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Stereoselective Epoxidation of 2,2-Dimethyl-2*H*-1-Benzopyran-6-Carbonitrile†

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Abstract—The chiral intermediate (3*S*,4*R*)-*trans*-3,4-dihydro-3,4-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-carbonitrile [(+)-*trans* diol **3**] was made by the stereoselective microbial epoxidation of 2,2-dimethyl-2*H*-1-benzopyran-6-carbonitrile **1**. This compound is a potential intermediate for the total synthesis of potassium-channel openers. Several microbial cultures were found which catalyzed the transformation of **1** to the corresponding (3*S*,4*S*)-epoxide **2** and (+)-*trans* diol **3**. The two best cultures, *Corynebacterium* sp. SC 13876 and *Mortierella ramanniana* SC 13840 gave reaction yields of 32 M% and 67.5 M% and optical purities of 88 and 96 %, respectively, for (+)-*trans* diol **3**.

A single-stage process (fermentation–epoxidation) for the biotransformation of **1** was developed using *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840. In a 25-L fermentor, the (+)-*trans* diol **3** was obtained in 38.6 M% yield with an optical purity of 90 % using *Corynebacterium* SC 13876. The reaction yield of 60.7 M% and optical purity of 92.5 % were obtained for (+)-*trans* diol **3** using *M. ramanniana* SC 13840.

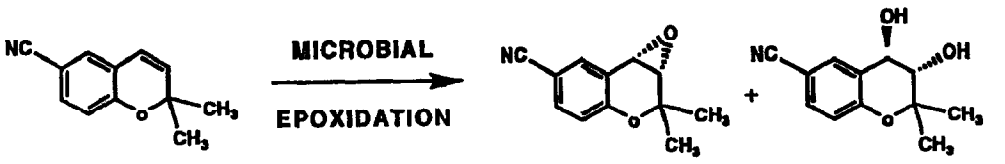
A two-stage process for the preparation of (+)-*trans* diol **3** was also developed using a 3 L cell-suspension (10 % w/v, wet cells) of *M. ramanniana* SC 13840. The reaction was carried out in a 5-L Bioflo fermentor. The concentration of substrate **1** was 2 g L^{-1} with glucose present at 10 g L^{-1}. After 48 h, (+)-*trans* diol **3** was obtained in 76 M% yield with an optical purity of 96 %. From the reaction mixture, (+)-*trans* diol **3** was isolated in 65 M% (4.6 g) overall yield. An optical purity of 97 % and a chemical purity of 98 % were obtained for the isolated (+)-*trans* diol **3**.

Introduction

The current interest in microbial production of chiral epoxides by stereoselective oxygenation lies in the preparation of intermediates for chemical synthesis.[1–7] The study of potassium (K) channel biochemistry physiology, and medicinal chemistry has flourished, and numerous papers and reviews have been published in recent years.[8–11] It has long been known that K channels play a major role in neuronal excitability,[11] and it is now clear that K channels 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.[12] A new class of highly specific pharmacological compounds has been developed which either open or block K channels.[9–14] K channel openers are powerful smooth muscle relaxants with *in vivo* hypotensive and bronchodilator activity, originally typified by cromakalim, nicorandil, and pinacidil.[12] Recently, the synthesis and antihypertensive activity of a series of novel K channel openers[14–18] based on monosubstituted *trans*-4-amino-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ol have been demonstrated. In this report we describe the stereoselective microbial oxygenation of 2,2-dimethyl-2*H*-1-benzopyran-6-carbonitrile **1** to the corresponding chiral epoxide **2** and chiral diol **3** (Scheme I). Chiral epoxide **2** and diol **3** are potential intermediates for the synthesis of K channel activators important as an antihypertensive and bronchodilator agent.

Results

Various microorganisms were screened for the stereoselective oxygenation of **1** to **2** and **3**. As shown in the Table 1, the reaction yield and stereoselectivity were



Scheme I. 2,2-Dimethyl-2*H*-1-benzopyran-6-carbonitrile **1**.
(3*S*,4*S*-*cis*)-3,4-Dihydro-2,2-dimethyl-2*H*-oxireno [c] [1] benzopyran-6-carbonitrile **2**.
(3*S*,4*R*)-*Trans*-3,4-dihydro-3,4-dihydroxy-2,2-dimethyl-2*H*-1-benopyran-6-carbonitrile **3**.

