Articles

Microbiological Transformations. 38. Clues to the Involvement of a General Acid Activation during Hydrolysis of Para-Substituted Styrene Oxides by a Soluble Epoxide Hydrolase from *Syncephalastrum racemosum*

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In the course of this work, we have determined the regioselectivity as well as the rate of biohydrolysis of various para-substituted styrene oxide derivatives catalyzed by a new epoxide hydrolase activity found in the soluble cell extract of the fungus *Syncephalastrum racemosum*. We have observed that this regioselectivity switched progressively from the benzylic C_α carbon atom to the terminal C_β carbon atom depending upon the electronic character of the para substituent. Hammett plotting of the ratio of water incorporation at both the benzylic and terminal carbon atoms, i.e., log α/β versus σ, gave linear relationships for the two (*R*)- and (*S*)-epoxide enantiomers with slopes $\rho_{\alpha/\beta} = -2.07$ and -1.35, respectively. Apparent kinetic constants K_m and V_{max} were determined for the biohydrolysis of the enantiomers of *R* absolute configuration, which were the better substrates. Hammett correlation was investigated for V_{max}/K_m for the reaction on both the C_α and C_β carbon atoms. Log $\alpha V_{max}/K_m$ vs σ gave a linear relationship with a slope $\rho_{\alpha V_{max}/K_m} = -1.8$, suggesting that, in the case of these enzyme/substrate couples, the rate-determining step is the oxirane ring cleavage. These results give, for the first time, interesting clues to the fact that a general acid activation of the epoxide is very probably involved in a concerted process together with its nucleophilic attack.

Epoxide hydrolases (EHs) catalyze the addition of water onto an epoxide ring, thus affording the corresponding vicinal diol. Due to their essential involvement in human detoxification processes, these enzymes have been mostly studied in mammals up to the recent years.[1] They were shown to exist predominantly in two different forms, i.e., microsomal (mEH) or cytosolic (soluble) (sEH) forms. However, similar EH activities have been identified recently in organisms as diverse as bacteria,[2] yeasts,[3] fungi,[4] plants,[5] and insects,[6] and these enzymes may thus be regarded as being ubiquitous in nature. Interestingly, they have been described in recent years to be very efficient biocatalysts for achieving the resolution of racemic epoxides and were shown to allow the preparation of several epoxides and diols thus obtained with good to excellent enantiomeric purity.[7] Therefore, these cofactor-independent enzymes can be regarded as being, as were lipases about 20 years ago, very promising new tools in the organic chemist's tool box, in particular for performing asymmetric synthesis. Thus, the study of their properties, i.e., of their catalytic behavior, molecular mechanism, and three-dimensional structure, is becoming a topic of utmost importance.

The mechanism implied in these enzymes has only been recently elicited, at least for murine mEH and sEH. It was shown that the reaction occurs via a two step process involving a trans-specific nucleophilic attack of the oxirane ring by an aspartic residue, which forms a covalent enzyme−substrate ester intermediate in a first step[8] (Scheme 1). This ester intermediate is subsequently hydrolyzed, in a second step, by a water molecule activated by an histidine−aspartate pair. The accurate understanding of the results obtained by performing such reactions is, however, complicated by the existence of two

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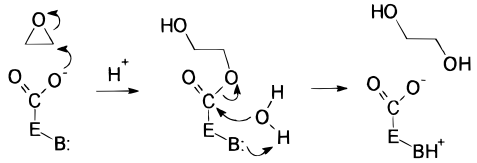
Scheme 1

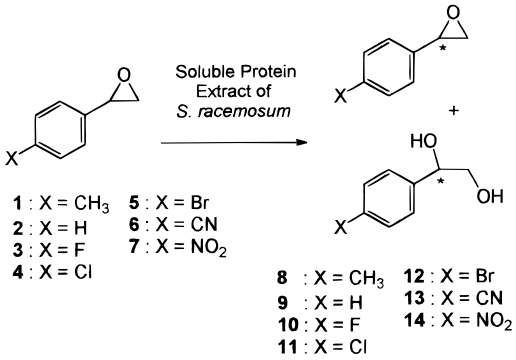


possible reaction centers at the oxirane ring. Thus, the aspartic residue may attack following two different regioselectivities, thus leading to (formal) incorporation of water at one—or at the other—carbon atom. Furthermore, this regioselectivity may be shared in different proportions on the two carbon atoms and may also be different for each of the two enantiomers.

