## **Enzymatic Transformations 63. High-Concentration Two Liquid-Liquid Phase** *Aspergillus niger* Epoxide Hydrolase-Catalysed Resolution: Application to Trifluoromethyl-Substituted Aromatic Epoxides

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Abstract: The aim of this work was to perform different studies allowing us to improve the methodology we had previously described to realise the Aspergillus niger epoxide hydrolase-catalysed resolution of eight trifluoromethyl-substituted styrene oxide derivatives. A two liquid-liquid phase methodology was developed, for the first time with such an enzyme, using iso-octane as a co-solvent. Different experimental parameters like the global volume substrate concentration, the substrate over enzyme ratio, the agitation rate and the purity of the enzyme were also optimised. This allowed us to set up very efficient bioreactors allowing performance of the hydrolytic kinetic resolution of the different substrates by operating at room temperature (27 °C), within a few hours only and at a very high global volume substrate con-

## Introduction

Epoxides – as well as their corresponding vicinal diols – are important chiral building blocks often involved in the production of a wide range of fine chemicals and pharmaceutical molecules. Owing to the fact that the biological activity of such products is often linked to only one enantiomer (eutomer), and due to the potential toxic side effects of the other antipode (distomer), the production of these molecules in enantiopure form is an ongoing industrial challenge.

As a consequence of several practical features, biocatalysed hydrolytic kinetic resolution (BHKR) of a racemic epoxide can be a very attractive alternative to potentially toxic and substrate-limited chemocatalytic methods. To date, several appropriate enzymes, i.e., epoxide hydrolases (EHs), have been discovered, showing interesting wide and complementary subcentration, i.e., 360 g/L (1.8M). The efficiencies of the *Aspergillus niger* epoxide hydrolase toward the different epoxides studied were compared and the productivity of the practical process was evaluated. These results amply demonstrated that, without contest, the biocatalysed hydrolytic kinetic resolution of epoxides is nowadays to be considered as a very efficient, mild, cheap and easy-to-use "green chemistry" methodology applicable for cost effective preparative (i.e., industrial) scale implementation.

**Keywords:** *Aspergillus niger* epoxide hydrolase; enzyme catalysis; fluorinated styrene oxide derivatives; high-concentration two-phase bioreactor; kinetic resolution

strate and/or enantioselectivity, thus providing the organic chemist with a set of potential tools for performing a specific epoxide resolution.<sup>[1]</sup>

Moreover, some of these enzymes have been cloned and overexpressed, and directed evolution techniques have allowed, in certain cases, one to improve some of their properties.<sup>[2]</sup> Thus, it was by now amply demonstrated that such a biocatalytic approach can, in principle, allow one to perform the resolution of a given racemic epoxide under very mild, cheap, easy-to-use and efficient experimental conditions. We, for instance, have shown that, using the now commercially available *Aspergillus niger (A. niger)* recombinant EH, the resolutions of several types of epoxides were efficiently performed, at room temperature and by using solely demineralised water as solvent, i.e., following a salt-free methodology.<sup>[3]</sup> Obviously, these practical features are very important as far as energy

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consumption and the overall downstream processing costs of a (potential) industrial process are concerned. Moreover, they clearly contribute to the world-wide effort for the development of "environmentally gentle" chemical processes, since the biocatalyst used can very easily be obtained in unlimited amounts, is natural, non-toxic and easily biodegradable.

One primordial key factor for such an industrial process is its volumetric productivity.<sup>[4]</sup> As a general feature, a major drawback in conventional aqueous biocatalysis is the low water solubility of most organic (hydrophobic) substrates (which is typically a few grams per litre) and the resulting poor space-time vield (STY) of the corresponding (theoretical) process. In the case of epoxide hydrolases, several approaches have been developed in order to overcome this stringent bottleneck which severely hampers large-scale industrial development. Different solutions, including addition of a water-miscible co-solvent as well as the use of two liquid-phase reactors, have been developed. For instance, we have shown that the A. niger EH could achieve the resolution of para-nitrostyrene oxide at a concentration of 54 g/L (330 mM) in the presence of 20% DMSO (a watermiscible solvent) over a 6 h period, whereas no reaction occurred in the absence of co-solvent.<sup>[5]</sup> Interestingly however, the same solvent appeared to be deleterious for the whole cell EH activity of either Rhodotorula glutinis<sup>[6,7]</sup> or Rhodococcus rubber SM 1789.<sup>[8]</sup> Subsequent studies demonstrated that, as a general rule, such solvents cause severe enzyme inhibition.<sup>[9]</sup>

