Microbiological Transformations 43. Epoxide Hydrolases as Tools for the Synthesis of Enantiopure α-Methylstyrene Oxides: A New and Efficient Synthesis of (S)-Ibuprofen

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Received October 19, 1998

Biohydrolysis of various α-methylstyrene oxide derivatives, differently substituted at the aromatic ring, was investigated using 10 epoxide hydrolases from different origins. Our results indicate that the enantioselectivity of these biohydrolyses strongly depends on the nature of the enzyme and of the substituent. Using some of these enzymes, this approach allows to prepare these epoxides in high optical purity. The potentiality to perform efficient preparative-scale resolution using such a biocatalyst was illustrated by the four-step synthesis of (S)-ibuprofen, a nonsteroidal antiinflammatory drug and household pain killer, one of the top-ten drugs sold worldwide. Using a combined chemoenzymatic strategy, we were thus able to set up a four-step enantioconvergent procedure allowing for the synthesis of this compound in optically pure form and with a 47% overall yield, including the resolution process, due to a possible recycling of the formed diol via chemical racemisation.

Introduction

A hot topic in synthetic organic chemistry is the preparation of optically pure epoxides as well as of their corresponding vicinal diols.[1] These compounds are highly versatile chiral synthons that are often used to achieve the synthesis of biologically active molecules. One of the emerging approaches to obtain such optically pure building blocks is the enantioselective hydrolysis of a racemic epoxide catalyzed by a "new" type of hydrolytic enzymes, i.e., epoxide hydrolases (EHs). This reaction will afford the corresponding vicinal diol together with the unreacted epoxide enantiomer, both of these products being, in certain cases, obtained in good to excellent enantiomeric purity.[2] Only recently, it became clear that these biocatalysts are ubiquitous in nature, since they have been detected in organisms as diverse as mammals,[3] plants,[4] or microorganisms.[5] As a consequence, it is now likely that a suitable enzyme can be found for achieving the enantioselective hydrolysis of either type of epoxide, and the screening of various EH for synthetically interesting chiral epoxides is therefore of paramount importance. Furthermore, it appears that, whereas the large preparative scale use of some of these enzymes, for instance, the once from mammalian origin, is severely hampered due to their low availability, several such biocatalysts are now easily available in large scale from microorganisms.[2]

In our laboratory, we have previously detected different EHs from fungal origin, and we have developed various studies aimed at exploring the potentialities of these enzymes on different types of substrates.[6] In this paper, we report the enantioselective hydrolysis of seven differently substituted α-methylstyrene oxide derivatives with 10 different soluble EHs. The choice of these substrates stemmed from the fact that they constitute interesting chiral building blocks allowing for the synthesis of various α-arylpropionic acid derivatives, a major class of nonsteroidal antiinflammatory drugs and household painkillers. Among these, ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid) and naproxen (2-(4-methoxynaphthyl)propanoic acid) are probably the best known and are sold worldwide in large quantities. These molecules are chiral, and it was shown that it is the *S* enantiomer that is responsible for the desired therapeutic effect.[7] Despite this fact, ibuprofen is currently administered as the racemate, although it has been demonstrated that its *R* enantiomer accumulates in fatty tissue as a glycerol ester, whose long-term effects are not

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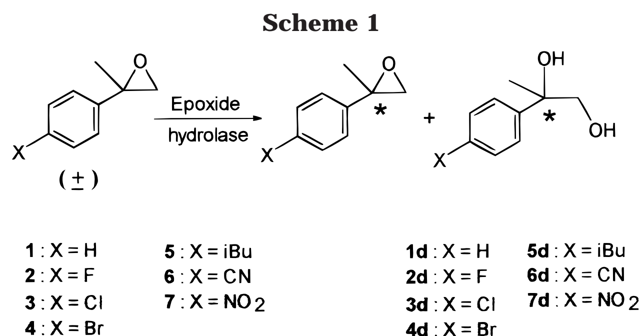
10.1021/jo982101+ CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/24/1999

Scheme 1



known.[8] Therefore, it is desirable to develop synthetic methods allowing for the preparation of these drugs in an enantiopure form. We illustrate in this work the synthetic potentialities of EH enzymes by describing a four-step synthesis of ibuprofen, using a combined chemoenzymatic strategy. This allowed us to prepare this drug in enantiopure form from racemic 4-isobutyl-α-methylstyrene oxide **5** with an overall yield as high as 47%, including the resolution process.

Results and Discussion

The various para-substituted α-methylstyrene oxide derivatives that we have studied are presented in Scheme 1. Their structures have been ascertained on the base of previously described syntheses described in the literature (see the Experimental Section). Theoretically, a nucleophile (or an enzyme) can attack an epoxide at either of the two carbon atoms of the oxirane ring, and it is obvious that this regioselectivity depends on the nature of the nucleophile, the structure of the epoxide, the reaction conditions, and for biocatalytic reactions, the nature of the enzyme. In this last case, it is to be stressed that both the regioselectivity and the kinetic parameters can be different from one enantiomer to the other, thus leading to quite puzzling results.[9] For instance, owing to a difference of regioselectivity for each enantiomer, hydrolysis of a racemic substrate can lead to one single, i.e. enantiopure, enantiomer of the diol product, following a so-called "enantioconvergent" process. However, in the case of the substrates studied in this work, the α-carbon atom is a severely sterically hindered tertiary carbon atom, and consequently, most nucleophiles should exclusively attack at the β-carbon atom.

In the course of this study, we used 10 different enzymatic extracts known to possess epoxide hydrolase activity, i.e., seven fungal strains and three overexpressed enzymes from various origins. The seven fungal EHs were obtained as a freeze-dried crude enzymatic extract from, respectively, the following: *Aspergillus niger* LCP 521; *Aspergillus terreus* CBS 116-46; *Chaetomium globosum* LCP 679; *Syncephalastrum racemosum* MUCL 28766; *Mortierella isabellina* ATCC 42613; *Cunninghamella elegans* LCP 1543 and *Beauveria bassiana* ATCC 7159 (previously named *Beauveria sulfurescens*). The other three soluble EHs are from human,[10] potato,[11] and rat[12] origin and were obtained as overexpressed proteins from baculovirus infected insect cells. They were obtained from these cells as freeze-dried extracts with a relatively high enzyme content (approximately 5%).