On the basis of the absolute configurations obtained for the formed diol, as well as from ^{18}O-labeling experiments, it is now well established that, for mammalian microsomal EH, the aspartic acid attack occurs highly preferentially at the *less* hindered carbon atom for both enantiomers.[9] Furthermore, series of styrene oxides and of *cis*-stilbene oxide derivatives, bearing aryl substituents of increasing electronic-withdrawing power, have been studied with the (partially purified) rat mEH.[10] From the observed variation of reaction rates, it has been concluded, via Hammett coefficient plotting, that (a) the rate-determining step was a nucleophilic attack and (b) a free carbocation ion was not involved in the mechanism. Interestingly, we have ourselves recently found results leading to the same conclusion with the sEH (crude soluble enzymatic extract) from the fungus *Aspergillus niger*, which catalyzes also the water incorporation at the less hindered carbon atom.[11] On the other hand, Bellucci et al. have observed that the hydrolysis of *trans*-3-bromo-1,2-epoxycyclohexane, catalyzed by rabbit mEH (crude microsomes), led to *trans*-2,3-cyclohexanol, presumably via intramolecular trapping of the transient oxyanion.[12] This was interpreted by the authors as evidence that the nucleophilic attack occurred at the oxirane ring without apparent electrophilic activation/protonation of the epoxide.

Unexpectedly, an opposite regioselectivity, i.e., indicating that the water molecule is partially (or even totally) incorporated at the *more* hindered (benzylic) center, has been observed for the opening of aryl epoxides by rabbit-[13] and murine-soluble[14] EH, as well as by whole cells of the fungus *Beauveria bassiana* (formerly *B. sulfurescens*).[15] On the basis of these regioselectivities, it has been suggested that, in these cases and in contrast to the above-cited examples, the oxirane ring cleavage would involve an electrophilic activation (via either protonation or hydrogen bonding) of the epoxide in the transition state.[13,15] However, direct experimental proofs that confirm this hypothesis, or kinetic studies similar to those carried out with mammalian mEH or *A. niger* EH, are not presently available. In the course of our present work, we have focused on a new fungal EH activity from the fungus *Syncephalastrum racemosum* MUCL 28766[16] (crude soluble protein extract), which, in contrast to what was observed with the fungus *A. niger*, led to a highly preferential attack at the *more* hindered carbon atom for styrene oxide itself. We report here our results about the regioselectivity of the water molecule incorporation, as well as the kinetic study of the hydrolysis of a series of differently para-substituted styrene oxides by this new biocatalyst.

Scheme 2



Results and Discussion

The various racemic para-substituted styrene oxides **1**−**7** (Scheme 2), prepared as previously described,[11] were submitted to biohydrolysis by a crude soluble enzymatic extract obtained from the fungus *Syncephalastrum racemosum* MUCL 28766. This extract was prepared from the soluble fraction of the cell extracts, similar to the one obtained from *A. niger*.[17]

The enantiomeric ratios (*E* values) for the reactions with X = CH_3, H, Cl, and NO_2 have been determined according to Sih et al.[18] Unfortunately, since these *E* values were very low (respectively, 1.1, 8, 5, and 1.6), this soluble enzymatic extract appeared not to be a good catalyst for the kinetic resolution of these racemic para-substituted styrene oxides. Interestingly, however, for all the epoxides studied, the residual enantiomer was always of *S* absolute configuration, which means that the preferred antipode was always of *R* configuration, whereas the absolute configuration of the diol formed in excess switched from *S* to *R*, respectively, for the CH_3, H, F, Cl, and Br, CN, NO_2 groups. This in fact indicates that the nature of the substituent born by the aromatic ring did strongly affect the regioselectivity of the hydrolysis. Thus, the water molecule was preferentially incorporated at the *more* hindered carbon atom for the substrate bearing weak electron-donating (CH_3) or -withdrawing groups, whereas incorporation at the *less* substituted

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Table 1. Regioselectivity of the Biohydrolysis of the *R* and *S* Enantiomers of Para-Substituted Styrene Oxides Using a Soluble Protein Extract of *S. racemosum*

	substituent						
	CH_3	H	F	CL	Br	CN	NO_2
σ_p[a]	−0.14	0	0.15	0.24	0.26	0.70	0.81

	substrate						
	(*R*)-**1**	(*R*)-**2**	(*R*)-**3**	(*R*)-**4**	(*R*)-**5**	(*R*)-**6**	(*R*)-**7**
α (% diol (*S*)[b])	93	90	87	67	56	29	11
β (% diol (*R*)[b])	7	10	13	33	44	71	89

	substrate						
	(*S*)-**1**	(*S*)-**2**	(*S*)-**3**	(*S*)-**4**	(*S*)-**5**	(*S*)-**6**	(*S*)-**7**
α (% diol (*R*)[b])	93	53	43	73	69	53	34
β (% diol (*S*)[b])	7	47	57	27	31	47	66

[a] σ_p values are from: March, J. *Advanced Organic Chemistry*, 3rd ed.; Wiley: New York, 1985. [b] The absolute configurations have been determined previously. See ref 10.

carbon atom was preferred for more powerful electron-withdrawing substituents.

