Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Enantioselective Hydrolysis of o-Nitrostyrene Oxide by Whole Cells of Aspergillus niger CGMCC 0496

Hao JIN[a] & Zu-yi LI[a]
[a] Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences 354 Fenglin Lu, Shanghai 200032, China
Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **66** (5), 1123–1125, 2002

Note

Enantioselective Hydrolysis of *o*-Nitrostyrene Oxide by Whole Cells of *Aspergillus niger* CGMCC 0496

Hao JIN and Zu-yi LI†

Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Lu, Shanghai 200032, China

Received October 22, 2001; Accepted January 16, 2002

The asymmetric biohydrolysis is described of *o*-nitrostyrene oxide with high selectivity by whole cells of *Aspergillus niger* CGMCC 0496. Both the epoxide and diol could be obtained in a high state of optical purity (over 98%). Product inhibition was found when using a high ratio of substrate to cells.

Key words: enantioselective hydrolysis; epoxide hydrolase; *Aspergillus niger*; *o*-nitrostyrene oxide

Chiral epoxides and their corresponding vicinal diols are important intermediates in the pharmaceutical and agrochemical industries. Several methodologies have been explored in order to prepare these chiral compounds such as using the salen manganese[1] direct oxidation of olefinic compound into a chiral epoxide. Some cobalt-salen catalysts[2] have also been found active in the hydrolytic kinetic resolution of racemic epoxides.

Apart from using complex metal catalysts, there are also some biocatalytic methods to produce these chiral compounds; for example, alkene epoxidation by monooxygenases[3] and chloroperoxidases.[4] One of the most promising biocatalytic ways for the synthesis of such chirons is by using epoxide hydrolases.

Epoxide hydrolases (EHs) are ubiquitous enzymes which can hydrolyze an epoxide to its corresponding vicinal diol with good to excellent enantioselectivity. EHs can be found from various sources such as mammals,[5] plants,[6] insects[7] and various microorganisms.[8–10] Most EHs studied have been of mammalian origin due to their important role in the detoxification process of xenobiotics and especially aromatic systems.[11] However, their application to asymmetric synthesis is severely hampered by their low availability.

In contrast, many EHs of microbial origin have recently been identified.[12,13] They seem to be one of the most promising tools for asymmetric synthesis, especially when using whole cells from a microbial culture, since these organisms can be incubated on a large scale. In this study, we investigated the possibility to achieve enantioselective hydrolysis of *o*-nitrostyrene oxide with whole cells of *A. niger* **CGMCC** 0496. Strain **CGMCC** 0496 of *A. niger* is available from *China General Microbiological Culture Collection Center* (Institute of Microbiology, the Chinese Academy of Sciences, Beijing, China) and was formerly named *A. niger* **5450**.[14]

o-Nitrostyrene oxide and its corresponding diol are useful intermediates for the production of pharmaceutical[15] and agricultural[16] products. They have recently been used to prepare photosensitive derivatives of biologically active compounds which can be studied in a complex system such as a living cell,[17] but the use of their optical form has been seriously hampered because no efficient way has existed to obtain them with high enantiomeric excess. The best results obtained have been a 46% yield with 89%ee[18] for chiral *o*-nitrostyrene and a 37% yield with 47% ee for the corresponding diol[19] in previous reports. In the course of our work, the kinetic resolution of *o*-nitrostyrene oxide was studied as shown in Fig. 1 by using EHs in the cells of *A. niger* **CGMCC** 0496.

The strain of *A. niger* **CGMCC** 0496 was maintained on an agar slant which contained K_2HPO_4 (1.0 g/l), KCl (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $FeSO_4 \cdot 7H_2O$ (0.01 g/l), fructose (10 g/l), corn steep (15 g/l), and agar (3 g/l) at pH 6.8–7.0. It was cultivated on a medium[20] of K_2HPO_4 (1.0 g/l), KCl (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $FeSO_4 \cdot 7H_2O$ (0.01 g/l), fructose (10 g/l), corn steep liquor (15 g/l) and tap water, at pH 6.8–7.0. Large-scale growth of the cultures was achieved by a two-stage process. The medium 100 ml in a 500-ml flask was first inoculated and then cultured for 20 h. In the second stage,

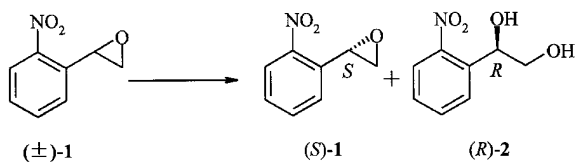

Fig. 1. Enantioselective Hydrolysis of *o*-Nitrostyrene Oxide by Whole Cells of *Aspergillus niger* **CGMCC** 0496.

