Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 15 (2004) 2801–2805

Enzymatic transformations. Part 58: Enantioconvergent biohydrolysis of styrene oxide derivatives catalysed by the *Solanum tuberosum* epoxide hydrolase

Maria I. Monterde,[a] Murielle Lombard,[a] Alain Archelas,[a] Annette Cronin,[b] Michael Arand[b] and Roland Furstoss[a,*]

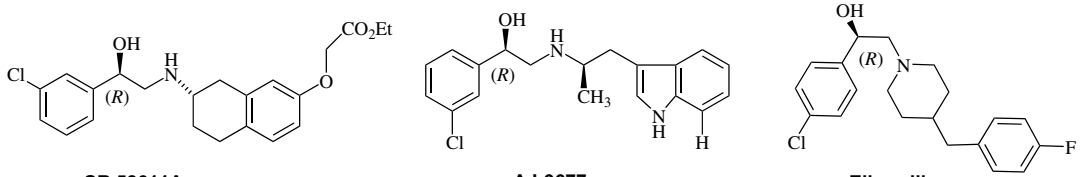
[a]*Groupe Biocatalyse et Chimie Fine, FRE 2712 associée au CNRS, Université de la Méditerranée, Faculté des Sciences de Luminy, Case 901, 163 avenue de Luminy, 13288 Marseille Cedex 9, France*
[b]*Institute of Pharmacology and Toxicology, University of Würzburg, Versbacher Strasse 9, D-97078 Würzburg, Germany*

Received 20 May 2004; accepted 10 June 2004
Available online 3 August 2004

Abstract—The biohydrolysis of four racemic styrene oxide derivatives has been explored, using the (recombinant) *Solanum tuberosum* epoxide hydrolase. Interestingly, this enzyme showed a marked tendency to operate a so-called *enantioconvergent* process, thus affording the corresponding (*R*)-diol in a nearly quantitative yield and good to excellent ee. We have demonstrated that this is due to the fact that the (*S*)-enantiomer of these epoxides was preferably attacked at the (benzylic) more substituted carbon atom, whereas the (*R*)-epoxide was attacked at the (terminal) less substituted carbon atom. The thus obtained *meta*- and *para*-chlorostyrene diol derivatives are important building blocks in the synthesis of various biologically active molecules. A nine cycles repeated batch reactor was performed starting from racemic *meta*-chlorostyrene oxide and afforded a 100% analytical yield (88% preparative) of the corresponding diol, obtained with ees as high as 97%.

1. Introduction

Due to the ongoing search for increased safety and efficiency of drugs, and to the release of new FDA's marketing guidelines, the involvement of contemporary pharmaceutical chemistry in the elaboration and commercialisation of enantiopure compounds is nowadays beyond discussion.[1] In this context, the synthesis of enantiopure epoxides, as well as of their corresponding vicinal diols is a most actively studied topic. This is due to the fact that these compounds are essential chiral intermediates for the production of biologically active molecules, such as SR 58611A or AJ-9677, two examples of various β3-adrenergic receptor agonists, which are known to require a 3-chlorophenyl moiety[2] or Eliprodil,[3] an effective NMDA receptor antagonist known to possess neuroprotective properties, whose structure involves a 4-chlorophenyl entity (Scheme 1).



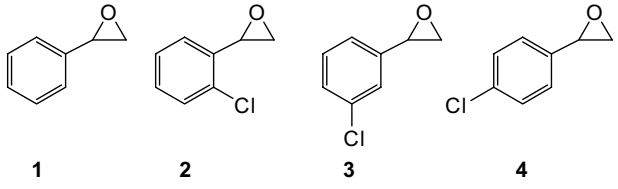
Scheme 1.