Regioselectivity of the Hydrolysis. To get a more accurate explanation of the reasons governing these differences, and to determine the regioselectivity involved for each one of the two antipodes, we performed separately, for all the substrates, the biohydrolysis of both enantiomers (Table 1). This determination could be achieved in a straightforward way, since the percentage of diol of opposite absolute configuration formed (as compared to the one of the starting substrate) corresponds to the percentage of attack at the stereogenic benzylic carbon atom (α attack), whereas the percentage of diol showing the same absolute configuration as the starting epoxide reflects the percentage of attack at the terminal carbon atom (*β* attack). The data obtained show a remarkable effect of the nature of the para-substituent on the regioselectivity, and this effect seems to depend essentially upon the electronic power of the substituent. Indeed, for hydrolysis of both (*R*)-**1** and (*S*)-**1** (bearing the electron-donating methyl group), the attack was highly preferential (93%) at the benzylic carbon atom C_α, whereas this percentage decreased when the electron-withdrawing character of the substituent group increases. Thus, for the powerful nitro electron-withdrawing group, the attack became highly preferential at the terminal C_β carbon atom, i.e., 89% for (*R*)-**7** and 66% for (*S*)-**7**. Plotting of the α/*β* ratio log versus the Hammett constant σ_p for each substituent is given in Figure 1. It can be seen that, for hydrolysis of the (*R*)-epoxides, a rather good linear relationship exists (correlation coefficient $r^2 = 0.96$) with a negative slope of $\rho_{\alpha/\beta} = -2.07$. This correlation indicates that, for this series of substrates, the variation of regioselectivity essentially depends on the electronic properties of the substituent.

Similarly, for the hydrolysis of almost all the (*S*)-epoxides, a rather good linear relationship (correlation coefficient $r^2 = 0.94$) with a different, but still negative, slope of $\rho_{\alpha/\beta} = -1.35$ was also obtained. As previously, the variation of regioselectivity seems to depend, for the major part, upon the electronic effect of the substituent. However, for the *S* enantiomer of styrene oxide itself, as well as for its *p*-fluoro-substituted derivative (*S*)-**3**, the data point repeatedly lied outside this correlation line. At the present time, we do not have any satisfactory explanation for this observation. One suggestion could

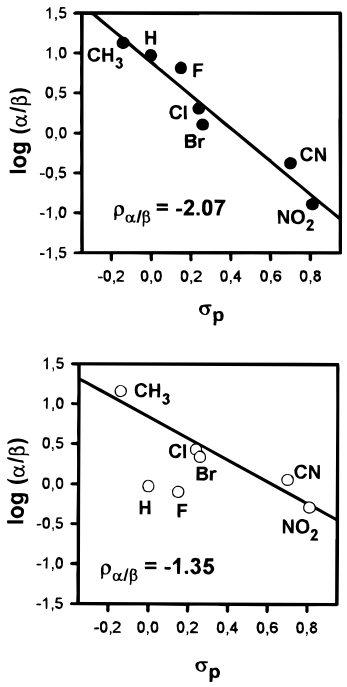


Figure 1. Hammett plot of log(α/*β*) versus σ_p for the hydrolysis of (*R*)-*p*-substituted styrene oxide (●) and (*S*)-*p*-substituted styrene oxide (○) catalyzed by a soluble enzymatic extract of *S. racemosum*.

be that a lack of steric hindrance at the para position could allow the substrate to adopt a different positioning into the enzymatic active site. In this context, it is interesting to emphasize that the fact that these correlations appear to be rather linear is a good indication that only one EH should be involved in this crude enzyme preparation.

