Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 3167–3175

TETRAHEDRON:
ASYMMETRY

Stereospecific enzymatic hydrolysis of racemic epoxide: a process for making chiral epoxide

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Received 17 June 1999; accepted 20 July 1999

Abstract

Among various microbial cultures evaluated, *Rhodotorula glutinis* SC 16293 and *Aspergillus niger* SC 16311 catalyzed the stereospecific hydrolysis of the racemic epoxide, *RS*-1-{2′,3′-dihydrobenzo[*b*]furan-4′-yl}-1,2-oxirane, **1** to the corresponding *R*-diol, *R*-1-{2′,3′-dihydrobenzo[*b*]furan-4′-yl}-ethane-1,2-diol, **3**. The *S*-epoxide, *S*-1-{2′,3′-dihydrobenzo[*b*]furan-4′-yl}-1,2-oxirane, **2** remained unreacted in the reaction mixture. A reaction yield of 45–50% (theoretical maximum yield is 50%) and an enantiomeric excess (ee) of >95% were obtained for unreacted *S*-epoxide **2** using each culture. Addition of 10% methyl *tert*-butyl ether to an aqueous reaction mixture during hydrolysis by *R. glutinis* improved the ee of the unreacted *S*-epoxide **2** to >99% (yield 48%) and that of the *R*-diol **3** to 79%. Unlike *R. glutinis*, hydrolysis of racemic epoxide **1** in the presence of 10% methyl *tert*-butyl ether by *A. niger* showed an adverse effect and gave *S*-epoxide **2** in 54% yield and 49% ee. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Epoxide hydrolase catalyzes the stereoselective hydrolysis of racemic epoxide to the corresponding chiral diol and unreacted chiral epoxide. Furstoss and his co-workers have used *Aspergillus niger* and *Beauveria sulfurescens* for the enantiospecific hydrolysis of epoxides including many substituted styrene epoxides.[1–7] Faber and his coworkers utilized epoxide hydrolases from *Rhodococcus*, *Nocardia* and other species for enantiospecific hydrolysis.[8–16] Enantioselective epoxide hydrolases from various fungal and other sources have been reported.[17–19] Weijers found the yeast *Rhodotorula glutinis* to be an effective microorganism for enantioselective hydrolysis of various epoxides.[20]

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PII: S0957-4166(99)00326-2

The present work describes screening of microorganisms for the stereospecific hydrolysis of the racemic epoxide, *RS*-1-{2′,3′-dihydrobenzo[*b*]furan-4′-yl}-1,2-oxirane, **1** to the corresponding *R*-diol **3** and unreacted chiral *S*-epoxide **2** (Fig. 1).