In the context of this work, our first aim was to develop a "matrix-type" approach allowing us to explore the various possibilities offered by the combination of each of the substrates with each of the specific enzymes. Our preliminary results indicated that all the substrates were hydrolyzed by the various enzymes, although with different rates. For each extract, the enzymatic activity was determined at three different pH values, i.e., pH 7.2, 8.0, and 9.0, relative to *p*-chlorostyrene oxide chosen as a reference substrate. The results are presented in Table 1. Generally, the enzymatic activity was strongly pH dependent. The highest values were always observed at pH 7.2, and increasing to pH 9 often led to a noticeable decrease of activity. The only exception was for *rat*-EH, whose activity stayed almost constant whatever the pH. Table 1 also indicates the rates of spontaneous chemical hydrolysis as a function of the pH. At pH 7.2, it was relatively important but slowed going to pH 9. Thus, the best compromise to achieve our experiments appeared to be at pH 8.0. Indeed, at this pH, the loss of enzymatic activity was still acceptable and the spontaneous hydrolysis stayed moderate. Owing to these results, more accurate studies using the extracts from *C. globosum, M. isabellina*, *C. echinulata*, and *B. bassiana* were not pursued due to low enzymatic activity.

The other extracts were, however, more promising and were therefore studied in more details, as indicated in Table 2. For each substrate/enzyme extract combination, five parameters were determined, i.e., (a) the specific activity; (b) the value of the observed enantioselectivity ratio E[13] (it is to be stressed that, because of the occurrence of chemical hydrolysis, the "real" *E* value of each enzyme must be somewhat higher); (c) the configuration of the recovered (slow reacting) epoxide enantiomer; (d) the enzymatic activity observed with this specific substrate at a 8 mM concentration (except for **7**, where it was measured at 4 mM because of its low solubility) (these activity values have been corrected for nonenantioselective spontaneous chemical hydrolysis, which occurred at a different extend for each substrate, as indicated in Table 2); and (e) the amount of enzymatic activity (expressed in units and calculated using **3** as reference substrate) used to perform this specific biohydrolysis. In certain cases, chemical hydrolysis being significant relative to the enzymatic hydrolysis, the amount of biocatalyst used to perform the reaction was enhanced in order to obtain a reasonable enzymatic/chemical hydrolysis ratio. This ratio is indicated in footnote f.

As a first result, it appears that in all cases, the diol with an absolute configuration opposite to the one of the residual epoxide was obtained. This indicates that, as expected, the nucleophilic attack always preferentially (if not exclusively) occurred at the C-β carbon atom of the oxirane ring. The structures of the formed diols have been ascertained either by comparison with literature

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Table 1. Enzyme Extract Activity[a,b] and Spontaneous Hydrolysis[c] of *rac*-3 as a Function of pH

pH	*A. n.*	*A. t.*	*C. g.*	*S. r.*	*M. i.*	*C. e.*	*B. b.*	*h*-EH	*p*-EH	*r*-EH	chem hyd
7.2	23.3	5.8	0.9	2.9	1.95	0.4	0.2	19.8	3.15	4.9	*3.7*
8.0	19.2	5.9	0.7	2.8	1.8	0.2	0.1	15.8	3.2	4.8	*1.2*
9.0	7.9	4.4	0.4	1.4	1.2	0.06	0.1	9.3	2.6	4.9	*0.6*

[a] Experimental conditions: $c_{extract}$ 5–20 mg/mL; $c_{substrate}$ 8.0 mM; 0.2 M Tris/HCl; t^0 27 °C; 1% DMF. [b] Enzymatic activity in nmol/min/mg per mL. [c] Chemical hydrolysis in nmol/min per mL.

Table 2. Biocatalyzed Hydrolysis of Epoxides 1–7 Using Various Epoxide Hydrolases

enzymatic extract	specific activity[a]	H 1	F 2	Cl 3	Br 4	iBu 5	CN 6	NO₂ 7
A. n.	19.2	**3**[b] (*S*)[c]	10 (*S*)	15 (*S*)	20 (*S*)	20 (*S*)	26 (*S*)	39 (*S*)
		9.6[d]	19.1	19.2	14.2	0.6	19.7	24.8
		192[e] (*9*)[f]	576 (*39*)	96 (*80*)	192 (*183*)	384 (*25*)	96 (*462*)	96 (*1859*)
A. t.	5.9	**2** (*S*)	3 (*S*)	1.3 (*R*)	3 (*R*)	8 (*R*)	1.4 (*R*)	1.1 (*S*)
		7.5	12.7	5.9	4.1	0.7	7.4	11.7
		118 (*14*)	177 (*26*)	119 (*99*)	118 (*106*)	177 (*42*)	118 (*693*)	118 (*3499*)
S. r.	2.8	**3** (*R*)	3 (*R*)	3 (*R*)	2 (*R*)	2 (*S*)	2 (*S*)	2 (*S*)
		0.4	0.6	2.8	2.4	0.12	1.3	1
		70 (*1*)	70 (*1*)	56 (*46*)	42 (*46*)	84 (*8*)	56 (*124*)	56 (*299*)
M. i.	1.8	**3** (*S*)	2 (*S*)	3 (*S*)	3.4(*S*)	3 (*S*)	5 (*R*)	4 (*R*)
		1.1	1.5	1.8	1.6	0.3	1.4	2.0
		54 (*3*)	54 (*3*)	36 (*30*)	36 (*42*)	54 (*22*)	54 (*199*)	36 (*599*)
h-EH	15.8	**8** (*R*)	5 (*R*)	1.5 (*S*)	2.5 (*S*)	1.1 (*R*)	4 (*R*)	2 (*R*)
		22.4	32.3	15.8	9.9	1.5	4.9	4.8
		158 (*21*)	158 (*22*)	158 (*132*)	158 (*128*)	474 (*98*)	158 (*230*)	79 (*359*)
p-EH	3.2	**3** (*S*)	2 (*S*)	2 (*S*)	2 (*S*)	3 (*R*)	7 (*R*)	3 (*R*)
		2.7	2.9	3.2	1.7	0.3	1.5	1.1
		64 (*5*)	64 (*4*)	64 (*53*)	64 (*48*)	96 (*17*)	32 (*68*)	32 (*169*)
r-EH	4.8	**6** (*R*)	14 (*R*)	1.4 (*R*)	1.4 (*S*)	2 (*R*)	10 (*R*)	4 (*R*)
		10.1	25.7	4.8	3.2	0.8	4.2	2.8
		96 (*19*)	96 (*35*)	*48* (*40*)	48 (*42*)	144 (*51*)	48 (*199*)	48 (*419*)
spont hydr (nmol/min)		10.7	14.7	1.2	0.77	0.47	0.21	0.13

