Microbiological Transformations. 33. Fungal Epoxide Hydrolases Applied to the Synthesis of Enantiopure *Para*-Substituted Styrene Oxides. A Mechanistic Approach

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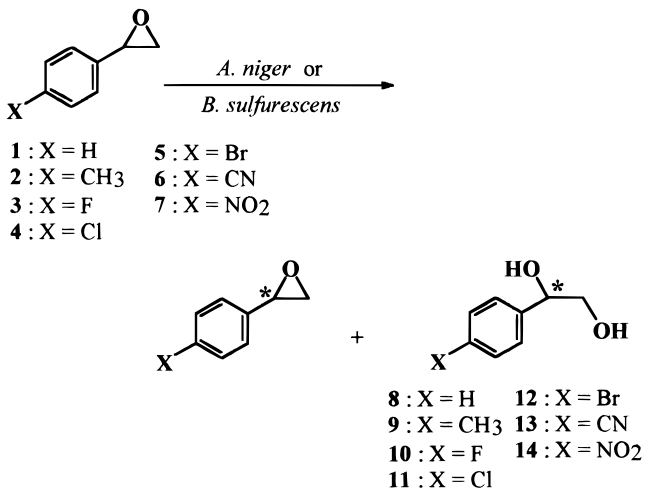
The biohydrolysis of differently *para*-substituted styrene oxide derivatives was studied, using whole cells of the fungi *Aspergillus niger* or *Beauveria sulfurescens*. These microorganisms proved to be equipped with epoxide hydrolases which are able to achieve these hydrolyses with high enantioselectivity. This allowed the preparation of the optically active epoxides and of the corresponding vicinal diols which were obtained with good to excellent enantiomeric purity. These two microorganisms proved to be enantiocomplementary. A mechanistic study, carried out using a crude lyophilized enzymatic extract from *A. niger*, indicated *via* Hammet coefficient plotting that this hydrolysis is very probably due to a general base-catalyzed process.

Epoxide hydrolases (EH) are very interesting enzymes which appear more and more to be ubiquitous in nature. They have been detected for example in organisms as diverse as mammals,[1] plants,[2] and microorganisms.[3,4] Because of their involvement in the metabolism of various xenobiotics, many of which are suspected to be carcinogenic, mammalian enzymes have been extensively studied during the past two decades. For example, it has been described very recently[5] that epoxide hydrolases were identified in 96% of prostate malignant tumors, and it has been suggested that the expression of these enzymes is one possible mechanism of anticancer drug resistance. Besides these important biological studies, at least two other aspects of these enzymes are of high interest, i.e., (a) their use as asymmetric biocatalysts for organic chemistry applications and (b) the determination of their intimate mechanism of action.

As far as chemistry is concerned, the value of epoxides and/or of their corresponding vicinal diols as synthetic intermediates, and the now largely admitted fact that most biologically active molecules ought to be studied—or even manufactured—in enantiomerically pure form, emphasizes the need and desirability of obtaining these intermediates in a high state of enantiomeric purity.[6,7] We have ourselves described recently a new preparative scale methodology based on the use of newly discovered fungal epoxide hydrolases from *Aspergillus niger* or *Beauveria sulfurescens*.[3]

Scheme 1



Another very interesting question about these enzymes is the detailed understanding of their mechanism of action. It is now well established that these enzymes act via a *trans* opening of the oxirane ring and that this attack occurs very preferentially at the less substituted carbon atom. Furthermore, the very elegant single turnover experiments recently conducted by Lacourciere and Armstrong[8] using rat liver microsomal epoxide hydrolase led to the conclusion that a two-step mechanism does in fact take place. A similar result has been very recently described by Hammock *et al.*[9] for soluble (cytosolic) recombinant murine epoxide hydrolase. However, it seems that these two types of enzymes may be somewhat different as far as the oxirane ring activation process is concerned. Indeed, whereas it is now well established that, for microsomal epoxide hydrolases (mEH) this mechanism corresponds to a *general base*

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Table 1. Biohydrolysis of *Para*-Substituted Styrene Oxide Derivatives Using *A. niger*

substr	yield of epoxide, % (reaction time, h)	ee of epoxide, % (abs conf)	product	yield of diol, %	ee of diol, % (abs conf)
1	28 (2)	99 (*S*)[3b]	**8**	50	65 (*R*)[3b]
2	34 (1)	96 (*S*)[a]	**9**	40	66 (*R*)[d]
3	35 (0.75)	98 (*S*)[16]	**10**	47	81 (*R*)[d]
4	33 (1)	>98 (*S*)[e]	**11**	49	79 (*R*)[b]
5	34 (1)	>98 (*S*)[f]	**12**	51	79 (*R*)[g]
6	38 (0.75)	>98 (*S*)[f]	**13**	42	76 (*R*)[g]
7	37 (1)	>98 (*S*)[c]	**14**	54	70 (*R*)[21]

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catalysis, a push-pull mechanism implying activation of the epoxide by protonation or hydrogen bonding has been postulated for cytosolic epoxide hydrolase. Thus, a *general acido-basic-catalyzed* process implying activation of the oxirane ring *via* either protonation or hydrogen bonding could be operative in this case. This hypothesis was essentially supported by the difference of regioselectivity observed for the opening of simple monosubstituted epoxides when catalyzed by either cEH or mEH.[10]

In this study, we now report our results aimed, on one hand, at exploring the possibility of using the two fungi *A. niger* and *B. sulfurescens* as asymmetric catalysts for organic synthesis and, on the other hand, getting some more insight into the mechanism of the two epoxide hydrolases contained in these fungal strains (Scheme 1).