* Corresponding author. Tel.: +33-491-82-91-55; fax: +33-491-82-91-45; e-mail: furstoss@luminy.univ-mrs.fr

0957-4166/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2004.06.032

Therefore, the elaboration of efficient and cost effective processes allowing for the preparation of these chiral synthons in enantiopure form are of utmost importance, with several methodologies already explored.[1] These include the hydrolytic kinetic resolution (HKR) of racemic epoxides, one of the most promising methods in this context being a biocatalytic approach. This environmentally safe methodology is particularly attractive since it allows us to minimise the cost of resources and the production of (potentially toxic) waste in industrial applications. It is based on the use of dedicated enzymes, that is, epoxide hydrolases (EH) (EC 3.3.2.3), which specifically catalyse the hydrolysis of an epoxide into the corresponding vicinal diol.[4–7] We, and others, have described over the last decade that, in many cases, this can be achieved in an enantioselective manner. Nowadays, an even more stringent industrial demand relates to the possibility to elaborate *enantioconvergent* processes, thus allowing us to approach the ideal '100% yield, 100% ee' goal starting from a racemic mixture. This is obviously of utmost importance for process cost efficiency, since it allows us to avoid the loss of 50% of the starting product, a feature intrinsic to any resolution process. In this context we have demonstrated that in some cases, it is possible to take advantage of the potential 'stereochemical flexibility' of the EHs approach, and to set up a single process implying combination of complementary enantio- and regioselectivities. This can be obtained either by combining two different but enantio- and regiocomplementary epoxide hydrolases[8] or by using one single enzyme.[9,10] In this last case, the chosen epoxide hydrolase has to display an opposite regioselectivity of the oxirane ring opening for each enantiomer of a racemic mixture. This aspect offered by EHs is an intriguing and potentially highly useful property of such enzymes. To the best of our knowledge, no chemical counterpart of this type has yet been described.

Herein we report on our recent work aimed at exploring the 'enantioconvergent potential' of the *Solanum tuberosum* epoxide hydrolase (*St*EH), a monomeric recombinant protein of plant origin,[11,12] applied to four styrene oxide derivatives **1**–**4** (Scheme 2).



Scheme 2. Structure of the racemic epoxides **1**–**4** studied.

2. Results and discussion

2.1. Preliminary experiments

Owing to the interest of enantiopure styrene oxide derivatives **1**–**4** as well as of their corresponding vicinal diols **1d**–**4d**, as key-step building blocks for the synthesis of various pharmaceutical compounds, we explored the behaviour of these four epoxides when submitted to biohydrolysis by the above-cited *St*EH.[†] This was in particular aimed at evaluating the influence of the chlorine atom positioning on the enantioconvergency, a phenomenon we had previously observed in preliminary studies conducted on substrate **4**.[8] Ideally, this would allow the 100% yield preparation of diols **1d**–**4d**, in their enantiopure forms.

Our initial experiments were designed to find the most suitable conditions for the biohydrolysis of *rac*-**1**–**4**. In a first set of experiments, four different conditions, that is, a combination of either deionised water or pH 7 phosphate buffer with the use of either DMF or DMSO as co-solvent (to increase the substrate solubility in the aqueous phase), were explored. Thus, as a model experiment, biohydrolysis of *meta*-chlorostyrene oxide **3** was carried out for each one of these possible combinations at 28 °C, 4 mM substrate concentration, and using a substrate over enzyme ratio (w/w) of 4.6. Figure 1 shows the evolution of the enantiomeric excess (ee) and conversion ratio (*c*) versus time dependent biohydrolysis of *rac*-**3**, using each one of these four media. The best results were obtained with the deionised H_2O/DMSO combination. This was therefore used for the following analytical studies. Increasing either the DMSO or DMF proportion (up to 30%), as well as using the three other combinations, led to noticeable decreases in the reaction rate.

