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Microbiological Transformations—XXIX. Enantioselective Hydrolysis of Epoxides Using Microorganisms: A Mechanistic Study

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Abstract—The regio- and stereochemistry of the hydrolysis of styrene oxide **1** by two fungi : *Aspergillus niger* and *Beauveria sulfurescens*, were studied using $H_2{}^{18}O$ labelling experiments. Also, the kinetic parameters of these hydrolyses were determined. We conclude that the epoxide hydrolases of these two fungi operate via different mechanisms.

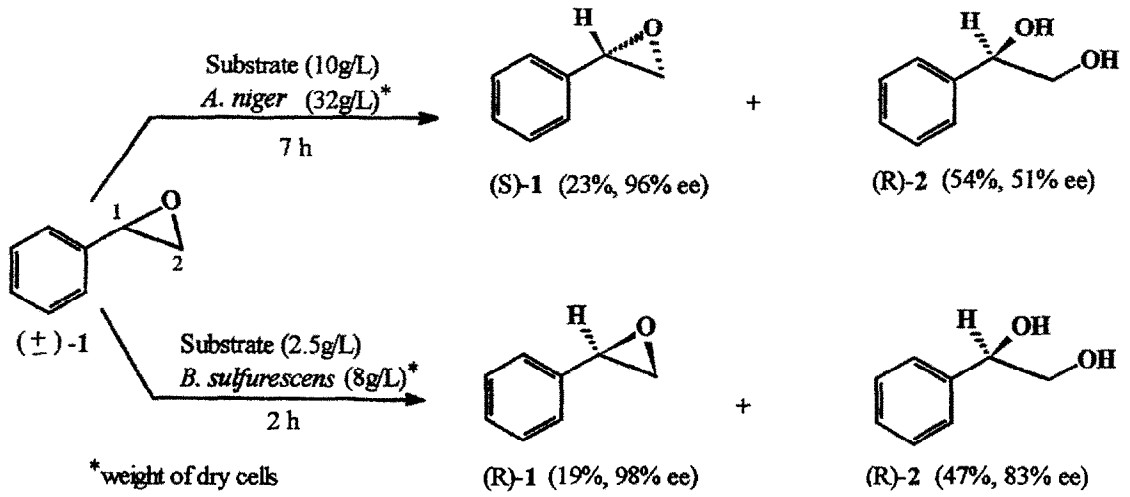
Introduction

Epoxide hydrolases are key enzymes for the metabolism of a broad class of epoxy derivatives in animals, plants and microorganisms. In fact, epoxides are by nature biologically toxic compounds and are often detoxified via enzymatic hydrolysis of the oxirane ring into chemically less reactive diols. An important amount of work has been carried out using subcellular fractions of rat, mouse, rabbit and human livers, where two distinct forms of epoxide hydrolases, i.e. a form located in the membranes (microsomal epoxide hydrases: mEH) and a form located in the cytosol (cytosolic epoxide hydrolase: cEH) have been purified and characterized.[1] Very recently, a higher plant epoxide hydrolase has also been reported and purified.[2,3] Unfortunately, these enzymes which have high theoretical value for organic synthesis cannot be used for large scale experiments because of their low availability. Therefore, we have developed microbiologically mediated epoxide hydrolyses allowing preparation of several gram-scale optically active epoxides via either enantioselective or diastereoselective differentiation.[4,5] Interestingly, up to now, there has been little published data pertaining to the presence or function of these enzymes in bacteria[6] and it has been generally assumed that bacteria are lacking epoxide hydrolases, a fact which accounts for the ability to accumulate epoxides by bacterial epoxidation of olefins. In these cases only the purification of epoxide hydrolases from two *Pseudomonas* strains have been described.[6c,j] Information on epoxide hydrolases from fungi[7] is even more scarce, and seems to indicate that the mechanism of hydration of fungi epoxide hydrolase may be quite different[7c,d] from those involved in the other EH.

Interestingly enough, both mammalian mEH and cEH, as well as plant, bacterial or fungal EH, were shown to exhibit a remarkable capability of chiral recognition, enabling them to discriminate between enantiomers of racemic epoxides. Furthermore, these enzymes were shown to be generally highly regio- and stereoselective.[1d,f,g]

Three main points can be gained out of the previously described work. First, it appears that for mammalian mEH and cEH, as well as for plant or bacterial enzymes, the reaction involves *trans*-addition of water with inversion of configuration at the oxirane carbon being attacked, and that as a general rule the addition occurs preferentially at the less sterically hindered carbon atom.[1a,2] However, in the case of two fungi, a mechanism of *cis*-hydration has been described. Kolattukudy and Brown[7c] have reported that *Fusarium pisi* contained an epoxide hydrolase that catalyzes the *cis*-hydrolyses of epoxy acids and, similarly, Marumo *et al.*[7d] described a selective *cis*-hydration of one enantiomer of 10,11-epoxyfarnesol by *Helminthosporium sativum*. Second, several kinetic studies conducted on mammalian mEH clearly indicate that, very often, one enantiomer which displays the lower K_m value, operates as a competitive inhibitor towards hydrolysis of the other antipode when the enzymatic hydrolysis is conducted on the racemic substrate. Since the antipode displaying a higher K_m value does show a higher V_{max} value, this results in a biphasic kinetic profile.[1e] Finally, careful examination of the various examples described so far confirm the fact that the active site model initially proposed by Belucci *et al.*[1c,8] and by Jerina *et al.*[9] — i.e. which implies the existence of a lipophilic pocket located at the right backside (when the oxirane ring is oriented with the oxygen atom on the topside) — can account for almost all the obtained results where a good enantioselectivity is observed.