[a] Specific enzymatic activity in nmol/min/mg, calculated using **3** as reference substrate. Experimental conditions: $c_{extract}$ (5–60 mg/mL); $c_{substrate}$ = 8 mM (4 mM for **7**); pH 8.0 (pH 9 for **5**) (0.2 M Tris/HCl); T = 27 °C; 1% DMF. [b] Calculated E value. [c] Absolute configuration of the residual epoxide. [d] Enzymatic activity in nmol/min/mg. [e] Amount (in units) of enzyme used to perform this biohydrolysis. [f] Ratio of enzymatic hydrolysis versus chemical hydrolysis.

data when available or, for the new diols **2d**, **6d**, and **7d**, by elemental analysis, accurate NMR spectroscopy, and exact mass determination as indicated in the Experimental Section.

Second, it can be observed that, for all substrates, the best results were obtained with the *A. niger* enzymatic extract, which in general afforded good enantioselectivity as well as high enzymatic activity. This extract showed a clear preference for hydrolyzing the *R* enantiomer, thus affording the recovered epoxide of *S* absolute configuration. Interestingly, the various *E* values clearly depend on the nature of the para substituent. As a rule of thumb, it seems that increasing the size of the substituent resulted in an appreciable increase of enantioselectivity, e.g., from $E = 3$ for **1** (R = H) to $E = 39$ for **7** (R = NO_2).

Good activities were also observed for the enzyme extract of *A. terreus*. However, the enantioselectivities were relatively low, the best *E* value ($E = 8$) being observed for **5**. Interestingly, this biohydrolysis was enantiocomplementary to the one performed with the *A. niger* extract for some of the substrates; i.e., the residual epoxide was of *R* configuration for **3**–**6**. In the case of the enzymatic extract obtained from *S. racemosum,* it is interesting to compare the results obtained in this study with those we have previously described using various para-substituted styrene oxide derivatives as substrates.[14] Indeed, we had observed that (a) although the enantioselectivity was rather low, hydrolysis led always to the residual (*S*) epoxide and (b) the regioselectivity of the oxirane opening switched progressively from the benzylic Cα carbon atom to the terminal carbon atom depending upon the electronic character of the para substituent. The results obtained in the present work show that (a) the presence of a methyl group at Cα does not improve the enantioselectivity of the reaction (E = 2–3), and (b) to the contrary of our previous observation, no regioselectivity switch was observed, the attack occurring always preferentially at the terminal carbon atom, whatever the nature of the para substituent. This lack of regioselectivity switch is best explained by the steric hindrance at Cα introduced by the additional methyl group. Interestingly in this case, the enantioselectivity, i.e., the choice for the fastest hydrolyzed substrate, switched from the *S* (epoxides **1**–**4**) to the *R* (epoxides **5**–**7**) absolute configuration depending on the nature of the aromatic substituent, thus leading to the formation of either the (*S*)-diol (for **1d–4d)** or the (*R*)-diol (for **5d–7d)** respectively.

Finally, the enantioselectivities observed with the three overexpressed enzymes (*human*-EH*, potato*-EH, and *rat*-EH) did vary from low to moderate. However, for the substrates showing reasonable enantioselectivities ($E > 5$), the residual enantiomer was always of the *R* configuration, and in this respect, these enzymes again appear to be enantiocomplementary to the *A. niger* extract. The best enantioselectivities ($E = 10$ for **6** and $E = 14$ for **2**) were observed with the *rat liver* EH, and for the latter substrate, it was better than for all the other extracts. Generally, the activities with the *potato* EH were moderate, but the activities with *human* EH and

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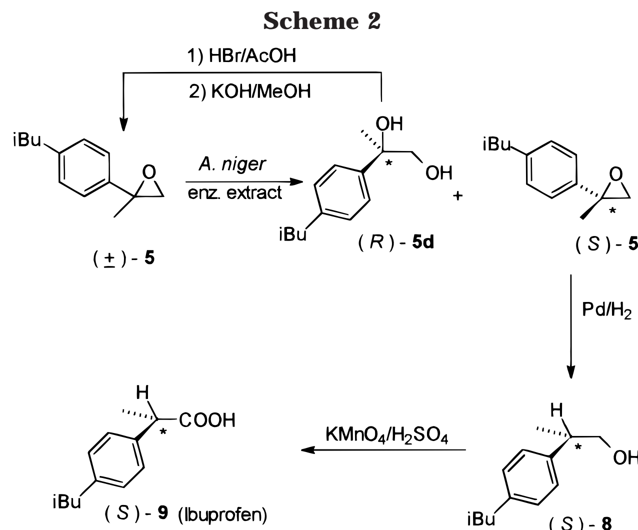
rat liver EH were rather good. However, it should be kept in mind that these enzymatic extracts were obtained from genetically engineered organisms (thus affording overexpression) and therefore contain relatively large quantities of EH (up to 5% enzyme), whereas this is not the case for the enzyme prepared from the wild-type strain, which has been shown to only contain about 0.1% enzyme for *A. niger*, for example.[15] This indicates that the specific activity of the overexpressed enzymes toward these substrates is quite low as compared to the *A. niger* EH.