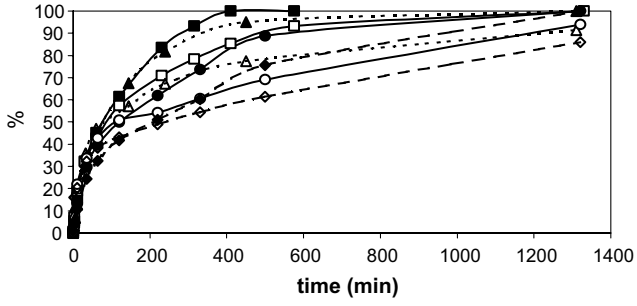


Figure 1. Analytical follow-up of the biohydrolysis of *rac*-**3** at 28 °C using *St*EH, under different experimental conditions: (a) H_2O, DMSO (1%): ■ ee, □ conversion ratio; (b) Buffer, DMSO (1%): ▲ ee, △ conversion ratio; (c) H_2O, DMF (1%): ● ee, ○ conversion ratio; (d) Buffer, DMF (1%): ◆ ee, ◇ conversion ratio.

2.2. Analytical scale biohydrolysis

Experiments conducted on substrates **1**–**4** using the *S. tuberosum* EH led to the results reported in Table 1. The initial rate measured for biohydrolysis of *rac*-**1** (4 mM aqueous solution, 20 °C) indicated an enzymatic activity of about 2 U/mg and, after about 40 min, the conversion ratio reached 53%. The unreacted (remaining) epoxide showed an ee as high as 94%. It was shown to be an (*R*)-configuration by comparison with a commercial sample. The *E* value, calculated on the basis of

[†] The *St*EH enzymatic extract used herein was prepared by overexpression in *Escherichia coli* BL21(DE3) (unpublished results). This was used as a crude extract for the analytical work and in a purified form for the reactor process.

Table 1. Parameters of the biohydrolysis of **1**–**4** (4 mM, 28 °C) using *S. tuberosum* EH

Substrate	t (h)	c (%)	Ee_{epoxi}	Ee_{diol}	Ee_{diol} (total conv.)[a]	E	Activity[b]	$\alpha(S)$	$\beta(S)$	$\alpha(R)$	$\beta(R)$	Chem. hydr %/h
rac-**1**[c]	0.7	53	94	95	86	30	2	98	2	7	93	1.5
rac-**2**	5.5	45	31	30	29	2.5	0.01	62	38	25	75	0.5
rac-**3**	2	57	61	94	91	6	0.4	97	3	6	94	0.5
rac-**4**	1.4	50	>99	88	74	70	1.8	97	3	8	92	1.2

[a] Ee of the formed diol at $c = 100\%$.
[b] U/mg enzymatic extract.
[c] Biocatalysis conducted at 20 °C.

the remaining substrate ee and of the conversion ratio, was about 30. After derivatisation into its acetonide and GC analysis, the resulting vicinal diol **1d** was shown (by chiral GC analysis) to also be 95% ee. However, this ee surprisingly only slightly decreased to about 86% at total conversion ($c = 100\%$). Moreover, the formed diol **1d** appeared to be also of (*R*)-configuration. This result obviously highlighted the occurrence of an unexpected enantioconvergent process.

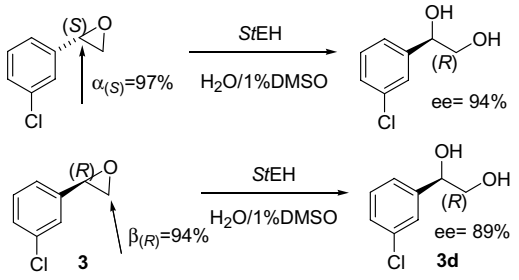
Racemic *ortho*-chlorostyrene oxide **2** behaved quite differently when compared to *rac*-**1**. Thus, the enzymatic activity was estimated to only be 0.01 U/mg. Therefore, about 5.5 h were necessary to reach a conversion ratio of 45%. Both the ee of the residual epoxide **2** as well as of the formed diol **2d** were rather low (30%) at this stage. A similar ee was found for **2d** at completion of the reaction. Interestingly however, both these products were shown to be of an (*R*)-configuration as in the case of **1**. The E value was estimated to be only about 2.5. Obviously, the introduction of a chlorine atom at the *ortho* position severely disturbed the enzymatic hydrolysis.