†This manuscript has been written as a tribute to Professor Bryan Jones' 60th birthday.

dependent upon the microorganism used during the epoxidation of **1**. *Corynebacterium* sp. SC 13876, *Rhodococcus erythropolis* SC 13845, *Pseudomonas dehalogens* SC 13873, *Cunninghamella enchinulata* ATCC 26269, *Mucor hiemalis* ATCC 8977b, *Hansenula polymorpha* SC 13865 and *M. ramanniana* SC 13840 catalyzed the epoxidation of compound **1** to **2**. This epoxide was further converted to (+)-(3*S*,4*R*)-*trans* diol **3**. Reaction yields of 32 molar percent (M%) and 67.5 M% and optical purities of 88 % and 96 % were obtained for (+)-*trans* diol **3**, respectively, with the two best cultures *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840.

Further research was conducted using *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 to convert

Table 1. Stereoselective microbial transformation of **1** to **2** and **3**

Microorganisms	Substrate 1 (mg/ml)	Epoxide 2 (mg/ml)	Epoxide 2 M% Yield	Optical purity of Epoxide 2 (%)	Diol 3 (mg/ml)	Diol 3 M% Yield	Optical purity of Diol 3 (%)
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

Reactions were carried out as described in the Experimental Section. The concentration of compounds **1**, **2** and **3** were determined by GC. The optical purities of compounds **2** and **3** were determined by chiral HPLC. ND: Not determined.

Table 2. Growth of *Corynebacterium* sp. SC 13876 and *Mortierella ramanniana* SC 13840 in a 25-L fermentor: Evaluation of cells for biotransformation of **1** to **2** and **3**

Cell harvest time (hr)	Substrate 1 (mg/ml)	Epoxide 2 (mg/ml)	Epoxide 2 M% Yield	Diol 3 (mg/ml)	Diol 3 M% Yield	Optical purity of 2 (%)	Optical purity of 3 (mg/ml)
Corynebacterium sp. SC 13867:							
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
Mortierella 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	0.08	7.3	0.85	72	ND	96

During fermentation, cells were harvested from 200 mL broth samples and suspended in 10 mL of 100 mM phosphate buffer, pH 6.8. Cell suspensions (10 %, w/v, wet cels) were used in the biotransformation of compound **1** (1 mg/mL) at 250 rpm and 25 °C for 72 h. The substrate **1** and products **2** and **3** concentrations were determined by GC and optical purities of **2** and **3** were determined by chiral HPLC.

compound **1** to (+)-*trans* diol **3**. Cells of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were grown in a 25-L fermentor containing 15 L of medium. To determine the activity of cells during growth, broth samples (200 mL) were taken at 30, 36, 42, and 48 h after inoculation. Cells were collected by centrifugation, suspended in buffer and the resulting cell-suspensions were used to carry out the biotransformation of compound **1** as described in the Experimental Section. Cells harvested after 42–48 h of growth gave a higher reaction yield and optical purity of (+)-*trans* diol **3**. Reaction yields of 32 M% and 72 M% and optical purities of 90 % and 96 % were obtained for (+)-*trans* diol **3** using cells (grown for 48 h) of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840, respectively (Table 2).

A single-stage fermentation–biotransformation process was developed for conversion of compound **1** to (+)-*trans* diol **3** 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^{-1} of substrate **1**, the biotransformation process was initiated by addition of 1 g L^{-1} of substrate **1** , 5 g L^{-1} of glucose and 5 L of additional medium as described in the Experimental Section. After a 120 h reaction period, (+)-*trans* diol **3** was obtained with a reaction yield of 38.6 M% and an optical purity of 90 % (Table 3). In contrast, *M. ramanniana* cultures were grown in a 25-L fermentor containing 15 L of medium in the absence of substrate **1**. After 24 h growth, the biotransformation was initiated by addition of 1 g L^{-1} of substrate **1** and 5 g L^{-1} of glucose. A higher reaction yield (60.7 M%) and optical purity (92.5 %) were obtained with *M. ramanniana* SC 13840 (Table 4). (3*S*,4*S*)-Epoxide **2** was obtained as an intermediate during biotransformation in both organisms (Tables 3 and 4). From 5-L of each reaction broth, (+)-*trans* diol **3** 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.