Other types of approaches were based on the use of a two liquid-liquid phase methodology, in which the immiscible organic phase was either the neat epoxide itself or various water-immiscible organic cosolvents. In this type of approach, the organic phase plays the role of an internal substrate reservoir and also offers the advantage both to protect the substrate epoxide from spontaneous hydrolysis and to extract part of the formed diol from the aqueous phase, thus minimising the inhibition phenomena very often linked to this reaction product. We have thus demonstrated that, by using the recombinant A. niger EH, the usable so-called "global volume substrate concentration"<sup>[10]</sup> could be as high as 80, 306 or even 500 g/L (respectively 0.36, 2 or 2.5 M) without noticeable substrate and/or product inhibition.<sup>[3a,11]</sup> The space-time yield thus reached good to excellent values (237  $g/Lh^{-1}$  in the last case) thus clearly opening the way to industrial implementation. Along this line, different studies based on the use of water-immiscible co-solvents, including methyl tert-butyl ether,<sup>[6]</sup> dodecane,<sup>[7,12]</sup> *n*-hexadecane<sup>[13]</sup> or iso-octane have also been</sup>explored.<sup>[14]</sup> One recent result described the use of an isolated (overexpressed) bacterial EH, which could be operated in the presence of octane as a co-solvent.<sup>[15]</sup> However, as a general feature and although the substrate concentration within the organic phase was claimed as being reasonable in different examples (i.e., 39 g/L in the last case), the global volume substrate concentration (i.e., 7.8 g/L in the last case) and therefore the overall reactor productivity was only moderate. As a consequence, the space-time yield (at the best a few  $g/Lh^{-1}$ ) of these reactors stayed dramatically incompatible with any further (industrial) implementation.

We here describe our studies on a specific trifluoromethyl-substituted epoxide family, which allowed us to set up, under appropriate co-solvent and optimised operational conditions, cost-effective reactors operating at room temperature (27 °C), at high global volume substrate concentration (i.e., several hundred g/L) and over a short reaction time, thus providing an excellent process productivity.

## **Results and Discussion**

In a preliminary work, we have recently described the *A. niger* EH-catalysed BHKR of the various trifluoromethyl-substituted styrene oxide epoxides *rac*-**1–8** (Scheme 1).<sup>[16]</sup> They were synthesised with moderate to excellent yield (46–99%) starting from the corre-



#### Scheme 1.

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sponding (commercially available) aldehyde or ketone.<sup>[17]</sup> Our exploratory results indicated that, in spite of the presence of fluorine atoms, which very often disturb chemical reactivity, the BHKR of these substrates was indeed possible, at rather low substrate concentrations, however (about 2 mM). In all cases, the unreacted epoxide and the corresponding diol of, respectively, (S) and (R) absolute configurations were obtained, as determined using a circular dichroism methodology.<sup>[16]</sup> In the present work we explored, using the same epoxides *rac*-**1**–**8**, the various experimental options described above for increasing the substrate concentration in this process.

## **Preliminary Experiments**

#### **Use of Water-Miscible Solvents**

Following our previously described results,<sup>[5]</sup> we first explored the possibility to enhance the substrate concentration in the presence of a water-miscible solvent. Epoxide rac-2 was used as a model substrate. Thus, its behaviour in a 20% (v/v) DMSO aqueous solution containing a constant (weight to weight) substrate over enzymatic powder S/E ratio (67)[18,19] was explored, at increasing substrate concentrations (i.e., 0.5, 5 and 10 g/L). Unfortunately, these attempts were unsuccessful, indicating in particular a severe phasetransfer rate limitation of the insoluble substrate to the aqueous/DMSO phase at a 10 g/L concentration. Thus, in contrast to our (above described) previous results, this limitation would obviously be worse in the absence of any water-miscible solvent (i.e., using our neat substrate approach).<sup>[11]</sup> We therefore explored an alternative two liquid-liquid phase methodology based on the use of an additional water-immiscible co-solvent.

## Two Liquid-Liquid Phase Approach using a Water-Immiscible Solvent

*Exploratory studies:* To explore this possibility, epoxide *rac-7* was used as a model substrate. Different water-immiscible solvents were checked, keeping in mind that such a solvent should not have deleterious effects on enzyme activity and should be chosen so that partitioning of substrate and product over the two phases facilitates downstream processing. All experiments were conducted at room temperature (27 °C). Among the solvents tested (diisopropyl ether, *tert*-butyl methyl ether, ethyl acetate, toluene, hexane and iso-octane) iso-octane proved to be by far the most appropriate, leading to the fastest resolution process. Thus, under vigorous (magnetic) stirring, a 10% (v/v) proportion of a *rac-7*/iso-octane solution (this concentration was adjusted so as to afford a 10 g/L global volume substrate concentration) was dispersed in a demineralised water phase, in the presence of an amount of A. niger EH corresponding to a substrate over enzyme (S/E) ratio of 58. Very satisfactorily, the resolution processed efficiently and a conversion ratio of nearly 50% was obtained after 120 min reaction. The recovered (less reactive) epoxide showed an  $ee(ee_s)$  higher than 99% and the calculated  $E_{app}$  value was 33.<sup>[20]</sup> Further experiments were conducted in order to explore the possibility to increase the global volume substrate concentration. Concentrations of 100 and 360 g/L (0.5 and 1.8 M, respectively) were assayed, keeping the 10% (v/v) isooctane/water proportion and an unchanged S/E = 58ratio. As shown in Figure 1 (upper and lower panels), the resolution proceeded very satisfactorily at 100 g/L concentration but, at 360 g/L, the concentration pro-



**Figure 1.** Concentration profile of the *A. niger* EH-catalysed resolution of *rac*-**7** at different global volume substrate concentrations. *Upper panel:* at 10, 100 and 360 g/L using a 10% iso-octane/water medium. *Lower panel:* at 360 g/L global volume substrate concentration using a 20% iso-octane/water medium.