Biohydrolysis of *meta*-chlorostyrene oxide *rac*-**3** similarly revealed a rather low E value ($E \sim 6$). However, contrary to the previous case, a much better enzymatic activity was observed (0.4 U/mg). Thus, after 2 h reaction, biohydrolysis of *rac*-**3** led to a conversion ratio of 57%. The remaining epoxide exhibited a 61% ee while the obtained diol exhibited an ee as high as 94%. Again, both these products were of an (*R*)-configuration, as determined by comparison with commercial samples. It is noteworthy however, that at total conversion, the formed diol still exhibited an ee of 91%.

Finally, *para*-chloro styrene **4** behaved similarly to **1**. An enzymatic activity of about 1.8 U/mg was estimated from the initial rate measurements and a 50% conversion ratio reached after 1.4 h. The remaining epoxide exhibited an ee higher than 99% whereas the obtained diol exhibited an ee of 94%. Again, both these products were shown to be of an (*R*)-configuration, as determined by comparison with commercial samples. At total conversion, the formed diol still exhibited an ee of 74%.

2.3. Regioselectivity coefficients

Throughout this study, the most surprising observation was that for each substrate, the *St*EH appeared to afford a good to excellent enantioconvergency. The most obvious explanation would be that, in each of these cases, the enzyme displayed an opposite regioselectivity for each enantiomer of the racemic mixture. Thus, the (*S*)-enantiomer should be essentially attacked at the *more* substituted (benzylic) carbon atom, thus leading to the (*R*)-diol by inversion of configuration, whereas the (*R*)-enantiomer should be essentially attacked at the *less* substituted carbon atom, affording the diol of unchanged (*R*)-configuration. A detailed analysis of the specific regioselectivity [i.e., the determination of the so-called regioselectivity coefficients $\alpha(R)$, $\beta(R)$, $\alpha(S)$ and $\beta(S)$] had therefore to be achieved for total understanding of the process.[9] This was conducted using each one of the two enantiomers of substrates **1**–**4**. Our results are reported in Table 1. Interestingly enough, it was observed that the (*S*)-enantiomer of substrates **1**, **3** and **4** were indeed very preferably attacked at the benzylic position [$\alpha(S) > 97\%$], whereas the (*R*)-enantiomer was mostly attacked at the terminal carbon atom [$\beta(R) > 92\%$]. This behaviour is illustrated for *rac*-**3** on Scheme 3. The same tendency stayed true, although to a lesser degree, for epoxide **2** where the regioselectivity was more balanced between the two α and β carbon atoms. We also found that, an opposite, spontaneous (chemical) hydrolysis essentially led to an attack at the benzylic position in all cases. However, we also have determined that this hydrolysis remained rather negligible (Table 1), thus precluding any noticeable interference with the enzymatic hydrolysis regioselectivity after a few hours.



Scheme 3. Enantioconvergent biohydrolysis of *rac*-**3** due to complementary regioselectivity.

2.4. Optimisation of substrate concentration

Owing to the high potential of the above-described results for setting up an enantioconvergent process, we decided to explore the possibility of performing a preparative scale biohydrolysis of one of these substrates.

The substrate of choice was *rac*-**3**, due to (a) the interest of the corresponding (*R*)-diol **3d** for the synthesis of various β3-adrenergic receptor agonists;[2] (b) its low *E* value (and therefore the moderate biohydrolysis rate difference between the two enantiomers); and (c) its reasonable enzymatic activity. Obviously, one important parameter for implementation of such a process is the determination of the maximum substrate concentration usable. We have shown previously, that when using the *A. niger* EH, in certain cases, a substrate concentration as high as 500 g/L could be used.[13] Exploration of this possibility was therefore conducted by increasing the concentration of *rac*-**3** up to 200 mM (34.5 g/L). Our exploratory results indicated that contrary to our previous results, a concentration limitation appeared at a value higher than 75 mM of substrate. Due to previous experiences, we felt that this limitation could be due to product inhibition. We therefore tested this hypothesis by conducting several experiments (at 2 mM substrate concentration), adding various concentrations of the (formed) (*R*)-**3** diol enantiomer at the start of the biohydrolysis. The results shown in Figure 2 clearly indicate that this concentration limitation was indeed due to product inhibition. They also indicate that a 75 mM (11.6 g/L) substrate concentration (which corresponds to a concentration of about 13 g/L of formed diol) was a reasonable compromise for further elaboration of an enzymatic reactor.

