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Journal of Molecular Catalysis B: Enzymatic 5 (1998) 447–458

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Microbiological transformations 41. Screening for novel fungal epoxide hydrolases

Philippe Moussou, Alain Archelas, Roland Furstoss *

Groupe Biocatalyse et Chimie Fine, ERS 157 associée au CNRS, Faculté des Sciences de Luminy, Université de la Méditerranée, Case 901, 163 avenue de Luminy, 13288 Marseille Cedex, 9, France

Received 17 December 1997; accepted 6 January 1998

Abstract

A search for new fungal epoxide hydrolases is described, which led to the selection of seven strains of interest. The biohydrolyses of various alkyl and aryl epoxides using whole cells of these seven strains are described. The enantio- and regio-selectivity observed proved to be variable depending upon the type of fungus and the substrate structure. However, a general trend was the preferential formation of the diol with (*R*) absolute configuration at the carbon atom bearing the bulkier substituent.

Keywords: Alkyl and aryl substituted epoxides; Enantiopure epoxides; Fungi; Epoxide hydrolase; Enantioselective hydrolysis

1. Introduction

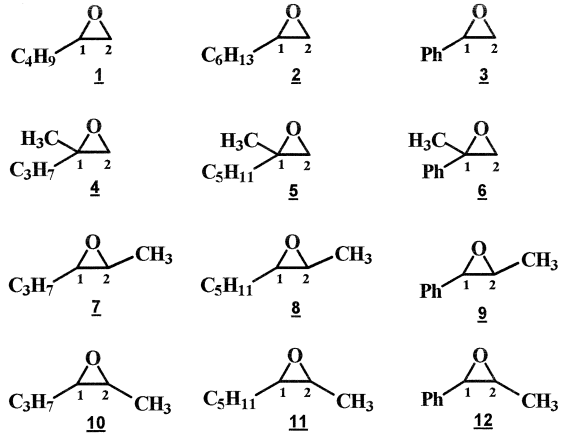
Chiral epoxides and vicinal diols are high-value intermediates for the synthesis of enantiopure compounds, and intensive work has been devoted in recent years for their production [1–4]. Their synthesis can be achieved using chemo-catalytic methods [5–7], or biocatalytic approaches, for example, alkene epoxidation by monooxygenases and chloroperoxydases, or bioreduction of α-halo-ketones into optically pure halohydrins. In this context, one of the most promising way for the synthesis of such chirons is the use of cofactor-independent epoxide hydrolases (EHs). Indeed, these enzymes have been proven to catalyse the enantioselective hydrolysis of various racemic epoxides, thus allowing to recover the unreacted epoxide enantiomer and the corresponding vicinal diol. However, most of the enzymes studied were from mammalian origin and their use for preparative scale application was therefore severely hampered due to their low availability. Only recently, EH from microbial origin—i.e., from bacterial, yeast or fungal sources—have been identified [8–10]. These appear to be potentially highly interesting tools for asymmetric synthesis since these organisms can be cultured in large-scale, making in principle these enzymes much easier available. In particular, we have shown that the fungi *Aspergillus niger* and *Beauveria bassiana* [1] were able to achieve —at a several gram-scale—the enantioselective

* Corresponding author. Fax: +33-04-91-82-91-45; e-mail: furstoss@gulliver.fr

[1] Formerly named *Beauveria sulfurescens*.

1381-1177/98/$ - see front matter
PII: S1381-1177(98)00007-1

hydrolysis of different types of epoxides derived from geraniol, limonene [8] or substituted styrene derivatives [11]. However, detailed searches for EH from such microbial sources are still scarce. In this context, and in order to increase the variety of available 'tools' for the organic chemist's toolbox, it was of high interest to search for new microbial EHs and to enlarge our knowledge on their enantio- and substrate selectivity. We described here our ongoing, screening efforts aimed to detect new fungal EHs, as well as our results focused on the possible biohydrolysis of diversely disubstituted alkyl- and aryl-epoxides using these new biocatalysts. Thus the mono-, *gem*-, *trans*- and *cis*- substituted epoxides **1**–**12** were studied in this context, in order to explore the influence of these various substitution patterns on the outcome of these reactions (Scheme 1). [2]



Scheme 1.

2. Experimental

2.1. General

The strains used in this work were purchased from the ATCC, CBS, DSM, IAM, IFO, LCP, MUCL, NRRL or VKMF collections, or selected from our laboratory collection. ^{1}H NMR spectra were recorded in $CDCl_3$ solution on a Brüker AC 250 apparatus. Chemical shifts are reported in ppm from TMS as internal standard. Optical rotation values were measured on a Perkin-Elmer 241C polarimeter at 589 or 578 nm. Vapor phase chromatography (GC) analyses were performed using one of the four following 'chiral' columns (0.22 mm, 25 m, Macherey-Nagel), i.e., (col. I): Heptakis (6-*O*-methyl-2,3-di-*O*-pentyl)-β-cyclodextrin, (col. II): Lipodex E [Octakis (3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin] in OV 1701 (1/1); (col. II*bis*): Lipodex E [Octakis (3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin] and (col. III): [Octakis (6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin] in OV 1701 (1/1).

2.2. Synthesis of substrates *1* to *12* [2]

(±)-Butyl-oxirane **1**, (±)-hexyl-oxirane **2** and (±)-phenyl-oxirane **3** were purchased from Fluka.

