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Microbiological transformations. Part 45: A green chemistry preparative scale synthesis of enantiopure building blocks of Eliprodil: elaboration of a high substrate concentration epoxide hydrolase-catalyzed hydrolytic kinetic resolution process

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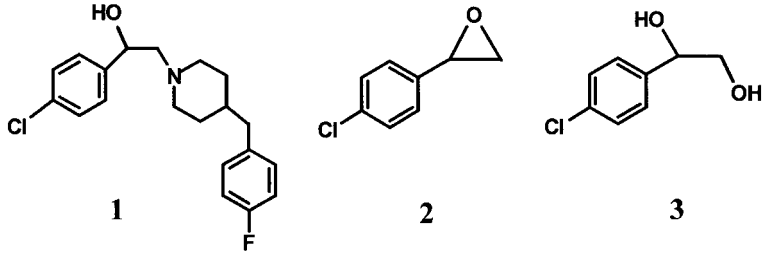
Abstract—The enantioselective hydrolysis of racemic *para*-chlorostyrene oxide **2** following a typical 'green chemistry' procedure based on the use of two different Epoxide Hydrolases is described. This allows the preparation of both enantiomers of **2** in very high enantiomeric purity. Furthermore, using a 'one-pot' sequential bi-enzymatic strategy enabling to overcome the 50% yield limitation intrinsic to any resolution process, *rac*-**2** could be transformed into nearly enantiopure (*R*)-**3** with an overall yield as high as 93%. The methodology developed was based on the use of a biphasic reactor at high substrate concentration, which is highly desirable for any potential industrial process. The obtained chirons are valuable building blocks for the synthesis of various biologically active targets, like (*R*)-Eliprodil.

1. Introduction

The search for novel methods enabling the synthesis of enantiopure pharmaceuticals is a major thrust area in contemporary fine organic synthesis, due to the increasing demand for safer and more efficient drugs.[1] This is aimed to avoid the possible (sometimes deleterious) side effects of the less active (or inactive) enantiomer of a given biologically active molecule.[2] At the start of this work, Eliprodil **1** (Scheme 1) was subject to phase three trials as a highly promising neuroprotective agent (acting as a glutamate antagonist), known to possess high efficiency against ischemic stroke.[3] Interestingly, it has been recently demonstrated that the in vivo activity of Eliprodil is essentially associated with its (−)-(*R*) enantiomer rather than to its (+)-(*S*) antipode, and that the pharmacodynamic properties of the two enantiomers were different.[4]

Another important long-term issue for contemporary industrial fine chemistry is the employment of environmentally gentle processes—the so-called 'green chemistry' approach—where the usage of toxic materials like heavy metal reactants/catalysts or organic solvents should be lowered or, ideally, eliminated.

During the last ten years, we (and others) have demonstrated that Epoxide Hydrolases (EH) (EC 3.3.2.3) are very interesting new tools for the organic chemist[5] since they allow



Scheme 1.

Keywords: enantioselective hydrolysis; biotransformation; hydrolytic kinetic resolution; epoxide hydrolase; *Aspergillus niger*; *Solanum tuberosum*; Eliprodil; chiral synthons.

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Table 1. Chemical hydrolysis (*experimental conditions*: 4 mM concentration of *rac*-**2**; HPLC analysis of the formed diol, hydrolysis expressed in nmol/h per mL) of *rac*-**2** as a function of pH and temperature

pH	0°C	6°C	28°C
6.7	4	7	36
7	3.5	5	26
7.5	2.5	4	20
8	2.2	3.5	16

efficient achievement of the Hydrolytic Kinetic Resolution (HKR) of various epoxides. It must in particular be emphasized that: (a) these hydrolases are cofactor independent; (b) they are ubiquitous in nature; (c) they can in particular be found in microorganisms, which makes them very easy to prepare in large scale (eventually after cloning and overexpression in an appropriate host); (d) they may be endowed with substrate- and/or enantio-complementarity, thus allowing to set up, in some cases, efficient enantiocomplementary processes. Studies using such enzymes have been flourishing over the last years[5] and we have ourselves developed strategies allowing for the preparation of enantiopure bioactive products.[6] The knowledge about these 'new' enzymes very recently culminated with the publication of the three first X-ray structures of cytosolic EHs from, respectively, the bacteria *Agrobacterium radiobacter*,[7] murine liver[8] and the fungus *Aspergillus niger*.[9]