Results and Discussion

The various *para*-substituted styrene oxide derivatives **2**−**7** were submitted to biotransformation by the fungi *A. niger* and *B. sulfurescens*.

Biohydrolysis Using *A. niger*. The results of the biohydrolysis of styrene oxide (**1**) and of its derivatives **2**−**7**, using whole cells of the fungus *A. niger*, are reported in Table 1. It clearly appears that, for all the epoxides studied, a rapid hydrolysis showing a high substrate enantioselectivity occurred. Indeed, after only 1 h of bioconversion (or even less for some of these substrates), all the residual epoxides were almost optically pure (ee > 98%) and all the formed diols also exhibited high enantiomeric excesses. Furthermore, it could be observed that the nature of the substituent born by the phenyl ring did not affect the absolute configuration of the products obtained. As in the case of styrene oxide (**1**), all the unreacted epoxides were of (*S*) absolute configuration, whereas the formed diols were all shown to be of (*R*)-configuration. We emphasize that all these epoxides and diols can thus be prepared easily, on a preparative multigram scale, by using this methodology. Furthermore the formed diols, which are always of (*R*) absolute configuration, can be in most cases easily and efficiently converted back to the corresponding (*R*) epoxides *via* chemical cyclization, without loss of stereochemical integrity. Interestingly, some of these products have not been described previously in optically pure form. As far as the regioselectivity of the oxirane opening is concerned, our previous experiments[11] conducted on styrene oxide (**1**) using ^{18}O-labeled water, have shown a high regioselectivity of the isotope incorporation—and therefore of the nucleophilic attack, which occurred at the less hindered carbon atom. The absolute configuration of the diols obtained from epoxides **2**−**7** indicates that, in all cases, the regioselectivity of the oxirane ring opening is similar, i.e., that the nucleophilic attack again takes place very preferentially at the methylene carbon atom.

Table 2. Biohydrolysis of *Para*-Substituted Styrene Oxide Derivatives Using *B. sulfurescens*

substr	yield of epoxide, % (reaction time, h)	ee of epoxide, % (abs conf)[a]	product	yield of diol, %	ee of diol, % (abs conf)[a]
1	34 (2)	98 (*R*)	**8**	45	83 (*R*)
2	30 (0.5)	>98 (*R*)	**9**	45	76 (*R*)
3	25 (2)	96 (*R*)	**10**	50	78 (*R*)
4	20 (24)	54 (*R*)	**11**	66	72 (*R*)
5	33 (2)	96 (*R*)	**12**	52	72 (*R*)
6	59 (24)	15 (*R*)	**13**	25	50 (*R*)
7	50 (24)	20 (*S*)	**14**	36	49 (*R*)

[a] See Table 1 for the determination of the absolute configurations.

Biohydrolysis Using *B. sulfurescens*. To the contrary of the results observed with *A. niger*, the data obtained by studying the biohydrolysis of the *para*-substituted styrene oxide derivatives (**2**−**7**) using whole cells of *B. sulfurescens* show a remarkable effect of the phenyl ring *para*-substitution (as compared to the nonsubstituted styrene oxide (**1**)) (Table 2). They indicate that substitution of the aromatic ring affects the reaction rate as well as the substrate enantioselectivity to an extent that seems to depend upon the electronic properties of the substituents. Thus, the *p*-CH_3 group (epoxide **2**), which is an electron-donating group (by hyperconjugation), caused a 4-fold increase of the reaction rate (as compared to that of **1**) without modification of the enantioselectivity. On the other hand, the weakly electron-withdrawing *p*-F (**3**) and *p*-Br (**5**) substituents did not cause any noticeable modification of either reaction rate or enantioselectivity. Surprisingly enough, substitution by a *p*-Cl group (**4**) led to a dramatic lowering of both reaction rate and enantioselectivity. At this stage of our study, we do not have any good explanation for this surprising fact: one could consider either the presence of two—or several—distinct enzymes or isoenzymes operating in this particular fungus and/or the influence of some permeation parameters which could be quite different for this particular substrate. Finally, the presence of the powerful cyano (**6**) or nitro (**7**) electron-withdrawing groups decreased significantly both reaction rate and enantioselectivity. Even more surprisingly, the *p*-NO_2 moiety caused a dramatic switch in the substrate enantioselectivity. Thus, predominant hydrolysis of the (*R*) enantiomer was observed in this case.