Assignment of the Absolute Configuration of the Epoxides and Diols. The absolute configuration of epoxide **1**[6b] and of diol **1d**[16] has been determined previously. The chloro- and bromo-substituted diols **3d** and **4d** were dehalogenated by treatment with sodium in ethanol,[17] thus affording the corresponding diol **1d** of known absolute configuration. Each of these diols could also be cyclized into their corresponding epoxide **3** or **4** (with retention of configuration), thus attributing to their absolute configuration. The absolute configuration of **5** (and therefore of **5d**) was proven by its transformation into known (*S*)-ibuprofene (see below). Until now, we have not been able to unequivocally prove the absolute configuration of the epoxides and diols **2**, **6**, and **7**. Therefore, these were tentatively assigned on the basis of three convergent features: (a) Their elution order on our chiral GC column (column I). Indeed, we observed previously that the (*R*)-epoxides from various para-substituted styrene oxide derivatives (of known absolute configuration) always exhibited the shortest retention time on this column.[6c] (b) Very probably, the *A. niger* enzyme should preferentially hydrolyze the *R* enantiomer. Indeed, in our hands, this enzyme always showed a high *R* enantiomer preference for 1-monosubstituted epoxides. (c) Both of these features appear to be confirmed for epoxides **1**, **3**, and **4** of known configuration (see above). Thus, a similar behavior of the other three epoxides seems reasonable and interestingly fits indeed with the GC elution order.

Synthesis of (*S*)-Ibuprofen 9. As pointed out previously, ibuprofen is one of the top-ten drugs sold worldwide, and it has been shown that only its *S* enantiomer is endowed with the desired biological activity. Despite this fact, it is presently sold as a racemate, and we therefore were interested in setting up a procedure allowing for the specific preparation of the biologically active enantiomer. One of the possible strategies was to achieve the enantioselective hydrolysis of racemic 4-isobutyl-α-methylstyrene oxide **5**, using an enzyme that would specifically hydrolyze the undesired (*R*)-**5**, and to further transform the thus-obtained (*S*)-**5** enantiopure epoxide into (*S*)-ibuprofen **9**, using classical chemical synthesis.

Examination of Table 2 indicates that the *A. niger* extract gives the best result, since the *E* value is quite reasonable ($E = 20$) and the residual epoxide has the desired *S* configuration. To set up the most efficient experimental conditions, small-scale trials were further performed, using this fungal extract, by varying different parameters. This led us to perform this biohydrolysis at 4 °C, which allowed us to both enhance enzyme stability

Scheme 2



and decrease spontaneous hydrolysis of the substrate. At this temperature, a 2- to 3-fold larger amount of epoxide could be resolved as compared to 27 °C. Thus, a preparative-scale experiment was carried out using 1.5 g (7.9 mmol) of racemic **5** in 30 mL buffer solution. This corresponds to a “concentration” of about 50 g/L (285 mM) of substrate, which in fact formed a two-phase system still allowing us to efficiently achieve this biohydrolysis, as we have previously described.[18] The ee of the unreacted epoxide was followed by chiral GC analysis, and when it reached a value higher than 95%, the reaction was stopped by extracting the remaining epoxide with pentane. The formed diol was further extracted from the reaction medium with ether and purified by column chromatography, leading to a 55% yield of (*R*)-**5d** (0.91 g; 4.38 mmol) showing a 70% ee (Scheme 2).

Crude (*S*)-**5** was directly converted into the corresponding alcohol (*S*)-**8** by catalytic hydrogenation. Preliminary experiments aimed at exploring this reduction indicated that the ee of the product was strongly dependent upon the reaction conditions used (temperature, solvent, and nature of the Pd catalyst). We obtained excellent results (less than 1% loss of optical purity) when performing this reduction at 0 °C, using *N*-methylformamide as a solvent and pure palladium (powder) as catalyst (submicron/Aldrich). Using these optimized experimental conditions, the reduction was shown to occur without any loss of stereochemical integrity, leading to the corresponding alcohol (*S*)-**8**. This could be easily purified by flash chromatography, affording 0.53 g of product (2.76 mmol, 35% overall yield for both the chiral resolution and the catalytic hydrogenation). Subsequently, (*S*)-**8** was oxidized with $KMnO_4/H_2SO_4$, leading to (*S*)-ibuprofen **9** in 76% yield (0.43 g, 2.09 mmol). Chiral GC analysis of the methyl ester of **9** showed an ee of 95% indicating that the optical purity of the product stayed unaffected all over the reaction scheme. It is to be stressed that, therefore, it is possible to prepare enantiopure (*S*)-ibuprofen simply by slightly increasing the biohydrolysis time. Following this strategy, the overall yield of **9**, starting from racemic **5**, is about 27%.

Recycling of Diol 5d. Obviously, one interesting improvement of this synthetic scheme would be to avoid the intrinsic 50% loss of material due to the resolution

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process. We have already described such strategies, based on using either an enzyme mixture[5a] or a chemoenzymatic approach.[19] In the present case, it was necessary to reuse the formed diol and to find a way to cyclize **5d** into epoxide **5** using experimental conditions that would lead, in the same time, to racemization. We found that this could be achieved by treatment of **5d** with HBr/AcOH and subsequent cyclization of the bromhydrin intermediate under basic conditions.[20] This indeed afforded *racemic* epoxide **5** in 80% overall yield, which could thus be resubmitted to enzymatic resolution. In other words, this synthetic combination makes the entire process enantioconvergent, allowing us to achieve the synthesis of enantiopure (*S*)-ibuprofen **9** following a four-step strategy that, starting from racemic epoxide **5**, can afford a 47% overall yield, including the resolution step.

Experimental Section

General Methods. The strains used in this work were purchased from the ATCC, CBS, LCP, or MUCL collections. NMR spectra (^{1}H and ^{13}C) were recorded in $CDCl_3$ at 250 and 100 MHz, respectively. Chemical shifts are reported in δ from TMS as internal standard. Determination of the enantiomeric excesses were performed using two different chiral columns (0.22 mm, 25 m, Macherey-Nagel), i.e.: heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-β-cyclodextrin (column I) and octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin in OV 1701 (column II). HPLC analyses were performed using a Spheri-5 silica column (220 × 4.6 mm, 5 μm).

