Synthesis of Four Chiral Pharmaceutical Intermediates by Biocatalysis

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ABSTRACT: Chiral intermediates were prepared by biocatalytic processes for the chemical synthesis of four pharmaceutical drug candidates. These include: (i) the microbial reduction of 3,5-dioxo-6-(benzyloxy) hexanoic ethyl ester to (3*S*,5*R*)-dihydroxy-6-(benzyloxy) hexanoic acid ethyl ester, an intermediate for a new anticholesterol drug; (ii) synthesis of (2*R*,3*S*)-(−)-*N*-benzoyl-3-phenyl isoserine ethyl ester, a taxol side-chain synthon; (iii) the microbial oxygenation of 2,2-dimethyl-2H-1-benzopyran-6-carbonitrile to the corresponding (3*S*,4*S*) epoxide and (3*S*,4*R*)-*trans* diol, intermediates for synthesis of potassium channel opener; (iv) the biotransformation of (exo,exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol to the corresponding chiral lactol and lactone, intermediates for thromboxane A_2 antagonist.

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KEY WORDS: Anticholesterol drug, biocatalysis, chiral intermediates, potassium channel opener, taxol side-chain synthon, thromboxane A_2 antagonist.

Currently there is much attention focused on the interaction of small molecules with biological macromolecules. The search for selective enzyme inhibitors and receptors, agonists or antagonists, has become one of the key areas for target-oriented research with the pharmaceutical industry. The increasing understanding of the mechanism of drug interaction on a molecular level has led to the increasing 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, and the other stereoisomer is either inactive or exhibits considerably reduced activity. Pharmaceutical companies are aware that, where appropriate, new drugs should be homochiral to avoid the possibility of unnecessary side effects due to undesirable stereoisomers.

Chiral drug intermediates can be prepared by three different routes. One is to obtain them from the naturally-occurring chiral synthon, mainly by fermentation processes. The second is to carry out the separation 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, the chiral synthon also can be prepared by asymmetric synthesis by either chemical or biocatalytic processes with microbial cells or enzymes derived therefrom. The advantages of microbially catalyzed reactions over chemical reactions are that they are stereoselective, can be carried out at ambient temperature, atmospheric pressure, and in aqueous solution. Furthermore, microbial cells or the enzymes derived from them can be immobilized and reused for many cycles.

In this report we describe biocatalytic processes for the synthesis of four chiral drug intermediates: (i) anticholesterol, (ii) anticancer, (iii) K-channel openers, and (iv) thromboxane antagonist programs (1–4).

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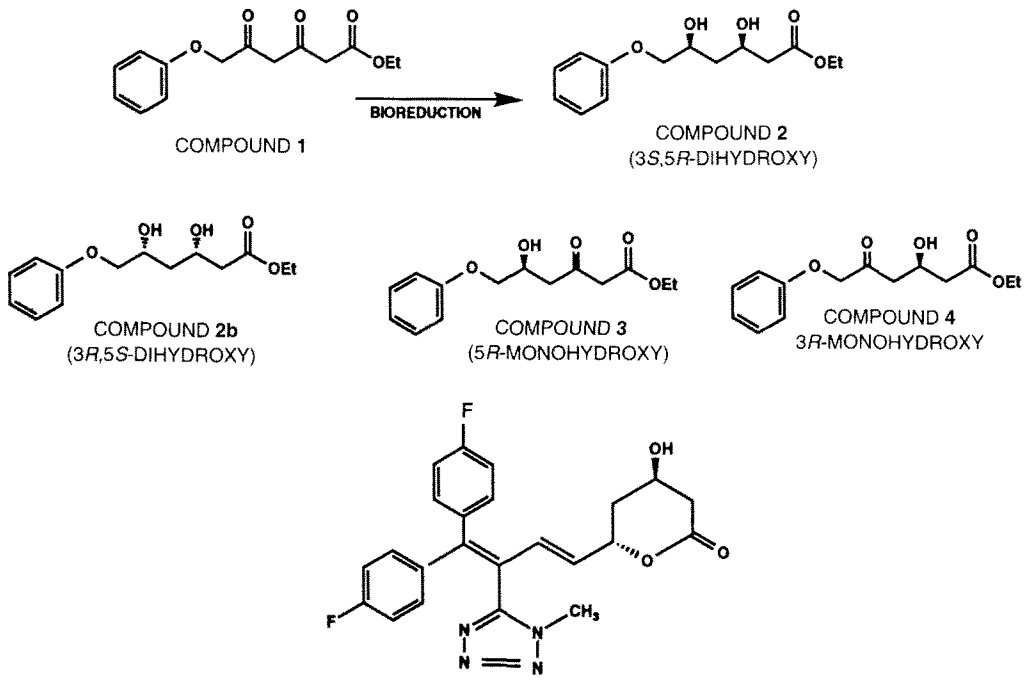
ANTICHOLESTEROL DRUG

Enantioselective microbial reduction of 3,5-dioxo-6-(phenylmethoxy)hexanoic acid, ethyl ester. Chiral β-hydroxy esters are versatile synthons in the preparation of natural products (5–9). Asymmetric reduction of ketones by Baker's yeast has been widely used to obtain chiral β-hydroxy esters because it is a cheap and readily available source of biocatalyst (10–14). Recently, we have demonstrated the stereoselective reduction of β-keto ester by *Geotrichum candidum* (15). In this report, we describe the stereoselective reduction of diketone **1** to the corresponding (3*S*,5*S*)-dihydroxy compound **2a** by *Acinetobacter calcoaceticus* SC13876 (Scheme 1). Compound **2a** is a key chiral intermediate for the chemical synthesis of {[4-[4 a, 6β(*E*)]]-6-[4,4-*bis* (4-fluorophenyl)-3-(1-methyl-1H-tetrazol-5-yl)-1,3-butadienyl} tetrahydro-4-hydroxy-2H-pyren-2-one (**5**). Compound **5**, a new anticholesterol drug, acts by inhibition of hydroxy methylglutaryl–Coenzyme A (HMG–CoA) reductase (16).

MATERIALS AND METHODS

Starting substrate **1** and reference compounds **2a–d**, **3**, and **4** were synthesized by our colleagues in the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ). The physicochemical properties, including spectral characteristics [H nuclear



SCHEME 1

magnetic resonance (NMR), C NMR, mass spectra), were in full agreement for all these compounds.

Microorganisms. Microorganisms were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the American Type Culture Collection (ATCC) (Rockville, MD). Microbial cultures 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 containing 1% malt extract, 1% yeast extract, 2% glucose, and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28°C and 280 rpm for 48 h. Cultures were harvested by centrifugation at 18,000 × *g* for 15 min, washed with 0.1 M potassium phosphate buffer (pH 7.0), and used for reduction studies.

Reduction of **1** *by cell suspensions.* Cells of various microorganisms were suspended separately in 10 mM potassium phosphate buffer (pH 7.0) at 20% (wt/vol, wet cells) cell concentration and supplemented with 2 mg/mL of **1** and 75 mg/mL glucose. Reduction was conducted at 28°C and 200 rpm. Periodically, 1-mL samples were taken and extracted with 4 mL of ethyl acetate. After centrifugation, the separated ethyl acetate phase was collected and dried with a nitrogen stream. The oily residue obtained was dissolved in 2 mL of methylene chloride, filtered through a 0.2-μm LID/X filter (Whatman, Inc., Fairfield, NJ), and analyzed by gas chromatography (GC).

Growth of A. calcoaceticus *in a fermentor. Acinetobacter calcoaceticus* SC 13876 was grown in a 25-L fermentor containing 15 L medium A or medium B (same as medium A, except 2% glucose was replaced with 4% glycerol). Growth consisted of several inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of *A. calcoaceticus* SC 13876 was inoculated into 100 mL of medium A or B contained in a 500-mL flask. Growth was carried out at 28°C and 280 rpm for 48 h on a rotary shaker. In the F2 stage, 10 mL of F1 stage culture was inoculated into 100 mL of medium A or 100 mL of medium B, and incubated at 28°C and 280 rpm for 24 h. Fermentors containing 15 L medium A or medium B each were inoculated with 100 mL of inoculum from a F2 stage. Fermentation was conducted at 25°C and 500 rpm with 15 LPM (liter per min) aeration for 24 h. Cells were periodically harvested by centrifugation from 200 mL of culture broth to determine the specific activity of cells during fermentation. Cell suspensions (20% wt/vol, wet cells) were prepared in 0.1 M potassium phosphate buffer (pH 6.0) and supplemented with 2 mg/mL of **1** and 75 mg/mL of glucose. The reduction was conducted in a 125-mL flask with a reaction volume of 10 mL at 30°C and 280 rpm for 2 h. Samples were analyzed for the reduction of **1** to **2** by GC. The specific activity was expressed as μmoles of **2a** produced per min per gram of dry cells. After 24 h fermentation, cells were collected and stored at –90°C until further use. About 1 kg of wet cell paste was collected from each fermentation.

Reduction of **1** *in a fermentor.* Frozen cells from these batches were used to conduct the reduction of **1** in a 5-L reactor. Cell suspensions (8% wt/vol, wet cells) in 3 L of 0.1 M potassium phosphate buffer (pH 6.0) were used. Compound **1** (6 g) and glucose (225 g) were added to the reactor, and the reduction was carried out at 30°C and 400 rpm with 4 LPM aeration for 24 h. The pH was maintained between 6.0 and

6.2. Periodically, samples were prepared as described previously and analyzed by GC to determine the % conversion of **1** to **2**. The optical purity of **2a** was determined by chiral high-performance liquid chromatography (HPLC).

Isolation of **2a**. At the end of bioreduction, the reaction mixture was centrifuged to remove cells, and the clear filtrate (2.8 L) containing **2a** was extracted three times with equal volumes of methylene chloride. The separated organic phase was washed with equal volumes of 0.7 M sodium bicarbonate, dried over anhydrous sodium sulfate, and evaporated under reduced pressure to obtain 4.8 g of viscous, oily liquid. About 3.5 g of pale-yellow solid (85% GC area % purity) was obtained when the oily liquid was dried under vacuum at room temperature. About 1 g of pale-yellow solid was purified by preparative HPLC. A 6-μm Waters (Milford, MA) Prep Nova-Pak cartridge, HR silica column (40 × 100 mm), with hexane/ethyl acetate (85:15) was used. The flow rate was 75 mL/min. The main component peak at 39 min was collected, and the solvent was removed on a rotary evaporator to obtain 0.7 g of pure compound **2a**.