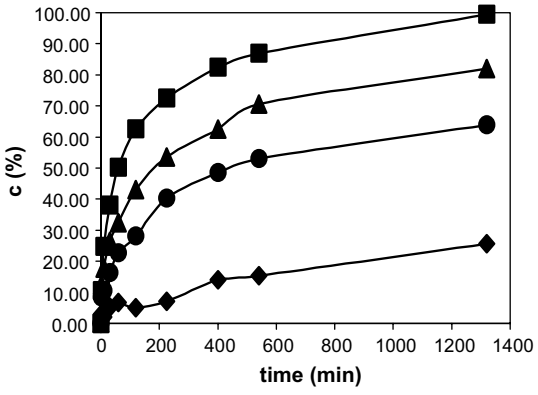


Figure 2. Influence of the initial (*R*)-**3** diol concentration on the biohydrolysis rate. [(*R*)-**3** diol]: ■ = 0 mM; ▲ = 75 mM (13 g/L); ● = 100 mM (17.2 g/L); ◆ = 200 mM (34.5 g/L).

2.5. Preparative scale biohydrolysis of *rac*-3 using a repeated batch reactor

Owing to the above described results, we decided to run a preparative scale repeated batch reactor, using the most appropriate experimental conditions for the biohydrolysis of *rac*-**3**. Thus, 340 mg of *rac*-**3** dissolved in 300 μL of DMSO were placed in a 100 mL reactor, together with 22 mL of plain water. The *St*EH was added (960 U)[‡,14] as an aqueous solution (10 mL, containing 10% of glycerol for enzyme stabilisation), which corresponded to an overall concentration of about 10 g/L of substrate. The mixture was then vigorously stirred (magnetic stirrer). In order to further minimise any spontaneous hydrolysis of the substrate and favour enzyme stability, the process was conducted at 20 °C (instead of 28 °C as used for the analytic experiments). Due to low solubility of **3** in water, this was done at the start of a biphasic system. However, the solution became homogeneous as the reaction proceeded, since the diol formed is totally soluble in water at that concentration. The reaction was followed by HPLC analysis, showing that total conversion occurred after a 1.5 h reaction time for this first run. The aqueous (homogeneous) solution was filtered through an ultrafiltration membrane, which allowed us to recover the enzyme (as a concentrated solution). This was re-used to perform the next biohydrolysis run on another 340 mg substrate sample, using the same reactor and experimental conditions. Nine runs were thus performed successively, using the same enzyme, which corresponds to a biohydrolysis of a total of 3 g (19.4 mmol) of *rac*-**3**. The nine aqueous solutions containing the product **3d** were pooled and extracted (ethyl acetate). This afforded 3.5 g of **3d** (100% analytical yield), which were purified by bulb-to-bulb distillation. A total amount of 3 g of pure **3d** (waxy solid) was thus obtained, which corresponds to an 88% preparative yield. As expected, the ee of the thus obtained (*R*)-**3d** was excellent (97%) {$[\alpha]_D^{22} = -23.4$ (*c* 1.55, EtOH); lit.[15] for (*S*)-**3d** $[\alpha]_D^{26} = +21.1$ (*c* 1.31, EtOH), ee = 91%}.