Kinetic Study. This surprising gradual switch in regioselectivity related to para substitution lies in sharp contrast with the results we have previously obtained with the *A. niger* EH, and led us to search for more information about the rates of these hydrolyses. To avoid the problems due to competition between the two enantiomers of a racemic substrate, and to the difference of regioselectivity observed in some cases for each one of these enantiomers, we performed this kinetic study using the faster hydrolyzed *R* enantiomer of epoxides **1**−**7** in enantiopure form. Also, due to the low solubility of these epoxides in water, the experiments were carried out using 10% of DMSO as a cosolvent.[17] The initial hydrolysis rates were measured as a function of the substrate concentration, up to their limit of solubility (i.e., 30, 37, 31, 23, 7, 36, and 5 mM, respectively, for epoxides **1**−**7**). The apparent kinetic parameters (K_m and V_{max}) were calculated via a nonlinear regression of the Michaelis−Menten equation and are reported in Table 2.

It appears as a general trend that, when the electron-withdrawing character of the substituent group increases, the maximal enzymatic rate (V_{max}) and the specificity constant (V_{max}/K_m) decrease (respectively, by a factor of 46 and 10 going from the electron-donating methyl group to the powerful electron-withdrawing nitro group). So, the gradual switch in regioselectivity related to para-substitution is combined with the V_{max} and the V_{max}/K_m decrease (Scheme 3).

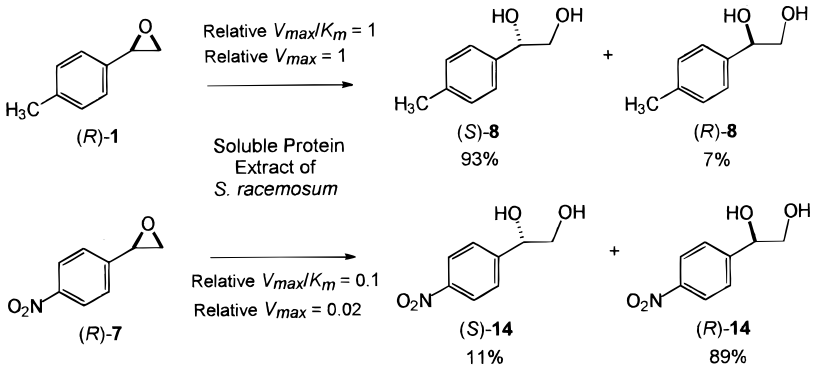
It is well documented that the interpretation of Hammett-type studies in enzymatic reactions may be com-

Table 2. Substituent Effects on the Apparent Kinetic Parameters of the Hydrolysis of Para-Substituted Styrene Oxides Catalyzed by a Soluble Protein Extract of *S. racemosum*[e]

	substrate						
	(*R*)-**1**	(*R*)-**2** l	(*R*)-**3**	(*R*)-**4**	(*R*)-**5**	(*R*)-**6**	(*R*)-**7**
K_m[a]	57 ± 6	100 ± 19	25 ± 5	17 ± 2	20 ± 9	29.5 ± 1.5	12.0 ± 1.2
V_{max}[a]	106 ± 8	57 ± 8	30 ± 4	17 ± 1	15 ± 5	9.2 ± 0.3	2.3 ± 0.2
V_{max}/K_m[b]	1.84± 0.06	0.57± 0.02	1.20± 0.13	0.99± 0.06	0.75± 0.08	0.31± 0.01	0.19± 0.005
V_{max}/K_m[c]	$r^2 > 0.99$	$r^2 > 0.99$	ND[d]	$r^2 > 0.99$	$r^2 > 0.99$	$r^2 > 0.99$	$r^2 = 0.97$

[a] K_m are expressed in mm, V_{max} in μmol·min^{-1}·g^{-1} of protein. Obtained from the substrate dependence of initial velocities by fitting the equation $v_i = a([S]/([S] + b)$, with $a = V_{max}$ and $b = K_m$. [b] Obtained from the substrate dependence of initial velocities by fitting the equation $v_i = a(b[S]/([S] + b)$, with $a = V_{max}/K_m$ and $b = K_m$. [c] Obtained from the substrate dependence of initial velocities for $[S] < K_m/10$ by fitting the equation $v_i = a[S]$, with $a = V_{max}/K_m$. [d] Not determined. [e] The obtained K_m values, which were in the range of 12−100 mM, were of the same magnitude (or higher) than the solubility limit of the epoxides **1**−**7**. Consequently, the K_m values have been obtained by fitting the Michaelis−Menten equation only with relatively high standard errors. However, the standard errors for the V_{max} and V_{max}/K_m values thus obtained were better. Furthermore, the V_{max}/K_m values calculated as the slopes of the linear correlations between the initial velocities and [S] for $[S] < K_m/10$ (for three different values of [S]), i.e., at low substrate concentrations when the enzyme is largely unbound, gave values similar to those previously obtained (differences between 4 and 24%). We therefore consider that the accuracy of these values was reasonably good.