Analytical methods. Analysis of **1** and **2** was carried out with a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatogram. A Restek Rtx (Bellefonte, PA) capillary column (15 m, 0.32 mm i.d., 0.33 μm capillary) was used under the following conditions: carrier gas, helium; split flow, 50 mL/min; injector temperature, 260°C; initial oven temperature, 230°C for 5 min; final oven temperature, 280°C, rate of 10°C/min; detector temperature, 250°C; flame-ionization detector (FID), hydrogen and air optimized. The retention times for **1** and **2** were 4.49 min and 3.76 min, respectively. The separation of *syn* (**2a**, **2b**) and *anti* (**2c**, **2d**) diastereomers of **2** was achieved by HPLC. A Whatman (Maidstone, England) Partisil ODS-3 column (4.6 × 250 mm, 5 μm particles) was used at ambient temperature. The mobile phase was 79% water with 21% acetonitrile, flow rate was 0.7 mL/min, and detection wavelength was 220 nm. Samples for analysis were dissolved in 50% water with 50% acetonitrile and filtered through a 0.2-μm LID/X filter. The retention times for *anti* and *syn* diastereomers were 26.4 and 28.5 min, respectively.

Separation of the enantiomers of *syn* **2** was achieved by chiral HPLC. A Bakerbond chiralcel OB column (4.6 × 250 mm) was used at ambient temperature, injection volume was 20 μL, mobile phase was a hexane/*n*-butanol/isopropanol (73:25.5:1.5) mixture, flow rate was 0.8 mL/min, and detection wavelength was 220 nm. The retention times for the (−) and (+) isomers (**2a** and **2b**) were 21.3 and 11.5 min, respectively.

All of the stereoisomers of **2** were separated by a coupled achiral and chiral column system. A C-18 column (J.T. Baker, Phillipsburg, PA) (polyspher RP-18, 150 × 0.4 mm i.d.) and a chiralcel o.d. column were used in series. The mobile phase was a methanol/*n*-butanol/hexane (5:1:94) mixture, flow rate was 0.8 mL/min, and the detection wavelength was 220 nm. The retention times for (+) *anti*, (+) *syn*, (−) *anti*, and (−) *syn* stereoisomers (**2c**, **2b**, **2d**, and **2a**) were 12, 17.4,19.6, and 40.1 min, respectively. The separation of **3** and **4** was achieved under the same conditions with retention times of 9.28 and 11.12 min, respectively.

Preparation of cell extracts. Frozen *A. calcoaceticus* cells (−70°C) were suspended in 0.2 M potassium phosphate buffer pH 6.0 (buffer A) at 10% (wt/vol, wet cells) concentration and homogenized to prepare cell suspensions. Cells were passed through a French Press at 18,000 × *g* for 30 min at 4°C. The supernatant solution obtained after centrifugation is referred to as cell extract. Cell suspensions of more than 100-mL vol were disintegrated with a Microfluidizer (Microfluidics, Inc., Newton, MA) at 12,000 psi, and disintegrated cells were centrifuged at 25,000 × *g* for 30 min to obtain cell extracts.

Reduction of **1** *to* **2a** *by cell extracts*. Reduction of **1** to **2a** by cell extracts was conducted in 50-mL and 1-L reactors. The reaction mixture contained cell extracts, glucose dehydrogenase (10 units/mL), glucose (100 mg/mL), and NAD^+ (0.7 mM). Glucose and glucose dehydrogenase were used to regenerate NADH required for the reduction reaction. Substrate **1** (33% solution in ethanol) was supplied at 5 mg/mL in a 50-mL reactor and 10 mg/mL in a 1-L reactor. The reaction was carried out at 25°C and 200 rpm (50-mL reactor) and at 33°C and 250 rpm (1-L reactor) for 24 h. The pH was maintained at 5.9 during reaction by using a Brinkmann (Westbury, NY) Metrohm pH stat. The isolation and purification of **2a** was carried out as described previously.

Reduction of **1** *to monohydroxy compounds* **3** *and* **4** *by cell extracts*. The reduction of **1** to **3** and **4** was carried out with cell extracts of *A. calcoaceticus* SC 13876. The reaction mixture contained 1 L cell extract, glucose (100 mg/mL), glucose dehydrogenase (10 units/mL), NAD^+, (0.7 mM), and substrate **1** (10 mg/mL). The reaction was carried out at 32°C and 200 rpm for 10 h. Incomplete reduction of **1** was carried out to obtain 4.8 g of monohydroxy **3** and **4** in the reaction mixture.

Isolation of **3** *and* **4**. The reaction mixture, containing 4.8 g of product, was adjusted to pH 7.0 and extracted three times with equal volumes of dichloromethane. After centrifugation, the separated organic phase was washed twice with 7% sodium chloride, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to obtain 4.5 g of brown liquid, which contained 2.1 g of the mixture of **3** and **4**. This was loaded on a silica column (35 × 2 cm) and successively washed with hexane/ethyl acetate (80:20 and 70:30). Finally, **3** and **4** were eluted with 200 mL hexane/ethyl acetate (60:40) into fractions 34–39. Solvent was removed from pooled fractions under reduced pressure to obtain 1.2 g of a mixture of **3** and **4** with 90% HPLC purity.

Purification of reductase. Cell extracts of *A. calcoaceticus* SC 13876 were prepared in a Microfluidizer as described earlier. Cell extracts (700 mL) loaded on a diethylaminoethyl-cellulose column (5 × 67 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 2 L of buffer A containing sodium chloride in a linear gradient of 0–0.5 M. Fractions of 15 mL were collected. Fractions that showed reductase activity (void volume fractions) were pooled, and protein was precipitated with ammonium sulfate by addition of salt up to 60% saturation with continu-

ous stirring at 4°C. The precipitated protein was collected by centrifugation at 25,000 × *g* for 30 min and dissolved in 8 mL of buffer A. The sample was loaded on a Sephacryl S-200 column (2.5 × 100 cm) previously equilibrated with buffer A. Fractions containing reductase activity were pooled and loaded onto a Mono Q column (0.5 × 5 cm) in a fast-performance liquid chromatographic (Pharmacia, Uppsala, Sweden) system. Fractions containing reductase activity were pooled, concentrated by an Amicon YM 10 filter to a 5-mL vol and stored at −70°C.

Enzyme assay. The reaction mixture in 0.1 M potassium phosphate buffer (pH 6.0) contained 0.7 mM NAD^+, 3.5 units/mL glucose dehydrogenase, 10 mg/mL glucose, and 2.35 mg/mL of substrate **1**. The reaction was initiated by addition of enzyme and was carried out at 25°C and 200 rpm on a rotary shaker. Periodically, samples were taken and extracted with two volumes of hexane/butanol (65:35) mixture. After centrifugation, the separated organic phase was analyzed by GC and HPLC to determine the concentrations of mono- and dihydroxy compounds **2**, **3**, and **4**. One unit of enzyme activity was defined as one μmole of monohydroxy compounds produced per minute. The specific activity was defined as units/mg protein. Concentration of protein was determined by Bradford's method (17). Bovine serum albumin was used as standard.

RESULTS

Among cultures evaluated, *A. calcoaceticus* SC 13876 and *Pichia methanolica* ATCC 58403 reduced **1** to **2a** in 85 and 91% reaction yield and 82 and 96% optical purity, respectively. In contrast, *Trichoderma glabarta* SC 9042, *Mortierella alpina* SC 2598, and *Hansenula polymorpha* ATCC 26012 reduced **1** to the undesirable *anti* enantiomer **2b** in 87 and 90% optical purity, respectively (Table 1). A number of other organisms tested, such as *Fusarium* sp. SC 6106, *Streptomyces azireus* SC 3060, and *Cunninghamella enchinulata* ATCC 6795, completely metabolized **1**.

Acinetabacter calcoaceticus SC 13876 was grown in a 25-L fermentor containing 15 L of medium A or medium B (same as medium A, except 2% glucose was replaced with 4% glycerol). Fermentation was conducted at 25°C and 500 rpm with 15 LPM aeration. During fermentation, cells were harvested from 200 mL broth by centrifugation every 6 h, and the reduction of **1** to **2a** was conducted by cell suspensions to determine the specific activity (μmoles of **2a** produced per min per gram dry cells). With glucose as carbon source, higher specific activity (0.55) was obtained in early growth periods (up to 24 h). The specific activity then decreased during the next 18-h growth period. The optical purity of **2a** and the *anti* diastereomer content were lower after the 42-h growth period. Glycerol-grown cells gave high specific activity (1.2) and higher optical purity (99.6%) of **2a**, compared to glucose-grown cells (Table 2).

The effect of temperature on the reduction of **1** to **2a** by cell suspensions of *A. calcoaceticus* SC 13876 was evaluated with 20% (wt/vol, wet cells) cells in buffer A and 2 mg/mL of **1**. As shown in Figure 1, the reaction rate was faster at 33–37°C than at 25°C. The content of *anti* diastereomer was higher (10%) at 25°C than at 30–37°C (2.5–3.5%). The effect of pH on reduction of **1** to **2a** by cell extracts of *A. calcoaceticus* SC 13876 was evaluated. The optimum pH was 5.5.

Reduction of **1** to **2a** was conducted in a 3-L preparative batch with 8% (wt/vol, wet cells) cell suspensions of *A. calcoaceticus* SC 13876. Substrate (6 g) and glucose (225 g) were used in the reaction mixture. After a 24-h reaction period, 80% yield and 97% optical purity of **2a** was obtained. About 6% *anti* diastereomer was produced in the reaction mixture (Table 3). Pale-yellow material (3.5 g) were obtained from the reaction mixture, and was further purified by preparative HPLC to obtain 0.7 g of **2a** in 99 GC area% purity and 99.5% optical purity.

Reduction of **1** to **2** was also carried out in a 1-L batch with cell extracts of *A. calcoaceticus* SC 13876. The reaction mixture contained 1 L cell extract, glucose (100 mg/mL), glucose dehydrogenase (10 units/mL), NAD^+ (0.7 mM), and substrate

TABLE 1
Stereoselective Microbial Reduction of 1 to 2a[a]

Microorganisms[b]	Reaction time (h)	Product **2a** (mg/mL)	Conversion to **2a** (%)	Optical purity desired **2a** (%)
Pichia methanolica ATCC 58403	24	1.82	91	82
Trichoderma glabarta SC 9042	48	0.8	40	19
Mortierella alpina SC 2598	48	1.58	78	17
Acinetobacter calcoaceticus SC 13876	24	1.7	85	96
Hansenula polymorpha ATCC 26012	24	1.52	76	9.8

[a]Substrate **1** and product **2a** concentrations were determined by gas chromatography, and the optical purity was determined by chiral high-performance liquid chromatography.
[b]ATCC, American Type Culture Collection (Rockville, MD).