(±)-gem-1-propyl-1-methyl-oxirane **4**. This was prepared according to the Corey–Chaykovsky method [12]. To a stirred solution of 8 g (36 mmol) of trimethyloxosulfonium iodide in 50 ml of DMSO, placed under argon, were added 1.4 g (36 mmol) of sodium hydride (60% mineral oil dispersion). After stirring for 30 min, a solution of 2.5 g (30 mmol) of 2-pentanone in 10 ml of DMSO was added and the reaction mixture was stirred at room temperature overnight. Purification by distillation under reduced pressure (30 mm Hg) of the crude mixture yielded 0.32 g (11% yield) of **4** as a colorless liquid. ^{1}H NMR [13] δ: 0.93 (t, 3H, $J = 6.9$ Hz, ω-CH_3); 1.30 (s, 3H, CH_3); 1.37–1.62 (m, 4H, $(CH_2)_2$); 2.57 and 2.61 (d, 1H, $J_{gem} = 4.9$ Hz, CH_2–O).

(±)-gem-1-pentyl-1-methyl-oxirane **5**. This was prepared from trimethyloxosulfonium iodide (8 g, 36 mmol), sodium hydride (60% mineral oil dispersion, 1.4 g, 36 mmol) and

[2] In order to keep homogeneity throughout this manuscript, the oxirane ring was numbered with the convention that carbon atom C(1) is bearing the 'bulkier' substituent, and this convention was kept for the diols, i.e., for instance for the epoxide **7** and the diol **7a**, C(1) is the carbon atom bearing the propyl group.

2-heptanone (3.4 g, 30 mmol) following the procedure described for **4**, except the purification step. After cooling and addition of water, the crude mixture of reaction was extracted with ether (3 times). The combined extracts were washed with brine, dried over $MgSO_4$ and evaporated to give crude product. Purification by flash chromatography (pentane/ether 96/4) yielded 2.9 g (77% yield) of **5** as a colorless liquid. ^{1}H NMR [13] δ: 0.89 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.30 (s, 3H, CH_3); 1.30–1.66 (m, 8H, $(CH_2)_4$); 2.57 and 2.61 (d, 1H, $J_{gem} = 4.9$ Hz, CH_2–O).

(±)-gem-1-phenyl-1-methyl-oxirane **6**. This was prepared from trimethylsulfonium iodide (8.2 g, 40 mmol), sodium hydride (60% mineral oil dispersion, 1.6 g, 40 mmol) and acetophenone (4 g, 33 mmol) in 100 ml of DMSO following the procedure described for **5**. Purification by flash chromatography (pentane/CH_2Cl_2 90/10) yielded 3.1 g (70% yield) of **6** as a colorless oil. ^{1}H NMR [11] δ: 1.69 (s, 3H, CH_3); 2.76 (d, 1H, $J_{gem} = 5.2$ Hz, H_2); 2.93 (d, 1H, $J_{gem} = 5.2$ Hz, H_2); 7.24–7.34 (m, $5H_{arom}$).

(±)-trans-1-propyl-2-methyl-oxirane **7**. To a stirred solution of 3 g (36 mmol) of *trans*-2-hexene in 10 ml of CH_2Cl_2, cooled in an ice bath, was added 7.5 g of *m*-chloroperoxybenzoic acid (70%, 1.1 equiv.) dissolved in 100 ml of CH_2Cl_2. After removing the ice bath and stirring for 3 h, the reaction mixture was washed with a saturated solution of $NaHSO_3$, aqueous NaOH 10%, brine, and dried over $MgSO_4$. Purification by distillation yielded 1.6 g (46% yield) of **7** as a colorless liquid. ^{1}H NMR [14] δ: 0.95 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.29 (d, 3H, $J = 5.2$ Hz, CH_3); 1.37–1.55 (m, 4H, $(CH_2)_2$); 2.63 (td, 1H, $J_{1-2} = 2.3$ Hz, $J = 5.1$ Hz, H_1); 2.74 (qd, 1H, $J_{1-2} = 2.2$ Hz, $J = 5.2$ Hz, H_2).

(±)-trans-1-pentyl-2-methyl-oxirane **8**. This was obtained by epoxidation of *trans*-2-octene (6 g, 53 mmol) using the same procedure described for **7**, except the purification step which was performed by flash chromatography (pentane/ether 96/4) (87% yield). ^{1}H NMR [15] δ: 0.89 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.29 (d, 3H, $J = 5.2$ Hz, CH_3); 1.32–1.55 (m, 8H, $(CH_2)_4$); 2.62 (td, 1H, $J_{1-2} = 2.1$ Hz, $J = 5.2$ Hz, H_1); 2.74 (qd, 1H, $J_{1-2} = 2.2$ Hz, $J = 5.2$ Hz, H_2).

(±)-trans-1-phenyl-2-methyl-oxirane **9**. This was obtained by epoxidation of *trans*-1-phenylpropene (0.41 g, 3.5 mmol) under biphasic conditions (CH_2Cl_2/phosphate buffer pH 8, 1/1) as described previously [11], and using the same procedure described for **8**. Purification by flash chromatography (pentane/ether 90/10) yielded 0.47 g (97% yield) of **9** as a colorless oil. ^{1}H NMR [11] δ: 1.45 (d, 3H, $J = 5.1$ Hz, CH_3); 3.04 (qd, 1H, $J_{1-2} = 2.0$ Hz, $J = 5.1$ Hz, H_2); 3.58 (d, 1H, $J_{1-2} = 2.0$ Hz, H_1); 7.17–7.47 (m, $5H_{arom}$).

(±)-cis-1-propyl-2-methyl-oxirane **10**. This was obtained by epoxidation of *cis*-2-hexene (3 g, 36 mmol) following the procedure described for **7**. Purification by distillation yielded 1.1 g (31% yield) of **10** as a colorless liquid. ^{1}H NMR [14] δ: 0.98 (t, 3H, $J = 7$ Hz, ω-CH_3); 1.27 (d, 3H, $J = 5.5$ Hz, CH_3); 1.40–1.60 (m, 4H, $(CH_2)_2$); 2.91 (td, 1H, $J_{1-2} = 4.2$ Hz, $J = 5.6$ Hz, H_1); 3.05 (qd, 1H, $J_{1-2} = 4.2$ Hz, $J = 5.5$ Hz, H_2).