Preparation of the Fungal Enzymatic Extracts. The fungal enzymatic extracts were prepared from each particular fungus. This was achieved in two steps (1) culture of the fungi and (2) breakage of the cells and extraction of the crude enzymatic epoxide hydrolase activity followed by lyophilization. The seven fungi were cultivated in a 5 L fermentor at 27 °C as previously described.[6d] The fungal cake was collected by filtration and washed with water. The following procedures were all carried out at 4 °C. The extracts were obtained in three different ways (methods A–C, see below).

Method A. This method was used in the case of *A. niger* (4.8 g of dry enzymatic extract) and with *M. isabellina* (2.1 g of dry enzymatic extract). The fungal cake was suspended in 0.8–1.5 L buffer solution (pH 7.0: 20 mM ammonium acetate; 1.0 mM EDTA; 1.0 mM cysteine) with the use of a mixer. Subsequently, a 200 mM solution of PMSF (protease inhibitor) in acetone was added such that the final concentration amounted to 0.5 mM. To break the cells, this suspension was passed through a cell disruptor (Constant Systems from CELL-D) at 27 kpsi and collected in an ice-cooled flask containing ice made from distilled water. The thus-obtained suspension was subjected to a centrifugation at 10000*g* for 20 min to remove the cell walls and unbroken cells. The supernatant was concentrated to 75 mL by means of ultrafiltration using a 10 kD filter. To this concentrated solution was added 500 mL of a pH 7.0 buffer solution (10 mM ammonium acetate; 0.1 mM EDTA; 0.1 mM cysteine), and the solution was again concentrated to 75 mL. The latter procedure was repeated two more times. The final concentrated solution was subjected to a 1.5 h ultracentrifugation at 135000*g* to remove the membranes. Sometimes, some lipidic material stayed floating on top of the solution. This could be removed by collecting the solution with a pipet and/or filtration of the solution through glass wool. The thus-obtained clear solution was freeze-dried, and the obtained powder was stored at 4 °C.

Method B. This method was used in the case of *A. terreus*, *C. echinulata*, and *B. bassiana*, which led respectively to 3.7, 3.1, and 4.1 g of dry enzymatic extract. It was similar to method A except that centrifugation at 135000*g* was omitted.

Method C. This method was used in the case of *C. globosum* and *S. racemosum*, which led, respectively, to 0.706 and 1.16 g of dry enzymatic extract. This method was similar to method A except that, after disruption of the cells, the suspension was directly subjected to ultrafiltration using a 0.1 μm filter. The suspension was concentrated to 200 mL, and subsequently, 1.0 L of a pH 7 buffer solution (10 mM ammonium acetate; 0.1 mM EDTA; 0.1 mM cysteine) was added. This suspension was concentrated again to 200 mL. The latter procedure was repeated one more time. The collected clear solution (that has passed through this filter) was concentrated to 75 mL by means of ultrafiltration using a 10 kD filter. The resulting solution was again diluted to 500 mL with a pH 7 buffer solution (10 mM ammonium acetate; 0.1 mM EDTA; 0.1 mM cysteine) and further concentrated to 75 mL. The latter procedure was repeated two more times. The final concentrated solution was freeze-dried, and the thus-obtained enzyme extract was stored at 4 °C.

Preparation of the Freeze-Dried Cells from Baculovirus-Infected Insect Cells.[21] The human, potato, and rat EH extracts were obtained from baculovirus infected insect cells in which these enzymes were overexpressed.[10–12] Two simple spinner-cultures of 500 mL volume, achieved in flat-bottomed flasks equipped with a magnetic stirrer, and containing 100 mL of rich medium (EX-CELL 401 medium supplemented with fetal calf serum (3%), and penicillin–streptomycin (1%)) were inoculated with SF21 insect cells (*Spodoptera frugiperda*) in order to obtain a cell density of 10^5 cell/mL.

After 96 h incubation at 28 °C under gentle stirring (50–70 rpm), the cell density reached 2–2.5 × 10^6 cell/mL, and they were infected with the required recombinant virus at a MOI of 0.1 pfu/mL, from a high-titer working stock of baculovirus, by simply adding the adequate inoculum to the insect cell culture. The culture mediums of the two flasks were again stirred 1 h at 28 °C before being poured into a 1.8 L Fernbach flask containing 200 mL of rich medium previously warmed at 28 °C. The medium was maintained at 28 °C during 72 h under gentle shaking (50 rpm) on an circular shaker and then centrifuged at 3000 tr/min during 5 min. The supernatant was discarded and the pellet was lyophilized in a carbonate ammonium buffer (0.1 M), thus leading to about 1 g of freeze-dried cells containing about 5% of overexpressed epoxide hydrolase.

Synthesis of Epoxides 1 to 7. Substrates **1**–**7** were synthesized from the corresponding acetophenone precursor by reaction with either $(CH_3)_3S^+ I^-$ (method I) or $(CH_3)_3SO^+ I^-$ (method II) in the presence of NaH. Better yields were obtained with method I for the substrates **1**–**5**. For **6** and **7** (strong electron-attracting substituents), better results were obtained using method II. In these cases, an excess of NaH relative to $(CH_3)_3SO^+ I^-$ should be avoided.

General Procedure. $(CH_3)_3S^+ I^-$ (or $(CH_3)_3SO^+ I)^-$ (0.12 mol) was dissolved in 200 mL of DMSO. To this solution was added 0.12 mol NaH under nitrogen at room temperature. After the solution was stirred for 20 min, 0.1 mol of the corresponding acetophenone in 40 mL of DMSO was added dropwise within 20 min. After being stirred for 10–15 h at room temperature, the reaction mixture was poured into 1 L water, and the epoxide was extracted with 2 × 150 mL of ether. The collected ether fractions were washed two times with 300 mL water, dried over $MgSO_4$, and concentrated into vacuo. The crude epoxide was purified by distillation under reduced pressure or by flash chromatography.