TABLE 2
Growth of *Acinetobacter calcoaceticus* on Glucose and Glycerol: Stereoselective Reduction of 1 to 2[a]

Fermentation time (h)	Yield of **2** (%)	Specific activity of cells	Optical purity of **2a** (%)	*Anti* diastereomers (%)
Glucose-grown cells				
18	66	0.58	93	8.7
24	74	0.55	90	7.4
30	64	0.47	90	7.2
36	60	0.46	88	5.6
Glycerol-grown cells				
18	44	0.89	99.2	6.8
24	47	0.82	99.2	6
30	63	1.2	99.6	6.3
36	68	1.45	99.2	7

[a]During fermentation of *A. calcoaceticus* SC 13876 on glucose and glycerol, samples of 200 mL broth cultures were taken, and harvested cells were used to conduct bioreduction of **1** to **2** by cell suspensions as described in the Materials and Methods section. Specific activity was expressed as μmoles of product **2** formed per min per gram dry cells.

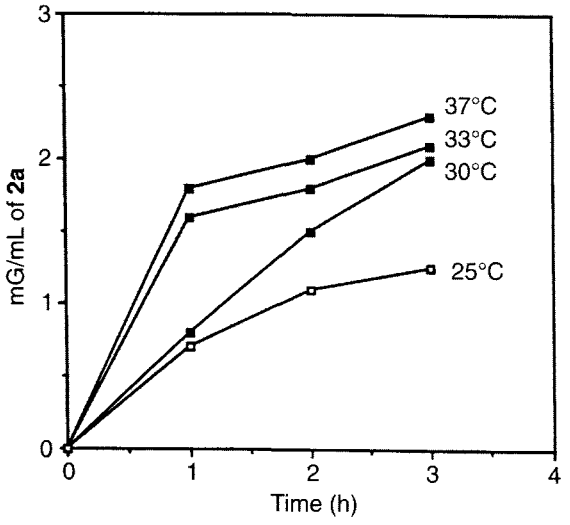


FIG. 1. Effect of temperature on the conversion of **1** to **2a** by cell suspensions of *Acinetobacter calcoaceticus* SC 13876.

1 (10 mg/mL). The reaction was carried out at 32°C and 200 rpm for 10 h. Glucose and glucose dehydrogenase were used to regenerate NADH required for reduction. A reaction yield of 92% and optical purity of >99% were obtained for **2a**. Lower *anti* diastereomer (2.5%) was obtained (Table 3). From the reaction mixture, 7.2 g of **2a** was isolated with 99% GC area % purity and 99.5% optical purity. In a separate experiment, with cell extract in a 1-L batch, partial reduction of **1** was carried out to obtain monohydroxy compounds. About 1.2 g of monohydroxy compound **3** and **4** mixture was isolated in 90% GC area % purity. The H NMR, C NMR, and HPLC analyses of isolated materials indicated that the product contained a 60:40 mixture of **3** and **4**. The isolated monohydroxy compounds **3** and **4** were further reduced to **2a** by cell extracts. On the basis of available information, the simultaneous reduction of both the 3-keto and 5-keto group of **1** has been proposed (Scheme 1). The kinetics of reduction of **1** by cell extracts is as shown in Figure 2. The concentration of monohydroxy compounds increased rapidly in the reaction mixture for the first three hours and then decreased. There was an initial one-hour lag period for the production of dihydroxy compound **2**, subsequently, all substrate was converted to **2** by cell extracts. Reductase that converted **1** to **2a** was purified 222-fold to homogeneity from cell extracts of *A. calcoaceticus* SC 13876 (Table 4). The sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified enzyme gave a single protein band corresponding to 35,000 daltons.

TABLE 3
Preparative-Scale Reduction of 1 to 2a by *Acinetobacter calcoaceticus* SC 13876[a]

Batch number	Product **2a** (g/L)	Yield of **2a** (%)	Optical purity of **2a** (%)	*Anti* diastereomers (%)
Cell suspensions (M313-109-61291)	1.6	80	97	6
Cell extracts (M313-166-7991)	9.2	92	99	2.5

[a]Reaction mixture in 3 L of 0.1 M phosphate buffer (pH 6) contained 240 g of cells (wet) of *A. calcoaceticus* SC 13876, 225 g glucose, and 6 g of substrate **1**. Reaction was carried out at 30°C, 400 rpm agitation, and 4 L/min aeration for 24 h. Bioreduction of **1** (10 g/L) by cell extracts was carried out in presence of glucose, NAD, and glucose dehydrogenase.

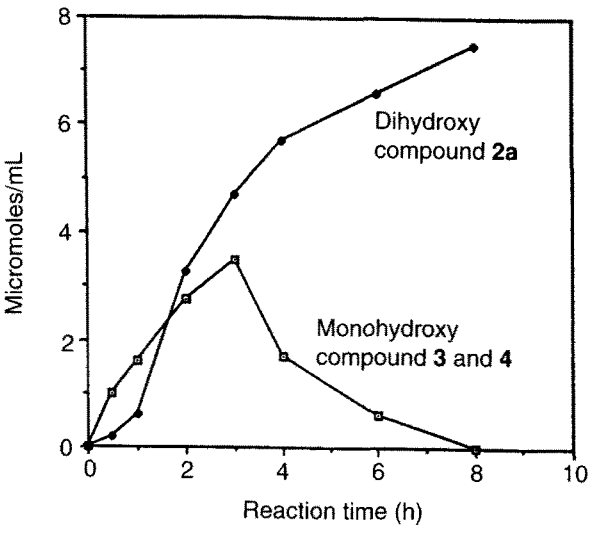


FIG. 2. Biotransformation of **1** to **2a** by cell extracts of *Acinetobacter calcoaceticus* SC 13876.

ANTICANCER DRUG

Synthesis of (2R,3S)-(−)-N-benzoyl-3-phenyl isoserine ethyl ester, a taxol side-chain synthon. Among the antimitotic agents, taxol (paclitaxel) **9** (18,19), a complex, polycyclic diterpene (20), exhibits a unique mode of action on the microtubule proteins that are responsible for the formation of the spindle during cell division. In contrast to other "spindle formation inhibitors," such as vinblastine and colchicine, both of which prevent the assembly of tubuline (21,22), taxol is the only compound known to inhibit the depolymerization process of microtubulin (23). Because of its biological activity and unusual chemical structure, taxol may represent the prototype of a new series of chemotherapeutic agents. Various types of cancers have been treated with taxol, and the results in treatment of ovarian cancer are very promising (19). In collaboration with the National Cancer Institute, we are developing taxol for treatment of various cancers. Taxol was originally isolated from the bark of the yew, *Taxus brevifolia* (18,19), and has also been found in other *Taxus* species (24,25) in relatively low yield. Alternative methods for production of taxol by cell suspension cultures and by semi-synthetic processes are being evaluated (25–28). We have described the enzymatic synthesis of a taxol side-chain intermediate. The stereoselective microbial/enzymatic reduction of **6** [2-keto-3-(*N*-benzoyl-amino)-3-phenyl propionic acid ethyl ester] to yield **7a** [(2*R*,3*S*)-(−)-*N*-benzoyl-3-phenyl isoserine ethyl ester] is demonstrated. The reduction of **6** could result in the formation of four possible alcohol diastereomers (**7a–7d**) (Scheme 2). In this segment, we describe the conditions under which only the single isomer **7a** was obtained. Coupling of alcohol **2a** to baccatin III (**8**) produces taxol (Scheme 3). Taxol **9** is one of the most important anticancer compounds to emerge from the screening of natural products in recent years.

MATERIALS AND METHODS

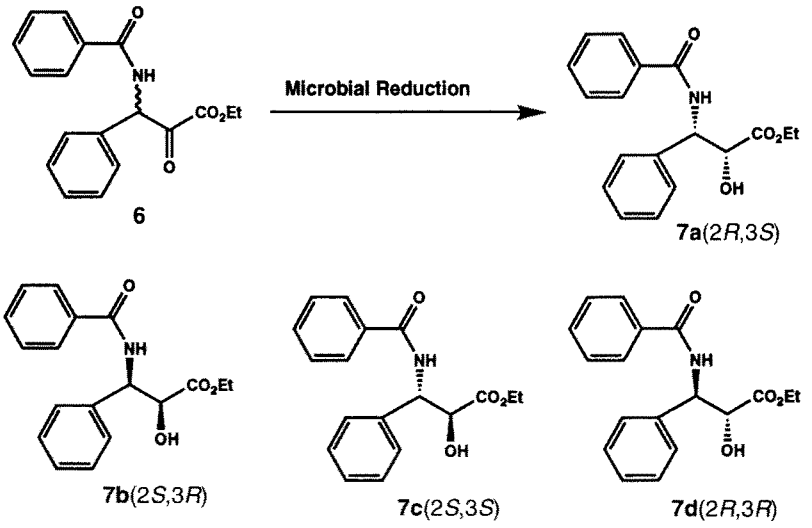
Starting substrate **6** and reference compounds **7a–7d** were synthesized in the Department of Chemical Process Research, Bristol-Myers Squibb Pharmaceutical Research Institute. The physicochemical properties, including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra), were in full accord for all these compounds. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker (Karlsruhe, Germany) AM-300 MHZ or AM-500 MHz spectrometers with tetramethylsilane as internal standard. Optical rotations were recorded on a Perkin-Elmer (Norwalk, CT) 241 polarimeter. Column chromatography was performed on silica gel (grade 60, 230–400 mesh; Merck, Darmstadt, Germany).

Microorganisms. Microorganisms were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the ATCC. Microbial cultures were stored at –90°C in vials.

Growth of microorganisms. For screening purposes, one vial of each organism 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 100 mM potassium phosphate buffer (pH 6.0), and then used for reduction studies.

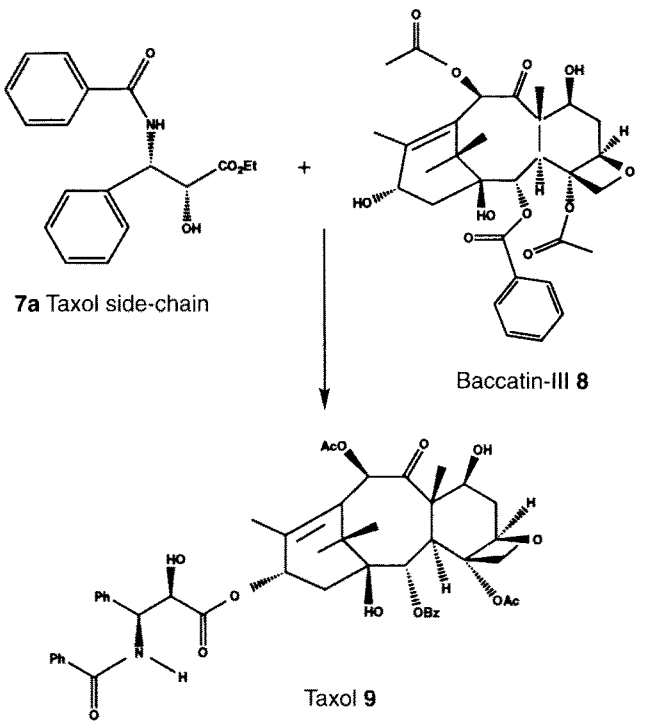
TABLE 4
Purification of Reductase from *Acinetobacter calcoaceticus* SC 13876

Step	Volume (mL)	Total activity (nmoles/mL)	Total protein (mg)	Specific activity (nmoles/min/mg)	Recovery (%)	Purification (fold)
Cell extracts	700	18400	560	32.8	100	1
DE-52 column chromatography	700	17700	140	126	96	3.8
Ammonium sulfate fraction (0–60%)	8	16800	71	230	91	7
Sephacryl S-200 column chromatography	30	11000	2.3	4750	59	144
Mono-Q column chromatography	20	4380	0.6	7300	24	222



SCHEME 2

Reduction of **6** *by cell suspensions.* Cells of various cultures were suspended separately in 100 mM potassium phosphate buffer (pH 6.0) at 20% (wt/vol, wet cells) cell concentration, and supplemented with 2.0 mg/mL of **6** and 35 mg/mL of glucose. Reduction was conducted at 28°C and 280 rpm. Periodically, samples of 1 mL were taken and extracted with four volumes of ethyl acetate. After centrifugation, the separated ethyl acetate phase was collected and dried with a nitrogen stream. The oily residue obtained was dissolved in 2 mL of mobile phase (hexane/isopropanol, 5:95, vol/vol), filtered through a 0.2-μm LID/X filter, and analyzed by HPLC.