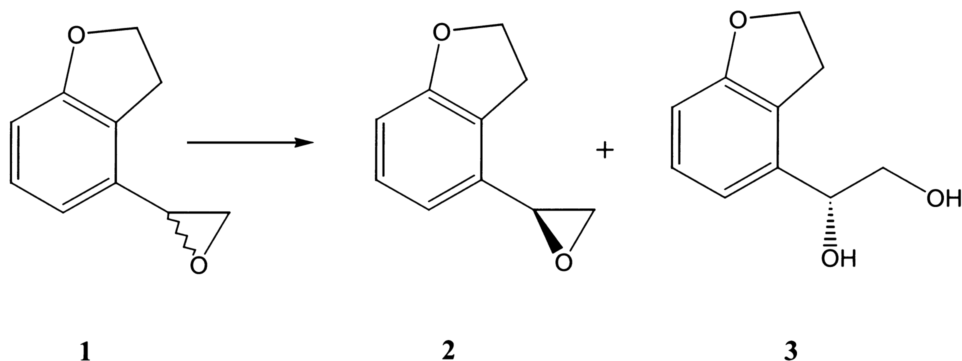

Figure 1. Hydrolysis of racemic epoxide to *S*-epoxide and *R*-diol

2. Results and discussion

2.1. Stability of racemic epoxide 1

Epoxides are unstable in acidic and basic conditions. It is important to select an appropriate condition to minimize the chemical hydrolysis of epoxide during the biotransformation process. The stability of the racemic epoxide **1** was determined under various conditions. About 100% and 91% of epoxide **1** was hydrolyzed in 24 h at pHs 5 and 6, respectively. Even under neutral conditions (pH 7), the epoxide was not very stable and 51% was hydrolyzed in 24 h. Alkaline conditions (pH >7) are better and there was less hydrolysis, for example, 38% and 30% in 24 h at pHs 8 and 9, respectively. Therefore, pH 8.0 was selected for conducting the enzymatic hydrolysis. Even at pH 8, 19% of epoxide **1** was hydrolyzed in 4 h. Therefore, it was necessary to find a microorganism that hydrolyzes the racemic epoxide with high stereospecificity at a faster rate to prevent (or at least minimize) the loss of unreacted desired *S*-epoxide **2** by chemical hydrolysis.

2.2. Stereospecific hydrolysis of racemic epoxide 1

Several fungi, yeast and bacterial cultures were screened for stereospecific hydrolysis of the racemic epoxide **1**. The summary of our results with various microorganisms is as shown in Table 1. Two *A. niger* strains (SC 16310, SC 16311) and *R. glutinis* SC 16293 selectively hydrolyzed the *R*-epoxide leaving behind the *S*-epoxide **2**. The enantiomeric ratio (*E*) values[21] for these microorganisms were ~25. The unreacted *S*-epoxide **2** was obtained in >95% ee and 45% yield (theoretical maximum yield is 50%). *Rhodococcus equi* SC 15835 did not hydrolyze the epoxide **1**. *Nocardia salmonicolor* SC 6310 hydrolyzed the racemic epoxide **1** at a slow rate and the enantiomeric excess of the *S*-epoxide **2** was only 30%.

From the initial screening studies, *R. glutinis* SC 16293 and two *A. niger* strains SC 16310 and SC 16311 were selected for further research. Hydrolysis of racemic epoxide **1** by these microorganisms proceeded rapidly. These microorganisms were studied in further detail for the stereospecific hydrolysis of racemic epoxide **1**.

Table 1
Screening for stereospecific microbial epoxide hydrolases

Microorganism	SC Number	Time h	Remaining Epoxide %	EE of remaining *S*-Epoxide %	E
Aspergillus niger	SC 2164	28	36%	73%	5
Aspergillus niger	SC 2564	28	56%	29%	3
Aspergillus niger	SC 2828	24	39%	67%	5
Aspergillus niger	SC 9719	28	41%	58%	4
Aspergillus niger	SC 15255	28	47%	34%	3
Aspergillus niger *	SC 16310	3	45%	95%	29
Aspergillus niger *	SC 16311	3	45%	95%	29
Aspergillus duricaulis	SC16280	45	75%	-2%	1
Scopulariopsis brumpti	SC 15091	28	44%	32%	2
Scopulariopsis brumpti	SC 16172	24	44%	57%	5
Rhodotorula glutinis *	SC 16293	4	44%	95%	25
Rhodococcus equi	SC 15835	28	100%	-2%	-1
Nocardia salmonicolor	SC 6310	45	62%	30%	4

* EE > 95%, only a small shoulder was observed for *R*-epoxide in chromatograms
Substrate concentration used for these are 2 g/l, it is 1 g/l for all others

2.3. *Hydrolysis of racemic epoxide **1** by* Rhodotorula glutinis *SC 16293*

Hydrolysis of racemic epoxide **1** by *R. glutinis* SC 16293 was carried out as described in the Experimental. The desired *S*-epoxide **2** was obtained in 40% yield and >95% ee when the substrate **1** was used at 2 g/l and cells were used at 100 g/l concentrations (Table 2). In an attempt to increase the substrate concentration to 5 g/l and keeping the same cell concentration (100 g/l), the extent of hydrolysis and the ee of *S*-epoxide **2** obtained was very poor. The cell concentration was increased to 200 g/l and substrate **1** concentration was increased to 5 and 10 g/l in hydrolysis reaction mixtures (Table 2). A reaction yield of 46% and an ee of 84% were obtained for unreacted *S*-epoxide **2** when the reaction was catalyzed at 5 g/l substrate concentration. At 10 g/l substrate concentration, a reaction yield of 56% and ee of 42% were obtained for *S*-epoxide **2**. In order to conduct enzymatic hydrolysis at high substrate concentration and improve the ee of *S*-epoxide **2**, the reaction was catalyzed at 300 g/l cell concentration. A reaction yield of 37% and an ee of 96% were obtained for the desired *S*-epoxide **2** (Table 3).