Scheme 3



plicated by theoretical difficulties.[19] Indeed, the observed V_{max} may represent different chemical events or the combination of different mechanistic steps and may, furthermore, be altered by nonproductive binding, strain, or induced fit. However, all these artifacts are canceled out for the V_{max}/K_m value, and this parameter may thus be considered as being relevant to the rate-limiting transition state, even if it still contains binding energy terms. We therefore examined the substituent effect on the V_{max}/K_m values as well as, because of the possible addition (of water) to either C_α and C_β carbon atoms, the substituent effect for each of these two reaction sites. Hammett plotting of log $\alpha V_{max}/K_m$ as well as of log $\beta V_{max}/K_m$ were performed as functions of the σ_p value of each substituent, as shown in Figure 2.

Our results indicate that, for the benzylic carbon atom (C_α), a good linear relationship exists for the $\alpha V_{max}/K_m$ values, showing substantial negative slopes, i.e., $\rho_{\alpha V_{max}/K_m} = -1.8$ (correlation coefficient $r^2 = 0.88$). On the basis of these results, one can deduce some information on the rate-limiting step as well as on the intimate mechanism of the reaction (see below). Also, despite the use of a crude protein extract, this again strongly suggests that only one EH was operating. On the other hand, the magnitude of the substituent effect on the $\beta V_{max}/K_m$ values was much smaller, and due to the insufficient accuracy of the V_{max}/K_m value determination, no conclusion could be drawn from these results.

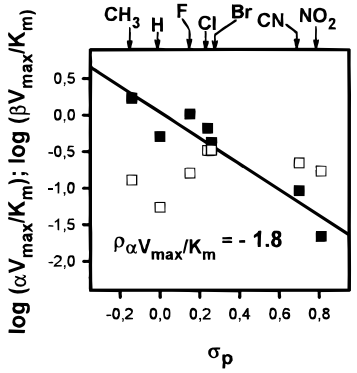


Figure 2. Hammett plot of log(αV_{max}) (●), log(βV_{max}) (○), log($\alpha V_{max}/K_m$) (■), and log($\beta V_{max}/K_m$) (□) versus σ_p for the hydrolysis of (*R*)-*p*-substituted styrene oxide catalyzed by a soluble enzymatic extract of *S. racemosum*. V_{max}/K_m values have been obtained from the substrate dependence of initial velocities by fitting the equation $v_i = a(b[S]/([S] + b)$, with $a = V_{max}/K_m$ and $b = K_m$.

Rate-Limiting Step. As mentioned above, it has been shown that for mammalian EHs the reaction occurs via a two-step process involving a covalent enzyme−substrate ester intermediate. Furthermore, using presteady and steady-state kinetic experiments, Tzeng et al. have concluded recently that, in the rat mEH-catalyzed hydration of glycidyl 4-nitrobenzoates, the rate-limiting factor was the second step, i.e., hydrolysis of the enzyme−substrate ester intermediate.[20] Surprisingly, the negative sign of the $\rho_{\alpha V_{max}}$ value we have obtained for the *R*

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enantiomers of **1**−**7**, together with its important magnitude of −2.4 (correlation coefficient 0.94), indicate that in our case the rate-limiting step cannot be hydrolysis of an enzyme−substrate ester intermediate, whatever mechanism is implied (acid or base catalysis). Indeed, these values are not compatible with those obtained for such (chemical) base and acid-catalyzed hydrolyses of para-substituted benzyl acetate derivatives (which can be considered as being analogues of the hypothetical enzyme−substrate ester intermediate formed when the nucleophilic attack of the enzyme occurs at the benzylic carbon atom C_α). Indeed, these have been plotted versus their σ_p Hammett coefficient, leading to ρ values of +0.743 and −0.054, respectively.[21] On the other hand, the $\rho_{\alpha/\beta}$ and $\rho_{\alpha V_{max}}$ values we have obtained (−2.07 and −2.4, respectively) are consistent with the Hammett correlation slopes calculated for the regioselectivity[22−24] as well as for the rates[23−25] of nucleophilic oxirane opening reactions cited in the literature. For example, the hydroxide-catalyzed hydrolysis of para-substituted styrene oxides derivatives was described to afford $\rho_{\alpha/\beta}$ and ρ_α values of, respectively, −1.1 and −0.9,[23] and the nucleophilic attack of benzylamine in ethanol led to a $\rho_{\alpha/\beta} = -2.02$ and a $\rho_\alpha = -1.15$.[24] Thus, our kinetic values, together with the observed regioselectivities, strongly suggest that, to the contrary of the result obtained by Tzeng et al. with rat mEH,[20] the rate-limiting reaction very probably is a nucleophilic attack at the oxirane ring in the case of the epoxide hydrolase studied here.