Table 3. Biotransformation of **1** to **2** and **3** by *Corynebacterium* sp SC 13876: single-stage process

Fermentation time (hours)	Substrate 1 (mg/ml)	Epoxide 2 (mg/ml)	Epoxide 2 M% Yield	Diol 3 (mg/ml)	Diol 3 M% Yield	Optical purity of epoxide 2 (%)	Optical purity of diol 3 (%)
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

Cells were grown in a 25-L Braun fermentor containing 10 L of medium supplemented with 0.2 g L of substrate **1** as an inducer. After 24 h of growth in a fermentor, the biotransformation process was initiated by addition of 15 g of substrate and additional 5 L of medium. Biotransformation was continued at 600 RPM agitation, 15 LPM aeeration at 28 °C. Periodically samples were removed and analyzed for substrate **1**, and products **2** and **3** concentrations by GC. The optical purities of **2** and **3** were determined by chiral HPLC. NA: Not applicable. ND: Not determined.

Table 4. Biotransformation of **1** to **2** and **3** by *Mortierella ramanniana* SC 13840: single-stage process

Fermentation time (hours)	Substrate 1 (mg/ml)	Epoxide 2 (mg/ml)	Epoxide 2 M% Yield	Diol 3 (mg/ml)	Diol 3 M% Yield	Optical purity of epoxide 2 (%)	Optical purity of diol 3 (%)
12	0.6	0.12	11.0	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

Cells were grown in a 25-L Braun fermentor containing 15 L of medium. After 24 h of growth period, the biotransformation process was initiated by addition of 15 g of substrate **1**. Biotransformation was continued at 600 RPM agitation, 15 LPM aeeration at 28 °C. Periodically samples were removed and analyzed for substrate **1**, and products **2** and **3** concentrations by GC. The optical purities of **2** and **3** were determined by chiral HPLC. ND: Not determined.

Table 5. Biotransformation of **1** to **2** and **3** by *Mortierella ramanniana* SC 13840: two-stage process

Reaction time (hours)	Substrate 1 (mg/ml)	Epoxide 2 (mg/ml)	Epoxide 2 M% Yield	Diol 3 (mg/ml)	Diol 3 M% Yield	Optical purity of epoxide 2 (%)	Optical purity of diol 3 (%)
12	1.8	0.15	6.9	0.05	2.1	96	ND
24	0.89	0.11	5.0	1.03	43.4	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

Cells were grown in a 25-L Braun fermentor containing 15 L of medium. After 48 h of growth, cell were harvested. Cell suspensions was prepared in a 3 L of 100 mM phosphate buffer (pH 6.8). Cell suspensionwas supplemented with 1 g L of susbrate and 5 g L glucose and the biotransformation was conducted in 5 L New Brunswick BioFlo fermentor at 28 °C and 250 RPM. After 24 h, additionsal substrate (1 g L) and glucose (5 g L) were supplied. Periodically, samples were removed and analyzed for substrate **1** and products **2** and **3** concentrations by GC. The optical purities of **2** and **3** were determined by chiral HPLC. ND: Not determined.

In the two-stage process, biotransformation of compound **1** to (+)-*trans* diol **3** was carried out in a 5-L fermentor using a cell suspension of *M. ramanniana* SC 13840. Cells harvested from a 25-L fermentor were suspended in 3 L of buffer at 10 % (w/v, wet cells) concentration. Substrate was then added to give 2 g L^{-1} concentration. A reaction yield of 76 M% and an optical purity of 96 % were obtained for (+)-*trans* diol **3** (Table 5). Isolation of chiral diol **3** was carried out as described in the Experimental Section; 4.6 g of product were recovered in 65 M% overall yield. Isolated (+)-*trans* diol **3** had an optical purity of 97 % as analyzed by chiral HPLC and 98 % chemical purity as analyzed by GC and HPLC.

Cell suspensions (10 % w/v, wet cells) of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were examined for the enzymatic conversion of epoxide **2** to diol **3**. *M. ramanniana* SC 13840 cells enzymatically converted epoxide **2** to the corresponding diol **3** in 12 h reaction time with a reaction yield of 90 M%. In the absence of cells, only 28 M% conversion of epoxide **2** to (+)-*trans* diol **3** 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, non-enzymatic conversion of epoxide **2** to (+)-*trans* diol **3** was observed with a 28 M% and 80 M% reaction yields after 24 and 72 h incubation period. The accumulation of toxic epoxide **2** during biotransformation of compound **1** to **3**, may be responsible for the poor yield of diol **3** with cells of *Corynebacterium* sp. SC 13876. Indeed, the initial addition of epoxide **2** (0.2 mg mL^{-1}) during biotransformation with *Corynebacterium* sp. SC 13876 inhibited the conversion of **1** to **3** by 40 % (Figure 1).