(±)-cis-1-pentyl-2-methyl-oxirane **11**. This was obtained by epoxidation of the corresponding *cis*-olefin formed by catalytic hydrogenation of 2-octyne [16]. To 450 mg of Lindlar catalyst in 60 ml of pentane, placed under hydrogen at room temperature, were added dropwise 4 g (36 mmol) of 2-octyne, and the mixture was stirred. After 2 h of reaction, no more hydrogen was consumed. The solid was then filtered on celite, and the pentane was partially evaporated. The crude product was dissolved in 10 ml of CH_2Cl_2, and the epoxide **11** was obtained using the same procedure described for **8** (yield 96%). ^{1}H NMR [15] δ: 0.89 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.29 (d, 3H, $J = 5.2$ Hz, CH_3); 1.32–1.55 (m, 8H, $(CH_2)_4$); 2.62 (td, 1H, $J_{1-2} = 2.1$ Hz, $J = 5.2$ Hz, H_1); 2.74 (qd, 1H, $J_{1-2} = 2.2$ Hz, $J = 5.2$ Hz, H_2). Using these experimental conditions, a small proportion(3%) of *trans*-1-

pentyl-2-methyl-oxirane was also formed (as indicated by GC analysis).

(±)-cis-1-phenyl-2-methyl-oxirane **12**. This was prepared by cyclisation of the corresponding bromohydrin formed by reduction of α-bromopropiophenone (1 g, 4.7 mmol) following the procedure described previously [11]. Purification by flash chromatography (pentane/ether 90/10) yielded 0.47 g (75% yield) of **12** as a colorless oil. ^{1}H NMR [11] δ: 1.09 (d, 3H, $J = 5.4$ Hz, CH_3); 3.33 (qd, 1H, $J_{1-2} = 4.2$ Hz, $J = 5.4$ Hz, H_2); 4.06 (d, 1H, $J_{1-2} = 4.2$ Hz, H_1); 7.25–7.39 (m, $5H_{arom}$). Using these experimental conditions, a small proportion (5%) of *trans*-1-phenyl-2-methyl-oxirane was also formed (as indicated by GC analysis).

2.3. Synthesis of reference material for the diols *1a–12a*

(±)-Butyl-ethanediol **1a** and (±)-hexyl-ethanediol **2a** were purchased from Fluka.

(±)-Phenyl-ethanediol **3a**, (±)-gem-1-phenyl-1-methyl-ethanediol **6a**, (±)-*erythro*-1-phenyl-2-methyl-ethanediol **9a**, (±)-*threo*-1-phenyl-2-methyl-ethanediol **12a**, have been synthesized previously by acid hydrolysis of the racemic epoxides [11].

(±)-1-Propyl-1-methyl-ethanediol **4a**, (±)-gem-1-pentyl-1-methyl-ethanediol **5a**, (±)-*erythro*-1-propyl-2-methyl-ethanediol **7a**, (±)-*erythro*-1-pentyl-2-methyl-ethanediol **8a**, (±)-*threo*-1-propyl-2-methyl-ethanediol **10a** and (±)-*threo*-1-pentyl-2-methyl-ethanediol **11a** were prepared as follows by hydrolysis of the corresponding epoxides. To 10 mg epoxide dissolved in 1 ml of a THF/water mixture (5/1), two drops of concentrated sulfuric acid were added. After stirring overnight, the solution was neutralized by addition of saturated $NaHCO_3$ solution, and extracted with ether. Evaporation of the washed (saturated NaCl solution) organic phase gave the crude racemic diol which was further analyzed by GC on a chiral stationary phase.

2.4. General procedure for the derivatisation of the diols

The ee determination of the diols were performed after derivatisation in acetonide for the diols **1a**–**6a**, **8a**–**9a**, after cyclisation into the corresponding *trans*-epoxide **8** for diol **11a**, and after derivatisation into the dimethylether derivative for diol **12a**.

As described previously [11], the acetonide derivative was obtained by reaction of the diol with 2,2-dimethoxypropane in the presence of a catalytic amount of APTS; and the dimethylether derivative was obtained by reaction of the diol in DMSO with CH_3I in presence of KOH.

Diol **11a** was cyclised into the corresponding epoxide **8** according to Golding et al. [17]. The crude diol **11a** obtained after extraction of the reaction mixture with ether and evaporation of the solvent was placed under nitrogen and two drops of HBr in glacial acetic acid (33%) was added. After stirring for 1 h, the mixture was neutralized by addition of water and solid Na_2CO_3, and extracted with ether (2 times). The combined organic layers were evaporated and dried under vacuum. After stirring 30 min with 50 μl of KOH in MeOH (1 N), the mixture was washed with brine and extracted with ether to give the epoxide **8**.

2.5. General procedure for the growth of fungi

The fungal strains were maintained on agar slants and grown on a medium consisting of 20 g of corn steep liquor (Roquette), and 10 g of glucose for 1 l of tap water (for the culture of *Chaetomium globosum*, 10 g/l of KH_2PO_4 and 5 g/l of K_2HPO_4 were added to obtain a pH 6). Approximately 0.5 cm^2 of agar bearing fungal mycelium and/or spores was used to inoculate 100 ml of medium in a baffled 500 ml Erlenmeyer flask. After 3 days of culture at 27°C, the biomass was filtered off and washed with water.

2.6. General procedure for biohydrolysis of epoxides **1**–**12**

A fraction of the biomass produced in an Erlenmeyer flask as described above was suspended in three different sealed Erlenmeyer flasks containing 10 ml of sodium phosphate buffer 0.1 M pH 8 at 27°C. Racemic epoxide was then added (9.5–12 μl, 8 mM) and the mixture was shaken on a linear shaker at 100 strikes/min. The reaction was monitored by determination of the epoxide ee in the headspace phase. For values of the epoxide ee below 99%, and after a maximal reaction time of 72 h, the reaction mixture was extracted with ether after NaCl saturation. The ee of epoxides and diols were determined by GC on a chiral stationary phase without further purification for the epoxides, and after purification on a silica gel column (elimination of the epoxide with pentane/ether 80/20 followed by elution of the diol with ether) and derivatisation or cyclisation for the diols.

