Pergamon

Chemosphere, Vol. 38, No. 10, pp. 2237-2242, 1999

0045-6535/99/ $ - see front matter

PII: S0045-6535(98)00442-1

FIRST EVALUATION OF THE BRAZILIAN MICROORGANISMS BIOCATALYTIC POTENTIAL

J. R. Cagnon, A. L. M. Porto and A. J. Marsaioli[1]*
Instituto de Química, UNICAMP, Campinas-SP, Brazil, CP. 6154, CEP: 13083-970. Fax: + 55 19 7883023

G. P. Manfio and S. Y. Eguchi
Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas-SP, Brazil.

(Received in Germany 19 May 1998; accepted 28 August 1998)

ABSTRACT

The biocatalytic potential of two novel Brazilian strains of *Aspergillus niger* and *Rhodotorula glutinis*, revealed enantioselective epoxide hydrolase activity in the asymmetrization of meso-epoxide and monosubstituted epoxides respectively. These two types of oxirane derivatives are not usually good substrates for biocatalytic enantioselective conversion. © 1999 Elsevier Science Ltd. All rights reserved

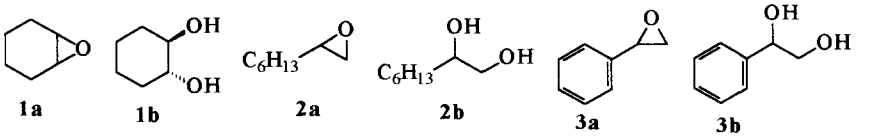
INTRODUCTION

A widespread strategy for the production of homochiral molecules of interest in biotechnological projects combines chemical synthesis and microbiologically mediated transformations [1]. Appreciation of the literature has pointed toward significant research characteristics that we can summarize as follows: there is a wide range of available substrates but each group of research has access or interest in a limited amount of microorganisms and reactions. It is thus evident that searching for new elicit reactions by eliciting specific enzymes and screening the potentiality of a microorganism library would certainly add information to this flourishing field [2]. Conscious of the great biodiversity of the Brazilian microorganisms we have first focused our efforts in evaluating the biocatalytic potential of the available microorganisms at the Fundação Tropical André Tosello, many of which have been isolated from Brazilian environments. Among the multiple reactions one can access using microorganisms we were particularly attracted to the epoxide hydrolyses mainly due the potentiality of hydrolytic enzymes which are promising as enantioselective biocatalysts independent of cofactors [3]. These enzymes have been found in plants [4], insects [5], bacteria [6] , filamentous fungi [7] and mammalian cells [8] and a good review has been recently reported by Archelas and Furstoss [9] one of the leading research group on the subject.

RESULTS AND DISCUSSION

We have envisaged the screening of epoxide hydrolases activity in yeasts, bacteria and fungi searching for enzymes presenting enantioselectivity towards *meso* and monosubstituted epoxides. Our

choice relied on the fact that *meso*-epoxides are normally considered nonsubstrates when epoxide hydrolases from microorganisms are concerned [9] and the great interest in this type of reactions is related to the asymmetrization that in this particular case would produce a *trans*-diol in a 100% theoretical yield. Our second topic of interest, was motivated by the lack of a good enantioselective epoxide hydrolase activity in fungal and bacterial enzymes for monosubstituted epoxides possessing alkyl chain longer than four carbon atoms, that would allow the kinetic resolution of racemic monosubstituted epoxides [10]. Bearing this in mind we have looked for appropriate substrates and the choice of **1a**, **2a** and **3a** (Figure 1) took into consideration some chemical aspects: Compound **1a** is a *meso*-epoxide normally asymmetrized by mammalian enzymes and only one microorganism, a particular strain of the yeast *Rhodotorula glutinis* had the suitable epoxide hydrolase to transform this substrate into **1b** with high enantiomeric excess [11]. The alkyl oxirane **2a** is a monosubstituted epoxide possessing an alkyl group of 6 carbon atoms and till the present moment no suitable biocatalyst was found for its kinetic resolution thus constituting a good substrate to monitor unusual enzyme activity in our screening purpose. Aryl oxirane **3a** represents an alternative monosubstituted epoxide with a 6 carbon atom side chain possessing a different stereoelectronic demand. Finally when looking for new enzymatic activity in microorganism cells one has to be careful about the absolute configuration of the stereogenic elements (stereogenic center in the present compounds) in both the product and substrate [6]. Thus choosing the above mentioned compounds possessing known absolute configurations (all compounds but for **1a**) would greatly reduce our task of determining the enantioselectivity of the epoxide hydrolases producing the diols and the remaining epoxides.