In this context, we have explored a way to prepare (*R*)-**1** in enantiopure form using an EH catalyzed procedure. Since *para*-chlorostyrene oxide **2** as well as the corresponding diol **3**, are two obvious possible key building blocks for achieving the synthesis of (*R*)-**1**, we embarked on a study aimed to prepare these chirons in enantiopure form. We describe here the preparation of each of the two enantiomers of **2** at a multi-gram scale by using two complementary EHs in high substrate concentration reactors. Moreover, we also report a 'one-pot' enantioconvergent procedure based on the sequential use of these two enzymes, which allowed to overcome the 50% maximum yield intrinsic to a classical resolution process and afforded (*R*)-**3** at very high yield and enantiomeric purity.

2. Results

2.1. Preliminary work

A rapid screening using our 'in house' available epoxide hydrolases led to the conclusion that EHs from *Aspergillus niger* (*An*EH)[10] and *Solanum tuberosum L.* (*St*EH)[11] were the two best candidates for achieving the resolution of **2**. Interestingly, they were seen to be enantio-complementary, the *An*EH preferentially hydrolyzing the (*R*) enantiomer whereas *St*EH preferred the (*S*) antipode.[12] Solubility of **2** in a pH 6.7 phosphate buffer was established by HPLC analysis to be ~5 mM (0.75 g/L) at 27°C. Therefore the optimum reaction parameters and overall reaction profile studies were first achieved at 4 mM substrate concentration using each of the two enzymes and will be discussed in a separate report.[13]

Since *rac*-**2** is also spontaneously hydrolyzed in the experimental conditions used, the rate of this hydrolysis was determined as a function of pH and temperature (Table 1) in order to allow correction of the experimental values obtained upon enzymatic hydrolysis and optimization of reaction conditions for preparative scale reactions. Owing to these results, the experiments were conducted at 0°C in order to avoid significant spontaneous hydrolysis.

2.2. Analytical scale studies

When *rac*-**2** (4 mM concentration) was submitted to react with *An*EH at 0°C, a conversion ratio of 51% was reached after 3 h, leading to the remaining (slow reacting) epoxide showing an ee of 98%. An *E* value of ~100 was calculated (using c and ee of remaining epoxide), whereas a value of ~40 was observed at 27°C. Such a temperature dependent *E* value enhancement has already been established previously.[14] The remaining epoxide **2** was shown to be of (*S*) absolute configuration by comparison with an authentic sample.[12] Higher reaction time led to enantiopure **2**, a fact which illustrates the attractive feature that such resolution processes allow to 'tune' the ee of the slow reacting enantiomer simply by tailoring the reaction time, i.e. the conversion ratio, a strategy which is not available starting from a prochiral substrate. After derivatization into its acetonide derivative, the formed diol **3** could also be analyzed by chiral gc and was shown to be of 90% ee. Its absolute configuration was determined after recyclization into the corresponding epoxide using a method avoiding loss of stereochemical integrity.[15] This indicated that it was of (*R*) absolute configuration, which leads to the conclusion that the (*R*)-**2** enantiomer was, at least preferentially, attacked by the enzyme at the β position, i.e. at the *less substituted* carbon atom, thus leading to *retention* of configuration. This also confirms that diol **3** can be a valuable chiral building block for **1**.

When *rac*-**2** (4 mM concentration) was submitted to *St*EH, a similar behavior was observed. Thus, within 2 h, a conversion ratio of 51% was reached, affording a remaining (slow reacting) epoxide showing an ee of 97% and the formed diol of 96% ee. Again an *E* value of ~100 could be calculated (whereas a value of ~40 was observed at 27°C). Interestingly, however, both the obtained epoxide and diol were