SCHEME 3

Two-stage process: growth of Hansenula *strains in a fermentor. Hansenula polymorpha* SC13865 and *H. fabianii* SC 13894 were grown in 25-L fermentors containing 15 L of medium A. Growth consisted of two inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of each culture was inoculated independently in 100 mL of medium A. The growth stage 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 of each organism was inoculated independently into 1.5 L of medium A in a 4-L flask and incubated at 28°C and 180 rpm for 24 h. Fermentors containing 15 L of medium A were inoculated with 1.5 L of F2 stage inoculum and grown at 28°C and 500 rpm with 10 LPM aeration. Cells were periodically harvested by centrifugation from 200 mL of culture broth during fermentation. Cell suspensions (20% wt/vol, wet cells) were prepared in 100 mM potassium phosphate buffer (pH 6.0) and supplemented with 2.0 mg /mL of compound **6** and 35 mg/mL of glucose. The reaction was conducted at 28°C and 280 rpm on a shaker. Periodically, samples were removed, extracted with four volumes of ethyl acetate, and the separated organic phase was evaporated under a gentle stream of nitrogen. The oily residue was dissolved in mobile phase, filtered through a 0.2-μm LID/X filter, and analyzed for the reduction of **6** to **7** by HPLC for the reaction yield and the optical purity of **7a**. Cells were harvested at an optimum activity period with the aid of a Cepa centrifuge (Schwarzwald, Germany), and the wet cell pastes were collected and stored at 60°C until further use.

Reduction of compound **6** *in a fermentor.* Cells (*H. polymorpha* SC 13865 and *H. fabianii* SC 13894) from these batches were used to conduct the reduction of compound **6** in a 5-L fermentor in an independent experiment. Cell suspensions (20% wt/vol, wet cells) in 4 L of potassium phosphate buffer (pH 6.0) were supplemented with 8 g of compound **6** and 140 g of glucose. The reduction was conducted at 28°C

and 250 rpm. Samples were removed periodically and extracted with four volumes of ethyl acetate. After centrifugation, the organic layer was collected and dried with a nitrogen stream. The oily residue was dissolved in mobile phase, filtered through a 0.2-μm LID/X filter, and analyzed by HPLC to determine the % conversion of **6** to **7a**. The optical purity of **7a** was determined by chiral HPLC. At the end of bioreduction, 4 L of broth (*H. polymorpha* SC 13865), containing product **7a** (7.2 g), was extracted with 8 L of ethyl acetate. The ethyl acetate layer was separated by centrifugation and washed with 4 L of 7% sodium chloride solution. The separated organic phase was dried with anhydrous sodium sulfate and evaporated under reduced pressure to obtain 6.4 g of pale-yellow solid. Product **7a** (5.2 g) was crystallized from acetonitrile with >99% chemical purity and >99.8% optical purity.

Single-stage fermentation and bioreduction. Growth of *H. fabianii* SC 13894 was conducted in a 5-L fermentor (contained 4 L of medium) at 28°C and 300 rpm with 3 LPM aeration for 48 h as described earlier. The bioreduction process was initiated by the addition of substrate (8 g) and glucose (140 g) at the end of the fermentation cycle (48 h). The bioreduction process was completed in 48 h. At the end of the bioreduction, 2 L of broth containing product **7a** (3.4 g) was extracted with 4-L ethyl acetate. The ethyl acetate layer was separated by centrifugation and washed with 2 L of 7% sodium chloride solution. The organic phase was separated, dried with anhydrous sodium sulfate, and evaporated under reduced pressure to obtain 3.2 g of pale-yellow solid. Product **7a** (2.4 g) was crystallized from acetonitrile to >98% chemical purity and >99.5% optical purity.

Preparation of cell extracts. Cultures of *H. polymorpha* SC 13865 and *H. fabianii* SC 13894 were grown in a 25-L fermentor as described earlier. Cell suspensions (10% wt/vol, wet cells) in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM dithiothreitol and 10% glycerol (buffer A) were disintegrated in a Microfluidizer M-110F (Microfluidics, Inc.) at 12,000 psi pressure at 4°C. The lysates were centrifuged at 20,000 × *g* for 30 min at 4°C, and the clear supernatant collected is referred to as cell extract.

Reduction of compound **6** *by cell extracts.* Cell extracts from *H. polymorpha* SC 13865 and *H. fabianii* SC 13894 were evaluated for the reduction of **6**. The reaction mixture contained 0.5 mM NADH and 100 mg of **6** in 50 mL of cell extract. The reactions were conducted at 28°C and 250 rpm on a shaker. A control experiment was carried out under similar conditions, except NADH was not added to the reaction mixture. Protein was determined by Bradford's method (17).

Analytical methods. Samples (5 mL) from the reaction mixture were extracted with two volumes of ethyl acetate, and the separated organic phase was filtered through a 0.2-μm LID/X filter. Collected samples were analyzed for substrate and product concentrations with a Hewlett-Packard 1070 HPLC. A Phenomenex Cyanopropyl (Torrance, CA) column (150 × 4.6 mm, 5μ) was used. The mobile phase consisted of 5% isopropanol in hexane. The ultraviolet detector wavelength was 230 nm. The retention times for substrate **6**, *syn* diastereomer (**7a**, **7b**), and *anti* diastereomer (**7c**, **7d**) of product were 26.8, 20.4, and 22.2 min, respectively.

Separation of the two enantiomers of the *syn* and *anti* diastereomers was achieved by HPLC with dual columns connected in series. The first column was a Pirkle column (dinitrophenylglycine, 250 × 4.6 mm, 5 μ; Alltech, Deerfield, IL), and the second column was Chiralcel OB (250 × 4.6 mm, 5 μ). The mobile phase consisted of 25:2.5:2.5:70 of isopropanol/*n*-butanol/methanol/hexane. The flow rate was 0.5 mL/min, and the detector wavelength was 230 nm. The retention times for the two enantiomers of the *syn* diastereomer of **7** were 20.4 and 23.4 min. The retention times for the two enantiomers of the *anti* diastereomer of **7** were 21.3 and 27.2 min.

RESULTS

As shown in Table 5, the reaction yield and stereoselectivity were dependent upon the microorganism used during the reduction of **6** to **7a**. Organisms from genus *Nocardia*, *Candida*, *Rhodococcus, Mortierella, Saccharomyces*, and *Hansenula* predominantly converted ketone **6** to the desired alcohol **7a** in high optical purity (>91%), and organisms from genus *Pullularia* and *Trichoderma* gave lower optical purity (75–88%) of the desired alcohol **7a**. *Pichia methanolica* ATCC 16623 reduced **6** to **7a** in the lowest optical purity (the undesirable enantiomer **7b** is produced). *Hanzenula polymorpha* SC 13865 and *H. fabianii* SC 13894 effectively reduced compound **6** to **7a**. Reaction yields of >80% and optical purities of >95% were observed for these biotransformations.

Cells of *H. polymorpha* SC13865 and *H. fabianii* SC13894 were grown in a 25-L fermentor containing 15 L of medium with glucose as the carbon source. Fermentors containing 15

TABLE 5
Microbial Reduction of 6 to 7a[a]

Microorganisms[b]	Reaction yield compound **7a** (%)	Optical purity compound **7a** (%)
Candida guilliermondi ATCC 20318	31	95
Rhodococcus erythropolis ATCC 4277	39	96
Saccharomyces cerevisiae ATCC 24702	35	94
Hansenula polymorpha SC 13865	80	99
Pseudomonas putida ATCC 11172	32	94
Mortierella rammanianna ATCC 38191	35	97
Hansenula fabianii SC 13894	85	95
Pichia methanolica ATCC 58403	80	26
Nocardia salmonicolor SC 6310	45	99

[a]The reaction yield and the optical purity were determined by high-performance liquid chromatography.
[b]ATCC, American Type Culture Collection (Rockville, MD).

TABLE 6
Growth of *Hansenula polymorpha* and *H. fabianii* in a 15-L Fermentor: Reduction of Compound 6[a]

Microorganism	Sample time (h)	*Anti* diastereomers (**7c**, **7d**) (%)	*Syn* diastereomers (**7a**, **7b**) (%)	Optical purity of desired *syn* (**7a**) (%)
H. polymorpha				
SC 13865	24	28	72	99.6
	32	25	75	98.9
	40	20	80	99.3
	48	20	80	99.3
H. fabianii				
SC 13894	24	12.7	87.3	93
	32	9.8	90.2	93.3
	40	10.8	89.2	93.6
	48	8.9	91.1	94

[a]Cultures were grown in a 15-L fermentor. Broth samples were taken at designated time, and reduction of **6** was carried out by cell suspensions (20%, wt/vol, wet cells) of organisms. After 48 h reaction time, the reaction yields of *anti* and *syn* diastereomers and the optical purity of **7a** were determined by high-performance liquid chromatography.

L of medium A were inoculated with 1.5 L inoculum and grown at 28°C and 500 rpm with 10 LPM aeration. During growth in a fermentor, broth samples (1 L) were taken at 24, 32, 40, and 48 h after inoculating the fermentor. Cells were collected by centrifugation, suspended in buffer, and reduction of **6** was carried out. Cells harvested after 40 h gave 80 and 91% reaction yields for *H. polymorpha* SC 13865 and *H. fabianii* SC 13894, respectively (Table 6). Lower optical purity (94%) of **7a** was obtained with *H. fabianii* SC 13894 compared with *H. polymorpha* SC 13865 (99% optical purity).