Several solvents at 10% (vol/vol) were evaluated in aqueous reaction mixture to improve the ee and yield (Table 3). Two solvents, cyclohexane and 1,1,2-trichlorotrifluoroethane (where the epoxide was not very soluble), were used in higher amounts. The two miscible solvents (DMSO and DMF) were used at 5%, 10% and 20%. Solvents had significant effects on both the extent of hydrolysis and ee of unreacted *S*-epoxide **2**. Most solvents, except methyl *tert*-butyl ether (MTBE), gave lower ee than that of reactions catalyzed in buffer without any solvent supplement. The extents of hydrolysis in the presence of solvents were always lower than that in buffer.

Methyl *tert*-butyl ether (MTBE) gave excellent results. A reaction yield of 45% and an ee of 99.9% were obtained for unreacted *S*-epoxide **2**. The hydrolysis reaction in the presence of MTBE gave an *E*-value of 68. Interestingly, 1,1,2-trichlorotrifluoroethane also gave a high *E*-value of 511. It seems that given enough time, this solvent could probably provide remaining *S*-epoxide **2** at very high ee, though chemical hydrolysis may reduce the yield significantly at longer times.

Table 2
Hydrolysis of racemic epoxide **1** by *Rhodotorula glutinis* SC 16293 in buffer

Epoxide Conc g/l	Cell Conc g/l	Time h	Remaining Epoxide %	EE of Remaining *S*-Epoxide %	EE of Product *R*-Diol %
2	100	1	67%	16%	ND
		2	59%	30%	ND
		4	44%	>90%	ND
		6	40%	>95%	ND
5	200	1	75%	26%	60%
		2	64%	40%	62%
		4	59%	59%	64%
		6	50%	72%	66%
		8	46%	84%	64%
10	200	1	83%	13%	60%
		2	76%	18%	58%
		4	69%	29%	62%
		6	61%	36%	62%
		8	56%	42%	64%

ND Not Determined

Table 3
Hydrolysis of racemic epoxide **1** by *Rhodotorula glutinis* SC 16293 in buffer–organic solvent mixture

Solvent Used	Solvent Amount, ml	Time h	Remaining Epoxide %	EE of Remaining *S*-Epoxide %	E
Buffer Only:		4	40%	86.17%	10
		5	39%	89.17%	10
		7	37%	96.65%	14
Water Immiscible Solvents:					
Cyclohexane	5	5	53%	45.45%	5
Toluene	1	5	66%	45.89%	29
1,1,2-Trichlorotrifluoroethane	5	5	76%	31.49%	511
Methyl tert-butyl ether (MTBE)	1	5	45%	99.88%	68
Methyl isobutyl ketone	1	5	68%	22.78%	4
n-Butanol	1	5	81%	3.19%	1
Water Miscible Solvents:					
Dimethyl Sulfoxide	0.5	5	46%	83.56%	14
	1	5	49%	80.88%	19
	2	5	44%	80.16%	11
Dimethyl Formamide	0.5	5	43%	79.99%	10
	1	5	45%	74.80%	9
	2	5	52%	62.58%	9

2.4. *Preparative scale hydrolysis of racemic epoxide **1** by* Rhodotorula glutinis

*Rhodotorula glutini*s cells were grown in a 25 l fermentor. Cells were harvested at different times and evaluated for the stereospecific hydrolysis of the epoxide **1** (Table 4). The cells grown for 24 h gave the fastest rate of hydrolysis and highest ee for *S*-epoxide **2**. Cells grown for longer times gave lower ee. This difference in activity was correlated to changes in several fermentation parameters, e.g. glucose concentration, optical density, pH, carbon dioxide evolution and dissolved oxygen concentration. The glucose concentration dropped to almost zero after 24 h. The optical density reached a maximum at 24

Table 4
Dependence of stereospecificity with the time of growth of *Rhodotorula glutinis* cells in large fermentor

Growth Time h	Remaining Epoxide %	EE of Remaining *S*-Epoxide %
24	38%	98.29%
48	40%	97.88%
72	41%	95.02%

h and then declined. The carbon dioxide evolution rate increased at the beginning, reached a maximum around 12 h and then declined to almost zero after 24 h. Subsequent fermentors were harvested at 24 h.