α-Methylstyrene Oxide 1. This epoxide was obtained as a colorless liquid in 88% yield. Bp: 55 °C (0.3 mm). ^{1}H NMR: δ 1.72 (s, 3H); 2.80 and 2.98 (2 × d, 2 × 1H); 7.2–7.4 (m, 5H). These data were consistent with the literature.[22] The two enantiomers can be separated by GC using column I (T = 60 °C: R = 12.6 min; S = 14 min).

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***p*-Fluoro-α-methylstyrene Oxide 2.**[23] This epoxide was obtained as a colorless oil in 79% yield. Bp: 54 °C (4.5 mm). ^{1}H NMR δ: 1.70 (s, 3H); 2.77 and 2.97 (2 × d, 2 × 1H); 7.0 and 7.33 (2 × t, 2 × 2H). ^{13}C NMR δ: 22.07; 57.18; 115.35 (d, J = 21 Hz), 127.24 (d, J = 8 Hz). The two enantiomers can be separated with column I (GC) at T = 70 °C: R = 10.0 min; S = 11.5 min.

***p*-Chloro-α-methylstyrene Oxide 3.** This epoxide was obtained as a colorless oil in 82% yield. Bp: 80 °C (0.2 mm). ^{1}H NMR δ: 1.70 (s, 3H); 2.75 and 2.97 (2 × d, 2 × 1H); 7.30 (s, 4H). These data were consistent with literature.[22] The two enantiomers can be separated by chiral GC (column I) (T = 90 °C: R = 14.6 min; S = 15.9 min).

***p*-Bromo-α-methylstyrene Oxide 4.** This epoxide was obtained as a colorless oil in 80% yield. Bp: = 92 °C (2.5 mm). ^{1}H NMR δ: 1.68 (s, 3H); 2.73 and 2.96 (2 × d, 2 × 1H); 7.1–7.5 (2 × d, 4H). ^{13}C NMR δ: 21.59; 56.36; 57.00; 121.43; 127.11; 131.44; 140.31. These ^{1}H and ^{13}C NMR data were consistent with the literature.[22] The two enantiomers can be separated by chiral GC (column I) (T = 100 °C: R = 20 min; S = 21 min).

***p*-Isobutyl-α-methylstyrene Oxide 5.** This epoxide was obtained as a colorless oil in 87% yield following method I. Bp: = 95 °C (4 mm). ^{1}H NMR δ: 0.9 (d, 6H); 1.7 (s, 3H); 1.8 (m, 1H); 2.45 (d, 2H); 2.80 and 2.95 (2 × d, 2 × 1H); 7.0–7.3 (2 × d, 4H). These data and bp were consistent with the literature.[22] The two enantiomers can be separated by chiral GC (column I) (T = 90 °C: R = 31.5 min; S = 32.4 min).

***p*-Cyano-α-methylstyrene Oxide 6.**[24] This epoxide is obtained as a low-melting solid in 77% following method II. Mp: = 41 °C. ^{1}H NMR δ: 1.76 (s, 3H); 2.75 and 3.06 (2 × d, 2 × 1H); 7.4–7.65 (2 × d, 4H). ^{13}C NMR δ: 21.30; 56.36; 57.29; 111.42; 118.81; 126.23; 132.33; 146.78. The ^{1}H NMR data were consistent with literature.[24] The two enantiomers can be separated by chiral GC (column I) (T = 110 °C: R = 21 min; S = 22 min).

***p*-Nitro-α-methylstyrene Oxide 7.**[24] This epoxide was obtained as a low-melting, pale yellow, solid in 35% yield following method II. Mp: = 33–34 °C. It was purified by flash chromatography (silica gel: pentane/CH_2Cl_2 (9/1)). ^{1}H NMR δ: 1.77 (s, 1H); 2.78 and 3.05 (2 × d, 2 × 1H); 7.5–8.25 (2 × d, 4H). ^{13}C NMR δ: 21.27; 57.20; 123.64; 126.25. The ^{1}H NMR data were consistent with literature.[24] The two enantiomers can be separated by chiral GC (column I) (T = 120 °C: R = 20.0 min; S = 21.3 min).

Synthesis of Diols 1d to 7d. The racemic diols **1d** to **7d** were synthesized by acid hydrolysis of the corresponding epoxides. This was achieved by adding a drop of concentrated sulfuric acid to a mixture of 200 mg of the racemic epoxide in a 10 mL water/2-methyl-2-propanol mixture (3/1). The reaction was followed by TLC, and the crude product was purified by means of flash chromatography (silica gel: dichloromethane/ether) (9/1 v/v).

2-Phenyl-1,2-propanediol 1d. This diol has been previously synthesized in our laboratory.[6b] The enantiomers of the acetonide of this diol can be separated by chiral GC (column I) (T = 60 °C: S = 46.3 min; R = 47.8 min) (column II) (T = 70 °C: R = 45.4 min; S = 47.0 min).

2-(*p*-Fluorophenyl)-1,2-propanediol 2d. This diol was obtained as a viscous oil. ^{1}H NMR δ: 1.47 (s, 3H); 2.42 (2 × d, 1H); 2.96 (s, 1H); 3.5–3.8 (o, 2H); 6.9–7.5 (m, 4H). ^{13}C NMR δ: 26.16; 71.09; 115.17 (d, J = 21 Hz); 126.85 (d, J = 8 Hz). Anal. Calcd for $C_9H_{11}O_2F$ (170.18): C, 63.5; H, 6.5; F, 11.2. Found: C, 63.32; H, 6.35; F, 11.2. Exact mass calcd for $C_9H_{11}O_2F$ 170.074 307 9; found 170.074 600 0. The acetonide of this diol can be separated by chiral GC with column I at T = 50 °C: S = 116 min; R = 126 min.