We have previously described that two fungi, *Aspergillus niger* (LCP521) and *Beauveria sulfurescens* (ATCC 7159), are able to perform highly enantioselective hydrolysis of racemic styrene oxide (Scheme I).[5] In addition, in the case of styrene oxide, these two strains were revealed to be enantiocomplementary since they achieve the hydrolysis of the styrene oxide enantiomers of opposite configuration. Since these results clearly open the way to the preparation of large scale quantities of enantiopure epoxides, it was of high interest to further study the stereochemical and kinetic features implied in these biotransformations and to compare them with the above described observations related to mammalian EH. We describe in this paper our findings concerning these points.



Scheme I.

Results and Discussion

Regio- and stereoselectivities implied in the enzymatic opening of (S)- and (R)-styrene oxide

Styrene oxide is one of the most frequently used substrates for the assay of mEH and cEH.[10] It was therefore interesting to compare the observed regio- and enantioselectivities of our microbiologically mediated hydrolysis versus mammalian enzyme mechanism using this model. Therefore, we have carried out separately, using both fungi, the complete biohydrolysis of both (*R*)- or (*S*)-styrene oxide. This allows a detailed examination of the regio- and stereoselectivities implied in the enzymatic opening of the oxirane ring. As can be seen in Table I, hydrolysis of (*R*)-styrene oxide using *A. niger* (over a 2 h reaction time) led exclusively to diol (*R*)-**2** which showed a 90 % *e.e.* This indicates that about 95 % (*R*)-**2** and 5 % (*S*)-**2** has been formed in the course of this reaction. In fact, we have checked separately the behavior of (*R*)-styrene oxide in the phosphate buffer (pH 8) used for these biohydrolyses. This indicated a rate of epoxide hydrolysis of about 2 % per hour via chemical hydrolysis. We have checked that 90 % of this hydrolysis leads to inversion of configuration at C1 due to attack of the water molecule at this carbon atom. Therefore, it may be assumed that the small amount of (*S*)-styrene oxide obtained during the biohydrolysis is essentially formed this way. Thus, the essential result is that a near total retention of configuration is observed with this fast reacting (*R*)-styrene oxide enantiomer. A similar feature is observed for the (*S*)-styrene oxide (slow reacting) antipode. Indeed, in this case, we have carried out the reaction over 24 h in order to be sure that complete hydrolysis was achieved. This led to a 60 % *e.e.* (*S*)-**2** diol, which indicated a 20 % formation of (*R*)-**2**. Due to the longer time needed for total hydrolysis, at least part of this inversion of configuration (which is difficult to calculate accurately) must be due to chemical hydrolysis. Thus, here again, the major process observed is retention of configuration.

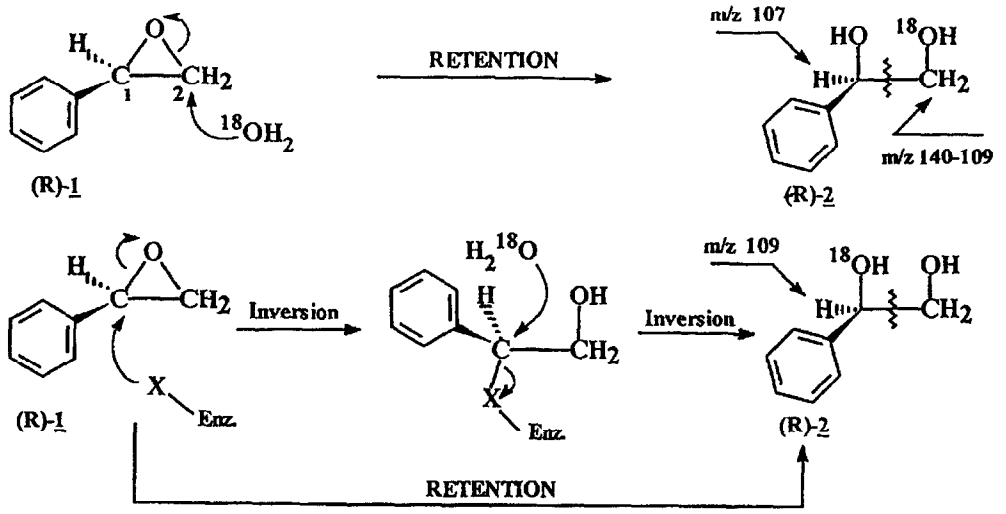
In our previously described work, we had observed that the results obtained using *B. sulfurescens* were surprisingly opposite to those obtained with *A. niger*. Indeed, in this case (1) the fast reacting enantiomer was the one of (*S*) absolute configuration and (2) the enzymatic hydrolysis led to total inversion of configuration at C1. The results obtained in the course of the present work, devoted to the separate study of each enantiomer of styrene oxide (Table 1), show that (*R*)-**2** is obtained with a 90 % *e.e.* from hydrolysis of (*S*)-styrene oxide. On the other hand, they indicate that biohydrolysis of the slow reacting antipode (*R*)-styrene oxide seems to be not selective, diol **2** remaining almost racemic all over the reaction. Here again, as stated above, at least one part of the observed inversion of configuration must be accounted for by chemical hydrolysis.