Preparative-scale reductions of ketone **6** to desired alcohol **7a** were carried out in a 5-L fermentor with cell suspensions of *H. polymorpha* SC 13865 and *H. fabianii* SC 13894 in independent experiments. In both batches, a reaction yield of 80–90% and an optical purity of >94% were obtained for compound **7a** (Table 7). From one batch (*H. polymorpha* SC 13865), 5.2 g of **7a** was isolated in 65% overall yield. The isolated compound gave 99.6% optical purity and 99.8% GC area % purity. The specific rotation of –21.7 was obtained for **7a** in chloroform.

A single-stage fermentation/bioreduction process was developed for conversion of compound **6** to compound **7a** with cells of *H. fabianii* SC 13894. Cells were grown in a 5-L fermentor, and after a 48-h growth cycle, the bioreduction process was initiated by addition of substrate and glucose and continued for a 48-h period. A reaction yield of 88% and an optical purity of 95% were obtained for the desired alcohol **7a** (Table 8). From the 2-L fermentation broth after bioreduction, 2.4 g of compound **7a** was isolated. The isolated compound gave 99.5% optical purity and 99.5% GC area % purity.

Cell extracts of *H. polymorpha* SC 13865 and *H. fabianii* SC 13894 were prepared and examined for the reduction of ketone **6**. Reaction mixtures in 50 mL of extract contained 0.5 mM NADH and 100 mg of substrate **6**. After a 48-h reaction time, a reaction yield of 80% and an optical purity of 98% were obtained for compound **7a** in both batches.

POTASSIUM CHANNEL OPENERS

Epoxidation of 2,2-dimethyl-2H-1-benzopyran-6-carbonitrile to the corresponding chiral epoxide and diol. The current interest in microbial production of chiral epoxides by stereoselective oxygenation lies in the preparation of intermediates for chemical synthesis (29–37). The study of potassium (K) channel biochemistry, physiology, and medicinal chemistry has flourished, and numerous papers and reviews have been published in recent years (38–41). It has long been known that K channels play a major role in neuronal excitability, and it is now clear that K channels also play a complex and critical role in the basic electrical and mechanical functions of a wide variety of tissues, including smooth muscle, cardiac muscle, and glands (42). A new class of highly specific pharmacolog-

TABLE 7
Preparative-Scale Reduction of 6 by Cell Suspensions of *Hansenula* Strains: Two-Stage Process[a]

Microorganisms	Reaction time (h)	*Anti* diastereomers (**7c**, **7d**) (%)	*Syn* diastereomers (**7a**, **7b**) (%)	Optical purity desired *syn* **7a** (%)
H. polymorpha				
SC 13865	72	20	80	99
H. fabianii				
SC 13894	48	10	90	94

[a]Cells were suspended in 4-L of 0.1 M phosphate buffer (pH 6) at 20% (wt/vol) concentration. Cell suspensions were supplemented with 2 g/L of substrate **6** and 35 g/L of glucose. The reduction of **6** was carried out at 28°C, 250 rpm in a 5-L NB Bioflo fermentor (New Brunswick Scientific, Edison, NJ).

TABLE 8
Reduction of 6 by *Hansenula fabianii* Culture: Single-Stage Process[a]

Reaction time (h)	*Anti* diastereomers (**7c**, **7d**) (g/L)	*Syn* diastereomers (**7a**, **7b**) (g/L)	Optical purity desired *syn* (**7a**) (%)
12	0.04	0.3	ND
24	0.09	0.63	ND
36	0.15	0.92	ND
46	0.18	1.18	93
60	0.2	1.5	94
72	0.21	1.7	94.5

[a]Cells were grown in a 25-L fermentor containing 15 L of medium at 28°C, 600 rpm agitation. After 48 h of growth period, substrate **6** (2 g/L) and glucose (35 g/L) were added to the fermentation broth, and the bioreduction process was continued. ND, not determined.

ical compounds has been developed which either open or block K channels (43–47). K channel openers are powerful smooth muscle relaxants with *in vivo* hypotensive and bronchodilator activity, originally typified by cromakalim, nicorandil, and pinacidil (48).

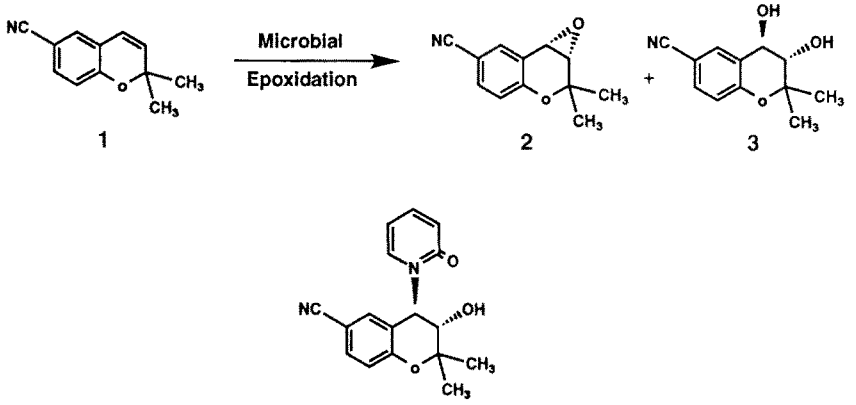
Recently, the synthesis and antihypertensive activity of a series of novel K channel openers (37–46), based on monosubstituted *trans*-4-amino-3,4-dihydro-2,2-dimethyl-2H-1-benzopyran-3-ol, have been demonstrated. In this section we describe the stereoselective microbial oxygenation of 2,2-dimethyl-2H-1-benzopyran-6-carbonitrile **10** to the corresponding (3*S*,4*S*-*cis*)-epoxide **11** and (3*S*,4*R*)-*trans* diol **12** (Scheme 4). Chiral epoxide **11** and diol **12** are potential intermediates for the synthesis of K channel activators that are important as antihypertensive and bronchodilator agents.

MATERIALS AND METHODS

Materials. Substrate **10** and standards of compounds **11** and **12** were synthesized by Chemical Process Development, Bristol-Myers Squibb Pharmaceutical Research Institute. The proton magnetic resonance (^{1}H NMR) and carbon magnetic resonance (^{13}C NMR) were recorded on a Bruker AM-300 spectrometer. The physicochemical properties, including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra), were in full accord for all these compounds.

Microorganisms. Microorganisms were obtained from our culture collection in the Microbial Technology Department of Bristol-Myers Squibb and from the ATCC. Microorganisms were stored at –90°C in vials.

Microbial epoxidation of compound **10**. For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A (2% glucose, 1% yeast extract, 1% malt extract, 0.3% peptone adjusted to pH 6.8) in a 500-mL Erlenmeyer flask. Cultures were grown at 28°C and 280 rpm agitation for 48 h in the presence of 0.2 mg/mL of compound **10**. Cultures were harvested by centrifugation at 20,000 × *g* for 15 min, washed with 100 mM potassium phosphate buffer (pH 6.8), and used for biotransformation studies. Cells of various 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 1 mg/mL of compound **10**. The reaction was carried out at 25°C and 200 rpm agitation for 120 h. Periodically, 1-mL samples were



SCHEME 4

taken and extracted with 4 mL of solvent consisting of toluene/tertiary butyl methyl ether (1:1 mixture). The separated organic phase was filtered through a 0.2-μm LID/X filter and analyzed for substrate and product concentrations by a Hewlett-Packard GC system. The optical purities of epoxide **11** and diol **12** were analyzed by chiral HPLC.

Growth of organisms in a fermentor. Corynebacterium sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor containing 15 L of medium A and 0.2 g/L of substrate **10** or medium C in the absence of substrate **10** (corn steep liquor, 20 mL; cerelose, 33 g; ammonium phosphate, 3 g; yeast extract, 1 g; soybean oil, 2.5 mL; and calcium carbonate, 1 g in 1 L distilled water). Growth of cells consisted of several inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, frozen vials of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were inoculated independently into 100 mL of medium A in a 500-mL Erlenmeyer flask. Growth was carried out at 28°C and 280 rpm agitation 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 agitation for 24 h. A fermentor with 15 L of medium A and 0.2 g/L of substrate **10** (*Corynebacterium* sp. SC 13876) or medium C in the absence of substrate **10** *(M. ramanniana)* was inoculated with 1.5 L of F2 stage inoculum. The fermentation was conducted for 48 h at 28°C and 600 rpm agitation with 15 LPM aeration at pH 6.8. To determine the epoxidation activity of cells during fermentation, cells were periodically harvested by centrifugation from 200 mL of culture broth. Cell suspensions (10% wt/vol of wet cells) were prepared in 100 mM potassium phosphate buffer (pH 6.8) and supplemented with 1 mg/mL of compound **10**. The reaction was carried out at 25°C and 250 rpm agitation on a rotary shaker (New Brunswick Scientific, New Brunswick, NJ). Samples were periodically taken and analyzed for the biotransformation of compound **10** to compounds **11** and **12** by GC. The activity of cells was expressed as mg of epoxide **11** and diol **12** formed per hour per gram of dry cells. After 48 h of fermentation, cells were harvested with the aid of a Cepa centrifuge, and wet-cell pastes were collected. Cells were used either to conduct the epoxidation reaction or were stored at −90°C until further use. About 400 g of wet-cell paste was collected from each fermentation.

Epoxidation of compound **10** *in a fermentor (two-stage process). Mortierella ramanniana* SC 13840 cells, harvested from these batches, were used to conduct the epoxidation of **10** in a 5-L BioFlo (New Brunswick Scientific Co., Edison, NJ) fermentor. Cell suspensions (10% wt/vol of wet cells) in 3 L of 100 mM potassium phosphate buffer (pH 6.8) were used. Substrate **10** (3 g) and glucose (15 g) were added to the cell suspension, and the epoxidation reaction was conducted at 28°C and 250 rpm agitation with 2.5 LPM aeration. After 24 h, additional substrate **10** (3 g) and glucose (15 g) were added, and biotransformation was continued. Periodically, samples (2 mL) were removed and extracted with 8 mL of solvent (toluene/tertiary butyl methyl ether, 1:1 vol/vol). After centrifugation, the organic phase was collected, filtered through a 0.2-μm LID/X filter, and a portion of the organic phase was analyzed by GC to determine the conversion of **10** to **11** and **12**. The remaining portion was dried under a gentle stream of nitrogen. The oily residue was dissolved in 1 mL of mobile phase that consisted of hexane/isopropanol mixture (1:1,vol/vol) and filtered through a 0.2-μm LID/X filter and analyzed by HPLC to determine the optical purity of epoxide **11** and diol **12**. At the end of the biotransformation, the reaction mixture (3 L), containing 5.6 g of chiral diol, was adjusted to pH 5.5 with 1 N sulfuric acid and extracted twice with 6 L of cyclohexane. The organic phase, containing the remaining substrate, was separated from the aqueous phase, which contained chiral diol **12**. The aqueous phase was extracted twice with 6 L of solvent (toluene/tertiary butyl methyl ether, 1:1 vol/vol). The separated organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain 0.3 L of rich toluene containing 4.9 g of chiral diol **12**. The toluene concentrate was further purified by flash-chromatography on a silica column to obtain 4.6 g of diol **12** in 65 M% overall yield with 98% chemical purity (GC area %) and 97% optical purity.