A preparative scale hydrolysis of racemic epoxide **1** was carried out in buffer containing 10% MTBE using *R. glutinis* SC 16293. After 5 h, a reaction yield of 48% and an ee of 99% were obtained for *S*-epoxide **2**. *R*-Diol was obtained with an ee of 79%. Interestingly, the ee of the *R*-diol in buffer–MTBE mixture reaction was higher than that obtained with buffer. Therefore, in the buffer–MTBE system the enzyme is not only more selective in binding to the *R*-epoxide giving higher enantiomeric excess of the remaining *S*-epoxide, the opening of the enzyme–*R*-epoxide substrate complex is more specific compared to that in buffer and thereby provides *R*-diol of higher ee.

*2.5. Hydrolysis of racemic epoxide **1** by* Aspergillus niger

Two *A. niger* strains SC 16310 and SC 16311 were found to be effective during screening. Further experiments were conducted to evaluate their potential for the stereospecific hydrolysis of the racemic epoxide **1**. *Aspergillus niger* SC 16311 produced a higher cell mass than SC 16310 under similar growth conditions. The results of hydrolysis of the racemic epoxide **1** by these cultures are shown in Table 5. The hydrolysis proceeded much faster and the ee (97%) and yield (45%) of the remaining *S*-epoxide were much higher with the strain SC 16311 when substrate was used at 2 g/l concentration. At a higher substrate concentration (5 g/l) using 100 g/l cell concentration, a reaction yield of 51% and ee of 84% were obtained with SC 16311.The results of increasing the cell concentration and use of solvent MTBE in the reaction mixture during hydrolysis by *A. niger* SC 16311 are shown in Table 6. As expected, the ee was improved to 96% at 45% remaining epoxide with higher cell concentration (200 g/l). The effect of MTBE, however, was quite different than that observed with *R. glutinis*. The presence of MTBE inhibits the hydrolysis of racemic epoxide **1** by *A. niger* SC 16311 and even after 5 h reaction time, 54% epoxide remained (only 28% remained with buffer at 5 h) and the ee of the remaining *S*-epoxide was only 49%. There are apparently contradictory reports in the literature on the effect of solvent on the hydrolysis of epoxides by *A. niger*. Hydrolysis of *para*-nitro styrene oxide was reported[6] to be improved in the presence of co-solvents, e.g. DMSO and DMF compared to that with the substrate and buffer alone. But another report from the same group[7] using the same strain noted that the hydrolysis of *para*-bromo-alpha-methyl styrene oxide is best carried out in buffer without any solvent.

Table 5
Hydrolysis of racemic epoxide **1** by *Aspergillus niger* SC 16310 and SC 16311

Microorganism	Substrate Conc g/l	Time h	Remaining Epoxide %	EE of Remaining *S*-Epoxide %	E
Aspergillus niger SC 16310	2	2	58%	48%	8
		4	43%	88%	14
		5	42%	90%	14
		6	38%	95%	13
	5	6	68%	40%	16
Aspergillus niger SC 16311	2	2	45%	97%	35
		4	38%	>95%	
		5	35%	>95%	
		6	31%	>95%	
	5	6	49%	84%	43

Table 6
Hydrolysis of racemic epoxide **1** by *Aspergillus niger* SC 16311 in buffer and buffer–MTBE

Description	Time h	Remaining Epoxide %	EE of Remaining *S*-Epoxide %
Aspergillus niger SC 16311	3	45%	96.20%
Cells 2 g, Buffer 10 ml	5	28%	100.00%
Aspergillus niger SC 16311	2	62%	21.82%
Cells 2 g, Buffer 10 ml, 1ml MTBE	5	54%	49.47%

3. Experimental

3.1. Chemicals

Racemic epoxide **1**, chiral *S*-epoxide **2**, racemic and chiral diol **3** and other authentic standards were prepared by the Process Research and Development Department of Bristol-Myers Squibb. The structural identity and purity of each compound was established by spectroscopic and other physical and chemical methods. Other chemicals were purchased from VWR and/or Aldrich.