Mechanistic Studies. As emphasized, another aim of this work was to gain more information about the mechanism involved during these oxirane ring biohydrolyses. Interestingly, whereas a huge amount of work has been devoted to the study of mEH, almost no

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Table 3. Substituent Effects on the Kinetic Parameters of Hydrolysis of *Para*-Substituted Styrene Oxide by the Epoxide Hydrolase of *A. niger*

substrate	Km[a]	Vm[a]	10^3(Vm/Km)	σ_p
(±)-**1**	4.7 ± 0.8	116 ± 10	34	
(*R*)-**1**	4.3 ± 0.6	221 ± 17	51	0
(*S*)-**1**	9.9 ± 1.6	28 ± 2	3	
(±)-**2**	2.4 ± 0.3	104 ± 5	43	−0.13
(±)-**4**	1.1 ± 0.1	128 ± 3	117	0.24
(±)-**6**	1.8 ± 0.2	191 ± 10	106	0.67
(±)-**7**	1.0 ± 0.2	195 ± 10	195	
(*R*)-**7**	0.8 ± 0.1	238 ± 17	297	0.78
(*S*)-**7**	4.7 ± 1.9	38 ± 10	8	

[a] Km are expressed in mM, Vm in μm min^{-1} g^{-1} of protein.

information about the mechanism involved in microbial EH is presently available. Our previous studies,[11] conducted on **1**, led us to suggest the occurrence of a general base-catalyzed process (similar to the one proposed for mEH) in the case of *A. niger*, whereas for *B. sulfurescens* we considered that activation of the oxirane ring *via* protonation or hydrogen bonding had to be implied, as in the case of soluble EH. These hypotheses were essentially based on the regio- and stereoselectivities observed. In order to confirm these hypotheses, another appealing way was to determine the Hammet correlation parameters of these biohydrolyses, using our *para*-substituted aromatic epoxides. Unfortunately, because of the physical state of fungal whole-cell cultures, the substrate distribution into the culture medium was heterogeneous, some substrate being preferentially adsorbed on the cell surface. Thus, it was not possible, using this methodology, to obtain accurate and reproducible data to conclude as to the influence of the electron-donating or -withdrawing power of the *para* substituents on the reaction rates (Table 1 or Table 2). However, we could achieve this study without any experimental problem using a soluble protein preparation from *A. niger*.[12]

Mechanism Implied in the Fungus *A. niger*. Using such an enzymatic extract, the biohydrolysis of the racemic derivatives **1**, **2**, **4**, **6**, and **7** was carried out and their initial hydrolysis rates were measured as a function of the substrate concentration. The apparent kinetic parameters (Km and Vm) were calculated *via* a nonlinear regression of the Michaelis equation and are reported in Table 3. Plotting of the relative rates (VmX/VmH) logarithm *versus* the Hammet constant σ_p for each substituent is given in Figure 1. This shows that a rather linear relation (correlation coefficient $r = 0.985$) for the five substrates studied exists indeed, and the observed linear relation suggests that the same mechanism operates throughout the series. Furthermore, the positive slope observed can be associated with a developing negative charge in the transition state during biohydrolysis. In the case of an oxirane ring opening mechanism, an electron-withdrawing group located on the phenyl moiety would be expected to stabilize, in the transition state, the development of a negative charge on the incipient oxygen anion at the benzylic carbon. The small magnitude of ρ (+0.3), which reflects the importance of the charge development at the reactive center, indicates a weak polar character of the "early" transition state structure. Therefore, this result strongly suggests

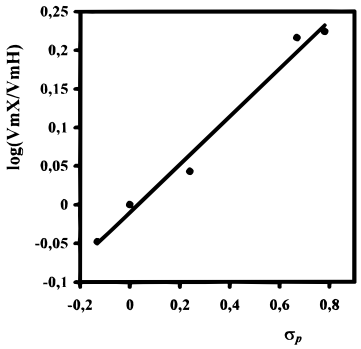

Figure 1. Hammet plot of log(Vmx/VmH) for biohydrolysis of (±)-*para*-substituted styrene oxide *vs* σ_p, using soluble enzyme extract of *A. niger* as biocatalyst.

that a nucleophilic mechanism was implied as the rate-determining step for the biohydrolysis of the epoxides studied. It should be noted that this conclusion is in agreement with the results previously reported by Blumenstein *et al.*[13] for the chemical base-catalyzed hydration (for the attack occurring at the methylene carbon) of the same aromatic epoxide derivatives, where a value of $\rho = +0.32$ was estimated. These results are also in accordance with a similar study previously carried out using rat liver mEH where a comparable correlation was obtained ($\rho = +0.2$) for substrates bearing an electron-withdrawing group.[14]

Some further interesting informations can be obtained from these kinetic measurements. Comparison of the various Km measurements shows a noticeable drop of these values when the electron-withdrawing power of the phenyl substituent increases, indicating that substrates with high σ_p show a better affinity for the enzyme. Furthermore, the specificity constant (Vm/Km) increases sharply with the σ_p value, showing a higher selectivity for these substrates (Table 3). Thus, this indicates that the observed *substrate selectivity* is the result of both higher affinity and higher catalytic rate.