Single-stage fermentation/biotransformation. Corynebacterium sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor with 15 L of medium A (in the presence of 0.2 g/L of substrate **10**) or medium C (in the absence of substrate **10**), respectively, as described previously. After 24 h of growth period, each fermentor was supplemented with substrate **10** (15 g) and glucose (75 g), and biotransformation was continued. Samples were removed periodically and analyzed for substrate **10**, epoxide **11**, and diol **12** concentrations by GC. Optical purities of **11** and **12** were determined by chiral HPLC. At the end of the biotransformation (120 or 48 h for *Corynebacterium* sp. SC 13876 or *M. ramanniana* SC 13840, respectively), 5 L of broth from each bioreaction batch were used to recover diol **12** as described previously: 2.02 g (30 M%) and 2.95 g (49 M%) of (+)-*trans* diol **12** were isolated, respectively, from *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 batches in 96% chemical (GC area %) purity.

Oxidation of epoxide **11**. *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor with 15 L of medium A containing 0.2 g/L of substrate **10** or with medium C in the absence of substrate **10**, as described previously. Cell suspensions (10% wt/vol, wet cells) of organisms were evaluated for the bioconversion of epoxide **11** to diol **12**. Cells (25 mL reactor) were supplemented with 25 mg of epoxide **11**, and biotransformation was carried out at 250 rpm and 25°C. One-mL samples were taken periodically and extracted with 4 mL of solvent (toluene/tertiary butyl methyl ether, 1:1 vol/vol). The separated organic phase was filtered through a 0.2-μm LID/X filter, and analyzed for epoxide **11** and diol **12** concentrations by a Hewlett-Packard GC system. Control experiments in the absence of cells also were carried out as described previously.

Epoxidation of compound **10** *by cell extracts. Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor with 15 L of medium A containing 0.2 g/L of substrate **10** or with medium C in the absence of substrate **10** as described previously. Cells were independently suspended in buffer A [50 mM (3-[*N*-morpholino] propanesulfonic acid) buffer pH 7.0 containing 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM ferrous ammonium sulfate] at 20% (wt/vol of wet cells) cell concentration. Cell suspensions were disintegrated by two passages through a French Press at 15,000 psi pressure at 4°C. Disintegrated cells were centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant solution is referred to as cell extract. Protein in cell extracts was determined by Bradford's method (17).

Cell extracts were analyzed for the ability to catalyze the oxygenation of **10** to **11** and **12**. The reaction mixture contained 0.8 mM NADH or NADPH and 5 mg of substrate in 10 mL of cell extract. The reaction was carried out at 20°C and 200 rpm on a rotary shaker. Control reaction mixtures in the absence of NADH or NADPH were carried out under similar conditions. After 48 h, the reaction mixture was analyzed by GC for the substrate **10** and products **11** and **12**.

Analytical methods. Analysis of compounds **10**, **11**, and **12** was carried out in a Hewlett-Packard 5890 gas chromatograph with a FID. An HP-2 fused-silica capillary column (25 m long × 0.32 mm i.d. × 0.17 mm thickness) was used at 250°C injection temperature, 250°C detector temperature, and 195°C oven temperature. The carrier gas was helium, and total run time was 8 min. The retention times for **10**, **11**, and **12** were 2.64, 3.44, and 6.14 min, respectively, under these conditions. The optical purity of compounds **11** and **12** were determined by chiral HPLC. Reaction samples (5 mL) were extracted with ethyl acetate (10 mL), and an aliquot of 5 mL was dried under a gentle stream of nitrogen. The residue was suspended in 2 mL hexane and passed through a silica cartridge (Bond Elut LRC; Analytichem, Harbor City, CA) previously equilibrated with hexane. The column was washed with 10 mL hexane, and compounds **10**, **11**, and **12** were eluted with 10 mL of 50% acetone in hexane. All solvent was evaporated under a gentle stream of nitrogen, and the residue was dissolved in 1 mL of 50% isopropanol in hexane. Samples were filtered through a 0.4-μm filter and analyzed by HPLC. Two columns were used in series. The first column was a C18 (J.T. Baker) column (ODS, 100 × 4.6 mm; Hewlett-Packard), and the second column was a Chiralcel OD (250 × 4.6 mm; Diacel Chemical Industries, Easton, PA). The mobile phase consisted of 95:4:1 hexane/*n*-butanol/cyclohexanol. The flow rate was 0.8 mL/min, and the detector wavelength was 254 nm. The retention times for the two enantiomers of racemic epoxide were 11 and 12.8 min. The retention times for the *cis* and *trans* diols were 29.4 and 33.2 min, respectively.

RESULTS

Various microorganisms were screened for the stereoselective oxygenation of **10** to epoxide **11** and diol **12**. As shown in Table 9, the reaction yield and stereoselectivity were dependent upon the microorganism used during the epoxidation of **10**. *Corynebacterium* sp. SC 13876, *Rhodococcus erythropolis* SC 13845, *Pseudomonas dehalogens* SC 13873, *H. polymorpha* SC 13865, and *M. ramanniana* SC 13840 catalyzed the epoxidation of compound **10** to **11**. This epoxide was further converted to (+)-(3*S*,4*R*) *trans* diol **12**. Reaction yields of 32 molar percent (M%) and 67.5 M% and optical purities of 88 and 96% were obtained for (+)-*trans* diol **12**, respectively, with the two best cultures, *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840.

Further research was conducted with *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC13840 to convert compound **10** to (+)-*trans* diol **12**. *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor with 15 L of medium A containing 0.2 g/L of sub-

TABLE 9
Stereoselective Biotransformation of 10 to Epoxide 11 and Diol 12[a]

Microorganisms	Substrate **10** (mg/mL)	Epoxide **11** (mg/mL)	Epoxide **11** M% Yield	Optical purity of epoxide **11** (%)	Diol **12** (mg/mL)	Diol **12** M% Yield	Optical purity of diol **12** (%)
Corynebacterium sp. SC 13876	0.35	0.21	19.3	87	0.38	32	88
Pseudomonas dehalogens SC 13873	0.72	0.08	7.4	ND	0.21	17.7	ND
Nocardia corallina SC 13897	0.7	0.1	9.2	ND	0.15	12.6	ND
Rhodococcus erythropolis SC 13845	0.52	0.18	16.5	75	0.32	27	76
Hansenula polymorpha SC 13865	0.8	0.05	4.6	82	0.12	10.1	ND
Mortierella ramanniana SC 13840	0.1	0.09	8.3	95	0.8	67.5	96

[a]Reactions were allowed to proceed for 120 h as described in the Materials and Methods section. The concentrations of compounds **10**, **11**, and **12** were determined by gas chromatography. The optical purities of compounds **11** and **12** were determined by chiral high-performance liquid chromatography. ND, not determined.

TABLE 10
Growth of *Corynebacterium* sp. SC 13876 and *Mortierella ramanniana* SC 13840 in a 25-L Fermentor: Evaluation of Cells for Biotransformation of 10 to Epoxide 11 and Diol 12[a]

Cell harvest time (h)	Substrate **10** (mg/mL)	Epoxide **11** (mg/mL)	Epoxide **11** M% Yield	Diol **12** (mg/mL)	Diol **12** M% Yield	Optical purity of epoxide **11** (%)	Optical purity of diol **12** (%)
Corynebacterium sp. SC 13876:							
30	0.85	0.12	11.0	0.04	3.4	86	87
36	0.81	0.1	9.2	0.16	13.5	86.5	88
42	0.3	0.16	14.7	0.3	25	87	88.9
48	0.21	0.18	16.5	0.38	32	88	90
M. ramanniana SC 13840:							
30	0.25	0.12	11.0	0.6	50.6	95	95
36	0.2	0.1	9.2	0.72	60.7	ND	95.5
42	0.06	0.07	6.5	0.82	69.2	ND	96
48	0.05	0.07	7.3	0.85	72	ND	96

[a]Biotransformation process was carried out as described in the Materials and Methods section. The substrate **10** and products **11** and **12** concentrations were determined by gas chromatography. The optical purities of epoxide **11** and diol **12** were determined by chiral high-performance liquid chromatography. ND, not determined.

strate **10** or with medium C in the absence of substrate **10**. Cell suspensions were used to carry out the biotransformation of compound **10**. Cells harvested after 42–48 h of growth gave a higher reaction yield and optical purity of (+)-*trans* diol **12**. Reaction yields of 32 and 72 M% and optical purities of 90 and 96% were obtained for (+)-*trans* diol **12** with cells (grown for 48 h) of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840, respectively (Table 10).

A single-stage fermentation/biotransformation process was developed for conversion of compound **10** to (+)-*trans* diol **12** with cells of *Corynebacterium* sp. SC 13876 or *M. ramanniana* SC 13840. Cells of *Corynebacterium* SC 13876 were grown in a 25-L fermentor containing 10 L of medium. After 24 h of growth in the presence of 0.2 g/L of substrate **10**, the biotransformation process was initiated by addition of 1 g/L of substrate **10**, 5 g/L of glucose and 5 L of additional medium. After a 120-h reaction period, (+)-*trans* diol **12** was obtained with a reaction yield of 38.6 M% and an optical purity of 90% (Table 11). In contrast, *M. ramanniana* cultures were grown in a 25-L fermentor with 15 L of medium in the absence of substrate **10**. After 24 h growth, the biotransformation was initiated by addition of 1 g/L of substrate **10** and 5 g/L of glucose. A higher reaction yield (60.7 M%) and optical purity (92.5%) were obtained with *M. ramanniana* SC 13840 (Table 12). (3*S*,4*S*)-Epoxide **11** was obtained as an intermediate during biotransformation in both organisms (Tables 11 and 12). From 5 L of each reaction broth, (+)-*trans* diol **12** was isolated in 30 M% (*Corynebacterium* sp. SC 13876) and 49 M% (*M. ramanniana* SC 13840) overall yield with 96% chemical purity and >92% optical purity in each batch.

In the two-stage process, biotransformation of compound **10** to (+)-*trans* diol **12** was carried out in a 5-L fermentor with a cell suspension of *M. ramanniana* SC 13840. Cells harvested from a 25-L fermentor were suspended in 3 L of buffer at 10% (wt/vol, wet cells) concentration. Substrate was then added to give 2 g/L concentration. A reaction yield of 76 M% and an optical purity of 96% were obtained for (+)-*trans* diol **12** (Table 13). Isolation of chiral diol **12** was carried out as described in the Materials and Methods section—4.6 g of product were recovered in 65 M% overall yield. Isolated (+)-*trans* diol **12** had an optical purity of 97% as analyzed by chiral HPLC and 98% chemical purity as analyzed by GC and HPLC.