2.7. Absolute configuration of the epoxides and diols

They were assigned via GC analysis on a chiral stationary phase by comparison with reference material. Relationships between chiral GC data and absolute configuration have been described previously for the epoxides **2**, **5**, **8**, **11** [18] and **3**, **6**, **9**, **12** [11] and the corresponding diols **2a**, **5a**, **8a**, **11a** and **3a**, **6a**, **9a**, **12a**.

2.7.1. Absolute configuration of epoxides ***1***, ***4***, ***7***, ***10*** and of their corresponding diol ***1a***, ***4a***, ***7a***, ***10a***

*Synthesis of optically active references **1** and **1a** and determination of their absolute configuration*. Optically active **1a** was produced by bioconversion of racemic butyl-oxirane **1** using the fungus *B. bassiana*. The fungus was grown in a 2 l fermentor containing 1 l of medium (glucose 10 g/l, corn steep liquor 20 g/l, tap water, in which 0.2 g of Pluronic PE 8100 (BASF) and 50 μl of antifoam silicone 426R (Prolabo) were added to prevent overflowing during the growth. Inoculation was performed with 10 ml of a 2 days old culture in an Erlenmeyer flask. The medium was maintained at 27°C, stirred at 700 rpm and aerated at 0.3–0.5 VVM. After 44 h culture, the biomass (6.8 g_{dwc}) was filtered off, washed with water, and placed back in the same fermentor filled with 1 l of sodium phosphate buffer 0.1 M pH 8. The medium was stirred at 700 rpm and maintained at 27°C. One gram of **1** was added to the suspension as a solution in ethanol (10 ml). The course of the bioconversion was monitored by determining the ee of the remaining epoxide. When the ee of the epoxide reached 98%, the medium was filtered off and the aqueous phase was extracted with 500 ml of pentane. Optically active epoxide **1** was not isolated because of its high volatility. Continuous extraction of the NaCl saturated aqueous phase with CH_2Cl_2 (3 days), and purification by flash chromatography (pentane/ether 30/70) and by bulb-to-bulb distillation (10^{-2} mbar) yielded 0.55 g of optically active butyl-ethanediol (*R*)-**1a**: ee 59%; $[\alpha]_D^{24} = +11$ ($c = 1$; MeOH); lit. [19] (*S*)-**1a** $[\alpha]_D^{24} = -16.4$ ($c = 1$; MeOH). A total of 10 mg (1 equiv.) of (*R*)-**1a**, ee 59%, and 16 mg of tosyl chloride (1 equiv.) were then dissolved in 1 ml of anhydrous THF. After stirring for 1 h, 28 mg of NaH (60% mineral oil dispersion, 6 equiv.) were added. After stirring overnight, the mixture was washed with water, and crude (*R*)-**1**, ee 46%, was extracted with ether. This epoxide was not isolated because of its high volatility.

*Synthesis of optically active references **4a** and **4** and determination of their absolute configuration*. Optically active 1-propyl-1-methyl-ethanediol **4a** was obtained by asymmetric dihydroxylation of 2-methyl-1 pentene (84 mg, 1 mmol), using AD-mix-β purchased from Aldrich, following the procedure described by Sharpless et al. [20]. Purification by bulb-to-bulb distillation (0.5 mbar) yielded 37 mg of (*R*)-**4a**, (31% yield) as a colorless oil: ee 76%; $[\alpha]_D^{25} =$

+5 ($c = 1.8$; $CHCl_3$); lit. [21] (*R*)-**4a** $[\alpha]_D^{25} = +7.2$ ($c = 1.8$; $CHCl_3$). ^{1}H NMR [13] δ: 0.94 (t, 3H, $J = 6.9$ Hz, ω-CH_3); 1.17 (s, 3H, CH_3); 1.31–1.51 (m, 8H, $(CH_2)_2$); 1.95 (s large, 2H, 2 OH); 3.40 and 3.46 (d, 1H, $J_{gem} = 10.9$ Hz). 10 mg of (*R*)-**4a**, ee 75%, was then cyclised to (*R*)-**4** following the procedure described for cyclisation of **1a**.

*Synthesis of optically active references **10a** and **7** and determination of their absolute configuration*. The asymmetric dihydroxylation of 84 mg of *trans*-2,3-hexene was performed with the commercial catalyst AD-mix-β according to Sharpless et al. [20] and yielded, after purification by bulb-to-bulb distillation (10^{-2} mbar), 28 mg of the *threo*-1-propyl-2-methyl-ethanediol **10a** (24% yield) as a colorless oil: ee 95%; $[\alpha]_D^{20} = +16$ ($c = 1.4$; $CHCl_3$); ^{1}H NMR [22] δ: 0.92 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.20 (d, 3H, $J = 6.5$ Hz, CH_3); 1.27–1.50 (m, 8H, $(CH_2)_4$); 2.16 (s large, 2H, 2 OH); 3.37 (m, 1H, H_1); 3.58 (m, 1H, H_2). Cyclisation of this diol **10a** according to Golding et al. [17] gave the *trans*-1-propyl-2-methyl-oxirane **7** with an ee of 94% (**7** was not isolated because of its high volatility, and was kept as an ether solution dried over $MgSO_4$). To a solution of **7** in dry ether were added, under nitrogen, 50 mg of $LiAlH_4$ in suspension in anhydrous ether (1 ml). After stirring for 3 h, the mixture was washed with a NaOH solution (5%) and the solid was eliminated by filtration. The mixture of 2- and 3-hexanol obtained was separated on a 'chiral' GC (col. III, 40°C), and by co-injection using commercially available (*R*)-2-hexanol, the (*R*) absolute configuration was assigned to the formed 2-hexanol (ee 87%). It could be deduced that the absolute configuration of the thus synthesized *threo*-1-propyl-2-methyl-ethanediol **10a** and epoxide **7** were (1*R*,2*R*).