The results obtained for the separate biohydrolysis of each enantiomer of styrene oxide show that the two fungi

Table 1. Preparative biohydrolysis of (*R*)- and (*S*)-styrene oxide to diol **2** using *A. niger* and *B. sulfurescens*

Substrate[a]	Microorganism	(*R*)-**2** %	(*S*)-**2** %	Isolated yield	*e.e.* %
(*R*)-styrene oxide	*A. niger*	95	5	87	90
(*S*)-styrene oxide	*A. niger*	20	80	60[b]	60
(*R*)-styrene oxide	*B. sulfurescens*	50	50	51[b]	0
(*S*)-styrene oxide	*B. sulfurescens*	95	5	89	90

[a]All these experiments were performed in a 1L medium containing the resting cells (8 g weight dry cells) using 1 g of (*R*)- or (*S*)-styrene oxide as starting compound.
[b]Due to the longer time needed for total hydrolysis, some substrate was lost with the fermentor air-flow.



Scheme II.

exhibit a rather different opening process resulting in either retention or inversion of configuration at the oxirane ring. If inversion of configuration must obviously take place in the anti mode, in agreement with a SN_2 type mechanism involving nucleophilic attack by water at C1, two enzymatic mechanisms leading to retention of configuration can be considered (Scheme II).

As previously emphasized, the more commonly encountered mechanism involves regiospecific attack of water at the less substituted carbon C2. However an unusual process, observed previously with other fungi[7c,d] would involve a backside attack (inversion of configuration) of a nucleophilic center of the enzyme on the oxirane ring, to form an intermediate complex, which could be further on solvolyzed via an SN_2 type reaction, thus leading apparently to a *cis*-hydration process. These two processes are of course indistinguishable on the base of our results obtained up to now. Therefore, in order to determine which C–O bond of the oxirane ring is cleaved during the enzyme catalyzed reaction, we decided to study the hydration of each enantiomer of styrene oxide, using ^{18}O labeled water. The amounts and positional distribution of ^{18}O incorporation into the obtained dihydrodiol **2** have been determined by GC–MS analysis. The ^{18}O content of the intact diol was determined by monitoring the molecular ions for both the ^{16}O and ^{18}O isotopes (M/M + 2). The ^{18}O distribution at C1 was obtained from the peak intensities of the isotopic ion $C_7H_8O^{+\cdot}$ (m/z 107 and m/z 109) arising from the fission of the C1–C2 bond[11] as shown in Scheme II. The ^{18}O content at C2 was determined from the difference in the atom percentage value for the molecular ion (m/z 138 and m/z 140) and for the isotopic fragment at $C_7H_8O^{+\cdot}$. In all cases, contributions of the ^{18}O ion intensity from natural isotope abundance were substrated. In order to minimize the chemical hydrolysis, these reactions were performed over a 3 h period. The results obtained (as well as the values corrected for chemical hydrolysis) are reported in Table 2. They indicate that, in the case of *A. niger*, a 95 % incorporation

Table 2. Amounts and positional distribution of ^{18}O incorporation into diol **2** after biohydrolysis of (*R*)- and (*S*)-styrene oxide in $H_2{}^{18}O$

Strains	Substrate	Total ^{18}O incorp. in diol **2** (%)	^{18}O content at C(1) (% of total)	^{18}O content at C(2) (% of total)	*e.e.* (%) of diol **2**
A. niger	(*R*)-styrene oxide	99	5 (≈0*)	95 (> 99*)	90
	(*S*)-styrene oxide	95	26 (15*)	74 (85*)	48
B. sulfurescens	(*R*)-styrene oxide	94	60 (50*)	40 (50*)	0
	(*S*)-styrene oxide	97	99 (> 99*)	1 (0*)	95

*Values corrected for non-enzymatic hydrolysis.

of ^{18}O is observed at C2 (corrected value 99 %) for the fast reacting enantiomer (*R*)-styrene oxide (the 5 % incorporation at C1 being essentially due, as previously stated on the basis of the stereochemical outcome interpretation, to chemical hydrolysis). Similarly, a 74 % ^{18}O incorporation is observed at C2 (corrected value 85 %) for the slow reacting enantiomer (*S*)-styrene oxide, the 26 % incorporation at C1 being again partly due to chemical hydrolysis. Therefore, our results ascertain the fact that retention of configuration observed in the course of enzymatic hydrolysis of styrene oxide using *A. niger* occurs via almost exclusive attack of water at the less hindered side of the oxirane, rather than via a double inversion process as previously observed.[7c,d]

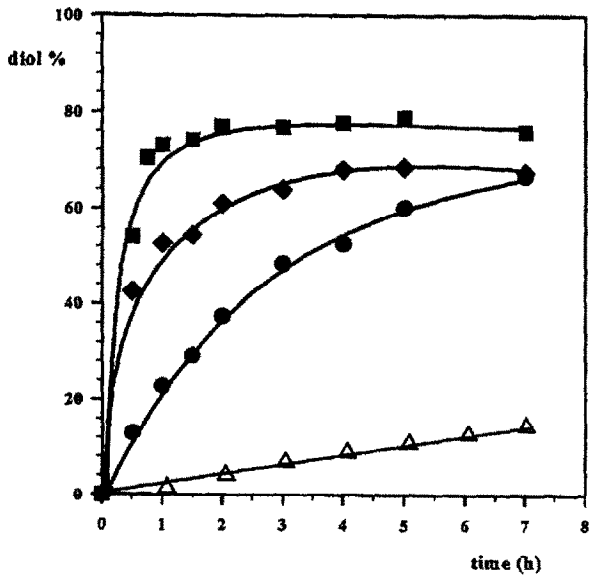


Figure 1. Time course evolution of the hydrolysis of *rac*- (◆), (*R*)- (■), (*S*)-styrene oxide (●) (1 g/L) using *A. niger* cells (8 g/L, weight of dry cells) and of non-enzymatic hydrolysis (Δ).