Cell suspensions (10% wt/vol, wet cells) of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were examined for the enzymatic conversion of epoxide **11** to diol **12**. *Mortierella ramanniana* SC 13840 cells enzymatically converted epoxide **11** to the corresponding diol **12** in a 12-h reac-

TABLE 11
Biotransformation of 10 to Epoxide 11 and Diol 12 by *Corynebacterium* sp. SC 13876: Single-Stage Process[a]

Fermentation time (h)	Substrate **10** (mg/mL)	Epoxide **11** (mg/mL)	Epoxide **11** M% Yield	Diol **12** (mg/mL)	Diol **12** M% Yield	Optical purity of epoxide **11** (%)	Optical purity of diol **12** (%)
24	0.95	0.016	1.5	0	0	ND	NA
48	0.84	0.12	11	0.12	8.9	ND	ND
72	0.35	0.15	13.8	0.23	17.1	87	ND
96	0.38	0.18	16.5	0.35	26	ND	88
120	0.3	0.06	5.5	0.52	38.6	ND	90

[a]Single-stage biotransformation process was carried out as described in the Materials and Methods section. Periodically, samples were removed and analyzed for substrate **10** and products **11** and **12** concentrations by gas chromatography. The optical purities of epoxide **11** and diol **12** were determined by chiral high-performance liquid chromatography. NA, not applicable; ND, not determined.

TABLE 12
Biotransformation of 10 to Epoxide 11 and Diol 12 by *Mortierella ramanniana* SC 13840: Single-Stage Process[a]

Fermentation time (h)	Substrate **10** (mg/mL)	Epoxide **11** (mg/mL)	Epoxide **11** M% Yield	Diol **12** (mg/mL)	Diol **12** M% Yield	Optical purity of epoxide **11** (%)	Optical purity of diol **12** (%)
12	0.6	0.12	11	0.02	1.68	ND	ND
24	0.4	0.2	18.4	0.32	27	ND	ND
36	0.2	0.21	19.3	0.5	42.2	92	ND
48	0.12	0.05	4.6	0.72	60.7	ND	92.5

[a]Single-stage biotransformation process was carried out as described in the Materials and Methods section. Samples were periodically removed and analyzed for substrate **10** and products **11** and **12** concentrations by gas chromatography. The optical purities of epoxide **11** and diol **12** were determined by chial high-performance liquid chromatography. ND, not determined.

tion time with a reaction yield of 90 M%. In the absence of cells, only 28 M% conversion of epoxide **11** to (+)-*trans* diol **12** was obtained in 24 h when incubated at pH 6.0. Under acidic conditions (pH 6.0) and in the presence or absence of *Corynebacterium* sp. SC 13876 cells, nonenzymatic conversion of epoxide **11** to (+)-*trans* diol **12** was observed with 28 M% and 80 M% reaction yields after 24 and 72 h incubation periods. The accumulation of toxic epoxide **11** during biotransformation of compound **10** to **12** may be responsible for the poor yield of diol **12** with cells of *Corynebacterium* sp. SC 13876. Indeed, the initial addition of epoxide **11** (0.2 mg/mL) during biotransformation with *Corynebacterium* sp. SC 13876 inhibited the conversion of **10** to **12** by 40% (Fig. 3).

The effects of pH and temperature on the oxygenation of compound **10** to diol **12** by both cultures were evaluated in 25-mL flasks. Cell suspensions (5% wt/vol of wet cells) were supplied with 25 mg of substrate **10**, and biotransformation was carried out on a rotary shaker for 48 h. The optimum pH for the bioconversion of compound **10** to diol **12** is 6.5–7.0, and the optimum temperature is 25°C.

Cell extracts of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were prepared and examined for the epoxidation of **10**. After a 72-h reaction time in the presence of NADH, a reaction yield of 4.2 M% for epoxide **11** and 6.7 M% for diol **12** was obtained with cell extracts of *Corynebacterium* sp. SC 13876. Optical purities of about 90% were obtained for both compounds. Cell extracts of *M. ramanniana* SC 13840 produced 12.6 M% diol **12** in 96% optical purity. NADPH could replace NADH as a cofactor with only 60% efficiency, and in the absence of cofactor (NADH or NADPH), the epoxidation of compound **10** to epoxide **11** was not observed.

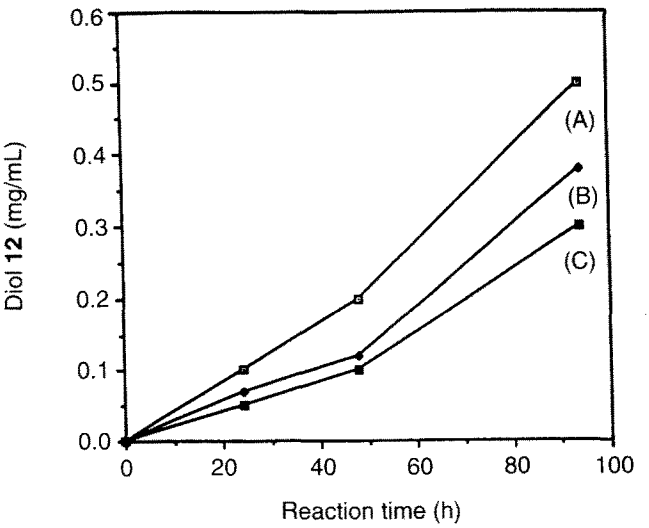


FIG. 3. Biotransformation of **10** to **12** by cell suspensions of *Corynebacterium* sp. SC 13876—effect of added epoxide. (A) Control, (B) epoxide added (0.1 mg/mL), and (C) epoxide added (0.2 mg/mL).

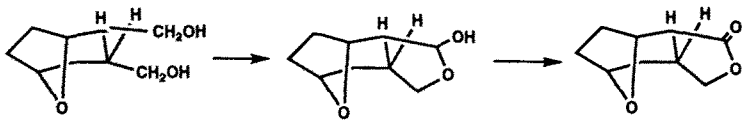
THROMBOXANE ANTAGONIST

Oxidation of (exo, exo)*-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol to the corresponding chiral lactol and lactone.* Thromboxane A_2 (TxA_2) is an exceptionally potent pro-aggregatory and vasoconstrictor substance produced by the me-

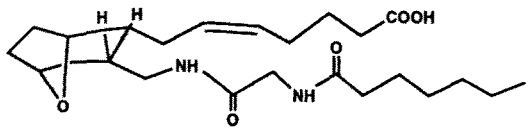
TABLE 13
Biotransformation of 10 to Epoxide 11 and Diol 12 by *Mortierella ramanniana* SC 13840: Two-Stage Process[a]

Reaction time (h)	Substrate **10** (mg/mL)	Epoxide **11** (mg/mL)	Epoxide **11** M% Yield	Diol **12** (mg/mL)	Diol **12** M% Yield	Optical purity of epoxide **11** (%)	Optical purity of diol **12** (%)
12	1.8	0.15	6.9	0.05	2.1	96	ND
24	0.89	0.11	5.0	1.03	43.3	ND	97
36	0.5	0.09	4.1	1.3	54.8	ND	95.8
48	0.06	0.025	1.15	1.8	76	ND	96

[a]Two-stage biotransformation process was carried out as described in the Materials and Methods section. Samples were periodically removed and analyzed for substrate **10** and products **11** and **12** concentrations by gas chromatography. The optical purities of epoxide **11** and diol **12** were determined by chiral high-performance liquid chromatography. ND, not determined.



DIOL **14** LACTOL **15** LACTONE **16**



Thromboxane A_2 Antagonist **13**

SCHEME 5

tabolism of arachidonic acid in blood platelets and other tissues. Together with the potent anti-aggregatory and vasodilator prostacyclin, it is thought to play a role in the maintenance of vascular homeostasis, and may contribute to the pathogenesis of a variety of vascular disorders. Approaches toward limiting the effect of TxA_2 have focused on either inhibiting its synthesis or blocking its action at its receptor sites by means of an antagonist (49–52). The lactol **15** is a key chiral intermediate for total chemical synthesis of [1*S*-[1α,2α(*Z*),3α,4α,]]-7-[3-[[[1-oxoheptyl)amine]acetyl]-methyl]-7-oxabicyclo-[2.2.1] hept-2-yl-5-heptanoic acid **13**, a new cardiovascular agent useful in the treatment of thrombolic disease (52–55).

We are describing the stereoselective oxidation of (*exo,exo*)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol **14** to the corresponding chiral lactol **15** and lactone **16** (Scheme 5). We have identified microorganisms with this capability and have conducted preparative-scale stereoselective oxidation of diol **14** to the corresponding lactol **15** and lactone **16** by utilizing cell suspensions of these microorganisms.

MATERIALS AND METHODS

Growth of microorganisms. Microorganisms were obtained from the culture collection of the Bristol-Myers Squibb and from the ATCC. Microorganisms were stored at −90°C in vials. For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A, which contained 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 agitation for 48 h on a rotary shaker. A 50-mL of inoculum from the flask was used to inoculate each organism tested in a 2-L flask with 600 mL of medium A. After 48 h of growth at 28°C and 280 rpm agitation, cultures were harvested by centrifugation, washed with buffer A (0.1 M potassium phosphate buffer, pH 7), and used for the oxidation of diol **14**.

Oxidation of diol **14** *by microbial cell suspensions.* Cells of various microorganisms were suspended separately in 10 mL of buffer A at 10% (wt/vol, wet cells) concentration and supplied with 10 mg of diol **14**. The oxidation reaction was conducted at 28°C and 280 rpm agitation for 72 h on a rotary shaker. After completion of biotransformation, cell suspensions were adjusted to pH 4 and extracted with four volumes of ethyl acetate. After centrifugation, the separated organic phase was dried under a nitrogen stream. The oily residue obtained was dissolved in methanol, filtered through 0.2-μm LID/X filter, and analyzed for substrate and product concentrations by GC.

Growth of microorganisms in a fermentor. Nocardia globerula ATCC 21505 and *Rhodococcus* sp. ATCC 15592 each were grown in a 380-L fermentor with 250 L of medium D (1.5% 1,2-propanediol, 1% yeast extract, 0.3% peptone adjusted to pH 6.8). Growth consisted of inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, frozen vials of each organism were inoculated in 100 mL of medium A, and growth was carried out in a 500-mL flask for 48 h as described earlier. 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 growth was carried out at 28°C and 180 rpm agitation for 24 h. A seed fermentor, containing 250 L of medium D, was inoculated with 1.5 L of F2 stage inoculum, and cultures were grown for 48 h at 28°C, 280 rpm agitation, and 250 LPM (L/min) aeration. Fermentors containing 250 L of medium D were inoculated with 13 L of cultures from the seed fermentor. Fermentations were conducted at 28°C, 250 rpm agitation, and 200 LPM aeration. After 48 h of growth, cells were harvested with a Sharples (Alphalavel Separation Inc., Warminster, PA) centrifuge, and wet-cell pastes (8 kg) were collected were stored at −60°C until further use.