Another valuable information about the mechanism leading to the observed *enantioselectivity* can be deduced from the kinetic parameters. Indeed, the Km and Vm values were determined separately for each enantiomer of **1** and **7**. For both these substrates, the (*R*) enantiomer showed a slightly lower Km (by a factor of 2.3 and 5.9, respectively) and a significantly higher Vm (7.9 and 6.2 times higher) than their (*S*) enantiomer. These results clearly indicate that the enantioselectivity is due to both a better affinity and a higher reaction rate for the (*R*) enantiomer. This may be due to a better positioning of the (*R*) enantiomer at the active site and/or to a higher stabilization of the transition state. The enantiomeric ratios *E*, estimated for **1** and **7** from the ratio of the Vm/Km of their enantiomers, were respectively 17 and 36. As reported in Table 1, these values are in good agreement with those obtained by the direct determination method using the conversion ratio and the ee of the remaining epoxide.[12]

Mechanism Implied in the Fungus *B. sulfurescens*. A similar study, aimed to confirm the fact that the intimate process involved with the fungus *B. sulfurescens* is different indeed, would have been obviously

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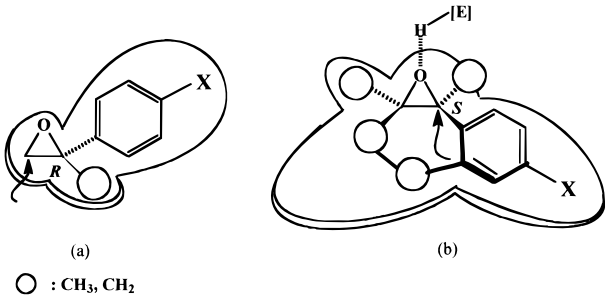
Scheme 2. Proposed Models for the Active Sites of Epoxide Hydrolases from (a) *A. niger* and (b) *B. sulfurescens*



very interesting. Unfortunately, we have not yet managed to prepare an active soluble enzyme extract from this fungus. Nevertheless, the results we have obtained using whole cells allow us to get some preliminary information about the mechanism implied. Thus, the results reported in Table 2 clearly show that the decrease of reaction rate is concomitant with the increase of the electron-withdrawing power of the *para* substituents. This observation is in accordance with an acid-catalyzed process in which some carbonium ion character would be created during the transition site. Another important observation, which confirms this hypothesis, is based on the regioselectivity of the oxirane opening. Indeed, one must notice that all the diols formed are, without exception, of (*R*) absolute configuration. Since the fast-reacting enantiomer is in most cases of (*S*) configuration, this implies an attack by the enzyme at the C(1) benzylic carbon atom, thus leading to inversion of configuration at this center. This regioselectivity is an accordance with the one previously observed during chemical acid-catalyzed opening of the same *para*-substituted styrene oxide derivatives.[13]

Active Site Topology. In a previous study,[15] the results we obtained using several aromatic epoxides bearing methyl substitution at the oxirane ring led us to propose an active site model for both EH involved in the two fungi employed in this study. For *A. niger*, we considered the existence of a lipophilic pocket located at the *right back side* of the active site (taking as a convention that the oxirane ring is oriented with the oxygen atom toward the top). The present results are in perfect agreement with this model, the phenyl ring of all the fast reacting (*R*)-enantiomers being located in the appropriate part of the space (Scheme 2). Obviously, positioning of the (*S*)-enantiomers into this model will be less favored. These results also lead to the conclusion that this pocket is able to accommodate bulky *para* substituents like cyano or nitro groups, since all these epoxides proved to be good substrates showing high reactivity and enantioselectivity.

In the case of *B. sulfurescens*, we proposed that the lipophilic pocket would be located at the right *front side*, the nucleophilic entity of the enzyme (presumably an aspartic residue) being situated beneath the benzylic carbon atom C(1). This model is supported by the fact that the fast-reacting enantiomers of the epoxides **1**−**6** are those of (*S*) absolute configuration. Again it appears that this pocket is able to accommodate the bulky *para* substituents born by the aromatic ring. As an exception, epoxide **7** (*p*-NO_2 group) does not fit into this model, since not only was the fast-reacting enantiomer shown to have the (*R*) configuration, but the nucleophilic attack of the oxirane ring also occurred preferentially at the less substituted C(2) carbon atom. This may be explained either (a) by a different positioning of this particular compound into the active site, due for instance to some specific bonding of the highly polar nitro group with some ionized peptide residues, or (b) by considering the existence of a second (or even several) EH in this fungus which would exhibit a higher catalytic power for this particular compound together with a reversed enantioselectivity.