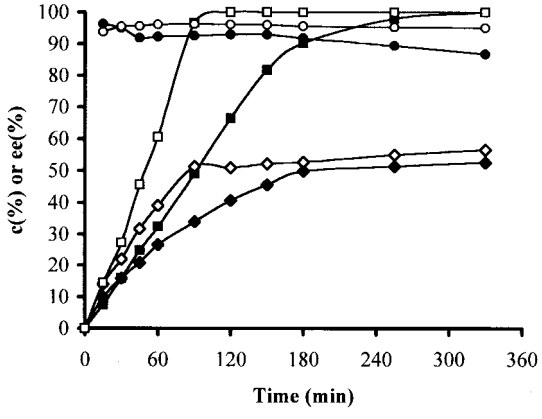


Figure 1. Analytical enzymatic resolution of *rac*-**2** at 0°C using *An*EH and *St*EH. ◆ c (*An*EH); ■ ee-epox. (*An*EH); ● ee-diol (*An*EH); ◇ c (*St*EH); □ ee-epox. (*St*EH); ○ ee-diol (*St*EH).

Table 2. Regioselectivity coefficients for the acid and enzyme catalyzed hydrolyses

	α(*S*) %	β(*S*) %	α(*R*) %	β(*R*) %
Chemical hydrolysis	67	33	67	33
*An*EH	3	97	1	99
*St*EH	97	3	8	92

determined to be of (*R*) absolute configuration. This indicated that (a) contrary to the result observed with the *A. niger* enzyme, the *St*EH showed a marked preference for hydrolyzing the (*S*)-epoxide (therefore being enantiocomplementary to the previous one) and (b) that, again opposite to the previous result, the water attack preferentially occurred at the *more substituted* (benzylic) carbon atom, leading to *inversion* of configuration. Fig. 1 shows the ee versus time dependent conversion of *rac*-**2** using the two different enzymes.

2.3. Determination of regioselectivity versus enantioselectivity

This 'stereochemical flexibility', i.e. the possible combination of various enantio- and regio-selectivities within one single EH catalyzed hydrolysis is one of the most intriguing and potentially useful properties of such enzymes, and we have previously studied this type of behavior in detail.[16] In order to accurately establish the specific regioselectivity implied for hydrolysis of *rac*-**2** by *An*EH and *St*EH (i.e. to determine the α(*R*), β(*R*), α(*S*) and β(*S*) coefficients[17]), each enantiomer of **2** was submitted independently, in enantiopure form, to hydrolysis by these two enzymes. In each case, the corresponding coefficients were determined from the ee of the formed diol, leading to the value shown in Table 2. For *An*EH, both enantiomers were essentially attacked at the β carbon atom. However, the *St*EH attacked predominantly (*R*)-**2** at the β position, whereas (*S*)-**2** was attacked at the benzylic α carbon atom, making this entire process highly enantioconvergent. The regioselectivity of spontaneous chemical hydrolysis was also determined starting from each enantiomer. This indicated a predominant hydrolytic attack at the benzylic carbon atom, a data which was taken into account for correction of the coefficients indicated in Table 2.

2.4. Preparative scale experiments

In order to set up an efficient preparative scale process for resolution of *rac*-**2**, the following priority of requirements were decided (a) high ee of the products (b) maximum possible substrate concentration (c) as high as possible yield (d) optimum amount of enzyme and (e) reasonable reaction time.[18] Also, the activity and stability profile of each one of the two enzymes had to be taken into account.[13]

Biphasic high concentration reactor using AnEH. Owing to the previously described priorities, the reaction was conducted in a pH 7 phosphate buffer at 0°C (the half life of the biocatalyst was determined to be of about 7 days at this temperature). The possibility to increase the substrate concentration (at a constant enzyme/substrate ratio) was explored by monitoring the ee of the remaining epoxide after 1 and 3 h reaction (Fig. 2).[18]

Interestingly, it appears that (a) the reaction could be performed using a substrate concentration much above its maximum solubility (about 5 mM, i.e. 0.75 g/L) (b) apparently no mass transfer limit does occur (c) above 400 mM substrate concentration, the (high) diol concentration seemed to slow down the reaction rate. Using the above defined experimental conditions, a preparative scale process was conducted at 2 M concentration (i.e. 306 g/L) using a reactor containing 4 g (26 mmol) of *rac*-**2**. This was used as a slurry together with a solution of 540 U[19] in a 9 mL phosphate buffer solution. The reaction reached a 50% conversion in about 8 h, after which the reaction mixture was extracted with pentane to isolate the remaining (*S*)-**2** epoxide and with ethyl acetate to extract the resulting (*R*)-**3** diol. Thus, after purification, 1.9 g (47% yield) of (*S*)-**2** was obtained which showed a 99% ee, together with 2.15 g (48% yield) of (*R*)-**3** (92% ee) (overall yield 95%). The space-time yield of this two-phase reactor was calculated