Both enantiomers of styrene oxide were similarly hydrolyzed separately using the fungus *B. sulfurescens*. Thus, a 99 % regiospecific ^{18}O incorporation was observed at C1 upon hydrolysis of the fast reactive enantiomer (*S*)-styrene oxide. This result, taken together with the total inversion of configuration observed in this case, ascertains the unprecedented fact that the epoxide ring is indeed attacked by a water molecule via a SN_2 type mechanism at the more substituted C1 carbon atom, whereas no significant reaction takes place at the less substituted one. In contrast, biohydrolysis of (*R*)-styrene oxide (slow reacting enantiomer) proceeded almost without any regioselectivity, the distribution of ^{18}O being almost equivalent between C1 and C2.

Kinetics of the biohydrolysis of (S)- and (R)-styrene oxide

Because of the possible complexity of the kinetical results obtainable from experiments conducted on racemic substrate — i.e. enantioselective inhibition phenomena — the previously reported conditions[5] for biohydrolysis of racemic styrene oxide were applied to the separate hydrolysis of (*R*)- or (*S*)-styrene oxide using both fungi.

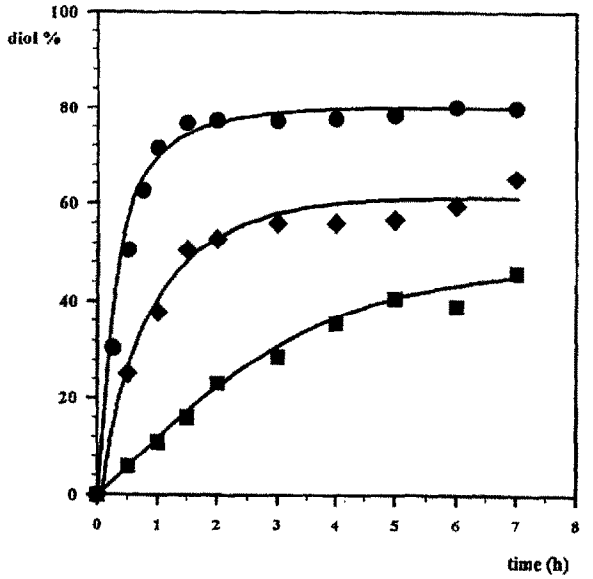


Figure 2. Time course evolution of the hydrolysis of *rac*- (◆), (*R*)- (■) and of (*S*)-styrene oxide (●) (1 g/L) using *B. sulfurescens* cells (8 g/L, weight of dry cells).

Time dependent analysis of *rac*-, (*S*)- and (*R*)-styrene oxide during biohydrolysis were performed. As can be seen from Figure 1, the enantiomer having a (*R*) configuration reacts about four times faster than its antipode (*S*)-styrene oxide in the case of *A. niger*. In contrast, with *B. sulfurescens* (Figure 2) the (*S*) enantiomer reacts approximately eight times faster than its antipode (*R*)-styrene oxide. However in both cases, and in sharp contrast to previously described results obtained using liver microsomes[10b,c] the observed kinetic profile in the case of *rac*-styrene oxide does not show any biphasic kinetic profile. Also, the average curve calculated from each enantiomer hydrolysis, measured separately, does approximately coincide with the observed time course study of the racemic compound. These results confirm the propensity of the enzyme systems to discriminate between the oxirane enantiomers and show that there is no strong competitive inhibition of the slower reacting enantiomer on the hydrolysis of its antipode as in the case of mEH.

To obtain some more information about the enantioselectivity of these biohydrolyses, we have tried to determine the apparent kinetic parameters for each enantiomer of styrene oxide using the two fungi. The initial hydrolysis rates were measured as a function of the substrate concentration versus rates of diol-2 formation. The K_m^{app} and V_{max}^{app} values were determined from the double reciprocal Lineweaver–Burk plots. In the case of *A. niger*, the results obtained indicate that biohydrolysis follows Michaelis–Menten kinetics. The kinetic parameters obtained show that there is no marked difference in affinity for the epoxide hydrolase towards each one of the enantiomers K_m^{app} 0.71 mM for (*R*)-styrene oxide and K_m^{app} 1.2 mM for (*S*)-styrene oxide but reveal a larger specific activity of the enzyme for the (*R*)-enantiomer (V_{max}^{app} 10 nmol/min × mg dry cells) than for the (*S*)-enantiomer (V_{max}^{app} 0.7 nmol/min × mg dry cells). These values confirm that there is no strong competitive inhibition of one enantiomer towards its antipode and that