† To whom Correspondence should be addressed. Phone: 86-21-64163300-1406; FAX: 86-21-64166128; E-mail: lizy@pub.sioc.ac.cn

Table 1. Kinetic Resolution of *O*-Nitrostyrene Oxide by *A. niger* **CGMCC** 0496 under Various Conditions

Reaction	Reaction time (min)	Weight of substrate (mg)	Weight of biomass[a] (g)	Yield of epoxide[b] (%)	E.e. of epoxide[c] (%)	Yield of diol[b] (%)	E.e. of diol (%)[d]
I[e]	55	100	20	33	85	42	—
II	68	100	20	34	98	38	>99
III	88	100	10	50	59	40	95
IV	360	100	10	45	74	41	96
V	170	200	10	52	58	42	96
VI	68	100	20	42	84	—	—

[a] In all cases, wet cells were suspended in 100 ml of a 0.1 M sodium phosphate buffer (pH 8).
[b] Isolated yield.
[c] E.e. of epoxides were determined with a chiral OD column (0.46 mmϕ × 25 mm) and an eluent of hexane:2-propanol = 9:1 v/v at a flow rate of 0.7 ml/min.
[d] E.e. of diols were determined with a chiral OJ column (0.46 mmϕ × 25 mm) and an eluent of hexane:2-propanol = 9:1 v/v at a flow rate of 0.7 ml/min.
[e] I was carried out at 30°C, and the others at 25°C.

1000 ml of the medium (in a 5-liter flask) was inoculated with 5% of the first-stage seed culture. The second stage lasted for 72 h. In both stages, the cultures were grown on a shaker maintained at 30°C and 100 rpm. *A. niger* cells were harvested by centrifugation (Hitachi CR20B2) at 7,000 rpm and 5°C for 30 min, before being washed twice with 100 ml of a 0.1 mol/l phosphate buffer (pH 8.0). The racemic substrate was prepared according to the literature.[21]

The reaction was carried out at 30°C, and when an appropriate degree of conversion have been reached, the reaction was stopped. The unreacted epoxide was recovered in a 33% yield with 85%ee.

Expecting an improvement in the enantioselectivity, we lowered the temperature to 25°C. Although this change may reduce the activity of the EH, and prolong the time for resolution. It also reduce the influence by other nucleophilic groups in the microorganisms which may affect ring-open of the epoxides.[22]

Biomass (20 g wet) of the *A. niger* **CGMCC** 0496 fungus was suspended in a 500-ml three-necked, round-bottomed flask which was filled with 100 ml of a 0.1 M sodium phosphate buffer (pH 8). The flask was equipped with a mechanical stirrer while keeping the temperature at 25°C. Epoxide (±)-**1** (100 mg) was added to the suspension as a solution in DMF (5 ml), and the mixture was stirred at 600 rpm. The course of bioconversion was monitored by TLC. After 68 min, when the conversion had reached an appropriate degree, the reaction was stopped by adding 50 ml of ethyl acetate.

The mycelia were filtered off, and the fungal cake was washed with ethyl acetate. The aqueous phase was saturated with sodium chloride and then extracted three times with ethyl acetate. The combined the organic layer was dried with Na_2SO_4, and the solvent was removed. Flash chromatography (petroleum ether/EtOAc, gradient from 4:1 to 1:1) of the residue gave (*S*)-**1**, 35 mg (35% yield), $[\alpha]_D^{19.5}$ −107.2 (c = 1.7; $CHCl_3$), e.e. = 98% {the absolute configuration was established by analogy with the spectroscopic behavior of (*S*)-styrene oxide};[18] and (*R*)-**2**, 43 mg (39% yield), $[\alpha]_D^{19.5}$ +53.4 (c = 1.1, EtOH) {lit.[19] $[\alpha]_D^{20}$ +9 (c 0.8, MeOH) for 47%e.e. (*R*)}, e.e. > 99%. The enantiomeric excess of the residual epoxide was determined by an HPLC analysis in a chiral OD column (0.46 mmϕ × 25 mm) with an eluent of hexane:2-propanol = 9:1 v/v at a flow rate of 0.7 ml/min. The enantiomeric excess of the diol product was determined by an HPLC analysis in a chiral OJ column (0.46 mmϕ × 25 mm) with an eluent of hexane:2-propanol = 9:1 v/v at a flow rate of 0.7 ml/min.