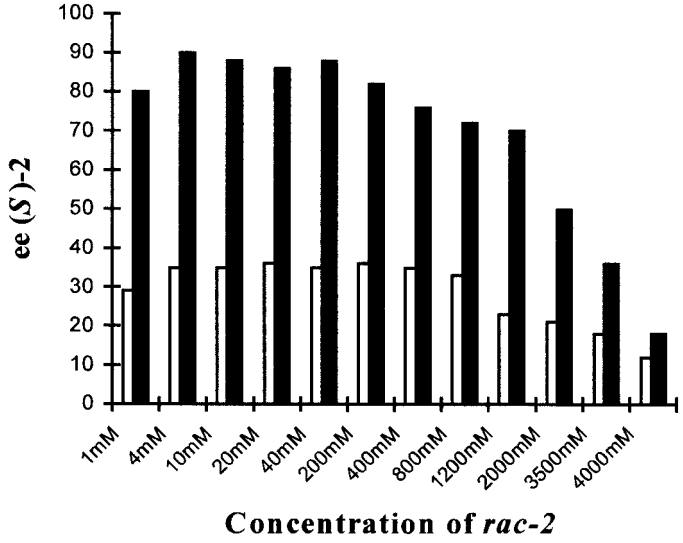


Figure 2. Scale-up of substrate concentration for *An*EH. *Experimental conditions:* Reaction volume 0.5 mL; pH 7; *t*° 0°C. The enzyme/substrate ratio was kept constant through out the scale-up at 0.02 U/μmol of *rac*-**2**. □ %ee of (*S*)-**2** at *t*=1 h ■ %ee of (*S*)-**2** at *t*=3 h.

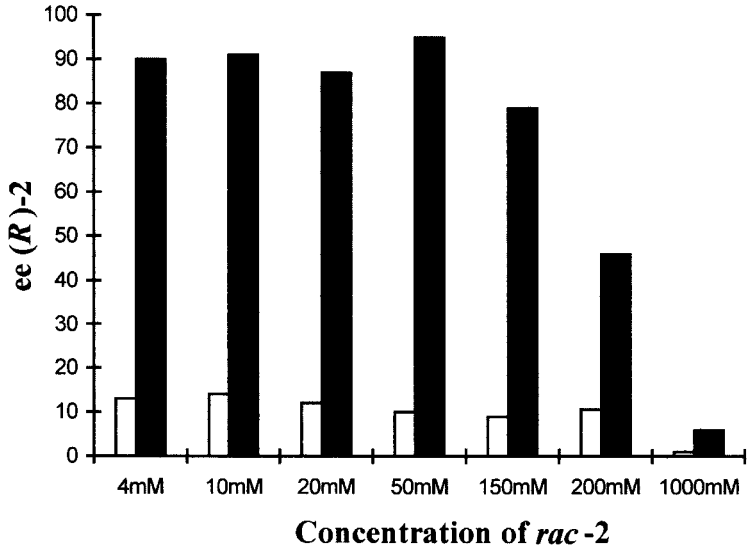


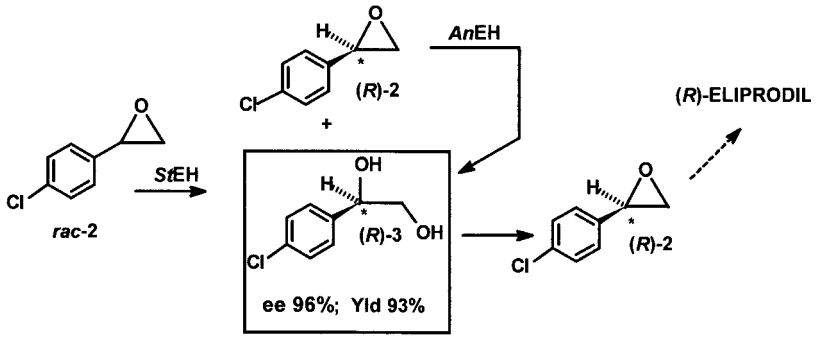
Figure 3. Scale-up of substrate concentration for *St*EH. *Experimental conditions:* reaction volume 0.5 mL; pH 7; *t*° 0°C. The enzyme/substrate ratio was kept constant through out the scale-up at 0.0063 U/μmol of *rac*-**2**. □ %ee of (*R*)-**2** at *t*=1 h ■ %ee of (*R*)-**2** at *t*=6 h.