the enantioselectivity observed is essentially due to a different specific activity of the enzyme for each enantiomer. These results are quite different from those described previously[1c] using mEH, where the tight-binding enantiomer (*R*)-styrene oxide (K_m = 0.08 mM) was shown to react slowly (V_{max} = 6.9 nmol/min × mg protein), whereas the looser-binding enantiomer (*S*)-styrene oxide (K_m = 0.22 mM) has a much higher V_{max} (22 nmol/min × mg protein). Interestingly, in spite of these differences, the observed enantioselectivity is similar in both cases, the (*R*)-styrene oxide enantiomer being hydrolyzed preferentially. In contrast, with *B. sulfurescens* the velocity versus substrate curves (Figures 3 and 4) show marked deviation from rectangular hyperbolas, reflecting the fact that the simple Michaelis–Menten velocity equation no longer describes the kinetic behavior. These results seem to indicate that a complex mechanism occurs during the biohydrolysis of styrene oxide with *B. sulfurescens* and did not allow us to determine the kinetic parameters K_m^{app} and V_{max}^{app}.

Model for the enzymatic active site

A model for the mammalian EH has been devised on the basis of several previously described studies.[1c,8,9] This model implies the existence of a lipophilic pocket located at the right backside if the oxirane ring is oriented with the oxygen atom towards the top. Another very interesting point relates to the enzymatic mechanism of these hydrolytic reactions. The basic mechanistic questions are how the enzyme activates water at the active site and how it destabilizes the oxirane ring towards nucleophilic attack. It has been suggested previously[12] that a histidine (in the case of mEH) or maybe a cysteine (in the case of cEH)[13] residue would be responsible for the water molecule activation. Very recently, expression of rat mEH[14] and cEH[15] in *Escherichia coli* were carried out. These results confirm that, in the case of mEH, a histidine residue is indeed involved in a general base catalysis for the deprotonation of the attacking water molecule, thus making it a stronger nucleophile while another residue (presumably lysine) would, in the transition state, act as a general acid catalytic assistance in dispersal of electron density on the oxirane oxygen for a nucleophilic addition mechanism, thus destabilizing the oxirane.[16] This information led to the model illustrated in Scheme III.[17]

It was interesting to check whether our results, obtained using microorganisms, do — or do not — fit into this model. The absolute configuration of the fast reacting antipodes (or diastereoisomers) of the various substrates we have studied up to now, and which show good enantioselection when hydrolyzed with *A. niger*, are presented in Scheme IV. As can be seen from these representations, they all fit into the proposed model, a fact which leads us to propose that the enzyme of *A. niger* can be described by a model very similar, if not identical, to the one of mEH. Although many more substrates need to be studied the present work suggests that *A. niger* could be used as a microbial model for mammalian metabolism. Obviously, in regard to the opposite regio- and stereochemistry observed for *B. sulfurescens*, the active site of the implied EH must be quite different in that case.

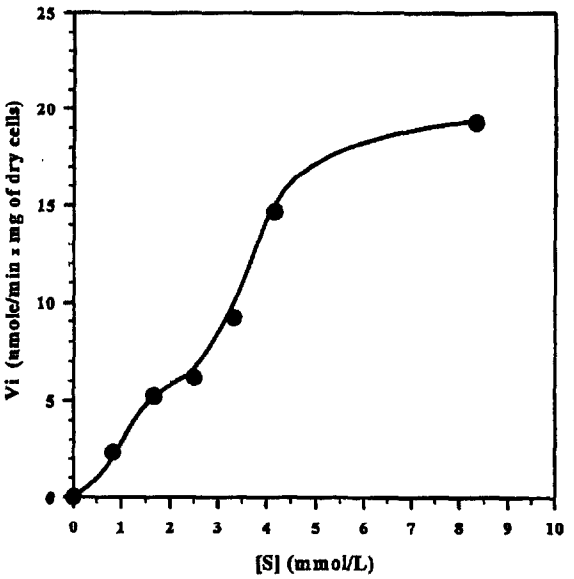


Figure 3. Kinetic of the biohydrolysis of styrene oxide using *B. sulfurescens* cells. Plot of initial velocity versus concentration of substrate (*S*)-styrene oxide.