1a **1b** **2a** **2b** **3a** **3b**

Figure 1.: - Oxirane derivatives used as substrates for epoxide hydrolases of *Aspergillus niger* CCT 4946, CCT3086, CCT2760, *Rhodotorula glutinis* CCT 2182, and *Corynebacterium aquaticum* CCT 3023.

The choice of three different strains of *Aspergillus niger* (fungus) , *Rhodotorula glutinis* (yeast) and *Corynebacterium aquaticum* (bacterium) among all the microoganisms available at the "Fundação Tropical André Tosello" was primarily based on the literature data and the species and genus showing more potentiality towards the targeted biocatalysts (epoxide-hydrolases). It should be mentioned that we were particularly attracted to *Aspergillus niger* CCT 4846, and *Rhodotorula glutinis* CCT 2182 because they were isolated from the Atlantic rain forest (Peruíbe, S.Paulo, Brazil) and from a Guava tree. Table 1 depicts our results and interestingly *A. niger* CCT 4846 produced (1*R*,2*R*)-*trans*-diol **1b** with an enantioselectivity (e.e. 70 %) usually assigned to mammalian and not to fungal enzymes [9,12]. This is the first epoxide hydrolase from a fungal source with a biocatalytic potential worth investigating.

Asymmetrization of **1a** was performed by Weijers in 90 % e.e. [12] using cells of *Rhodotorula glutinis* but the strain used in our laboratory did not reproduce the above encouraging results. We will therefore channel our future efforts to a better evaluation of the *Aspergillus niger* CCT 4846 epoxide hydrolase potentiality.

Concerning the resolution of monosubstituted oxirane we had negligible to low selectivity using substrates **2a** and **3a** and the three *Aspergillus niger* strains and *Corynebacterium aquaticum* cells. On the other hand, the resolution of **2a** with *R. glutinis* produced (2*R*)-1,2-octanediol **2b** in 145 min with 80% of enantiomeric excess and 48% of conversion. The retrieved epoxide showed an enantiomeric excess of 69% thus the calculated selectivity was E = 19. Latest reports on this subject reveal that one of the best results is the hydrolysis of monosubstituted epoxide possessing 2 to 4 carbons with *Rhodotorula glutinis* cells [11] as biocatalyst, notwithstanding the selectivity is poor and increasing the carbon number of the alkyl chain reduced the enantioselectivity. Bacterial epoxide hydrolases were investigated by Faber *et al.* [10] using **2a** as substrate and the best results were obtained with *Nocardia* (diol 2b, 54% e.e. and epoxide 2a, 54 % e.e. and low selectivity). Thus we believe that our results are so far the best for the enantioselective hydrolysis of **2a.** Compound **3b** was a not produced enantioselectively and a search in the literature has pointed to the fact that **3a** as well as other styrene derivatives are prone to undergo spontaneous hydrolyses [11].