to be about 20 g/L/h and the turnover number about 12000 mol of substrate/mole enzyme/h.[20]

Biphasic high concentration reactor using StEH. Similar experimental conditions were chosen for performing the resolution of *rac*-**2** using *St*EH. The only difference lies in the fact that the reaction was conducted at pH 6.7, as it was demonstrated to be optimum for reactivity and stability of this enzyme. The possibility to increase the substrate concentration was also explored as shown on Fig. 3.[18] Here again it appears that (a) the reaction can be performed at substrate concentrations much above its maximum solubility in the medium (b) no mass transfer limitation occurred. However it clearly appeared that, to the contrary of what was observed with the *An*EH, substrate concentration above 200 mM were deleterious to the enzyme activity. In accordance with this inhibition effect, the preparative scale experiment was conducted at 0.2 M (30.6 g/L) substrate concentration. Thus, 4 g of *rac*-**2** were hydrolyzed as a slurry (under mechanical stirring) using a solution of 150 U of *St*EH in 125 mL phosphate buffer. The reaction reached a 50% conversion in about 18 h, after which the reaction mixture was worked up as described above. After purification, 1.9 g (47% yield) of (*R*)-**2**, which showed an ee higher than 99%, was obtained together with 2.1 g (47% yield) of (*R*)-**3** (97% ee).

2.5. Enantioconvergent process using a sequential bi-enzymatic strategy

As mentioned previously, the major drawback of any resolution process is its intrinsic 50% yield limitation. Fortunately, several ways allowing to overcome this industrially utmost important problem have been described, including dynamic resolution, combined chemoenzymatic processes or bi-enzymatic protolcols.[6,21] In the present case, it clearly appeared that it could be possible to exploit the enantio- and regio-complementarity of our two enzymes in order to reach the ideal '100% yield, 100% ee' goal. For this purpose, we did set up an optimized procedure using the two enzymes sequentially rather than simultaneously. This choice was based on three interesting and complementary facts, i.e.: (a) as emphasized above, the *St*EH appeared to be more sensitive to inhibition by the (*R*)-diol formed. Therefore, since the concentration of formed **3** would increase about twofold if an equivalent amount (in Units) of *An*EH would be present simultaneously, this would lead to a negative effect on the biohydrolysis rate; (b) since *St*EH was shown



Scheme 2.

to be enantioconvergent, formation of the (*R*) diol would be nearly exclusive even after consumption of (*S*)-**2**; (c) finally, since (owing to the *E* value of about 100) direct use of *An*EH at the start of the reaction would lead to hydrolysis of some (*S*)-**2** enantiomer to the (*S*) diol, this would lower the ee of **3** at total conversion. To the contrary, addition of *An*EH *after* consumption of (*S*)-**2** by the *St*EH would obviously avoid this problem.

Therefore, in a typical experiment, biohydrolysis was started using 150 U *St*EH in 125 mL buffer solution. After about 50% conversion (18 h at 0°) 500 U *An*EH were added into the reaction vessel and the reaction was led to completion (about 2 days in total). The reaction broth was extracted with ethyl acetate in order to isolate the resulting diol, affording 4.18 g (*R*)-**3** (93% yield) which showed an ee of 96%. This overall strategy is shown in Scheme 2.