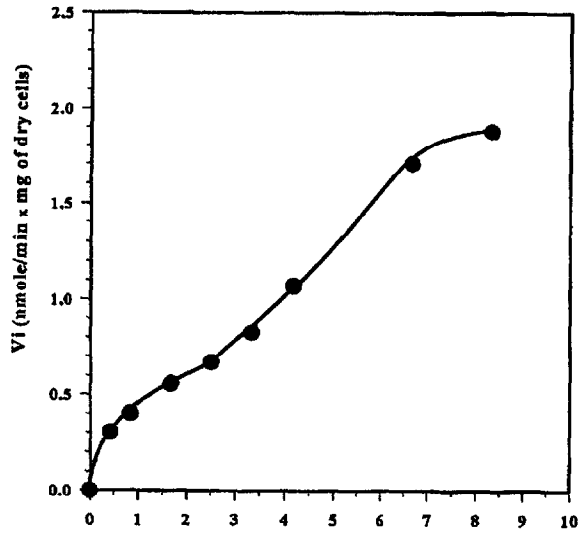
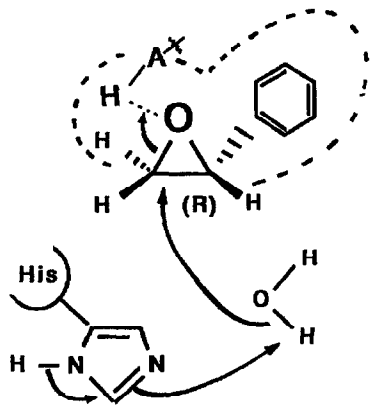
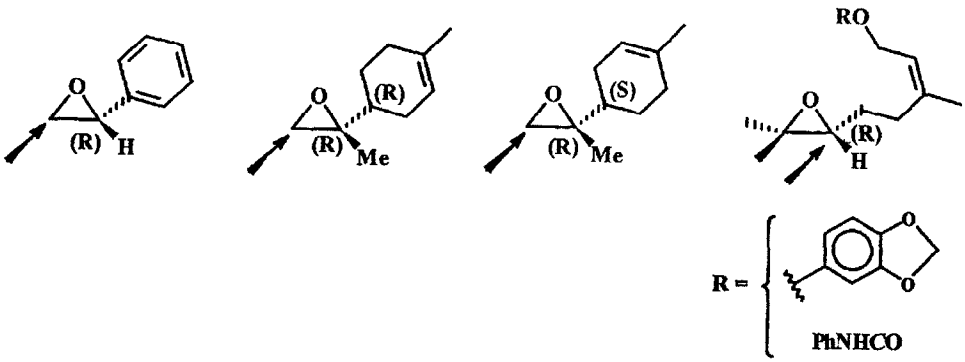


Figure 4. Kinetic of the biohydrolysis of styrene oxide using *B. sulfurescens* cells. Plot of initial velocity versus concentration of substrate (*R*)-styrene oxide.



Scheme III.



Scheme IV.

Conclusion

Our experiments conducted using ^{18}O labeled water show clearly that *A. niger* and *B. sulfurescens* operate each with a different regioselectivity during opening of the oxirane ring. First, it must be emphasized that, whereas in the case of *A. niger* the water molecule is incorporated at the less hindered site of the oxirane ring, as in the case of mammalian EH, *B. sulfurescens* surprisingly leads to incorporation of labeled water at the more substituted carbon atom. As far as the stereochemical course of these enzymatic hydrolyses is concerned it appears that, similarly to the results obtained for mammalian mEH and cEH, enzymatic hydration of both enantiomers of styrene oxide always occurred via a *trans* opening process implying nucleophilic attack of a water molecule on the oxirane ring. This leads to a retention of configuration in the case of *A. niger*, and to an inversion of configuration for *B. sulfurescens* mediated hydrolyses. Interestingly, this is in sharp contrast with results previously described using two other fungi where a *cis*-hydration was observed.[7c,d] We also have shown in the course of this study that no strong competitive inhibition is operating for either enantiomer, an observation which is quite different from the results described for mammalian mEH.

Finally, it has to be emphasized that the enantioselectivity we have observed using our substrates and the fungus *A. niger* can be well predicted by using the active site model previously proposed for mammalian mEH, thus making this fungus a good candidate for microbial models of mammalian metabolism. Interestingly, *B. sulfurescens* led to very unusual and unprecedented regio- and stereochemical results. Work is in progress in our laboratory to further explore the potentialities of these highly attractive microbiological biotransformations and to purify the epoxide hydrolases implied in these hydrolyses.

Experimental Section

General

The strain of *A. niger* used in this work is registered at the Museum d'Histoire Naturelle (Paris) under n° LCP 521 (Lab. de Cryptogamie, 12 rue Buffon, 75005 Paris, France). Corn steep liquor (CSL) is from Roquette S.A. Vapor-phase chromatography analyses were performed by using a chiral 25 m capillary column heptakis (6-*O*-methyl-2,3-di-*O*-pentyl)-β cyclodextrin at 80 °C for the determination of enantiomeric excess of diol **2** via its acetonide derivative [elution order : (1*R*)-**2** t_R = 21.5 min ; (1*S*)-**2** t_R = 22.6 min; α = 1.05]. HPLC analyses were carried out with an UV detector at 220 nm and a normal-phase column (12.5/0.4 cm) filled with 5 µm silica gel using hexane–ethanol 97/3 as eluent (1 mL/min). Purification of products was achieved by flash chromatography (silica gel 60 H from Merck and solvent mixtures consisting of pentane and ether in the range of 100 % pentane to 100 % ether).

Complete biohydrolysis of (R)- *and* (S)-*styrene oxide with* A. niger *and* B. sulfurescens

The fermentations were carried out in a 2 L fermentor as previously described.[5] After incubation (40 h for *A. niger*, 48 h for *B. sulfurescens*) the mycelium was filtered off, washed with water, and then placed back in the same fermentor filled with 1 L of a pH 8 phosphate buffer (0.1 M) solution. The medium was stirred at 700 rpm and maintained at 27 °C. (*R*)- or (*S*)-styrene oxide (1 g) as a solution in ethanol (10 mL) was added to the medium. The fast reacting enantiomers hydrolyses (*R* for *A. niger* and *S* for *B. sulfurescens*) were stopped after 2 h by addition of ether. In order to be sure that complete hydrolysis of slow reacting enantiomers (*S* for *A. niger* and *R* for *B. sulfurescens*) was achieved, incubation was stopped after 24 h by addition of ether. The medium was filtered off, and the fungal cake was separately extracted two times with ether. After decantation, the aqueous phase was saturated with NaCl and then continuously extracted with dichloromethane (48 h). The combined organic layers were dried ($MgSO_4$) and purification of diol **2** was achieved by flash chromatography. Preparative yields and *e.e.*s of **2** are given in Table 1.