Entry	Biocatalyst	Substrate		Products			
		epoxide	Conv. (%)	Diol	e.e (%)	epoxide	e.e.(%)
			Fungi				
1	*Aspergillus niger* CCT 4846	**1a**	>98	(1*R*,2*R*)-**1b**	70	*meso*	
2	*Aspergillus niger* CCT 3086	**1a**	>98	(±)-**1b**	00	*meso*	
3	*Aspergillus niger* CCT 2760	**1a**	>98	(±)-**1b**	00	*meso*	--------
4	*Aspergillus niger* CCT 4846	(±) **2a**	36	(2*R*)-**2b**	56	(2S)-**2a**	29
5	*Aspergillus niger* CCT 3086	(±) **2a**	40	(2*R*)-**2b**	45	(2S)-**2a**	60
6	*Aspergillus niger* CCT 2760	(±) **2a**	15	(2*R*)-**2b**	27	(2S)-**2a**	26
7	*Aspergillus niger* CCT 4846	(±)**3a**	>98	(±)-**3b**	00	--------	--------
8	*Aspergillus niger* CCT 3086	(±) **3a**	>98	(±)-**3b**	00	--------	--------
			Yeast				
9	*Rhodotorula glutinis* CCT 2182	**1a**	23*	(1*R*,2*R*)-**1b**	44	*meso*	--------
10	*Rhodotorula glutinis* CCT 2182	(±) **2a**	48*	(2*R*)-**2b**	80	(2S)-**2a**	69
11	*Rhodotorula glutinis* CCT 2182	(±) **3a**	>98	(±)-**3b**	00	---------	--------
			Bacterium				
12	*C. aquaticum* CCT 3023	**1a**	>98	(±)-**1b**	00	*meso*	--------
14	*C. aquaticum* CCT 3023	(±) **3a**	00	----------	--------	----------	--------

Entry 1: **1b** $[\alpha]_D^{20}$ −12.3° (c 2.0, $CHCl_3$), Lit.[14] (1*R*,2*R*)-**1b** $[\alpha]_D^{20}$ -39° (c 1.6, H_2O); Entry 10: **2b** $[\alpha]_D^{20}$ +6.6° (c 3.2, EtOH) Lit.[15] (2*R*)-**2b** $[\alpha]_D^{20}$ +6,78° (c 5.0, EtOH) e.e. 46%. * Isolated yield.

Table 1: - Biocatalytic Hydrolysis of 1a, 2a and 3a using *Rhodotorula glutinis*, *Corynebacterium aquaticum* and three strains of *Aspergillus niger*

The diols **1b**, **2b** and **3b** produced biosynthetically were identified by coinjection with synthetic standards obtained by chemical hydrolyses of the corresponding epoxides (**1a**, **2a** and **3a**) and by GC/MS It should be mentioned that the racemic synthetic standards were fully characterized by spectrometric data comparison (MS, IR, ^1H NMR and ^{13}C NMR with those available in the literature [13]). The absolute

configurations of **1b** [14], **2a** [15] and **2b** [15] are known, thus a simple comparison of the specific optical rotations of the enriched enantiomeric mixtures with the reported values for the pure enantiomers allowed the determination of the absolute configurations of the predominant enantiomer in the mixtures.

CONCLUSION

This paper reports the discovery of novel enzymatic properties in microorganisms isolated from exotic environments, one of our major purposes when this research group was established. We were successful in our first screening work demonstrating that there is an unusual enantioselective fungal epoxide hydrolase activity on cyclohexene oxide by cells of *Aspergillus niger* CCT 4846. This microorganism was isolated from the Atlantic Rain Forest one of the world's premier tropical rainforests. Additionally an enantioselective epoxide hydrolase for monosubstituted epoxide was detected in a new strain of *Rhodotorula glutinis* isolated from a *Guava* tree [18].

EXPERIMENTAL SECTION

GENERAL

The reactions were monitored by silica gel TLC (Aluminum foils, 60-F_{254}-Merck) and the visualization was obtained by spraying with *p*-anisaldehyde / sulphuric acid followed by heating at about 120^0 C with a hot gun. Flash column chromatography were performed with Merck 60 (230-400 mesh) using N_2 gas for pressure. Optical rotation values were measured with a Polamat A polarimeter . The reported data (see table 1) refer to the Na-line value using a 1 dm cuvette. Enzymatic reactions were monitored by GC(FID) HP-5890 chromatograph, using hydrogen as carrier gas and or GC/MS -analyses using a HP-5890/5970 system using helium as carrier gas and for the latter the MS were taken at 70 eV. Scanning speed was 0.84 scan/s from m/z 40 to 550. The capillary columns used were either 1) J&W Scientific DB-5 fused silica capillary column (25m x 0.2mm x 0.33 μm) or 2) Chrompack chiral column CP-chirasil-Dex CB (25m x 0.25 mm x 0.25 μm).