The results confirmed our prediction that the enantioselectivity of *A. niger* **CGMCC** 0496 would be enhanced at the lower temperature.

Reducing the ratio of the biocatalyst/substrate (w/w) was next attempted, and reactions **III**, **IV** (10 g/100 mg) and **V** (10 g/200 mg) were carried out.

According to Table 1, the best reaction choice was **II**, using 20 g/100 mg of wet biomass to substrate at 25°C. After 68 min, both the epoxide and diol could be recovered with very high enantiomeric excess and high isolated yield. Using a higher ratio of substrate to cells slowed the rate of the reaction. Even after a prolonged incubation, such as for 6 h, e.e. of the residual epoxide by reaction **IV** did not reach 90%. This may probably have been due to product inhibition.[23] In order to find more evidence to confirm our prediction, reaction **VI** was carried out. The conditions for **VI** followed those for **II**, except for adding 7 mg of diol (*R*, e.e. > 98) to the substrate. We found that more residual epoxide was obtained when the reaction was stopped at the same time as that for reaction **II**, so product inhibition must be considered during the resolution procedure.

Comparing with the results for ractions **II**, **III**, **IV** and **V**, it can be seen that the ratio of substrate to cells was an important factor for biohydrolysis to overcome product inhibition. To our surprise, e.e. of the diols from reactions **III**, **IV** and **V** was lower than that from **II**, and we cannot ascribe this decrease in enantioselectivity to spontaneous substrate hydroly-

sis, because no hydrolysis was apparent in a blank test even after 7 hrs. The lower enantioselectivity of the reaction may probably have been due to modification of the enzyme enantioselectivity by product inhibition, which suggests that the enantioselectivity could be enhanced or decreased due to product inhibition kinetics.[24)]

The results of our work show that outstanding resolution of *o*-nitrostyrene oxide could be achieved by whole cells of the *A. niger* **CGMCC** 0496 fungus with careful selection of the reaction temperature and biocatalyst/substrate ratio. Product inhibition was found when the ratio biocatalyst/substrate ratio for the resolution was reduced.

Acknowledgments

The authors are grateful to the National Natural Science Foundation of China (No. 200032020 and No. 29770020) and Chinese Academy of Sciences for financial support and to Ms. Zhuo-Ding Ding for performing the HPLC analysis.