3. Conclusion

Herein we have reported the biohydrolysis of the substituted styrene oxide derivatives **1**–**4** catalysed by the *S. tuberosum* epoxide hydrolase, overexpressed in an appropriate *E. coli* strain. We observed that each one of the two enantiomers of these substrates were attacked by this enzyme with a different and complementary regioselectivity. Thus, the (*S*)-enantiomers were preferably attacked at the benzylic position, whereas the (*R*)-enantiomers were mostly attacked at the terminal carbon atom. As a consequence, this led to a so-called enantioconvergent process. Based on this very interesting property, we were able to perform the preparative scale biohydrolysis of 3 g (19.4 mmol) of *rac*-**3** in a repeated batch reactor, which was run over nine cycles using the same enzyme sample. The corresponding diol (*R*)-**3d** was thus obtained with an excellent yield (100% analytic, 88% preparative), in nearly enantiopure form (ee 97%). This illustrates the highly interesting potential offered by the enantio-dependent regioselectivity of this enzyme, which allows to efficiently overcome the 50% yield limitation theoretically linked to any resolution process. It should be stressed that this biohydrolysis was performed using plain water instead of a buffer solution, thus affording a so-called salt-free process. Moreover, it should also be emphasized that, the best of our knowledge such an enantio- and regio-complementary behaviour has never been described for a metal-based catalysed hydrolytic kinetic resolution of an epoxide. Work is currently in progress in our laboratory in

[‡] This enzymatic activity was measured against styrene oxide, using the same methodology as the one we have described previously for the *A. niger* EH.[14]

order to further explore the potential of this new epoxide hydrolase.

Acknowledgements

This work was partly supported by the European Community Contract 'Engineering integrated biocatalyst for the production of chiral epoxides and other pharmaceutical intermediates' (No QLK3-CT2000-00426). Two of us (M.M. and M.L.) are very grateful to the EC for financial support in this context.

References

1. Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B.; Keßeler, M.; Stürmer, R.; Zelinski, T. *Angew. Chem., Int. Ed. Engl.* **2004**, *43*, 788–824.
2. Harada, H.; Hirokawa, Y.; Suzuki, K.; Hiyama, Y.; Oue, M.; Kawashima, H.; Yoshida, N.; Furutani, Y.; Kato, S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1301–1305.
3. Pabel, J.; Höfner, G.; Wanner, K. T. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1377–1380.
4. Archelas, A.; Furstoss, R. *Curr. Opin. Chem. Biol.* **2001**, *5*, 112–119.
5. de Vries, E. J.; Janssen, D. B. *Curr. Opin. Biotechnol.* **2003**, *14*, 414–420.
6. Smit, M. S. *Trends Biotechnol.* **2004**, *22*, 123–129.
7. Steinreiber, A.; Faber, K. *Curr. Opin. Chem. Biol.* **2001**, *12*, 552–558.
8. Manoj, K. M.; Archelas, A.; Baratti, J.; Furstoss, R. *Tetrahedron* **2001**, *57*, 695–701.
9. Moussou, P.; Archelas, A.; Baratti, J.; Furstoss, R. *Tetrahedron: Asymmetry* **1998**, *9*, 1539–1547.
10. Steinreiber, A.; Mayer, S. F.; Faber, K. *Synthesis* **2001**, *13*, 2035–2039.
11. Stapleton, A.; Beetham, J. K.; Pinot, F.; Garbarino, J. E.; Rockhold, D. R.; Friedman, M.; Hammock, B. D.; Belknap, W. R. *Plant J.* **1994**, *6*, 251–258.
12. Morisseau, C.; Beetham, J. K.; Pinot, F.; Debernard, S.; Newman, J. W.; Hammock, B. D. *Arch. Biochem. Biophys.* **2000**, *378*, 321–332.
13. Monfort, N.; Archelas, A.; Furstoss, R. *Tetrahedron* **2004**, *60*, 601–605.
14. Mateo, C.; Archelas, A.; Furstoss, R. *Anal. Biochem.* **2003**, *314*, 135–141.
15. Schaus, S. E.; Brandes, B. D.; Larrow, J. F.; Tokunaga, M.; Hansen, K. B.; Gould, A. E.; Furrow, M. E.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2002**, *124*, 1307–1315.