3.2. Microorganisms

Microorganisms were obtained from culture collection. The SC number denotes the number in the BMS culture collection. Some microorganisms were obtained from ATCC or other sources.

3.3. Analytical methods

The amounts of epoxide and diol were determined by GC on Hewlett–Packard Ultra 2 GC column (crosslinked 5% phenyl methyl silicone, 25 m×0.32 mm×0.17 mm film) using helium as a carrier gas. The samples were dissolved in ethyl acetate and analyzed directly.

Chiral purity and enantiomeric excess of epoxide was determined by HPLC. The samples were dissolved in hexane:isopropanaol (90:10) and subjected to HPLC on a Pirkle covalent SS column (25 cm×0.46 cm, Regis) with hexane:ethanol (99:1) as eluent at 0.7 ml/min. The enantiomeric excess of the

diol was analyzed by HPLC on Chiralcel OD column (25 cm×0.46 cm, Daicel) using hexane:isopropanol (95:5) as eluent at 1 ml/min.

3.4. Stability of epoxide under different conditions

A solution of racemic epoxide (**1**, 20 μl) in dimethyl sulfoxide (DMSO, 80 μl) was added to 10 ml of buffer in a 50 ml Erlenmeyer flask and placed in a shaker at 200 rpm at 28°C. At specified times, the entire mixture was extracted with ethyl acetate (20 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate, filtered and analyzed by GC to determine the amount of epoxide remaining and diol formed.

3.5. Growth of microorganisms

The F7 medium for growing microorganisms was made as follows. Malt extract 10 g, yeast extract 10 g, peptone 1 g and dextrose 20 g were dissolved in distilled water to a total volume of 1 l, adjusted to pH 7.0 and autoclaved at 121°C for 20 min.

Sterilized F7 medium (100 ml in 500 ml flask) was inoculated with microorganisms from vials and allowed to grow by shaking at 200 rpm at 28°C for 72 h. The filamentous fungi (*Aspergillus*, *Scopulariopsis*, etc.) were harvested by filtration. Yeast and bacteria (*Rhodotorula*, *Rhodococcus*, etc.) were harvested by centrifugation (16 000*g* for 20 min). The cells were washed with 100 mM phosphate buffer pH 7.0, filtered or centrifuged.

For large scale growth of cultures, a two stage process was used. A 100 ml F7 medium (in a 500 ml flask) was inoculated first and grown for 24 h. In the second stage, 1000 ml F7 medium (in a 4 l flask) was inoculated with 2% of the first stage culture. The second stage was grown for 65 h. In all stages, cultures were grown on a shaker maintained at 28°C and 200 rpm. *Rhodotorula glutinis* cells were harvested by centrifugation (Sorvall, GS-3) at 8000 rpm, 4–8°C for 30 min. The cells were washed twice with 100 mM phosphate buffer pH 7.0. *Aspergillus niger* cells were harvested by filtration. The cells were washed twice with 100 mM phosphate buffer pH 7.0. All cells were stored in a −70°C freezer before use.

3.6. Growth of Rhodotorula glutinis SC 16293 in a fermentor

Rhodotorula glutinis SC 16293 cells were grown in 15 l fermentor for 72 h. Cells were withdrawn from the fermentor at 24 h, 48 h and 72 h, centrifuged, washed with 100 mM phosphate buffer pH 7.0 and stored in the freezer at −70°C before use. Activity of cells was determined by routine assay as described below.

3.7. Hydrolysis of racemic epoxide **1**

Various microorganisms were screened for hydrolysis of racemic epoxide **1**. The cells were suspended in 10 ml of 100 mM phosphate buffer pH 8.0 in a 50 ml Erlenmeyer flask. A solution of racemic epoxide **1** in DMSO (one to four times the volume of epoxide) was added and placed on a shaker at 200 rpm at 28°C. At various times, a 1 ml sample of the reaction mixture was extracted with ethyl acetate (2 ml). The ethyl acetate layer was dried over anhydrous sodium sulfate, filtered and analyzed by GC to determine the amount of epoxide remaining and diol formed. The solvent ethyl acetate was removed from another portion of the extract and the residue was redissolved in a mixture of hexane:isopropanol (90:10) and

analyzed by HPLC to determine the enantiomeric purity. A comparative reaction in buffer with no cells provided information on the amount of chemical hydrolysis at the specified time.