The effects of pH and temperature on the oxygenation of compound **1** to diol **3** by both cultures were evaluated in 25-mL flasks. Cell suspensions (5 % w/v of wet cells) were supplied with 25 mg of substrate **1** and biotransformation was carried out on a rotary shaker for 48 h. The optimum pH for the bioconversion of compound **1** to diol **3** is 6.5–7.0 and the optimum temperature is 25 °C.

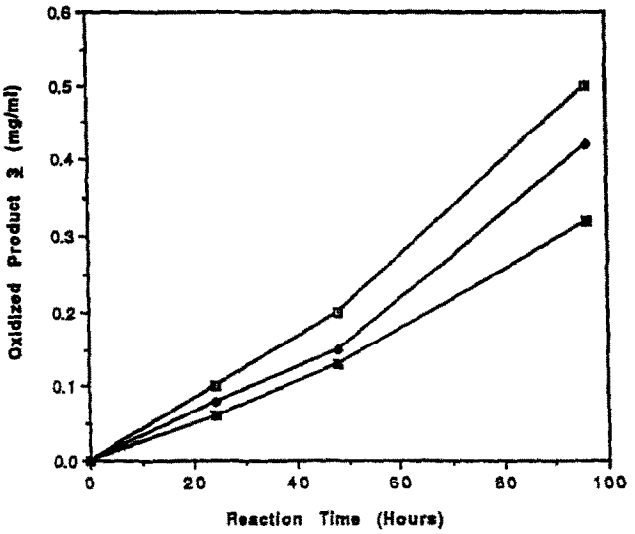


Figure 1. Biotransformation of **1** to **3** by cell suspensions of *Corynebacterium* sp. SC 13876: effect of added epoxide. (A) Control —□—; (B) epoxide added (0.1 mg mL^{-1})—◆—; (C) epoxide added (0.2 mg mL^{-1})—■—.

Cell extracts of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 were prepared and examined for the epoxidation of **1** (as described in the Experimental Section). After a 72 h reaction time in the presence of NADH, a reaction yield of 4.2 M% for epoxide **2** and 6.7 M% for diol **3** was obtained using 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 **3** 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 **1** to epoxide **2** was not observed.

Discussion

Cytochrome P-450 and the non-heme iron monooxygenases such as ω-hydroxylase from

Pseudomonas oleovorans and methane monooxygenase from methanotrophic bacteria function mainly as hydroxylating enzymes. However, in the absence of any steric constraints, olefin epoxidation is kinetically more favorable than hydroxylation.[20,21] These enzymes have received much more attention in recent years for the epoxidation of terminal olefins.[20–29] Stereoselectivity of epoxidation reactions catalyzed by ω-hydroxylase has been demonstrated.[29] A cytochrome P-450 dependent hydroxylating system has been demonstrated in *Corynebacterium* sp. which catalyzed the conversion of octane to 1-octanol.[30] In addition to alkane-utilizing bacteria, Van Ginkle *et al.*[31] isolated alkene-utilizing organisms which catalyzed the stereoselective epoxidation of alkenes. These organisms require alkane or alkene as growth substrate to induce monooxygenase to catalyze the epoxidation reactions. Recently, it has been discovered that *Nocardia corallina* does not require growth on hydrocarbons to induce monooxygenase. Glucose-grown cells also catalyzed the epoxidation reactions. *N. corallina* has been successfully used in the preparation of aryl glycidyl ethers, chiral intermediates required in the synthesis of β-blockers.[32]