Mechanism of the Oxirane Ring-Opening Step. In theory, two limiting types of mechanism may operate for the oxirane cleavage: (a) a base-catalyzed nucleophilic event, without apparent activation of the oxirane ring, and (b) an acid-catalyzed event, implying an electrophilic activation of the oxirane ring (by hydrogen-bonding or protonation for example) *concerted* with the nucleophilic attack.

The negative sign, as well as the significant magnitude of the $\rho_{\alpha V_{max}/K_m}$ value (−1.8) we have obtained using the enzymatic extract from *S. racemosum*, strongly suggest the occurrence of an electron demand (i.e., the development of a positive charge) at the benzylic carbon atom. In other words, this suggests that, in the course of this process, the C−O bond breaking is more advanced than the ester−enzyme bond formation in the transition state and, therefore, that an *electrophilic activation* of the epoxide must be occurring. Thus, a "push-pull" mechanism, i.e., a *general acid activation concerted with a nucleophilic attack of the oxirane ring*, in which the "pull" step would be predominant, seems to be operating in this case. Consequently, the major factor influencing the regioselectivity of the oxirane ring nucleophilic attack should be the electronic stabilization of this incipient positively charged carbon atom implied in the transition state. Nevertheless, protonation of the epoxide oxygen atom by a free hydronium ion may be eliminated because the magnitude of $\rho_{\alpha V_{max}/K_m}$ (−1.8) is significantly lower than the one obtained for the acid hydrolysis of para-substituted styrene oxides ($\rho_\alpha = -4.2$).[23]

Conclusion

We have studied in this work the biohydrolysis of various racemic or enantiopure para-substituted styrene oxide derivatives using a crude enzymatic extract prepared from the fungus *S. racemosum*. This led us to observe (a) that, in contrast to previously described examples with several mammalian mEH as well as of a fungal (*A. niger*) EH, the regioselectivity of these reactions is directed toward the *more substituted* carbon atom for substrates bearing weak electron-donating or -withdrawing groups, (b) that a striking progressive switch of regioselectivity was observed when the electron-withdrawing power increased, and (c) that the values of V_{max} and V_{max}/K_m decreased when the electron-withdrawing power increased.

Analysis of these results via Hammett relationships yields a consistent indication to the existence, in this case, of a positive charge in the oxirane cleavage rate-determining transition state. Thus, this enzymatic mechanism seems to be best described by a *concerted process implying a general acid activation* of the oxirane ring, together with a nucleophilic attack. Interestingly, this mechanism thus appears to be very probably different from the one presumed to be operating for such microsomal enzymes. Interestingly, this difference of mechanism is reminiscent of the proteases, for which there are the divergent general baselike (e.g., chymotrypsin) and general acid-like (e.g., pepsin) mechanisms. Although this conclusion must still be taken with caution, because of the use of a crude enzyme and of the lack of any absolute proof, we consider this explanation to be the most reasonable in view of the overall results we have obtained. To the best of our knowledge, this is the first time that such experimental outcomes point to the highly probable occurrence of such a mechanism for an epoxide hydrolase. However, since other explanations, such as variable substrate positioning into the active site, can of course not be totally ruled out for the moment, further work is in progress in our laboratory for gaining additional experimental clues to support this hypothesis.

Experimental Section

General Methods. The strain of *S. racemosum* used in this work was purchased at the MUCL collection (no. 28766). Corn steep liquor (CSL) is from Roquette SA. ^{1}H NMR spectra were recorded in $CDCl_3$ solution on a Bruker AC 250. Optical rotation values were measured on a Perkin-Elmer 241C polarimeter at 589 nm. The absolute configurations of epoxides and diols were assigned via GC analysis on a chiral stationary phase by comparison with previously described data.[11] Vapor-phase chromatography analyses were performed using a chiral 25 m capillary column [heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-*β*-cyclodextrin]. Determination of the ee of the diols were performed after derivatization into their acetonide as described previously.[11] HPLC analyses were carried out using an UV detector and a C18 reversed-phase column (250 × 4.6 mm, 5 μm) using water−acetonitrile as eluent.