Conclusion

In conclusion, these results show clearly that the two fungi *A. niger* and *B. sulfurescens* are able to achieve an enantioselective hydrolysis of several *para*-substituted aromatic epoxides. This allows us to obtain both the (*S*) and the (*R*) enantiomers of epoxides **1**−**6** in a high state of enantiomeric purity. Interestingly, these microbiological transformations can easily be carried out on a preparative scale, thus allowing production of several grams of products. Several of these epoxides (**3**,[16] **4**,[17] and **7**[18]) are known to be valuable chiral building blocks involved in the synthesis of compounds showing biological activities. Our results also confirm that the interesting enantiocomplementarity which we previously did observe during the biohydrolysis of styrene oxide (**1**) with these two fungi was not affected by the presence of a *para* substituent. Indeed, the residual epoxide antipodes were always of (*S*) absolute configuration with *A. niger*, whereas the ones of (*R*)-configuration were obtained using *B. sulfurescens*, except for epoxide **7** where the (*S*) enantiomer was less reactive. As far as the enzymatic mechanism is concerned, we have shown, using a Hammet correlation approach, that a general *base*-catalyzed process is very probably implied in the case of *A. niger*. In contrast, the reaction rates and regioselectivities observed using whole cells of *B. sulfurescens* strongly suggest the implication of a general *acido-basic* catalyzed mechanism. Work is in progress in our laboratory to order to explore the scope and limitations of these interesting biotransformations as well as to study the possibilities of building up biotechnological processes using such fungal enzyme extracts.

Experimental Section

General. The strain of *A. niger* used in this work is registered at the Museum d'Histoire Naturelle (Paris) under no. LCP 521 (Lab. de Cryptogamie, 12 rue Buffon, 75005 Paris, France) and *B. sulfurescens* was purchased at the ATCC collection under no. 7159. Corn steep liquor (CSL) is from Roquette SA. NMR spectra (^1H and ^{13}C) were recorded in $CDCl_3$ solution on a Bruker AC 250. Chemical shifts are reported in δ from TMS as internal standard. Optical rotation values were measured on a Perkin-Elmer 241C polarimeter at 589 nm. Vapor phase chromatography analyses were performed using either a 25 m capillary column (OV 1701) or a chiral column [heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-β-cyclodextrin]. Determination of the enantiomeric excesses of diols was performed after derivatization into their acetonides. HPLC analyses were carried out using an UV detector and a

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column (25.5/0.4 cm) filled with 5 μm silica gel using hexane−ethanol as eluent. Separation and purification of the products were 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) or by bulb-to-bulb distillation.

Synthesis of Substrates 2−7. (±)-*p*-Methylstyrene Oxide (2). This epoxide was obtained by epoxidation of the corresponding olefin using *m*-chloroperoxybenzoic acid under biphasic conditions (CH_2C1_2/phosphate buffer) for 5 h (86% yield) as described previously.[19] ^{1}H-NMR δ: 2.34 (s, 3H, CH_3); 2.78 (dd, 1H, H_{2cis}, J_{gem} = 5.4 Hz; J_{1-2cis} = 2.6 Hz); 3.12 (dd, lH, H_{2trans}, J_{gem} = 5.4 Hz, $J_{1-2trans}$ = 4.l Hz); 3.82 (dd, lH, H_l, J_{1-2cis} = 2.6 Hz, $J_{1-2trans}$ = 4.1 Hz); 7.2 (m, 4H_{arom}). ^{13}C-NMR δ: 21.4 (*p*-CH_3); 51.4 (C-2); 52.6 (C-1); 125.7; 129.6; 134.8; 138.2 (C-Ar).

(±)-*p*-Fluorostyrene Oxide (3). This epoxide was obtained using the same procedure as described for **2** (72% yield). ^{1}H-NMR δ: 2.76 (dd, 1H, H_{2cis}, J_{gem} = 5.3 Hz, J_{1-2cis} = 2.5 Hz); 3.14 (dd, lH, H_{2trans}, J_{gem} = 5.4 Hz, $J_{1-2trans}$ = 4.1 Hz); 3.14 (dd, 1H, H_l, J_{1-2cis} = 2.6 Hz, $J_{1-2trans}$ = 4.0 Hz); 6.9−7.1 (m, 2H_{arom}); 7.2−7.4 (m, 2H_{arom}). ^{13}C-NMR δ: 51.2 (C-2); 51.9 (C-l); 115.3−115.7 (d, C-Ar, J = 21.8 Hz); 127.1−127.2 (d, C-Ar, J = 8 Hz); 133.3 (C-Ar); 160.7−164.7 (d, C-Ar, J = 246 Hz).

(±)-*p*-Chlorostyrene Oxide (4). This epoxide[20] was obtained using the same procedure as described for **2** (70% yield). ^{1}H-NMR δ: 2.75 (dd, lH, H_{2cis}, J_{gem} = 5.5 Hz, J_{1-2cis} = 2.6 Hz); 3.15 (dd, 1H, H_{2trans}, J_{gem} = 5.4 Hz, $J_{1-2trans}$ = 4.1 Hz); 3.84 (dd, 1H, H_l, J_{1-2cis} = 2.6 Hz, $J_{1-2trans}$ = 4.0 Hz); 7.18−7.26 (m, 2H_{arom}); 7.30−7.34 (m, 2H_{arom}). ^{13}C-NMR δ: 51.3 (C-2); 51.8 (C-l); 126.8; 128.7; 133.9; 136.2; 136.2 (C-Ar).