SYNTHESIS OF THE STANDARDS

(±)-cyclohexene oxide 1a, (±)-1,2-epoxyoctane 2a, (±)-styrene oxide 3a: were obtained from the commercial olefins (Aldrich) following standard procedure (m-CPBA/CH_2Cl_2, $NaHCO_3$, 0^0 C) The epoxides **1a**, **2a** and **3a** were purified by flash silicagel column chromatography using hexane and hexane:ethyl acetate (9:1) as eluent and their spectroscopic data compared to those in the literature[12]. Column 2) **1a**: temperature program 60°C iso, pressure at the head of the column 0.6 bar Rt (*meso*)-**1a** = 12.2 min.; **2a**: temperature program : 70° C iso, pressure at the head of the column 0.6 bar, Rt (1*S*,2*S*)-**2a** = 14.6 min., (1*R*,2*R*)-**2a** = 15.0 min; **3a**: temperature program :70° C iso, pressure at the head of the column 0,6 bar, Rt (*S*)-**3a** = 6.4 min, (*R*)-**3a** = 6.9 min.

(±)-*trans*-1,2-cyclohexanediol 1b, (±)-1,2-octanediol 2b, (±)-1,2-styrenediol 3b: were obtained from the the corresponding epoxides by acid catalysed hydrolysis. The diol **1b** was purified by crystallization

in hexane, diol **2b** was purified by silicagel flash column chromatography (hexane : diethyl acetate, 3:1 to 1:1). Their spectroscopic data were compared with those in the literature[13]. Column 2) **1b**: temperature program :130° C iso, pressure at the head of the column 0,6 bar, Rt (1*S*,2*S*)-**1b** = 9.0 min, (1*R*,2*R*)-**1b** = 9.8 min.; **2b**: temperature program: 130°C iso, pressure at the head of the column: 0.6 bar , Rt (2*S*)-**2b** = 14.6 min, (2*R*)-**2b** 15.2 min.; **3b**: temperature program : 150° C iso, pressure at the head of the column 0.6 bar, Rt: (1*S*)-**3b** = 12.4 min, (1*R*)-**3b** = 13.1 min

GROWTH CONDITIONS FOR MICROORGANISM CULTURES

The microorganisms *Rhodotorula glutinis* CCT 2182, *Aspergillus niger* CCT 4846, *A. niger* CCT 3086, and *A. niger* CCT 2760 and *Corynebacterium aquaticum* CCT 3023 were obtained from the Culture Collection, Fundação de Pesquisa e Tecnologia André Tosello (Brazil). Each microorganism was grown at 30° C in culture shaker-flasks in an appropriate medium as follows:*R. glutinis* CCT (48 h) - yeast extract (3g/L), malt extract (3g/L), glucose (10g/L), peptone (5g/L); *A.niger* CCT (60 h) - malt extract (8g/L); *C. aquaticum* CCT (16 h) - peptone (5g/L), bacto-beef extract (3g/L). The bacteria and yeast cells were harvested by centrifugation (3000 rpm) and washed twice with potassium phosphate buffer pH 7.0 (0.07M). The fungi cells were harvested by filtration and washed with potassium phosphate buffer.

General procedure for the asymmetric hydrolysis of epoxides

Hydrolysis of the epoxide was performed in 100 mL bottles on rotary shaker (140 rpm). To the bottles containing phosphate buffer pH 7.0 (20 mL, 0.07M) and washed cells (2.0 to 4.5 g wet weight),the epoxide was added (20 to 25 mg). The mixture was shaken at 30°C and the reaction was monitored by chiral GC. Upon reaching the appropriate conversion degree, the cells were centrifuged, except for the fungi cells which were filtrated, the formed diol and remaining epoxide were extracted from the supernatants (previously saturated with NaCl) with ethyl acetate and dried. Larger scale hydrolysis (100 mg of substrate) was performed in 1 L bottles containing 200 ml of phosphate buffer pH 7.0 and washed cells (8.5 to 10.0 g wet weight).

e.e. - determination:- Enantiomeric excesses were determined by GC (HP GC-5890 equipped with FID) and the results are depicted on Table 1. The conversions were determined using guaiazulene as an internal standard and correction factors were used to calculate the percent composition taking into consideration the specific detector response [16]. In some cases we report the isolated yield values. E (selectivity) value was calculated following Sih equation [17].