*Synthesis of optically active references **7a** and **10** and determination of their absolute configuration*. Optically active **7a** was produced by bioconversion of racemic epoxide **7** (2.5 g) using the fungus *Mortierella isabellina* (61 g_{dwc} of biomass obtained from 10 l of medium after 40 h of growth) following the procedure described above for bioconversion of **1**. Purification by bulb-to-bulb distillation (10^{-2} mbar) yielded 1.68 g of *erythro*-1-propyl-2-methyl-ethanediol **7a** as a colorless oil: ee 71%; $[\alpha]_D^{24} = +14$ ($c = 1.4$; $CHCl_3$); ^{1}H NMR [22] δ: 0.94 (t, 3H, $J = 6.8$ Hz, ω-CH_3); 1.14 (d, 3H, $J = 6.4$ Hz, CH_3); 1.27–1.58 (m, 4H, $(CH_2)_2$); 2.63 (s large, 2H, 2 OH); 3.63 (td, 1H, $J_{1-2} = 3.1$ Hz, $J = 7.6$ Hz, H_1); 3.78 (qd, 1H, $J_{1-2} = 3.1$ Hz, $J = 6.4$ Hz, H_2). The (1*R*,2*S*) absolute configuration of this optically active diol **7a** was established as for its *threo* stereoisomer **10a** after cyclisation of 100 mg of **7a** to *cis*-1-propyl-2-methyl-oxirane **10** (ee 95%) and reduction to a mixture of 2- and 3-hexanol as described above.

*2.7.2. Chiral gc data for the epoxides **1**, **4**, **7**, **10** and their corresponding diol **1a**, **4a**, **7a**, **10a***

(**1**) col. I, 40°C, t_{ret} (*R*) 2.9 min and t_{ret} (*S*) 3.1 min
(**4**) col. I, 40°C, t_{ret} (*R*) 2.2 min and t_{ret} (*S*) 2.4 min
(**7**) col. I, 35°C, t_{ret} (1*S*,2*S*) 2.3 min and t_{ret} (1*R*,2*R*) 2.5 min
(**10**) col. III, 40°C, t_{ret} (1*S*,2*R*) 4.4 min and t_{ret} (1*R*,2*S*) 4.9 min
(**1a**) acetonide derivative, col. I, 40°C, t_{ret} (*R*) 13.8 min and t_{ret} (*S*) 14.7 min
(**4a**) acetonide derivative, col. I, 35°C, t_{ret} (*R*) 9.8 min and t_{ret} (*S*) 10.3 min
(**7a**) col. II, 90°C, t_{ret} (1*R*,2*S*) 9.1 min and t_{ret} (1*S*,2*R*) 10.1 min
(**10a**) col. I, 80°C, t_{ret} (1*S*,2*S*) 7.5 min and t_{ret} (1*R*,2*R*) 8.0 min

3. Results and discussion

*3.1. Screening for new epoxide hydrolase activities. Biohydrolysis of racemic epoxides **1** and **7***

Our search for microbial sources of EH was carried out using only fungal strains, according to the general observation that incubation of

alkenes with fungi frequently lead to the formation of vicinal diols, whereas with bacteria accumulation of the corresponding epoxide was rather observed. This screening was performed on an analytical scale, using both racemic butyl-oxirane (±)-**1** and *trans*-1-propyl-2-

Table 1
Biohydrolysis of butyl-oxirane **1** and *trans*-1-propyl-2-methyl-oxirane **7**