2-(*p*-Chlorophenyl)-1,2-propanediol 3d. This diol is a very viscous oil. ^{1}H NMR δ: 1.47 (s, 3H); 2.43 (t, 1H); 2.97 (s, 1H, OH); 3.45–3.8 (o, 2H); 7.25–7.45 (2 × d, 4H). ^{13}C NMR δ: 26.16; 70.96; 74.75; 126.75; 128.47; 133.18; 143.69. The ^{1}H and ^{13}C NMR were consistent with the literature.[22] The enantiomers of the acetonide derivative of this diol can be separated by chiral GC with column I at T = 65 °C: S = 185 min; R = 201 min.

2-(*p*-Bromophenyl)-1,2-propanediol 4d. White solid. Mp: = 59 °C. ^{1}H NMR δ: 1.50 (s, 3H); 2.05 (t, 1H); 2.72 (s, 1H); 3.5–3.8 (o, 2H); 7.2–7.5 (2 × d, 4H). ^{13}C NMR δ: 25.98; 70.83; 74.60; 121.20; 127.01; 131.47; 144.05. The ^{1}H and ^{13}C NMR were consistent with the literature.[25] The enantiomers of the acetonide derivative of this diol can be separated by GC (column I) (T = 75 °C: S = 208 min; R = 222 min).

2-(*p*-Isobutylphenyl)-1,2-propanediol 5d. This diol was obtained as a white solid. Mp: = 92 °C. ^{1}H NMR δ: 0.88 (d, 6H); 1.54 (s, 3H); 1.4–2.0 (s (br), 2H); 1.83 (m, 1H); 2.45 (d, 2H); 3.45–3.85 (2 × d, 2 × 1H); 7.1–7.4 (2 × d, 4H). ^{13}C NMR δ: 22.62; 26.23; 30.40; 45.17; 71.39; 75.00; 125.04; 129.41; 140.90; 144.71. The ^{1}H and ^{13}C NMR were consistent with the literature.[22] The acetonide derivative of this diol could be analyzed by chiral GC analysis (column I) (T = 95 °C: S = 82.9 min; R = 84.6 min).

2-(*p*-Cyanophenyl)-1,2-propanediol 6d. This diol was obtained as a viscous oil. ^{1}H NMR δ: 1.53 (s, 3H); 2.05 (t, 1H); 2.85 (s, 1H); (o, 2H); 7.48–7.7 (2 × d, 4H). ^{13}C NMR δ: 26.13; 70.66; 74.97; 110.90; 118.97; 126.34; 132.34; 151.04. Anal. Calcd for $C_{10}H_{11}NO_2$ (177.20): C, 67.8; H, 6.3; N, 7.9; O, 18.1. Found: C, 67.46; H, 6.38; N, 7.64; O, 18.78. Calcd for $C_{10}H_{11}NO_2$ 177.078 978 7; found 177.078 300 0. The acetonide derivative of this diol could not be separated efficiently enough using the chiral GC columns available. Therefore, this diol was converted back into the epoxide by reaction with *p*-toluenesulfonyl chloride and NaH in ether, and the thus-obtained epoxide was analyzed as described above.

2-(*p*-Nitrophenyl)-1,2-propanediol 7d. This diol is a very viscous slightly yellow oil. ^{1}H NMR δ: 1.53 (s, 3H); 2.50 (t, 1H); 3.13 (s, 1H); 3.6–3.9 (o, 2H); 7.45–8.25 (2 × d, 4H). ^{13}C NMR δ: 26.21; 70.72; 75.04; 123.69; 126.46; 152.88. Anal. Calcd for $C_9H_{11}NO_4$ (197.19): C, 54.8; H, 5.6; N, 7.1; O, 32.5. Found: C, 54.15; H, 5.72; N, 7.14; O, 32.85.. Calcd for $C_9H_{11}NO_4$ 197.068 808 0; found 197.069 000 0. As for **6d**, the acetonide derivative could not be separated efficiently enough on the chiral GC columns available. Thus, the diol was transformed back into the epoxide using *p*-toluenesulfonyl chloride and NaH in ether and was analyzed as described above.

Kinetic Measurements. Lyophilized enzymatic extract (5–60 mg) was dissolved in 1.0 mL of buffer (0.4 M Tris/HCl) of the desired pH. The substrate was injected as a 0.8 M solution in DMF (10 μL) into the buffer solution and stirred at 27 °C. The reaction was followed by periodically withdrawing 0.1 mL samples. These were saturated with NaCl and extracted with 0.3 mL of ethyl acetate. The amount of diol present in the organic phase (and therefore the degree of conversion) was determined by HPLC analysis (silica gel: hexane/ethanol 95/5 (v/v)). The ee of the epoxide was determined by GC, and the E value was calculated using the ee of the epoxide and the calculated conversion ratio. The initial rate of the reaction was determined by plotting the amount of formed diol against the time (t) and extrapolation to t = 0.

Synthesis of Ibuprofen. (*R*/*S*)-4-Isobutyl-α-methylstyrene Oxide 5. To a solution of 20.8 g (102 mmol) of $(CH_3)_3S^+I^-$ in 200 mL of DMSO was carefully added 4.1 g of NaH (approximately 60% = 103 mmol) in an N_2 atmosphere. After the solution was stirred for 15 min, 15 g (85 mmol) of 4-isobutylacetophenone in 50 mL of DMSO was added dropwise within a 20 min period at room temperature. The solution was stirred overnight. Subsequently, the reaction mixture was poured into 1 L of tap water, and the epoxide was extracted with 2 × 200 mL pentane. The collected pentane fractions were

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washed with 2 × 300 mL of water, dried over $MgSO_4$, and concentrated in vacuo. The oily residue was subjected to distillation, which gave pure (*R/S*)-**5** as a colorless oil (bp = 95 °C (4 mm)). This afforded 14.1 g (74 mmol; 87% yield).