(±)-*p*-Bromostyrene Oxide (5). This epoxide[23] was obtained using the same procedure as described for **2** (70% yield). ^{1}H-NMR δ: 2.74 (dd, 1H, H_{2cis}, J_{gem} = 5.5 Hz, J_{1-2cis} = 2.5 Hz); 3.14 (dd, 1H, H_{2trans}, J_{gem} = 5.4 Hz, $J_{1-2trans}$ = 4.1 Hz); 3.83 (dd, 1H, H_l, J_{1-2cis} = 2.6 Hz, $J_{1-2trans}$ = 4.1 Hz); 7.13−7.17 (d, 2H_{arom}); 7.45−7.48 (d, 2H_{arom}). ^{13}C-NMR δ: 51.2 (C-2); 51.8 (C-l); 122.0; 127.1; 131.6; 136.7 (C-Ar).

(±)-Cyanostyrene Oxide (6). To a stirred solution of 4 g (18 mmol) of trimethyloxosulfonium iodide in 50 mL of dry DMSO, placed under argon, was added 0.72 g (18 mmol) of sodium hydride (60% mineral oil dispersion). After the solution was stirred for 30 min (formation of the dimethyloxosulfonium methylide), a solution (2 g, 15 mmol) of *p*-cyanobenzaldehyde in 10 mL of DMSO was added and the reaction mixture stirred at room temperature for 2 h. After cooling and addition of water, the mixture was extracted with ether (three times). The combined extracts were washed with brine, dried over sodium sulfate, and evaporated to yield 2.5 g of crude product. Purification by flash chromatography (pentane/ether) yielded 1.66 g (76% yield) of **6** as a white solid, mp 34 °C, lit.[21] mp 38 °C. ^{1}H-NMR δ: 2.76 (dd, lH, H_{2cis}, J_{gem} = 5.5 Hz, J_{1-2cis} = 2.4 Hz); 3.2 (dd, lH, H_{2trans}, J_{gem} = 5.4 Hz, $J_{1-2trans}$ = 4.2 Hz); 3.9 (dd, lH, H_l, J_{1-2cis} = 2.5 Hz, $J_{1-2trans}$ = 4.0 Hz). ^{13}C-NMR δ: 51.5 (C-l and C-2); 111.7 (CN); 118.6; 126.1; 132.2; 143.3 (C-Ar).

(±)-*p*-Nitrostyrene Oxide (7). Racemic **7** was prepared by cyclization of the corresponding bromohydrin formed by reduction of ω-bromopropiophenone. To a stirred solution of 5 g (20 mmol) of ω-bromo-4-nitroacetophenone in 50 mL of MeOH, cooled in an ice bath, was added 0.83 g (22 mmol) of sodium borohydride. After the ice bath was removed stirring was continued for 3 h, 2.76 g (20 mmol) of potassium carbonate was added in the same flask. After 20 h of stirring, 30 mL of water was added and the mixture was extracted with ether (three times), washed twice with brine, and dried over magnesium sulfate. Evaporation of the solvent yielded 5.7 g of crude product. Purification by flash chromatography (pentane/ether) yielded 3 g (91% yield) of **7** as a yellow solid, mp 84−85 °C. ^{1}H-NMR δ: 2.79 (dd, 1H, H_{2cis}, J_{gem} = 5.5 Hz, J_{1-2cis} = 2.4 Hz); 3.24 (dd, lH, H_{2trans}, J_{gem} = 5.5 Hz, $J_{1-2trans}$ = 4.2 Hz); 3.97 (dd, lH, H_l, J_{1-2cis} = 2.4 Hz, $J_{1-2trans}$ = 4.0 Hz); 7.45 (d, 2H_{arom}, J = 8.7 Hz); 8.22 (d, 2H_{arom}, J = 8.8 Hz). ^{13}C-NMR δ: 51.5 (C-l); 51.7 (C-2); 123.8; 126.3; 145.3; 147.9 (C-Ar).

Synthesis of Racemic Diols 9−14. These diols were prepared as follows by hydrolysis of the corresponding epoxides. To 1 g of epoxide dissolved in a mixture of THF (50 mL) and water (10 mL) were added two drops of concentrated sulfuric acid. After 24 h of stirring, the hydrolysis was complete (checked by TLC) and the solution was neutralized by addition of saturated $NaHCO_3$ solution and extraction with ether (three times). Evaporation of the washed (saturated salt solution) and dried ($MgSO_4$) extracts yielded a crude residue which was further purified by flash chromatography.

(±)-9: yield 65%, mp 70 °C. ^{1}H-NMR δ: 2.32 (s, 3H, CH_3); 3.49 (m, 4H, (2OH+H_2)); 4.73 (s large, lH, H_l); 7.11−7.25 (m, 4H_{arom}). ^{13}C-NMR δ: 21.0 (C-3); 67.9 (C-2); 74.5 (C-l); 126.0; 129.1; 137.4; 137.4 (C-Ar). Anal. Calcd for $C_9H_{12}O_2$ (152.19): C, 71.03; H, 7.95. Found: C, 70.65; H, 7.81.