Entry	Fungus	Substrate					
		(±)-**1**			(±)-**7**		
		Cells[a] g/l	ee% (Absolute configuration)[b]		Cells[a] g/l	ee% (Absolute configuration)[b]	
		(Time, h)	Epoxide	Diol	(Time, h)	Epoxide	Diol
1	*Absidia coerula* LCP 1894	7 (6)	36 (*S*)	37 (*R*)	5 (48)	~ 0	~ 0
2	*Acremonium alternatum* LCP 3010	2 (23)	9 (*S*)	48 (*R*)	12 (48)	~ 0	16 (1*R*,2*S*)
3	*A. candidus*	6 (20)	~ 0	nd[c]		nd	
4	*A. flavus*	5 (20)	5 (*S*)	nd		nd	
5	*A. niger* ATCC 9124	7 (9)	53 (*S*)	52 (*R*)		nd	
6	*A. niger* LCP 166	9 (0.9)	71 (*S*)	53 (*R*)		nd	
7	*A. niger* LCP 167	8 (1.3)	70 (*S*)	53 (*R*)		nd	
8	*A. niger* LCP 518bis	8 (1.5)	69 (*S*)	53 (*R*)		nd	
9	*A. niger* LCP 521	9 (0.5)	69 (*S*)	57 (*R*)	7 (72)	7 (1*R*,2*R*)	26 (1*R*,2*S*)
10	*A. niger* LCP 1965	9 (1.3)	71 (*S*)	55 (*R*)		nd	
11	*A. ochraceus* ATCC 1008	7 (20)	~ 0	nd		nd	
12	*A. oryzae*	3 (23)	~ 0	nd		nd	
13	*A. oryzae* var. *Viridis* IAM 2750	21 (7)	23 (*S*)	62 (*R*)		nd	
14	*A. sojae* IAM 2631	20 (7)	12 (*S*)	55 (*R*)		nd	
15	*Aspergillus* sp.	4 (23)	36 (*S*)	33 (*R*)	7 (48)	2 (1*R*,2*R*)	15 (1*R*,2*S*)
16	*A. terreus*	6 (23)	61 (*S*)	11 (*R*)		nd	
17	*A. terreus* CBS 116-46	7 (8)	30 (*S*)	42 (*R*)	8 (48)	~ 0	53 (1*R*,2*S*)
18	*A. wentii*	9 (24)	10 (*S*)	24 (*R*)	7 (48)	9 (1*R*,2*R*)	30 (1*R*,2*S*)
19	*B. bassiana* ATCC 7159	7 (2)	47 (*S*)	77 (*R*)	7 (72)	31 (1*S*,2*S*)	52 (1*R*,2*S*)
20	*Botrytis cinerea*	5 (4)	50 (*S*)	68 (*R*)	5 (48)	6 (1*S*,2*S*)	33 (1*R*,2*S*)
21	*C. globosum* LCP 679	4 (7)	52 (*S*)	40 (*R*)	7 (48)	47 (1*S*,2*S*)	52 (1*R*,2*S*)
22	*Colletotrichum dermatium*	1 (5)	21 (*S*)	52 (*R*)	1 (48)	~ 0	~ 0
23	*Corynespora casiicola* DSM 62474	7 (8)	57 (*S*)	36 (*R*)	5 (48)	3 (1*S*,2*S*)	28 (1*R*,2*S*)
24	*Cor. casiicola* DSM 62475	8 (5)	36 (*S*)	28 (*R*)		nd	
25	*Cun. echinulata* LCP 1901	7 (8)	53 (*S*)	19 (*R*)	7 (48)	9 (1*S*,2*S*)	6 (1*S*,2*R*)
26	*Cun. echinulata* NRRL 3655	8 (6)	49 (*S*)	17 (*R*)		nd	
27	*Cun. elegans* LCP 1543	6 (11)	41 (*S*)	28 (*R*)	7 (48)	11 (1*S*,2*S*)	6 (1*S*,2*R*)
28	*Cun. verticillata* VKM F 430	5 (8)	24 (*S*)	35 (*R*)		nd	
29	*Curvularia lunata* NRRL 2380	7 (5)	61 (*S*)	46 (*R*)	8 (48)	5 (1*R*,2*R*)	42 (1*S*,2*R*)
30	*Diplodia gossypina* ATCC 10936	7 (23)	44 (*S*)	40 (*R*)	12 (48)	~ 0	18 (1*S*,2*R*)
31	*Dreschlera sorokiniana* ATCC 16092	8 (23)	10 (*S*)	nd	10 (48)	~ 0	27 (1*S*,2*R*)
32	*Fusarium solani* sp. *pisi* MUCL 906	4 (12)	35 (*S*)	70 (*R*)	8 (48)	2 (1*R*,2*R*)	31 (1*S*,2*R*)
33	*Geotrichum candidum*	6 (23)	~ 0	nd		nd	
34	*M. isabellina* ATCC 42613	6 (23)	11 (*S*)	60 (*R*)	8 (10)	36 (1*R*,2*R*)	76 (1*R*,2*S*)
35	*Mucor plumbeus* CBS 110-16	8 (10)	31 (*S*)	36 (*R*)	7 (48)	9 (1*R*,2*R*)	39 (1*S*,2*R*)
36	*Pelliculosa filamentosa* IFO 6254	4 (7)	31 (*S*)	31 (*R*)		nd	
37	*Penicilium lilacinum* ATCC 10114	7 (10)	9 (*S*)	36 (*R*)	14 (48)	2 (1*R*,2*R*)	19 (1*S*,2*R*)
38	*Penicilium simplicissimum* VKM F 16	8 (23)	8 (*S*)	nd		nd	
39	*Rhizopus arrhizus* ATCC 11145	6 (5)	30 (*S*)	54 (*R*)	6 (48)	18 (1*R*,2*R*)	13 (1*S*,2*R*)
40	*Scopulariopsis brevicaulis* VKM F 406	14 (3)	77 (*S*)	27 (*R*)	8 (24)	32 (1*R*,2*R*)	27 (1*S*,2*R*)
41	*Stachybotrys atra*	5 (23)	~ 0	nd		nd	
42	*S. racemosum* MUCL 28766	7 (6)	48 (*S*)	44 (*R*)	8 (27)	42 (1*R*,2*R*)	24 (1*S*,2*R*)

[a] Dry weight.
[b] The absolute configuration was assigned via chiral GC analysis by comparison with authentic samples.
[c] nd: Not determined.

methyl oxirane (±)-**7** as substrate, on a selection of 42 different strains. Each fungus was routinely grown in Erlenmeyer flasks on a complex medium containing glucose and corn steep liquor. Racemic substrates were incubated using a resting cell suspension in a sodium phosphate buffer (pH 8.0, 0.1 M) at 27°C. The reactions were monitored by periodical analysis of the head-space phase using a chiral GC column allowing to determine the ee of the remaining epoxide. After some degree of conversion (within a maximal period of 72 h) the diol formed, as well as the remaining epoxide, were isolated and analyzed for ee and absolute configuration. The results obtained are described in Table 1 and indicate the following.

(a) Most of the fungi (37 out of 42) were able to hydrolyse epoxide (±)-**1** and, in all cases, this hydrolysis was enantioselective since the remaining epoxide showed an ee ≠ 0.

(b) All the fungi (23 out of the 42) tested with *trans*-disubstituted epoxide (±)-**7** led to hydrolysis of this substrate, but only 12 among them led to a residual epoxide with an ee ≥ 5%. In general, the reaction rate was significantly lower for this substrate than for the terminal epoxide (±)-**1**.