Bacteria which degrade α-pinene form pinene oxide as the first catabolic product. The monooxygenase present in pinene degrading *Pseudomonas fluorescens* NCIMB 11671 appears to be specific for methyl-substituted internal double bond.[33] Bacteria isolated on styrene as carbon source contained alkene-specific monooxygenase capable of stereoselective epoxidation. Hartmans *et al.*[34] described a FAD-containing styrene monooxygenase which required the aryl substituent for activity. In the metabolism of aromatic compounds, procaryotic organisms introduce both atoms of molecular oxygen into the aromatic ring to produce an unstable cyclic endoperoxy intermediate which is converted to stable *cis*-dihydrodiol.[35] In contrast, eucaryotic organisms catalyze the metabolism of aromatic compounds by introduction of a single atom of molecular oxygen by cytochrome P-450 monooxygenase system to give arene oxide intermediates. Arene oxides are further converted to *trans*-dihydrodiol.[22,23,35] Oxidation of xenobiotics by cytochrome P-450 enriched extracts of *Streptomyces griseus* and the biotransformation of precocene II to the corresponding (–)-*cis* and (+)-*trans* precocene II 3,4-dihydrodiol with *S. griseus* has been reported by Sariaslani *et al.*[36,37] Recently, Boyd *et al.*[38] have demonstrated the biotransformation of both aromatic and non-aromatic heterocyclic ring compounds to the corresponding *cis*-diols by *Pseudomonas putida*. In this report we have described the stereoselective microbial epoxidation of **1**. Chiral epoxide **2** is subsequently hydrolyzed to the corresponding chiral *trans* diol **3**. The reaction is catalyzed by an NAD(P)H-dependent oxygenase.

Experimental Section

Materials

Substrate **1** and standards of compounds **2** and **3** were synthesized by Chemical Process Development, Bristol-Myers Squibb Pharmaceutical Research Institute [14–18]. The proton magnetic resonance (^{1}H-NMR) and carbon magnetic resonance (^{13}C-NMR) were recorded on a Brucker 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.

2,2-Dimethyl-2H-1-benzopyran-6-carbonitrile

Prepared as described earlier,[17] mp 45–47 °C, NMR ($CDCl_3$) δ 1.45 [s, 6H, $C(Me)_2$], 5.72 (d, J = 10 Hz, 1H, H-3), 6.3 (d, J = 10 Hz, 1H, H-4), 6.8 (d, J = 10 Hz, 1H, H-8), 7.3 (s, 1H, H-5), 7.4 (d. J = 6 Hz, 1H, H-7). ^{13}C-NMR ($CDCl_3$, 75.46 MHz) δ 156.6, 133.15, 132.01, 129.9, 121.5, 119.13, 117.05, 103.6, 75.6, 28.2; Anal. Calcd $C_{12}H_{11}N_1O_1$: C, 76.68; H, 6.06; N, 7.45; Found C, 75.56; H, 5.98; N, 7.57. Mass spectrum m/z 186 (m + H$^+$), 185 (m$^+$).

(3S,4S-cis)-3,4-Dihydro-2,2-dimethyl-2H-oxireno[c][1]-benzopyran-6-carbonitrile

This compound was synthesized using Jacobson's catalysts,[16] mp 142–144 °C; NMR ($CDCl_3$) δ 1.15 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 3.35 (d, J = 4 Hz, 1H, H-3), 3.92 (d, J = 4 Hz, 1H, H-4), 6.84 (d, J = 10 Hz, H-8), 7.28 (s, 1H, H-5), 7.42 (d, J = 6 Hz, H-7). ^{13}C-NMR ($CDCl_3$, 75.46 MHz) d 156.4, 134.3, 133.7, 121.0, 118.93, 118.67, 104.18, 74.6, 62.2, 49.76, 25.4, 22.9; $[\alpha]_D^{25}$ = –89.3 (C = 1.01, MeOH). Anal. Calcd for $C_{12}H_{11}NO_2$: C, 71.56; H, 5.52; N, 6.95; Found: C, 71.46; H, 5.46; N, 7.04. Mass spectrum, m/z 201, (m$^+$).

(3S,4R)-trans-3,4-Dihydro-3,4-dihydroxy-2,2-dimethyl-2H-1-benzopyran-6-carbonitrile