(±)-10: yield 73%, mp 56−57 °C, lit.[22] mp 53 °C. ^{1}H-NMR δ: 3.20 (s large, 2H, OH); 3.63 (m, 2H, H_2); 4.77 (dd, 1H, H_l, J_{1-2} = 3.2 Hz and 8.11 Hz); 7.0 (m, 2H_{arom}); 7.3 (m, 2H_{arom}). ^{13}C NMR δ: 68.1 (C-2); 74.2 (C-1); 115.3 and 115.7 (d, C-Ar, J = 21.4 Hz); 127.8 and 127.9 (d, C-Ar, J = 8.2 Hz); 136.2; 160.6 and 164.5 (d, C-Ar, J = 246 Hz).

(±)-11: yield 75%, mp 83−84 °C. ^{1}H-NMR δ: 2.89 (s large, 1 H, OH); 3.37 (s large, 1H, OH); 3.57 (dd, 1H, H_2, J_{1-2} = 8.2 Hz, J_{gem} = 11.3 Hz); 3.70 (dd, lH, H_2, J_{1-2} = 3.2 Hz, J_{gem} = 11.4 Hz); 4.75 (dd, lH, H_l, J_{1-2} = 3.3 Hz, J_{1-2} = 8.1 Hz); 7.21−7.40 (m, 4H_{arom}). ^{13}C-NMR δ: 67.9 (C-2); 74.0 (C-l); 127.4; 128.7; 133.7; 138.9 (C-Ar). Anal. Calcd for $C_8H_9O_2Cl$ (217.06): C, 55.80; H, 5.27. Found: C, 55.65; H, 5.25.

(±)-12: yield 78%, mp 103−104 °C. ^{1}H-NMR δ: 3.58 (m, 2H, H_2); 3.92 (t, 1H, OH, J_{OH-H2} = 5.7 Hz); 4.48 (d, lH, OH, J_{OH-Hl} = 3.9 Hz); 4.71 (m, lH, H_l); 7.36 (d, 2H_{arom}, J = 8.4 Hz); 7.48 (d, 2H_{arom}, J = 8.5 Hz). ^{13}C-NMR δ: 68.7 (C-2); 74.6 (C-1); 121.2; 129.3; 131.8; 143.2 (C-Ar). Anal. Calcd for $C_8H_9O_2$-Br (217.06): C, 44.3; H, 4.2. Found: C, 44.38; H, 4.22.

(±)-13: yield 71%, viscous oil. ^{1}H-NMR δ: 2.25 (t, 1H, OH, J = 5.5 Hz); 2.91 (d, 1H, OH, J = 3.4 Hz); 3.63 (m, 1H, H_2); 3.80 (m, 1H, H_2); 4.89 (m, 1H, H_1); 7.50 (d, 2H_{arom}, J = 8.4 Hz); 7.66 (d, 2H_{arom}, J = 8.3 Hz). ^{13}C-NMR δ: 67.7 (C-2); 73.9 (C-1); 111.6 (CN); 118.7; 126.8; 132.3; 145.9 (C-Ar). Anal. Calcd for $C_9H_9O_2N$ (163.17): C, 66.25; H, 5.56; N, 8.58. Found: C, 66.21; H, 5.52; N, 8.45.

(±)-14: yield 60%, mp 78 °C. ^{1}H-NMR δ: 3.55 (m, 2H, H_2); 3.93 (t, 1H, OH, J = 6.0 Hz); 4.65 (d, lH, OH, J = 4.0 Hz); 4.79 (m, 1H, H_l); 7.62 (d, 2H_{arom}, J = 8.5 Hz); 8.11 (d, 2H_{arom}, J = 8.8 Hz). ^{13}C-NMR δ: 68.4 (C-2);74.5 (C-l); 123.8; 128.2; 146.3; l5l.6 (C-Ar).

General Procedure for Derivatization of the Diols into the Corresponding Acetonides. The corresponding diol (5−10 mg) and 2,2-dimethoxypropane (100 μL) were stirred in the presence of a catalytic amount of TsOH for 30 min. After neutralization with a saturated $NaHCO_3$ solution, the reaction mixture was extracted with ether, dried over $MgSO_4$, and directly analyzed by chiral GLC.

Biohydrolysis of Styrene Oxide Derivatives (1−7) with *A. niger* and *B. sulfurescens*. General Procedure. The fungal strains were cultured in a 2 L fermentor as previously described.[3] 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. The appropriate racemic styrene oxide derivative (1 g) was added to the medium as a solution in ethanol (10 mL). The course of the bioconversion was followed by withdrawing two aliquots (2 mL) at regular time intervals. One of these samples was extracted with pentane (2 mL) and subjected to direct chiral GC analysis, which allowed for determination of the ee of the

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residual epoxide. After saturation with NaCl, the second sample was extracted with ether (2 mL), and after purification through silica gel (using a prepacked silica gel Extrasep column) and derivatization to the acetonide, the ees of the diols were determined by chiral GC analysis. The bioconversion was stopped by addition of ether (500 mL). The medium was filtered off, and the fungal cake was separately extracted two times with ether (2 × 200 mL). 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 the products was achieved by flash chromatography or/and by bulb-to-bulb distillation. Preparative yields and ees of residual epoxides and formed diol are given in Tables 1 and 2. All these bioconversions were performed several times, in particular in the case of the chloro derivative **4** using *B. sulfurescens* as biocatalyst.