References

1) Bousquet, C. and Gilheany, D. G., Chromium catalyzed asymmetric alkene epoxidation. Greater selectivity for an E-alkene versus its Z-isomer. *Tetrahedron Lett.*, **36**, 7739–42 (1995).
2) Brandes, B. D. and Jacobsen, E. N., Synthesis of enantiopure 3-chlorostyrene oxide *via* an asymmetric epoxidation-hydrolytic kinetic resolution sequence. *Tetrahedron: Asymmetry*, **8**, 3927–3933 (1997).
3) Ortiz de Montellano, P. R., Fruetel, J. A., Collins, J. R., Camper, D. L., and Loew, G. H., Theoretical and experimental analysis of the absolute stereochemistry of cis-b-methylstyrene epoxidation by cytochrome P450cam. *J. Am. Chem. Soc.*, **113**, 3195–6 (1991).
4) Lakner, F. J., Cain, K. P., and Hager, L. P., Enantioselective epoxidation of w-bromo-2-methyl-1-alkenes catalyzed by chloroperoxidase. Effect of chain length on selectivity and efficiency. *J. Am. Chem. Soc.*, **119**, 443–444 (1997).
5) Hammock, B. D., Grant, D. F., and Storm, D. H., Epoxide hydrolases. In Comprehensive Toxicology, Vol. 3 , eds. Sipes, I., McQueen, C., and Gandolfi, A., Pergamon Press, Oxford, England, pp. 283–305 (1990).
6) Blee, E. and Schuber, F., Regio- and enantioselectivity of soybean fatty acid epoxide hydrolase. *J. Biol. Chem.*, **267**, 11881–11887 (1992).
7) Graham, S. M. and Prestwich, G. D., Synthesis and inhibitory properties of pheromone analogs for the epoxide hydrolase of the gypsy moth. *J. Org. Chem.*, **59**, 2956–66 (1994).
8) Faber, K., Mischitz, M., and Kroutil, W., Microbial epoxide hydrolases. *Acta Chem. Scand.*, **50**, 249–58 (1996).
9) Weijers, C. A. G. M., Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by Rhodotorula glutinis. *Tetrahedron: Asymmetry*, **8**, 639–647 (1997).
10) Chen, X. J., Archelas, A., and Furstoss, R., Microbiological transformations. 27. The first examples for preparative-scale enantioselective or diastereoselective epoxide hydrolyses using microorganisms. An unequivocal access to all four bisabolol stereoisomers. *J. Org. Chem.*, **58**, 5528–32 (1993).
11) Seidegaard, J. and DePierre, J. W., Microsomal epoxide hydrolase. Properties, regulation and function. *Biochim. Biophys. Acta*, **695**, 251–70 (1983).
12) Mischitz, M., Kroutil, W., Wandel, U., and Faber, K., Asymmetric microbial hydrolysis of epoxides. *Tetrahedron: Asymmetry*, **6**, 1261–72 (1995).
13) Moussou, P., Archelas, A., and Furstoss, R., Microbiological transformations. 40. Use of fungal epoxide hydrolases for the synthesis of enantiopure alkyl epoxides. *Tetrahedron*, **54**, 1563–1572 (1998).
14) Jin, H. and Li, Z.-Y., Biocatalytic resolution of *para*-nitrostyrene oxide by resting cells of different *Aspergillus niger* strains. *Chinese J. Chem.*, **19**, 272–275 (2000).
15) Andor, H., Zsuzsa, H., Marton, K., Jozsef, B., Sandor, E., and Istvan, P., Hungarian patent, Hung. 154,951 (Jul. 25, 1968).
16) Gerhard, J. and Klaus, L., German patent, Ger. Offen. 2,230,179 (Jan. 17, 1974).
17) Xia, J., Huang, X., Sreekumar, R., and Walker, J. W., Photolabile 'caged' fatty acids containing a 1-(2′-nitrophenyl)-1,2-ethanediol moiety. *Bioorg. Med. Chem. Lett.*, **7**, 1243–1248 (1997).
18) Naruta, Y., Tani, F., Ishihara, N., and Maruyama, K., Catalytic and asymmetric epoxidation of olefins with iron complexes of ''twin-coronet'' porphyrins. A mechanistic insight into the chiral induction of styrene derivatives. *J. Amer. Chem. Soc.*, **113**, 6865–6872 (1991).
19) Wei, Z.-L., Lin, G.-Q., and Li, Z.-Y., Microbial transformation of 2-hydroxy and 2-acetoxy ketones with Geotrichum sp. *Bioorg. Med. Chem.*, **8**, 1129–1137 (2000).
20) Morisseau, C., Venturi, G., Moussou, P., and Baratti, J., Effect of carbon and nitrogen sources on the production of a highly enantioselective epoxide hydrolase from *Aspergillus niger*. *Biotechnol. Tech.*, **12**, 805–809 (1998).
21) Rafizadeh, K. and Yates, K., One-pot synthesis of nitrostyrene oxides. *Org. Prep. Proced. Int.*, **17**, 140–142 (1985).
22) Archer, I. V. J., Epoxide hydrolases as asymmetric catalysts. *Tetrahedron*, **53**, 15617–15662 (1997).
23) Pedragosa-Moreau, S., Archelas, A., and Furstoss, R., Microbiological transformations. 28. Enantiocomplementary epoxide hydrolyses as a preparative access to both enantiomers of styrene oxide. *J. Org. Chem.*, **58**, 5533–5536 (1993).
24) Rakels, J. L. L., Caillat, P., Straathof, A. J. J., and Heijnen, J. J., Modification of the enzyme enantioselectivity by product inhibition. *Biotechnol. Prog.*, **10**, 403–409 (1994).