ACKNOWLEDGMENTS

The authors are indebted to FAPESP (Fundação de Amparo a Pesquisa do Estado de S.Paulo for finantial support that allowed the establishment of our biocatalyses research laboratory in 1997. J.R. Cagnon is thankful to FAPESP for a post doctoral fellowship and A. L M. Porto is thankful to CNPq for scholarship. We acknowledge Prof. J. Jones from UFRJ, Brazil for the gift of starting material.

REFERENCES

1. For a textbook see: Faber, K. *Biotransformations in Organic Synthesis*; 2nd ed., Springer: Heidelberg, 91995).
2. Azerad, R. *Bull. Soc. Chim. Fr.*, **132**, 17-51 (1995).
3. Furstoss, R.; Pedragosa-Moreau, S.; Archelas *Bull. Soc. Chim. Fr.*, **132**, 769-800 (1995).
4. Blee, E.; Schuber, F. *Eur. J. Biochem.*, **230**, 229-234 (1995).
5. Linderman, R. J.; Walker, E. A.; Haney, C.; Roe, R. M. *Tetrahedron*, **51**, 10845-10856 (1995).
6. Faber. K.; Mischitz, M.; Kroutil, W. *Acta Chem. Scand.*, **50**, 249-259 (1996).
7. Morisseau, C.; Nellaiah, H.; Archelas, A.; Furstoss, R.; Baratti, J. C. *Enzyme Microb. Technol.*, **20**, 446-452 (1997).
8. Wixtrom, R. N.; Van Pee, K. H. Membrane-bound and soluble fraction epoxide hydrolases: methodological aspects. In: *Biochemical Pharmacology and Toxicology*, (Zakim, D. and Vessey, D. A. eds.) Wiley, NY, 1:1-93 (1985).
9. Archelas, A.; Furstoss, R. *Annu. Rev. Microbiol.*, **51**, 491-525 (1997).
10. Osprian, I.; Kroutil, M.; Faber, K. *Tetrahedron: Asymmetry*, **8**, 65-71 (1997).
11. Weijers, C. A. G. M. *Tetrahedron: Asymmetry*, **8**, 639-647 (1997).
12. a) Bellucci, G.; Chiappe, C.; Cordoni A.; Marioni, F. *Tetrahedron Lett.*, **35**, 4219-4222 (1994).; b) Bellucci, G.; Chiappe, C.; Ingrosso, G.; Rosini, C. *Tetrahedron: Asymmetry*, **6**, 1911-1918 (1995).
13. The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra and The Aldrich Library of FT-IR Spectra
14. Commercially available compounds in the enantiomerically pure form (Aldrich and Fluka).
15. Mischitz, M, Kroutil, W. Wandel, U. and Faber, K. *Tetrahedron: Asymmetry*, **6**, 1261-1272 (1995).
16. McNair, H.M.; Bonelli, E.J. *Basic Gas Chromatography* 5nd ed., Varian Aerograph: Berkeley, 1969.
17. Chen, C-S.; Fugimoto, Y.; Girdaukas G.; Sih, C.J. *J. Am. Chem. Soc.*, **104**, 7294-7299 (1982).
18. While in press the following articles reporting epoxide hydrolases with good enantioselectivity for **2a** were published. a) Weijers, C.A. G.M.; Botes, A.L.; van Dyk, M.S.; Bont, J.A.M. . *Tetrahedron: Asymmetry*, **9**, 467-473 (1998). b) Moussou, P.; Archelas, A.; Furstoss, R. . *Tetrahedron*, **54**, 1563-1572 (1998).