Optical rotations and chiral GLC data of the isolated products after biohydrolysis are as follows:

(1*S*)-**2**, $[\alpha]^{20}_D$ +23.0 (*c* 0.61, $CHCl_3$); (1*R*)-**2**, $[\alpha]^{20}_D$ +25.5 (*c* 1.3, PhH); lit.[19] $[\alpha]^{20}_D$ (+) (PhH) for (1*R*)-**2** [70 °C, t_R(1*R*) 16.0 min and t_R(1*S*) 17.5 min].

(1*S*)-**3**, $[\alpha]^{20}_D$ +15.6 (*c* 0.97, $CHCl_3$); (1*R*)-**3**, $[\alpha]^{20}_D$ −15.1 (*c* 0.45, $CHCl_3$); lit.[16] $[\alpha]^{20}_D$ +16.7 (*c* 1.03, $CHCl_3$) for (1*S*)-**3** [80 °C, t_R(1*R*) 6.2 min and t_R(1*S*) 7.3 min].

(1*S*)-**4**, $[\alpha]^{20}_D$ +19.3 (*c* 1.16, $CHCl_3$) [100 °C, t_R(1*R*) 8.6 min and t_R(1*S*) 9.2 min].

(1*S*)-**5**, $[\alpha]^{20}_D$ +13.6 (*c* 1.46 $CHCl_3$); (1*R*)-**5**, $[\alpha]^{20}_D$ −12.9 (*c* 1.04, $CHCl_3$) [110 °C, t_R(1*R*) 9.9 min and t_R(1*S*) 10.4 min].

(1*S*)-**6**, $[\alpha]^{20}_D$ +7.3 (*c* 1.46, $CHCl_3$) [120 °C t_R(1*R*) 11.2 min and t_R(1*S*) 11.8 min].

(1*S*)-**7**, $[\alpha]^{20}_D$ +37.6 (*c* 1.99, $CHCl_3$); lit.[21] $[\alpha]^{20}_D$ +37.4 (*c* 1.99, $CHCl_3$) for (1*S*)-**7** [130 °C, t_R(1*R*) 14.2 min and t_R(1*S*) 15.1 min].

(1*R*)-**9**, $[\alpha]^{20}_D$ −44.7 (*c* 0.75, $CHCl_3$) using *A. niger*; $[\alpha]^{20}_D$ −50 (*c* 0.87, $CHCl_3$) using *B. sulfurescens* [100 °C, t_R(1*R*) 14.1 min and t_R(1*S*) 14.6 min].

(1*R*)-**10**, $[\alpha]^{20}_D$ −49 (*c* 1.07, $CHCl_3$) using *A. niger*; $[\alpha]^{20}_D$ −49.2 (*c* 1.01, $CHCl_3$) using *B. sulfurescens* [100 °C, t_R(1*R*) 8.2 min and t_R(1*S*) 8.5 min].

(1*R*)-**11**, $[\alpha]^{20}_D$ −31.4 (*c* 0.97, EtOH) using *A. niger*; lit.[20] $[\alpha]^{20}_D$ −27.60 (*c* 0.96, EtOH) for (1*R*) [110 °C t_R(1*R*) 16.6 min and t_R(1*S*) 17.0 min].

(1*R*)-**12**, $[\alpha]^{20}_D$ −37.2 (*c* 1.03, $CHCl_3$) using *A. niger*; $[\alpha]^{20}_D$ −36.5 (*c* 1.00, $CHCl_3$) using *B. sulfurescens* [110 °C, t_R(1*R*) 29.5 min and t_R(1*S*) 30.4 min].

(1*R*)-**13**, $[\alpha]^{20}_D$ −17.1 (*c* 0.53, EtOH) using *A. niger* [120 °C t_R(1*R*) 32.0 min and t_R(1*S*) 33.0 min].

(1*R*)-**14**, $[\alpha]^{20}_D$ −15.0 (*c* 0.71, MeOH) using *A. niger*; lit.[21] $[\alpha]^{20}_D$ −20.0 (*c* 1.15, MeOH); for (1*R*)-**14** [130 °C t_R(1*R*) 36.8 min and t_R(1*S*) 37.8 min].

Biohydrolyses by Soluble Enzyme Extract from *A. niger*. All enzymatic reactions were carried out at 25 °C in a minivial (3 mL) containing phosphate buffer (1.84 mL, 0.1 M, pH 7) and 8−53 mg of protein/mL.[12] Reactions were initiated by addition of 0.16 mL of the epoxide in ethanol (range of 0.125−24 mM). The course of the biohydrolysis was followed by withdrawing samples (0.25 mL) at different time intervals. After saturating with NaCl, samples were extracted with CH_2Cl_2 (0.5 mL). The produced diols were quantified by HPLC analysis using a specific calibration curve for each diol. Initial rates of hydration were calculated at each initial substrate concentration from the amounts of diol formed. The apparent kinetic parameters (Km and Vm) were calculated by nonlinear regression of the Michaelis' equation (see Table 3).

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