3. Conclusion

In the course of this work, we have achieved the enantioselective hydrolysis of racemic *para*-chlorostyrene oxide **2** using two different Epoxide Hydrolases. This enabled the preparation of both enantiomers of **2** in nearly enantiopure form. Furthermore, using a 'one-pot' sequential bi-enzymatic strategy, *rac*-**2** could be transformed into (*R*)-**3** with an overall yield as high as 93%. Thus, this strategy enabled to overcome the 50% yield limitation intrinsic to any resolution process and to approach the ideal '100% yield, 100% ee' goal. The methodology developed within this work was based on the use of a biphasic reactor at high substrate concentration, which is highly desirable for any potential industrial process. Moreover, this was performed using a typical 'green chemistry' procedure, a strategy which will be of utmost importance in the coming century due to increasing environmental pressure on chemical industry. The obtained chirons are valuable building blocks for the synthesis of various biologically active targets, like (*R*)-Eliprodil. Work is in progress in our laboratory in order to further evaluate the scope and limitations of such methodologies, with the intention of enhancing the utility of microbial Epoxide Hydrolases as industrial catalysts.

4. Experimental

4.1. General methods

The *Aspergillus niger* strain used in this work is registered at the Museum d'Histoire Naturelle under n°. LCP 521 (Lab. de Cryptogamie, 12 rue Buffon, 75005 Paris, France). 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 of epoxide-**2** and diol-**3** were performed using chiral column Chirasil-Dex CB (0.25 mm×25 m, Chrompack). Other conditions were as follows: 1/100 split, injector and FID at 250°C, column at 125°C for **2** and 150°C for acetonide derivative of **3**, Helium at 1 mL/min. Under these conditions, (*R*)-**2**, (*S*)-**2**, (*R*)-**3** and (*S*)-**3** eluted at 11, 11.6, 9.8 and 10.7 min, respectively. Reverse phase HPLC was used to quantify **3**. 20 μL of aqueous sample were injected into a Lichrosorb RP 18 column (Merck) and eluted using a mixture of water/acetonitrile (3:1) at 1.5 mL/min (UV detector at 220 nm). Under these conditions, **3** eluted at 5 min (and **2** eluted at 30 min).

4.2. Preparation of the enzymatic extracts

The *A. niger* and the potato enzymatic extracts were prepared as previously described.[6c]

4.3. Synthesis of *rac-p*-chlorostyrene oxide 2

This epoxide was obtained by epoxidation of the corresponding olefin using *m*-chloroperoxybenzoic acid under biphasic conditions (CH_2Cl_2/phosphate buffer) as previously described[12] (77% yield). ^{1}H NMR δ: 2.75 (dd, 1H, 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_1, 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-1); 126.8; 128.7; 133.9 136.2; 136.2 (C–Ar).

4.4. Analytical reactors

To 3960 μL of pH6.7 buffer containing the appropriate amount of enzyme (*An*EH=0.275 mg/mL at 0.26 U/mg solid or *St*EH=1.837 mg/mL at 0.06 U/mg solid) maintained at 0°C, 40 μL of DMF solution containing 400 mM *rac*-**2** and 100 mM *meta*-bromoacetophenone (MBAP) were added to give the final concentration at 4 mM *rac*-**2**, 1 mM MBAP, DMF<1% (0.0179 U/mM substrate for *An*EH and 0.2756 U/mM substrate for *St*EH). Aliquots of 400 μL were withdrawn at different time intervals; 200 μL methanol were added to stop the reaction. The epoxide was extracted with 500 μL isooctane and analyzed by GC for conversion ratio and ee. A fraction of the diol was also used for conversion ratio determination by HPLC. The remainder was extracted with ethyl acetate and derivatized for determination of diol ee.

4.5. Preparative enzymatic resolution of (*R*/*S*)-2 using *An*EH

To 9 mL of sodium phosphate buffer (pH 7, 100 mM, at 0°C), 2.3 g of *An*EH preparation (~540 U, at 0.234 U/mg solid) were added and NaOH solution was used to adjust the pH to 7. The resultant solution was ~9.8 mL. To this, 4 g of *rac*-**2** were added and the slurry stirred at 400 rpm using a magnetic paddle. A few microliters of the reaction mixture were withdrawn at appropriate time intervals to check for the enantiomeric excess of the remaining epoxide and the formed diol. The reaction was stopped around 8 h and the reaction mixture extracted with pentane (3×40 mL). The collected organic factions were dried over $MgSO_4$ and concentrated in vacuum. After purification by bulb-to-bulb distillation 1.9 g (47% yield) of (*S*)-**2** was isolated $[\alpha]^{20}_D$=+26 (*c* 1.29; $CHCl_3$). The remaining aqueous phase was extracted with ethyl acetate (3×40 mL), dried over $MgSO_4$ and concentrated in vacuum. After flash chromatography purification (silica gel 60H from Merck and solvent mixtures consisting of pentane and diethyl ether in the range of 100% pentane to 100% diethyl ether) 2.15 g

(48% yield) of (*R*)-diol **3** were isolated $[\alpha]^{20}_{D}=-58$ (*c* 1.26; $CHCl_3$), mp 82.5°C.