Synthesis of Epoxides 1−7 and Diols 8−14. Racemic epoxides **1**−**7** and diols **8**−**14** have been previously synthesized in our laboratory[11] (except **2** and **9** purchased from Janssen). Optically pure epoxides (*S*)-**2** and (*R*)-**2** were purchased from Fluka. (*S*)-Epoxides **3**−**7** have been previously obtained with ee > 98% in our laboratory by bioconversion of the racemates using the whole cells of *A. niger*.[11] (*R*)-**7** (ee > 99%) has been previously synthesized in our laboratory by cyclization of the optically pure diol (*R*)-**14** obtained by biohydrolysis of (±)-**7**,

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using an enzymatic extract from *A. niger*, followed by recrystallization.[26] The epoxides (*S*)-**1**, (*R*)-**1**, and (*R*)-**3**−**6** were obtained following a two-step synthesis: (i) catalytic asymmetric dihydroxylation of the corresponding olefin using commercial AD-mix-α or AD-mix-β (Aldrich) according to Sharpless,[27] and (ii) cyclization of the diol according to Kolb and Sharpless.[28] The procedure for the cyclization of activated diols was used for the diols **8** and **10**, substituted by the CH_3 and F groups. The procedure used for the inactivated diols was followed for diols **11**−**13**. The diols and the epoxides were obtained, respectively, with yields of 73−94% and 73−86% after purification by bulb-to-bulb distillation. The ^{1}H NMR spectra of all these compounds were identical to those previously described.[11] Optical rotation and chiral GC analysis of the isolated products are as follows: (*R*)-**1**, +27 (*c* 0.98, PhH, ee 95%); (*S*)-**1**, $[\alpha]^{22}_D$ −27 (*c* 1.01, PhH, ee 95%) [lit.[11] $[\alpha]^{20}_D$ +25.5 (*c* 1.3, PhH, ee > 98%) for (*R*)-**1**]; (*R*)-**3**, $[\alpha]^{20}_D$ −17 (*c* 1.03, $CHCl_3$, ee 97%) [lit.[11] $[\alpha]^{20}_D$ −15.1 (*c* 0.45, $CHCl_3$, ee 96%)]; (*R*)-**4**, $[\alpha]^{20}_D$ −24 (*c* 1.08, $CHCl_3$, ee 97%) [lit.[11] $[\alpha]^{20}_D$ +19.3 (*c* 1.16, $CHCl_3$, ee > 98%) for (*S*)-**4**]; (*R*)-**5**, $[\alpha]^{20}_D$ −22 (*c* 1.00, $CHCl_3$, ee 98%) [lit.[11] $[\alpha]^{20}_D$ −12.9 (*c* 1.04, $CHCl_3$, ee > 98%)]; (*R*)-**6**, $[\alpha]^{20}_D$ −6 (*c* 1.26, EtOH, ee 96%) [lit.[11] $[\alpha]^{20}_D$ +7.3 (*c* 1.09, EtOH, ee > 98%) for (*S*)-**6**]; (*R*)-**8**, $[\alpha]^{23}_D$ −67 (*c* 0.90, $CHCl_3$, ee > 97%) [lit.[11] $[\alpha]^{20}_D$ −50 (*c* 0.87, $CHCl_3$, ee 76%)]; (*R*)-**10**, $[\alpha]^{23}_D$ −63 (*c* 1.06, $CHCl_3$, ee > 98%) [lit.[11] $[\alpha]^{20}_D$ −49 (*c* 1.07, $CHCl_3$, ee 81%)]; (*R*)-**11**, $[\alpha]^{23}_D$ −60 (*c* 1.00, $CHCl_3$, ee > 98%) [lit.[11] $[\alpha]^{20}_D$ −52.1 (*c* 1.03, $CHCl_3$, ee 79%)]; (*R*)-**12**, $[\alpha]^{23}_D$ −47 (*c* 1.02, $CHCl_3$, ee > 98%) [lit.[11] $[\alpha]^{20}_D$ −37.2 (*c* 1.03, $CHCl_3$, ee 79%)]; (*R*)-**13** $[\alpha]^{23}_D$ −23 (*c* 0.80, EtOH, ee > 98%) [lit.[11] $[\alpha]^{20}_D$ −17.1 (*c* 0.53, EtOH, ee 76%)].