Biohydrolysis of (R)- *and* (S)-*styrene oxide in* $H_2{}^{18}O$

Biohydrolyses in $H_2{}^{18}O$ (95 % isotopic enrichment, ISOTEC) were all carried out in a minivial (1 mL) at 27 °C containing phosphate buffer pH = 8 (400 µL, 0.1 M in $H_2{}^{18}O$) and 20 mg of fungal cake obtained as described above. After 2 h stirring 4 µL (c = 1 g/L) of a solution of (*R*)- or (*S*)-styrene oxide (100 mg in 1 mL EtOH) was added via a syringe and the mixture was stirred for 3 h at 27

°C. After saturation with NaCl, each minivial was extracted three times with 0.5 mL portions of dichloromethane. The combined organic layers were dried ($MgSO_4$) and analyzed by HPLC (for determination of analytical yield) and by GC–MS.

Mass spectral analysis

Mass spectra were performed on a Hewlett–Packard 5890 GC coupled to a 5989 A MS Engine. The whole system was controlled by a HPUX-MS-Chemstation. Analyses were performed in electron impact (EI 70 eV) with the mass spectra scanned from 25 to 250. The scan time was 1.17 scans/second. Gas chromatographies were carried out in a HP5 column (30 m × 0.25 mm). The injection was performed within 1 min in splitless mode. Helium was used as a carrier gas at 2 bars and the programmed temperature was from 70 to 200 °C (10 °C/min). The ^{18}O content of a fragmentation was calculated as the ratio of peak intensities (m + 2)/(m + (m + 2)) and was corrected by substracting the corresponding value observed for unlabeled material. At least three scans of the peak doublets of interest were averaged for each sample. The relative distribution of ^{18}O was obtained from the peak intensities of the molecular peak and the isotopic ion $C_7H_8O^{+\cdot}$. The other isotopic ion $CH_2{}^{18}OH^{+\cdot}$ cannot be used for ^{18}O analysis because fragments of m/z 31 can result from decomposition of heavier fragments.

Enzymatic hydrolyses

Time course of hydrolysis of (R)-, (S)- *and* rac-*styrene oxide with* A. niger *and* B. sulfurescens. Hydrolyses of (*R*)-, (*S*)- and *rac*-styrene oxide were carried out in Erlenmeyer flasks (0.5 L) containing phosphate buffer (0.1 L, 0.1 M, pH 8) and 10 % by weight of a fungal cake obtained as described previously from a 2 L fermentor. A solution of styrene oxide (*R*), (*S*) or racemic (100 mg) in EtOH (1 mL) was poured into the medium, and the flasks were stirred with reciprocal shaking (120 cpm) at 27 °C. The course of the bioconversion was followed by withdrawing samples (1 mL) at time intervals. After saturation with NaCl, samples were extracted with ethyl acetate (1 mL). After centrifugation at 3000 rpm (3 min) the produced diol **2** was quantified by HPLC analysis using racemic phenylethanediol as an external standard. The amount of diol **2** formed at each time was used to obtain the kinetic curves reported in Figure 1 for *A. niger* and Figure 2 for *B. sulfurescens*.

Determination of $K_m{}^{app}$ and $V_{max}{}^{app}$. All enzymatic reactions were carried out at 27 °C in Erlenmeyer flasks (0.25 L) containing phosphate buffer (0.05 L, 0.1 M, pH = 8) and 5 % by weight of an *A. niger* or *B. sulfurescens* cake obtained as described previously. Reactions were initiated by addition of pure (*R*)- or (*S*)-styrene oxide (range of 5 to 50 μL) with a syringe. The course of the bioconversion was followed as described above for hydrolyses of (*R*)-, (*S*)- and *rac*-styrene oxide. Initial rates of hydrolysis of each epoxide were calculated at each substrate concentration from the amount of diol formed. The data, corrected for nonenzymatic hydrolysis by subtraction of the rate observed for a blank control experiment without fungus (the chemical hydrolysis rate is the same with inactivated fungus), were used to obtain Lineweaver–Burk plots and to determine the $K_m{}^{app}$ and $V_{max}{}^{app}$.

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References

1. (a) Wistuba, D.; Schurig, V. *Chirality* **1992**, *4*, 178; (b) Wistuba, D.; Träger, O.; Schurig, V. *Chirality* **1992**, *4*, 185; (c) Belluci, G.; Chiappe, C.; Cordoni, A.; Marioni, F. *Tetrahedron: Asymmetry* **1993**, *4*, 1153; (d) Bellucci, G.; Marioni, F.; Chiappe, C.; Capitani, I. *J. Chem. Soc., Chem. Commun.* **1989**, 1170; (e) Bellucci, G.; Chiappe, C.; Conti, L.; Marioni, F.; Pierini, G. *J. Org. Chem.* **1989**, *54*, 5978; (f) Bellucci, G.; Berti, G.; Chiappe, C.; Fabri, F.; Marioni, F. *J. Org. Chem.* **1989**, *54*, 968; (g) Bellucci, G.; Chiappe, C.; Marioni, F.; Simonelli, C. *Xenobiotica* **1989**, *19*, 279; (h) Prestwich, G. D.; Graham, S. McG.; Konig, W. A. *J. Chem. Soc., Chem. Commun.* **1989**, 575; (i) Belluci, G.; Chiappe, C.; Marioni, F. *J. Chem. Soc. Perkin Trans. I* **1989**, 2369.