Preparative-Scale Enzymatic Resolution of (*R*/*S*)-5. *A. niger* enzymatic extract (2.6 g) was dissolved in 30 mL of buffer (pH 8.0; 0.4 M Tris/HCl) at 4 °C. To this solution was added 1.5 g (7.9 mmol) of **5**. The solution was shaken for 24 days at 4 °C (in a cold room), and the ee of the remaining substrate **5** was followed by chiral GC analysis. When it reached 96%, the reaction was stopped by extraction of the epoxide with pentane (3 × 10 mL). Subsequently, the diol was extracted from the remaining aqueous phase with diethyl ether (3 × 15 mL). The formed diol **5d** was purified by column chromatography (silica gel, dichloromethane). The collected pentane fractions not only contained (*S*)-**5** but also some diol **5d** (and some uncharacterized compounds that were presumably present in the enzymatic extract). They were concentrated in vacuo and used without further purification since, on a silica gel column, **5** rapidly rearranged into the corresponding (racemic) aldehyde. Distillation also led to formation of this aldehyde, which we were unable to separate from the epoxide. The total isolated amount of (*R*)-**5d** was 0.91 g (4.38 mmol) corresponding to a 55% yield (ee 70%). $[\alpha]^{20}_D$: −11 (*c* 1.17; $CHCl_3$) (lit.[22] $[\alpha]^{20}_D$ −4.5 (*c* 1.55; $CHCl_3$)). The corresponding acetonide could be analyzed by chiral GC (column I) (T = 95 °C: S = 82.9 min and R = 84.6 min).

(*S*)-2-(4-Isobutylphenyl)propanol 8. A suspension of 0.2 g of Pd powder (submicron/Aldrich) in 20 mL of *N*-methylformamide was stirred for 20 min in a H_2 atmosphere at room temperature. The mixture was cooled to 0 °C, and the crude (*S*)-**5** epoxide obtained above was added. The reaction mixture was stirred overnight at 0 °C under H_2. Subsequently, the reaction mixture was poured into 150 mL of water. The aqueous phase was extracted with pentane (2 × 35 mL). The collected pentane fractions were dried over $MgSO_4$ and concentrated in vacuo. The residue was subjected to flash chromatography (silica gel: pentane/dichloromethane 3/1 (v/v)). The formed alcohol (*S*)-**8** (0.53 g; 2.76 mmol) was obtained as a colorless oil. A fraction of **5d**, present in the crude epoxide, was eluted from the chromatography column with dichloromethane and added to the previously obtained diol. The overall yield of **8**, starting from racemic **5**, amounted to 35%. $[\alpha]^{20}_D$: −16 (*c* 1.0, $CHCl_3$) (lit.[26] $[\alpha]^{20}_D$ −14.8 (*c* 1.6; $CHCl_3$)). Alcohol **8** was analyzed by chiral GC (column III) (90 °C: R = 133 min and S = 139 min); the measured ee was 95%. ^{1}H NMR δ: 0.9 (d, 6H); 1.25 (d, 3H); 1.34 (t, 1H); 1.82 (m, 1H); 2.42 (d, 2H); 2.8 (m, 1H); 3.67 (2 × d, 2H); 7.0−7.2 (2 × d, 4H).

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(*S*)-Ibuprofen 9. A solution of 2.76 mmol (0.53 g) of (*S*)-**8** (ee 95%) in 12 mL acetone/H_2SO_4 (3 N) was cooled in an ice bath under stirring. To this solution was added 5.0 mmol of $KMnO_4$ portionwise at 0 °C. After the solution was stirred for 1 h (at 0 °C), the ice bath was removed and the mixture was stirred for 2 more hours at room temperature. Subsequently, solid $NaHSO_3$ was added until the solution became colorless. The mixture was dissolved in 50 mL of water and the aqueous phase was extracted two times with 25 mL of ether/pentane (1/2, v/v). The collected organic phase was extracted with a 2% NaOH solution (2 × 50 mL), and the thus-obtained collected aqueous phase was acidified to pH 1 with HCl (35%). The turbid aqueous phase was extracted with $CHCl_3$ (2 × 50 mL), and the collected organic fractions were dried over $MgSO_4$ and concentrated in vacuo. The residue was subjected to flash chromatography (silica gel: dichloromethane/ether, 4/1, v/v), which gave 0.43 g of **9** (2.09 mmol, 76% yield) as a sticky low-melting solid. Mp: 48 °C. $[\alpha]^{20}_D$: +55 (*c* 2, EtOH). The commercially available (*S*)-ibuprofen from Aldrich (ee 98%) shows an mp of 51−53 °C and an optical rotation $[\alpha]^{20}_D$ +59 (*c* 2, EtOH). ^{1}H NMR δ: 0.88 (d, 6H); 1.50 (d); 1.81 (m, 1H); 2.45 (d, 2H); 3.69 (q, 1H); 7.0−7.3 (2 × d, 4H); 9.8 (s (very broad), 1H).

A small amount of **9** was converted into its methyl ester with $SOCl_2$ in methanol. This could be analyzed by chiral GC (column I) (80 °C: S = 118 min and R = 121 min) and showed an ee of 95%.

Synthesis of Racemic Epoxide 5 from Diol (*R*)-5d. The method we have used was described by Golding et al.[20] To 8.0 mL of acetic acid saturated with HBr was added 0.91 g (4.38 mmol) of (*R*)-**5d** (ee = 70%) at room temperature. After being stirred for 30 min, the reaction mixture was poured into 40 mL of water and neutralized with solid Na_2CO_3. The solution was extracted with ether (2 × 20 mL), and the combined ether fractions were dried over $MgSO_4$ and concentrated in vacuo. The residue was dissolved in 3 mL of methanol, and NaOH (4.4 mmol) in 4 mL of methanol, was added for 15 min at room temperature. After being stirred for 30 min, the reaction mixture was poured into 25 mL water and was extracted with pentane (2 × 15 mL). The combined pentane fractions were dried over $MgSO_4$ and concentrated in vacuo. The residue was distilled (bulb-to-bulb distillation), which gave **5** as a colorless oil. The yield amounted to 80% (0.66 g, 3.50 mmol), and the ee was shown to be about 0%, showing that complete racemization occurred during this process.

Acknowledgment. This work has been achieved in the context of the EC BIOC4-CT95-0005. Financial support to one us (M.C.) in the frame of this contract is greatly acknowledged.

JO982101+