Enzyme Preparation. *S. racemosum* was grown in a 2 L fermentor (SETRIC) containing 1.4 L of culture medium (20 g of CSL, 10 g of glucose in 1 L of tap water). Before sterilization (20 min at 115 °C), 0.28 g of Pluronic PE 8100 (BASF) and 70 μL of antifoam silicone 426R (PROLABO) were added to prevent overflowing during the growth. The medium was maintained at 27 °C, stirred at 700 rpm, and aerated at 20 ± 5 L/h. The broth was inoculated with 5 mL of a suspension of mycelium and spores in physiologic water (NaCl 0.9%) from an agar slant of *S. racemosum*. After incubation for 70 h, the mycelium (dry mass 9.8 g) was filtered off, washed with water, and suspended in 700 mL of 20 mM ammonium acetate buffer pH 7.0 containing 1 mM cysteine and 1 mM EDTA. After addition of 1.75 mL of a 200 mM solution of phenylmethylsulfonyl fluoride in acetone, the cells were broken by two passages through a Cell Disrupt (CELL-D) at a pressure of 1800 bar. Unbroken cells and cells debris were separated by centrifugation (9600*g* for 30 min at 4 °C) and discarded. After addition of solid ammonium sulfate at 25% of the saturation to the supernatant, the solution was stirred for 30 min and centrifuged at 9600*g* for 30 min. After having discarded insoluble particles on the surface of the supernatant, ammonium sulfate at 80% of the saturation was added to this one, and this solution was again stirred for 30 min and centrifuged at 9600*g* for 30 min. The pellet was dissolved in 25 mL of 10 mM ammonium acetate buffer pH 7.0 containing 0.1 mM cysteine, 0.1 mM EDTA, and desalted by passing on a Sephadex G25 column (6 × 24 cm) equilibrated with the same buffer. The protein solution obtained was lyophilized and stored at 4 °C.

Biohydrolysis of Racemic Para-Substituted Styrene Oxides 1−7 Using the Enzymatic Extract of *S. racemosum*. All bioconversions were carried out at 27 °C in a stirred (750 rpm) minivial (3 mL) containing 0.9 mL of a pH 8.0 sodium phosphate buffer (0.1 M) solution and 14.6 mg of protein. Reactions were initiated by addition of 0.1 mL of the racemic epoxide in DMSO (final concentration in the reaction medium of 3.9−4.9 mM). After 3−7.5 h of reaction, 200 μL of the medium was extracted with pentane (100 μL) and subjected to direct chiral GC analysis, which allowed us to determine the ee and the absolute configuration of the residual epoxide. After addition of 600 μL of MeOH to a second 600 μL sample to stop the reaction and extraction of the residual epoxide with 600 μL of pentane, the produced diol was quantified by HPLC analysis using an external calibration to determine the conversion rate *c*. The enantiomeric ratio *E* was then calculated using the Sih equation $E = \ln[(1 - c)(1 - ees)]/\ln[(1 - c)(1 + ees)]$.[18] The ee and the absolute configuration of the diol were further determined by chiral GC analysis after saturation with NaCl, ether extraction of diol, purification through silica gel, and derivatization.

Biohydrolysis of Optically Pure Para-Substituted Styrene Oxides 1−7 Using the Enzymatic Extract of *S. racemosum*. The biohydrolyses were carried out as described above in the presence of 10% DMSO for enzymatic extract concentrations of 20−50 mg/mL and for substrate concentrations of 3.5−4.5 mM. For each biohydrolysis, at two different conversion rates, between 15 min and 2 h of the reaction, the ee of the formed diols were analyzed as described above. Under these conditions, the nonenzymatic hydrolysis was insignificant.

Determination of the Kinetic Parameters. All reactions were carried out at 27 °C in a stirred (750 rpm) Eppendorf tube (1.5 mL) containing 1−4 mg of enzymatic extract dissolved in 360 μL of a pH 8.0 sodium phosphate buffer (0.1 M) solution. Reactions were initiated by addition of 40 μL of the (*R*)-epoxide in DMSO. The final concentration ranges of the epoxides were between 0.6 mM and their maximal solubility in the reaction medium, i.e., 30, 37, 31, 23, 7, 36, 5 mM, respectively, for the epoxides **1**−**7**. After 10 min, the reactions were stopped by addition of 400 μL of CH_3CN. After centrifugation (3 min, 10000 rpm), the produced diols were quantified by HPLC analysis of the medium using an external calibration for each diol. For all these reactions, the conversion was below 5%. Initial rates of hydration were calculated at each initial substrate concentration from the amounts of diol formed. The apparent kinetic parameters (K_m, V_{max} and V_{max}/K_m) were calculated by nonlinear regression of the Michaelis−Menten equation (see Table 2), using the commercial Jandel software Sigmaplot (Marquardt−Levenberg algorithm).

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