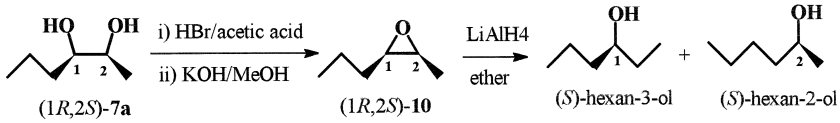
(c) Despite the diversity of the 42 fungi screened, biohydrolysis of (±)-**1** led in all cases to the same residual epoxide of (*S*) absolute configuration and to the (*R*)-**1a** diol. Interestingly, such a behaviour did not occur for *trans*-epoxide (±)-**7**, where the four possible stereochemical outcomes were observed. Indeed we observed the formation of either (1*R*,2*S*) or (1*S*,2*R*) diols **7a**, which resulted from the preferential hydrolysis of either (1*R*,2*R*) or (1*S*,2*S*)-**7** enantiomer.

Out of this screening, we selected seven fungi according to two criteria: (i) the possibility of gaining high ee for the remaining epoxide and/or for the formed diol (ii) the possibility to obtain either enantiomer of **7** and **7a**, i.e., to select enantiocomplementary biocatalysts. These seven strains are all available from the following culture collections: *A. niger* LCP 521, *A. terreus* CBS 116-46, *B. bassiana* ATCC 7159 *C. globosum* LCP 679, *Cunninghamella elegans* LCP 1543, *M. isabellina* ATCC 42613, *Syncephalastrum racemosum* MUCL 28766. We have previously described various results obtained with two of these fungi (i.e., *A. niger* and *B. bassiana*) [11]. Studies using these strains were achieved using epoxides **2**–**6** and **8**–**12** as substrates.

3.2. Determination of the absolute configuration of the remaining epoxide

The absolute configuration of the remaining epoxides **1**–**12**, as well as of diols **1a**–**12a**, were assigned via chiral GC analysis by comparison with reference material. The synthesis and the absolute configuration of these references have been previously described [11,18], except for epoxides **1**, **4**, **7**, **10** and their corresponding diols. Two different strategies were used to prepare these optically enriched samples and to elucidate their absolute configuration.

(a) Optically active diols **1a** (ee 59%) and **4a** (ee 76%) were respectively prepared by bioconversion of *rac*-**1** with *B. bassiana* and by asymmetric dihydroxylation of 2-methyl-pentene using AD-mix-β according to Sharpless et al. [20]. Their (*R*) absolute configuration was assigned by comparison of their optical rotation with previously described data [19,21]. These diols were further cyclised—by tosylation followed by reaction with sodium hydride—to afford the reference epoxides (*R*)-**1** and (*R*)-**4**

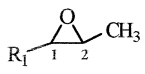
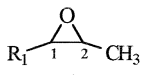


Scheme 2.

which were thus obtained with an ee of 46% and 75%, respectively.

(b) Optically active diol **7a** (ee 71%) obtained by biohydrolysis of rac-**7** with *M. isabellina* was cyclised back into the corresponding *cis* epoxide **10** according to Golding et al. [17]. $LiAlH_4$ reduction of this epoxide led to a mixture of hexan-2-ol and 3-ol (Scheme 2). The

Table 2
Biohydrolysis of racemic epoxides **1–12**

				Absolute configuration of the residual epoxide / the formed diol						
	Entry	R_1	Epox. / Diol	***A.n.***	***A.t.***	***B.b.***	***C.g.***	***C.e.***	***M.i.***	***S.r.***
	*1*	butyl	**1** / **1a**	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*
	2	hexyl	**2** / **2a**	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*	*S* / *R*
	3	phenyl	**3** / **3a**	*S* / *R*	*R* / *R*	*R* / *R*	*S* / *R*	*S* / *R*	*R* / *R*	*S* / *S*
	*4*	propyl	**4** / **4a**	*S* / *R*	nd	*S* / *R*	*R* / *S*	*R* / *S*	*S* / *R*	*R* / *S*
	5	pentyl	**5** / **5a**	*S* / *R*	*R* / *S*	*S* / *R*	*R* / *S*	ne	*R* / *S*	*R* / *S*
	6	phenyl	**6** / **6a**	*S* / *R*	*R* / *S*	*S* / *R*	*R* / *S*	ne	*S* / *R*	ne
 *trans*	*7*	propyl	**7** / **7a**	1*R*,2*R* / 1*R*,2*S*	–	1*S*,2*S* / 1*R*,2*S*	1*S*,2*S* / 1*R*,2*S*	1*S*,2*S* / 1*S*,2*R*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*S*,2*R*
	8	pentyl	**8** / **8a**	1*R*,2*R* / 1*R*,2*S*	1*S*,2*S* / 1*R*,2*S*	1*S*,2*S* / 1*R*,2*S*	1*S*,2*S* / 1*R*,2*S*	1*S*,2*S* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*
	9	phenyl	**9** / **9a**	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*	1*R*,2*R* / 1*R*,2*S*
 *cis*	*10*	propyl	**10** / **10a**	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*
	11	pentyl	**11** / **11a**	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*
	12	phenyl	**12** / **12a**	1*S*,2*R* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*R*,2*S* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*	1*S*,2*R* / 1*R*,2*R*

nd: Not determined.
ne: ee epoxide $< 3\%$.
–: Reaction too slow for measuring the formation of diol.
A.n.: *A. niger*.
A.t.: *A. terreus*.
B.b.: *B. bassiana*.
C.g.: *C. globosum*.
C.e.: *Cun. elegans*.
M.i.: *M. isabellina*.
S.r.: *S. racemosum*.

absolute configuration of the alkan-2-ol thus obtained was determined as being (2*S*) by chiral GC analysis via co-injection with an optically pure commercially available reference. Owing to the facts (i) that the absolute configuration at C(2) stayed unchanged along these reactions and (ii) that the relative configuration of epoxide **10**/diol **7a** were *cis*/*erythro*, it can be deduced that their absolute configuration must be (1*R*,2*S*). An identical approach was used to assign the absolute configuration of epoxide **7** and diol **10a**. An optically active (ee 94%) sample of **7** was synthesized via (i) asymmetric dihydroxylation of *trans*-2-hexene using AD-mix-β which, according to Sharpless et al. [20], affords *threo*-1-propyl-2-methyl-ethanediol **10a** (ee 95%), and (ii) cyclisation to **7** (ee 95%). The (1*R*,2*R*) configuration of these products was elicited (by comparison with an authentic sample of optically pure commercially available hexan-2-ol) after $LiAlH_4$ reduction as described above.