Compound **2** (0.8 g) was dissolved in 25 mL of tetrahydrofuran (THF). To this solution 1 mL of water and 2 drops of perchloric acid were added. The reaction mixture was stirred at room temperature for 30 min. The progress of the reaction was followed by gas chromatography. After all epoxide was converted, 20 mL of water was added and the resulting reaction mixture was extracted twice with 25 mL of dichloromethane. The organic phase was washed with 0.7 M sodium bicarbonate solution. The organic phase was then dried over anhydrous sodium carbonate. The solvent was evaporated under reduced pressure to produce 0.7 g (87.5 % yield) of white waxy solid of 98 % chemical purity as analyzed by GC. NMR ($CDCl_3$) δ 1.22 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 3.18 (s, 2H, OH), 3.62 (d, J = 8.5 Hz, 1H, H-3), 4.68 (d, J = 8.5 Hz, 1H, H-4), 6.82 (d, J = 10 Hz, 1H, H-8). 7.31 (s, 1H, H-5), 7.4 (d, J = 1 Hz, 1H, H-7). ^{13}C NMR ($CDCl_3$, 75.46 MHz) d 156.16, 133.14, 132.55, 124.7, 119.35, 117.97, 103.56, 75.46, 68.48, 49.45, 26.97, 26.61; Mass spectrum, m/z 219, (m$^+$). $[\alpha]_D^{25}$ = + 16.2 (C = 1.02, MeOH); Anal. Calcd for $C_{12}H_{13}NO_3$: C, 65.71; H, 5.93; N, 6.38; Found: C, 65.82; H, 6.01; N, 6.45.

Microorganisms

Microorganisms (Table 1) were obtained from our culture collection in the Microbial Technology Department of

Bristol-Myers Squibb (New Brunswick, NJ) and from the American Type Culture Collection (Rockville, MD). Microorganisms were stored at –90 °C in vials.

Microbial epoxidation of compound 1

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 and 0.02 % substrate **1** adjusted to pH 6.8) contained in a 500-mL Erlenmeyer flask. Cultures were grown at 28 °C and 280 rpm (revolutions per min) agitation for 48 h in the presence of 0.2 mg mL^{-1} of compound **1**. 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 % (w/v, wet cells) cell concentration and supplemented with 1 mg mL^{-1} of compound **1**. The reaction was carried out at 25 °C and 200 rpm agitation for 120 h. Periodically, samples of 1 mL were taken and extracted with 4 mL of solvent consisting of toluene:*t*BME (1:1 mixture). The separated organic phase was filtered through a 0.2 μm LID/X filter (Whatman Inc., Fairfield, NJ) and analyzed for substrate and product concentration using a Hewlett Packard gas chromatographic (GC) system. The optical purities of epoxide **2** and diol **3** were analyzed by chiral HPLC.

Growth of Corynebacterium *sp. SC 13876 and* M. ramanniana *SC 13840 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 containing 0.2 g L^{-1} of substrate **1** or medium B in the absence of substrate **1** (corn steep liquor, 20 mL; cerelose, 33 g; ammonium phosphate, 3 g; yeast extract, 1 g; soy bean oil, 2.5 mL; calcium carbonate, 1 g in 1 L of distilled water). Growth of cells consisted of several inoculum development stages and fermentation.

Inoculum development consisted of F_1 and F_2 stages. In the F_1 stage, frozen vials of *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 independently were inoculated into 100 mL of medium A contained in a 500-mL Erlenmeyer flask. Growth was carried out at 28 °C and 280 rpm agitation for 48 h. In the F_2 stage, 100 mL of F_1 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 containing 15 L of medium A containing 0.2 g L^{-1} of substrate **1** (*Corynebacterium* sp. SC 13876) or medium B in the absence of substrate **1** (*M. ramanniana*) was inoculated with 1.5 L of F_2 stage inoculum. The fermentation was conducted for 48 h at 28 °C and 600 rpm agitation with 15 Lpm (liter per min) 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 % w/v of wet cells) were prepared in 100 mM potassium phosphate buffer (pH 6.8) and supplemented with 1 mg mL^{-1} of compound **1**. The reaction was carried out at 25 °C and 250 rpm agitation on a rotary shaker (New Brunswick Scientific, New Brunswick, NJ).

Periodically samples were taken and analyzed for the biotransformation of compound **1** to compounds **2** and **3** by gas chromatography. The activity of cells was expressed as mg of epoxide **2** and diol **3** formed per h per g 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 either used to conduct the epoxidation reaction or stored at –90 °C until further use. About 400 g of wet cell paste were collected from each fermentation.