4.6. Preparative enzymatic resolution of (*R/S*)-2 using *St*EH

4.2 g of enzyme powder (∽150 U, at 0.037 U/mg solid) were added to 125 mL buffer (pH 6.7, 100 mM, at 0°C) and pH adjusted. The resultant solution was 127.5 mL. To this, 4 g of *rac*-**2** were added and this was stirred at 400 rpm using a magnetic paddle at the bottom and an external impeller from the top. A few microliters of the reaction mixture were withdrawn at appropriate time intervals to check for the enantiomeric excess of the remaining epoxide and the formed diol. The reaction was stopped around 18 h and the reaction mixture was extracted with pentane (4×140 mL) for the epoxide and the remaining aqueous phase with ethyl acetate (4×100 mL) for the diol. The same work-up described above using *An*EH allowed isolation of 1.9 g (47% yield) of (*R*)-**2** $[\alpha]^{20}_{D}=-24$ (*c* 1.2; $CHCl_3$) and 2.1 g (47% yield) of (*R*)-diol **3** $[\alpha]^{20}_{D}=-60$ (*c* 1.29; $CHCl_3$), mp 84.6°C.

4.7. Preparative enantioconvergent process using *An*EH and *St*EH

1.9 g of *St*EH enzyme powder (∼150 U, at 0.079 U/mg solid) was added to 125 mL buffer (pH 6.7, 100 mM, at 0°C) and pH adjusted to 6.7. The resultant was 127.5 mL. To this, 4 g of *rac*-**2** were added and stirred using a magnetic paddle at the bottom and an external impeller from the top. A few microliters of the reaction mix was withdrawn at appropriate time intervals to check for the enantiomeric excess of the remaining epoxide and the formed diol. After 25 h, when almost all of (*S*)-**2** was consumed, 2.18 g *An*EH powder (∽500 U, at 0.23 U/mg solid) were added and the pH was noted to be at 7.1. The reaction was stopped around 45 h when the concentration of the remaining epoxide was lower than 1 mM. The reaction mixture was extracted with ethyl acetate (4×140 mL) for the (*R*)-diol **3**: 4.18 g (93% yield) was isolated after flash chromatography purification (silica gel 60H from Merck and solvent mixtures consisting of pentane and diethyl ether in the range of 100% pentane to 100% diethyl ether) $[\alpha]^{20}_{D}=-61.4$ (*c* 1.29; $CHCl_3$), mp 83.7°C.

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β(*R*) coefficients were used in the case of the (*R*)-epoxide hydolysis.

18. Since the reaction system was highly heterogeneous, the conversion ratio could not be determined even with a fair sense of approximation. Ee was taken as an index to measure the reaction extent at two different time intervals. The analysis achieved after 1 h gave an indication of the effect of substrate concentration and the analysis at 3 h (for *An*EH) or at 6 h (for *St*EH) indicated the effect of the formed diol on the overall reaction.
19. The enzyme used throughout this work was obtained as a very crude extract (specific activity towards *para*-chlorostyrene oxide **2** was about 0.2 U/mg). Our recent work (unpublished results) now allows us to prepare a much more purified enzymatic extract which shows specific activity of about 22 U/mg. Therefore, the weight of *An*EH necessary to perform these experiments could be cut down by a factor of 100.
20. We estimate the specific activity of pure *An*EH to be of about 90 U/mg pure enzyme. Interestingly, comparison of the turnover number for a similar substrate (*meta*-chlorostyrene oxide) using Jacobsen catalyst gives a value of 1.5 mol of substrate/mole of catalyst/h: Brandes, B. D.; Jacobsen, E. N. *Tetrahedron: Asymmetry* **1997**, *8*, 3927–3933.
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