Oxidation of compound **14** *in a fermentor.* Frozen cells from these batches were used to conduct the oxidation of diol **14** in a 5-L fermentor. Cells (400 g) were suspended in 4 L of buffer A and homogenized to prepare cell suspensions. Compound **14** (20 g) was supplied to the cell suspensions, and the oxidation was conducted at 28°C, 400 rpm agitation, and 4 LPM aeration for 168 h. Samples were taken periodically, ex-

TABLE 14
Oxidation of Diol 14 to Lactol 15 and Lactone 16 by Cell Suspensions of Microorganisms[a]

Microorganisms	Reaction time (h)	Lactol **15** (μg/mL)	Lactone **16** (μg/mL)	Conversion (%)	Optical purity of lactone **16** (%)
Geotrichum candidum SC 5469	66	187	216	40	ND
Nocardia restrictus ATCC 14807	24	26	497	52	ND
Nocardia globerula ATCC 21505	24	8	1042	100	95
Rhodococcus sp. ATCC 15592	24	6	1035	100	98
Rhodococcus rhodochrous	24	320	155	45	
ATCC 15904	66	423	670	100	94
Rhodococcus rhodochrous	24	231	478	70	
ATCC 19150	66	5	985	100	96

[a]Reactions were carried out as described in the Materials and Methods section. The concentrations of diol **14**, lactol **15**, and lactone **16** were determined by gas chromatography. The optical purity of lactone 16 determined by chiral high-performance liquid chromatography. ND, not determined.

tracted with ethyl acetate and analyzed by GC to determine the % conversion of diol **14** to lactol **15** and lactone **16**. The optical purity of lactol **15** and lactone **16** were determined by chiral HPLC.

Oxidation of diol **14** *by cell extracts.* Cell suspensions (20% wt/vol, wet cells) of *N. globerula* ATCC 21505 and *Rhodococcus* sp. ATCC 15592 in buffer B (0.2 M Tris–HCl, pH 7.5 containing 5 mM dithiothreitol) were disintegrated by sonication at 4°C. The lysate was centrifuged at 20,000 × *g* for 20 min at 4°C, and clear supernatant solution collected is referred to as cell extract. Proteins in cell extracts were precipitated and fractionated by solid ammonium sulfate at 4°C (0–40, 40–55, and 55–65% saturation). All fractions were evaluated for the diol **14** and lactol **15** oxidizing activity. The reaction mixture contained 10 mg of diol **14** or lactol **15**, 20 mg of NAD^+, and fractionated enzyme extracts.

Analytical methods. Analysis of compounds **14–16** was carried out by GC. An HP-1 capillary column (cross-linked methyl silicone gum phase, 12 m × 0.2 mm × 0.33 mm film thickness) was used at 120°C oven, 250°C detector, and 250°C injector temperatures. The retention times for compounds **14**, **15**, and **16** are 8.15, 4.48, and 6.8 min, respectively.

The separation of the two enantiomers of racemic **15** and **16** was achieved by chiral HPLC. A Bakerbond chiralcel OB column (4.6 × 250 mm) was used at 10°C. Mobile phase was 70% hexane with 30% isopropanol, flow rate was 0.5 mL/min, and detection wavelength was 210 nm. Both diode array and refractometer detectors were used. The retention times for the (+) isomer and (−) isomer of compound **15** are 14.5 and 11.5 min, respectively. The retention times for the (+) isomer and (−) isomer of compound **16** were 28.2 and 32.6 min, respectively.

RESULTS

Among cultures evaluated, *N. globerula* ATCC 21505, *Rhodococcus* sp. ATCC 15592, and *R. rhodochrous* ATCC 19150 catalyzed the oxidation of diol **14** to the corresponding lactone **16** in 99–100% reaction yield and 95–98% optical purity at 1 mg/mL substrate concentration (Table 14).

The effect of pH on the oxidation of diol **14** to lactol **15** and lactone **16** by cell suspensions of *N. globerula* ATCC 21505 was evaluated in a 100-mL reactor volume at 28°C, 280 rpm for 40 h. A 10% cell (wt/vol, wet cells) concentration and 4 mg/mL of diol **14** were used. The optimum pH for the oxidation is 7.5. The effect of cell concentration was evaluated in a 100-mL volume at 28°C, 280 rpm for 18 h. Diol **14** (4 mg/mL) was supplied. The amount of lactol **15** and lactone **16** produced from diol **14** is directly dependent upon cell concentration.

Nocardia globerula ATCC 21505 and *Rhodococcus* sp. ATCC 15592 each were grown in a 380-L fermentor with 250 L of medium D. Samples were taken at various times during growth, and cells were evaluated for the oxidation of diol **14** by the cell suspensions. As shown in Table 15, the reaction yield of 99% was obtained after 18, 24, and 48 h growth

TABLE 15
Growth of *Norcardia globerula* in a 380-L Fermentor: Evaluation of Cells for Oxidation of Diol 14 to Lactol 15 and Lactone 16[a]

Cells harvest time (h)	Reaction time (h)	Lactol **15** (μg/mL)	Lactone **16** (μg/mL)	Conversion (%)
18	24	273	1156	28
	48	87	2424	50
	72	72	4891	99
24	24	246	1206	29
	48	190	2868	61
	72	96	4895	99
48	24	187	1379	31
	48	144	2782	58
	72	91	4875	99

[a]Rection mixture in 10 mL of 0.1 M phosphate buffer (pH 7) contained 1 g of *N. globerula* cells and 50 mg of diol **14**. Reactions were carried out at 28°C, 280 rpm on a rotary shaker. The concentration of diol **14**, lactol **15**, and lactone **16** were by gas chromatography and the optical purities were determined by chiral high-performance liquid chromatography.

TABLE 16
Oxidation of Diol 14 to Lactol 15 and Lactone 16 by Cell Suspensions of *Norcacdia globerula* in a 5-L Fermentor[a]

Reaction time	Lactol **15**	Lactone **16**	Conversion	Optical purity of lactone **16**
(h)	(μg/mL)	(μg/mL)	(%)	(%)
5	200	15	4	ND
24	424	660	22	95.8
48	294	1372	33	95.7
72	275	2374	52	96
96	52	3475	70	96

[a]Reaction mixture in 3 L of 0.1 M phosphate buffer (pH 7) contained 300 g of wet cells of *N. globerula* and 15 g of diol **14**. Reaction was carried out at 30°C, 500 rpm agitation and 3 L/min aeration. The concentrations of lactol **15** and lactone **16** were determined by gas chromatography. The optical purity of lactone **16** was determined by chiral high-performance liquid chromatography. ND, not determined.

period. This indicates that diol-oxidizing enzyme activity remained the same throughout the fermentation process. Subsequently, cells were grown in a fermentor for 48 h. Similar results were obtained when *Rhodococcus* sp. ATCC 15592 cultures were grown in a fermentor.

Nocardia globerula ATCC 21505 cells, collected from the fermentor, were evaluated for the oxidation of diol **14**. A 10% (wt/vol, wet cells) cell suspension was supplemented with 5 mg/mL of diol, and the reaction was carried out in a 5-L fermentor at 30°C, 500 rpm agitation, and 3 LPM aeration. After a 96-h reaction, 70% reaction yield was obtained for lactone **16**, and an optical purity of 96% was obtained (Table 16).

Oxidation of diol **14** to lactol **15** and lactone **16** was also evaluated by cells of *Rhodococcus* sp. ATCC 15592 grown in a 380-L fermentor: 10% (wt/vol, wet cells) suspensions were supplemented with 5 mg/mL of diol, and the reaction was carried out in a 5-L fermentor at 30°C, 600 rpm agitation, and 3 LPM aeration. After 120-h reaction, 34% yield of lactone **16** and 12% yield of lactol **15** were obtained. The optical purity of 98.4 and 96.7% were obtained for lactone **16** and lactol **15**, respectively (Table 17). The lactone **16** was purified by silica gel chromatography, and the optical rotation of purified lactone was determined. A specific rotation of +117 [ref. (specific rotation of standard reference compound **16**) +116] was

TABLE 17
Oxidation of Diol 14 to Lactol 15 and Lactone 16 by Cell Suspensions of *Rhodococcus* sp. ATCC 15592 in a 5-L Fermentor[a]

Reaction time (h)	Lactol **15** (μg/mL)	Lactone **16** (μg/mL)	Conversion (%)	Optical purity (%)	
				Lactol **15**	Lactone **16**
20	300	105	8	98.3	ND
40	490	210	14	98.4	ND
66	552	560	22	96.7	98
96	550	1075	34	96.5	98.5
120	596	1725	46	96.7	98.4

[a]Reaction mixture in 3 L of 0.1 M phosphate buffer (pH 7) contained 300 g wet cells of *Rhodococcus* sp. ATCC 15592 and 15 g of diol **14**. Reaction was carried out at 30°C, 600 rpm agitation and 3 L/min aeration. The concentrations of lactol **15** and lactone **16** were determined by gas chromatography, and optical purities were determined by chiral high-performance liquid chromatography. ND, not determined. ATCC, American Type Culture Collection (Rockville, MD).

TABLE 18
Oxidation of Diol 14 and Lactol 15 by Cell Extracts of *Rhodococcus* sp. ATCC 15592[a]

Fraction	Substrate	Lactol **15** (μg/mL)	Lactone **16** (μg/mL)	Optical purity of lactone **16** (%)
Cell extracts	Diol	45	0	
	Lactol		178	98.2
0–40% Ammonium sulfate saturation	Diol	60	0	
	Lactol		90	
40–80% Ammonium sulfate saturation	Diol	0	0	
	Lactol		480	99

[a]Reactions were carried out as described in the Materials and Methods section. The concentrations of lactol **15** and lactone **16** were determined by gas chromatography, and optical purity of lactone **16** was determined by chiral high-performance liquid chromatography.

obtained in methanol as solvent. The purified lactone **16** gave enantiomeric excess of >99% by chiral HPLC assay.

Cell extracts of *Rhodococcus* sp. ATCC 15592 were prepared and fractionated by ammonium sulfate as described in the Methods and Materials section. Cell extracts and all fractions were assayed for the diol **14** and lactol **15** oxidizing activity. Both activities were obtained in cell extracts and 0–40% ammonium sulfate fractions. Only lactol **15** oxidizing activity was detected in 40–55% and 55–65% ammonium sulfate fractions. This indicates that there may be different enzymes involved in diol and lactol oxidation (Table 18).

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