2. Blée, E.; Schuber, F. *J. Biol. Chem.* **1992**, *267*, 11881.

3. Blée, E.; Schuber, F. *Biochem J.* **1992**, *282*, 711.

4. Chen, X.-J.; Archelas, A.; Furstoss, R. *J. Org. Chem.* **1993**, *58*, 5528.

5. Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. *J. Org. Chem.* **1993**, *58*, 5533.

6. (a) Hechtberger, P.; Wirnsberger, G.; Mischitz, M.; Klempier, N.; Faber, K. *Tetrahedron: Asymmetry* **1993**, *4*, 1161; (b) Weijers, C. A. G. M.; de Bont, J. A. M. *Enzyme Microb. Technol.* **1991**, *13*, 306; (c) Jacobs, M. H. J.; van den Wijngaard, A. J.; Pentenga, M.; Janssen, D. B. *Eur. J. Biochem.* **1991**, *202*, 1217; (d) Escoffier, B.; Prome, J. C. *Bioorg. Chem.* **1989**, *17*, 53; (e) Weijers, C. A. G. M.; de Haan A.; de Bont J. A. M. *Appl. Microbiol. Biotechnol.* **1988**, *27*, 337; (f) Weijers, C. A. G. M.; de Haan A.; de Bont J. A. M. *Microbiol. Sci.* **1988**, *5*, 156; (g) Michaels, B. C.; Fulco, A. J.; Ruettinger, R. T. *Biochem. Biophys. Res. Commun.* **1980**, *92*, 1189; (h) Buchanan, J. F.; Fulco, A. J. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1254; (i) Niehaus, Jr W. G.; Kisic, A.; Torkelson, A.; Bednarczyk, D. J.; Schroepfer, Jr G. J. *J. Biol. Chem.* **1970**, *245*, 3802; (j) Allen, R. H.; Jacoby, W. B. *J. Biol. Chem.* **1969**, *244*, 2078.

7. (a) McMillan, D. C.; Fu, P. P.; Cerniglia, C. E. *Appl. Environ. Microbiol.* **1987**, *53*, 2560; (b) Wackett, L. P.; Gibson, D. T. *Biochem. J.* **1982**, *205*, 117; (c) Kolattukudy, P. E.; Brown, L. *Arch. Biochem. Biophys.* **1975**, *166*, 599;

(d) Suzuki, Y.; Imai, K.; Marumo, S. *J. Am. Chem. Soc.* **1974**, *96*, 3703; (e) Suzuki, Y.; Marumo, S. *Tetrahedron Lett.* **1972**, *19*, 1887; (f) Hartmann, G. R.; Frear, D. S. *Biochem. Biophys. Res. Commun.* **1963**, *10*, 366.

8. (a) Bellucci, G.; Ferretti, M.; Lippi, A.; Marioni, F. *J. Chem. Soc. Perkin Trans. I* **1988**, 2715; (b) Bellucci, G.; Berti, G.; Ferretti, M.; Mastrorilli, E.; Silvestri, L. *J. Org. Chem.* **1985**, *50*, 1471; (c) Belluci, G.; Bianchini, R.; Cetera, P.; Mastrorilli, E. *J. Org. Chem.* **1982**, *47*, 3105; (d) Bellucci, G.; Berti, G.; Ingrosso, G.; Mastrorilli, E. *J. Org. Chem.* **1980**, *45*, 299.

9. Sayer, J. M.; Yagi, H.; van Bladeren, P. J.; Levin, W.; Jerina, D.M. *J. Biol. Chem.* **1985**, *260*, 1630.

10. (a) Splinter, K.; Bauer, D.; Seidel, W. *Exp. Pathol.* **1990**, *39*, 195; (b) Watabe, T.; Hiratsuka, A.; Ozawa, N. *Biochem. Pharmacol.* **1983**, *32*, 777; (c) Watabe, T.; Ozawa, N.; Yoshikawa, K. *Biochem. Pharmacol.* **1981**, *30*, 1695.

11. Audier, H. E.; Dupin, J. F.; Jullien, J. *Bull. Chem. Soc. Fr.* **1968**, *9*, 3844.

12. Dubois, G. C.; Appella, E.; Levin, W.; Lu, A. Y. H.; Jerina, D. M. *J. Biol. Chem.* **1978**, *253*, 2932.

13. Mullin, C. A.; Hammock, B. D. *Arch. Biochem. Biophys.* **1982**, *216*, 423.

14. Bell, P. A.; Kasper C. B. *J. Biol. Chem.* **1993**, *19*, 14011.

15. Knehr, M.; Thomas, H.; Arand, M.; Gebel, T.; Zeller, H.-D.; Oesch, F. *J. Biol. Chem.* **1993**, *268*, 17623.

16. Very recently : Lacourcière, G. M.; Armstrong, R. N. *J. Am. Chem. Soc.* **1993**, *115*, 10466. It has been demonstrated that the oxygen atom incorporated into the product, though ultimately derived from water, is proximally derived from the enzyme by way of an ester intermediate.

17. Amstrong, R. N. *CRC Crit. Rev. Biochem.* **1987**, *22*, 987.

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