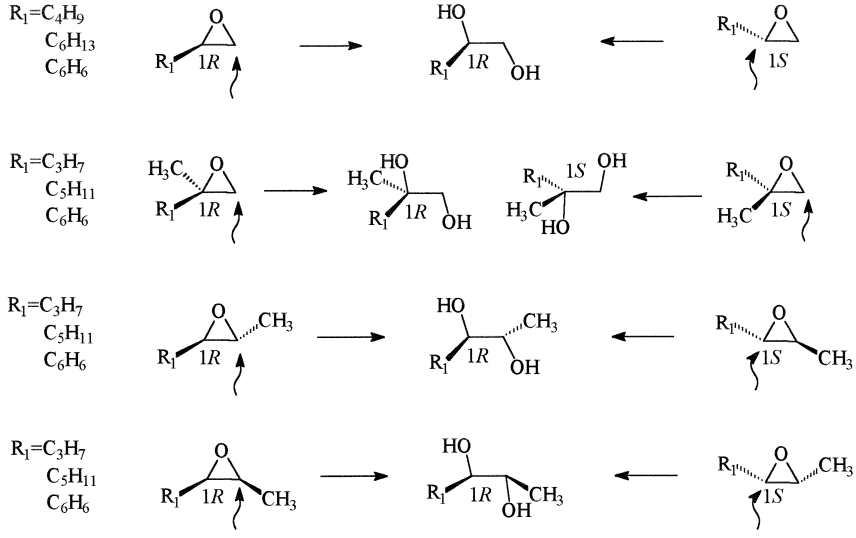
3.3. Biohydrolysis of racemic epoxides *1*–*12*

Biohydrolysis of epoxides **2**–**6** and **8**–**12** was carried out in a way similar to the one used for achieving the screening with epoxides **1** and **7**. Only the ee of the remaining epoxides and of the formed diol were determined, together with their absolute configuration. The conversion ratio *c* was not determined since, in the case of biotransformations conducted using whole-cells, this is very difficult because of the heterogeneity of the medium [23]. Consequently, as mentioned previously, the determination of the *E* values of these biohydrolyses was not possible with sufficient accuracy at that stage [11].

The results obtained, summarised in Table 2, show the following trends.

(a) Whatever the strain used, biohydrolysis of 1,2-disubstituted epoxides **7**–**12** led in all cases to an inversion of configuration at the stereogenic center attacked, which confirms that the reaction occurred via a *trans* opening process. This fact is in line with the mechanistic scheme presently accepted for mammalian EHs [24].

(b) The enantioselectivity was found to depend upon the substrate structure as well as upon the strain used. (see for example, entries 2 and 3, or entries 8 and 9). Thus, for a same fungus, changes in substrate structure may induce changes in enantioselectivity (see for example, entries 7–12 for *C. globosum*). Conse-



Scheme 3.

quently, no general rule could be drawn which would allow to make any prediction for a specific substrate/fungus couple.

(c) The monosubstituted and 1,2-disubstituted epoxides led, in almost all cases, to preferential formation of the (1*R*) diol (59 cases out of 62 reactions, see entries 1–3 and 7–12). Thus it appeared that (i) when the (1*R*) epoxide was the preferred substrate, water incorporation occurred preferentially at C(2), which led to retention of configuration at C(1) and thus to formation of the (1*R*) diol; (ii) when the (1*S*) epoxide was the preferred substrate, water incorporation occurred preferentially at C(1), thus leading again to formation of the diol of (1*R*) absolute configuration, due to inversion of configuration at that carbon atom (Scheme 3).

(d) On the contrary, for the 1,1-disubstituted epoxides **4**–**6**, water incorporation on the faster epoxide enantiomer always occurred preferentially at the less hindered terminal carbon atom C(2), independently of the enantioselectivity (Scheme 3).

4. Conclusion

The results obtained in the course of this study indicate that epoxide hydrolases were found in several different fungal strains and these microorganisms may therefore be considered as highly interesting sources for such new enzymatic activities. Seven strains were selected from a screening performed on 42 fungi, and were further used to study the biohydrolyses of various racemic alkyl or aryl monosubstituted, as well as of *gem*-, *trans*- or *cis*-disubstituted epoxides. The results we have obtained indicate that the enantio- and regio-selectivity of these hydrolyses were variable depending on the fungus and on the substrate structure. Nevertheless, a general trend could be drawn from these results. Thus, as an almost general rule, biohydrolysis of monosubstituted, *trans*- or *cis*-disubstituted epoxides led to the preferential formation of the diol bearing the 'bulkier' substituent on a carbon atom of (*R*) absolute configuration, whatever the enantioselectivity was. Thus, enantio- and regio-selectivity of the biohydrolysis of these epoxides appear to be strongly related. This was, however, not true for the 1,1-disubstituted substrates, where the preferential attack on the faster enantiomer always occurred at the less substituted carbon atom, independently of the substrate absolute configuration.

As an overall result, one can conclude that the fungi we have selected and studied in the course of this work appear to possess complementary epoxide hydrolases activities and, using these new biocatalysts, it should therefore be possible to achieve, at a preparative scale, the resolution of various different substrates. Such results will be described elsewhere [18].

Acknowledgements

This work is part of the PhD thesis presented by P. Moussou (March 97, University of Aix-Marseille II, France). Financial support by the Société Rhône-Poulenc (BIOAVENIR program —including the PhD stipend to P.M.) is greatly acknowledged.

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