Epoxidation of compound 1 in a fermentor (two-stage process)

M. ramanniana SC 13840 cells harvested from the above batches were used to conduct the epoxidation of **1** in a 5-L BioFlo fermentor. Cell suspensions (10 % w/v of wet cells) in 3 L of 100 mM potassium phosphate buffer (pH 6.8) were used. Substrate **1** (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 **1** (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:*t*BME, 1:1 by volume). 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 **1** to **2** and **3**. The remaining portion was dried under a gentle stream of nitrogen. The oily residue was dissolved in 1 mL of mobile phase consisting of hexane:isopropanol mixture (1:1, v/v) and filtered through 0.2 μm LID/X filter and analyzed by HPLC to determine the optical purity of epoxide **2** and diol **3**. 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 remaining substrate was separated from the aqueous phase containing chiral diol **3**. The aqueous phase was extracted twice with 6 L of solvent (toluene:*t*BME, 1:1 by volume). 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 **3**. The toluene concentrate was further purified by flash-chromatography on a silica column to obtain 4.6 g of diol **3** 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 containing 15 L of medium A (in the presence of 0.2 g L^{-1} of substrate **1**) or medium B (in the absence of substrate **1**), respectively, as described above. After 24 h of growth

period, each fermentor was supplemented with substrate **1** (15 g) and glucose (75 g) and biotransformation was continued. Periodically samples were removed and analyzed for substrate **1**, epoxide **2** and diol **3** concentration by GC. Optical purities of **2** and **3** were determined by chiral HPLC. At the end of the biotransformation (120 h 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 **3** as described above. 2.02 g (30 M%) and 2.95 g (49 M%) of (+)-*trans* diol **3** were isolated, respectively, from *Corynebacterium* sp. SC 13876 and *M. ramanniana* SC 13840 batches in 96 % chemical (GC area %) purity.

Oxidation of epoxide 2

Corynebacterium sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor containing 15 L of medium A containing 0.2 g L^{-1} of substrate **1** or medium B in the absence of substrate **1** as described previously. Cell suspensions (10 % w/v, wet cells) of organisms were evaluated for the bioconversion of epoxide **2** to diol **3**. Cells (25 mL reactor) were supplemented with 25 mg of epoxide **2** and biotransformation was carried out at 250 rpm and 25 °C. Periodically samples of 1 mL were taken and extracted with 4 mL of solvent (toluene:*t*BME, 1:1 by volume). The separated organic phase was filtered through a 0.2 μm LID/X filter (Whatman Inc., Fairfield, NJ) and analyzed for epoxide **2** and diol **3** concentration by Hewlett Packard gas chromatographic (GC) system. Control experiments in the absence of cells were also carried out as described above.

Epoxidation of compound 1 by cell extracts

Corynebacterium sp. SC 13876 and *M. ramanniana* SC 13840 cultures were grown in a 25-L fermentor containing 15 L of medium A containing 0.2 g L^{-1} of substrate **1** or medium B in the absence of substrate **1** as described previously. Cells independently were suspended in buffer A (50 mM MOPS buffer pH 7.0 containing 1 mM dithiothreitol, 10 % glycerol, 1 μM PMSF, 1 μM ferrous ammonium sulfate) at 20 % (w/v 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 was referred to as cell extracts. Protein in cell extracts was determined by Bradford's method.[39]

Cell extracts were analyzed for the ability to catalyze the oxygenation of **1** to **2** and **3**. The reaction mixture contained 0.8 mM NADH or NADPH and 5 mg of substrate in 10 mL of cell extracts. 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 **1** and products **2** and **3**.

Analytical methods

Analysis of compounds **1**, **2** and **3** was carried out by using a Hewlett Packard 5890 gas chromatograph with a flame ionization detector (FID). An HP-2 fused-silica capillary column (25 m long x 0.32 mm Int. diameter x 0.17 μm thickness) at 250 °C injection temperature, 250 °C detector temperature and 195 °C oven temperature was used. The carrier gas was helium and total run time was 8 min. The retention times for **1**, **2**, and **3** were 2.64, 3.44 and 6.14 min, respectively, under the above conditions. The optical purity of compounds **2** and **3** 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 of hexane and passed through a silica cartridge (Analytichem Bond Elut LRC) previously equilibrated with hexane. The column was washed with 10 mL hexane and compounds **1**, **2**, and **3** 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 column (ODS, 100 × 4.6 mm, Hewlett–Packard) and the second column was a Chiralcel OD (250 × 4.6 mm, Daicel Chemical Industries). The mobile phase consisted of 95:4:1 of hexane:*n*-butanol:cyclohexanol. The flow rate was 0.8 mL min^{-1} and the detector wavelength was 254 nm. The retention times for the two enantiomers of racemic epoxide were 11 min and 12.8 min. The retention times for the *cis* and *trans* diols were 29.4 min and 33.2 min respectively.

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