For further studies and better quantitation of epoxide and diol, at different times, the entire reaction mixture was saturated with NaCl and extracted with ethyl acetate (20 ml). The ethyl acetate layer was analyzed as before.

Hydrolysis of racemic epoxide **1** with *R. glutinis* in a buffer–organic solvent system was carried out by suspending cells (3 g) in 10 ml of 100 mM phosphate buffer pH 8.0 in a 50 ml Teflon flask. A solution of 50 μl of racemic epoxide **1** in organic solvent was added to the cell suspension. The flask was closed and placed in a shaker at 250 rpm at 28°C. After 5 h, 2 g of sodium chloride and ethyl acetate (total volume of ethyl acetate and organic solvent 20 ml) were added. The mixture was transferred to a tube and the extraction was done by a vortex mixture. The layers were separated by centrifugation. The organic layer was dried over anhydrous sodium sulfate, filtered and analyzed.

3.8. *Preparative scale hydrolysis of racemic epoxide **1** by* Rhodotorula glutinis

The cells (150 g) were suspended in 500 ml of 100 mM phosphate buffer pH 8.0 in a jacketed three-necked flask fitted with an air driven stirrer, condenser and stopper. The reaction flask was kept at 28°C and stirred throughout the entire period. A solution of racemic epoxide **1**, 2.5 g in 50 ml of methyl *tert*-butyl ether was added to the stirred cell suspension. The progress of the hydrolysis was monitored by withdrawing samples and analyzing the ee. After 5 h reaction time, 1.2 g of epoxide remained. At 5.5 h, the reaction was stopped. NaCl (180 g) was added and the mixture was extracted twice with ethyl acetate (1 l each). The two ethyl acetate layers were combined, dried over anhydrous Na_2SO_4, filtered and solvent was removed in the rotary evaporator. HPLC analysis showed that the reaction product contained only the *S*-epoxide and the *R*-diol. There was no peak for the *R*-epoxide and the calculated total amount of remaining epoxide was 1.2 g (48% yield).

A portion (2.2 g) of the reaction product was dissolved in a mixture of heptane:ethyl acetate (1:1) and repeatedly extracted with 0.10 M solution of $Na_2B_4O_7$ in water. The organic layer provided 0.95 g of *S*-epoxide **2** with ee of more than 99% while the aqueous layer provided 1.02 g of *R*-diol **3** with ee of 79%.

3.9. *Hydrolysis of racemic epoxide **1** by* Aspergillus niger *SC 16310 and SC 16311*

The cells (1 g) were suspended in 10 ml of 100 mM phosphate buffer pH 8.0 in a 50 ml Erlenmeyer flask and reactions were carried out as described earlier.

Hydrolysis of racemic epoxide **1** by *A. niger* SC 16311 in buffer and buffer–methyl *tert*-butyl ether system was carried out by suspending cells in 10 ml of 100 mM phosphate buffer pH 8.0 in a 50 ml Teflon flask. For buffer only reaction, 50 μl of racemic epoxide **1** was added. For buffer–MTBE reaction, a solution of 50 μl racemic epoxide **1** in 1 ml of methyl *tert*-butyl ether (MTBE) was added. Flasks were shaken at 250 rpm at 28°C. At various times, 1 ml was taken out from each and extracted with ethyl acetate (2 ml). The ethyl acetate layer was dried (Na_2SO_4), filtered and analyzed as before.

Acknowledgements

We are indebted to Dr. C. A. M. Weijers for providing a specimen of *R. glutinis*. We are thankful to Mr. D. Brzozowski for his help in screening and in growing various cultures, to Mr. S. Schwarz for providing

cultures from the collection, to Dr. J. Wasylyk for analysis and to Dr. W. He for his help in the isolation of products from the preparative scale reaction.

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