyl)-4[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]butan-1-one by *Mortierella ramanniana* (29). We have developed an enzymatic process for the preparation of chiral alcohol (required for the synthesis of calcium channel antagonist) by the stereoselective reduction of 4,5-dihydro-4-(methoxy phenyl)-6-(trifluoromethyl-1H-1)-benzazepin-2-one (30). A chiral intermediate of (3*S*,5*R*)-dihydroxy-6-(benzyloxy)hexanoic acid, ethyl ester was enzymatically prepared by the stereoselective reduction process (31). Chiral dihydroxy compound is an intermediate for our new anticholesterol drug. Recently, we have prepared C-13 side-chain of an anticancer drug, paclitaxel, by the stereoselective microbial reduction of 2-keto-3-(*N*-benzoylamino)-3-phenyl propionic acid ethyl ester to yield (2*R*,3*S*)-(−)-*N*-benzoyl-3-phenyl isoserine ethyl ester (32). In this report, we have described the preparation of chiral chloroalcohol **2** by the stereoselective reduction process, as well as the preparation of two different chiral intermediates needed for the synthesis of β-3-receptor agonists. We have developed a process for the stereoselective reduction of **1** to the corresponding (*R*)-alcohol **2** . In a batch process in the absence of resin, a reaction yield of 85–95% was obtained under either aerobic or anaerobic conditions. Substrate was used at 1–2 g/L. To increase the substrate concentration up to 10 g/L and to develop an efficient recovery process, the biotransformation process was carried out in the presence of resin. In this process, substrate was adsorbed onto resin XAD-7 and supplied to the reaction. At the end of the biotransformation process, product-rich resin was easily separated from cell suspensions of organisms, and rich resin was extracted with solvent to recover product.

Optically pure α-methyl-substituted amino acids are chiral synthons for drugs (33–35). α-Methyl-substituted amino acids can be prepared by enzymatic resolution using esterases (36), acylases (37), hydantoinases (38), or amidases (39–41). In this report, we have demonstrated the preparation of α-methyl-substituted amino acids by a resolution process using amidase from *M. neoaurum* ATCC 25795. Cell suspensions of organisms and partially purified amidase were used in biotransformation to obtain (*S*)-amino acids in >49 M% yield (theoretical maximum yield is 50 M%). The enzymatic resolution of **5** by cell suspension of organisms gave lower optical purity (74%) of the corresponding (*S*)-amino acid compared to purified enzyme, which gave 94% optical purity of the desired product. In a resolution process, the maximum theoretical yield obtained is 50%. Hence, we have developed an alternative process in which α-methyl-substituted amino acid was also prepared by the asymmetric synthesis by the hydrolysis of the prochiral molecule **7** to the corresponding (*S*)-monoester by PLE. A reaction yield of >95% was obtained for the desired (*S*)-monoester.

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