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file clearly indicated the occurrence of a phase-transfer limitation. This was due to the rather surprising (and specific) physico-chemical properties of fluorinecontaining compounds.<sup>[21]</sup> Thus, increasing the substrate concentration led to formation of numerous substrate droplets firmly sticking to the reaction vessel surface, thus making the substrate inaccessible to dispersion/emulsification in the medium. This could be overcome simply by increasing the isooctane/water ratio to 20 % which allowed us to avoid this "substrate sticking" phenomenon (Figure 1, lower panel). Thus, even at substrate concentrations as high as 100 and 360 g/L the resolution could be achieved within 210 and 240 min (3.5 and 4 h) respectively.

Optimisation of the S/E ratio: Due to the interest to minimise the amount of enzyme used (i.e., to optimise the S/E substrate over enzyme ratio) which obviously would allow one to lower the overall cost of a large-scale process, we further explored this option using epoxide rac-4. This was achieved at a fixed substrate concentration of 250 g/L (1.22 M), using a 20% iso-octane/demineralised water liquid medium and by varying the S/E ratio from 50 to 200. The concentration profile of these experiments is provided in Figure 2. It could be observed that, even at an S/E value as high as 200, the BHKR proceeded smoothly within 240 min. From a practical point of view, this translates into the fact that, under these experimental conditions, the BHKR of 1 kilogram of rac-4 could be conducted at room temperature and within about 4 h by using only 5 g of partly purified enzymatic powder (containing about 25% pure enzyme). In this particular case, the catalyst over substrate molecular ratio (i.e., the E/S mol% value), a parameter which is very often used for transition metal catalysts, was about  $5.7 \cdot 10^{-4}$  mol%, a value which very favourably com-



**Figure 2.** Influence of the S/E ratio on the resolution of *rac*-**4** at a global volume substrate concentration of 250 g/L.  $\blacktriangle$ : S/E=50;  $\blacklozenge$ : S/E=100;  $\blacksquare$ : S/E=150;  $\blacklozenge$ : S/E=200.

pares with those generally in operation with these chemical catalysts.  $\ensuremath{^{[22]}}$ 

### SemiPreparative Scale Experiments

On the base of these very satisfactory preliminary results it could be concluded that, at least for epoxides 4 and 7, the use of iso-octane as a water-immiscible solvent allowed us to overcome the above discussed phase-transfer bottleneck. Moreover, this allowed a two liquid-liquid phase methodology to be run at a very high global volume substrate concentration, and also to noticeably minimise the amount of enzyme used. These results greatly encouraged us to pursue this study in order to explore the applicability of these findings to the other epoxides. Each of these substrates was submitted to exploratory semi-preparative trials. The experimental parameters were set so as to provide a 50 g/L global volume substrate concentration. Thus, 1.25 g of epoxide was submitted to BHKR in a 50 mL (total) volume mechanically stirred microreactor, using 25 mL liquid medium (10% v/v iso-octane/demineralised water) and an S/E ratio of 100. The obtained results are provided in Table 1, indicating that all (but 6) epoxides were very efficiently processed under these experimental conditions. Neither substrate nor product inhibition was observed, except in the case of 5, where severe product inhibition was detected. The only exception was for epoxide 6, where the EH showed low activity. In this case, the reaction had to be run at 10 g/L substrate concentration only, using a S/E = 50 value. All resolutions were achieved within a few hours, the  $ee_s$  reaching a value of at least 98% in all cases. Obviously, as in any kinetic resolution, an enantiomerically pure (unreacted) epoxide could be obtained by allowing the reaction to proceed further. The E<sub>app</sub> value was determined for each substrate using both  $ee_s$  and  $ee_p$ . It appeared to be reasonable to excellent for the ortho- and parasubstituted derivatives, whereas it was rather low for the meta-substituted substrates 2 and 7 which only exhibited  $E_{app}$  values of about 5 and 17, respectively. This tendency is in perfect coherence with our general (unpublished) observation about the influence of the aromatic ring substitution pattern of styrene oxide derivatives. The absolute configuration of the recovered epoxide and of the formed diol [that is, (S) and (R), respectively] remained unchanged as compared to our previous results obtained in the absence of water-immiscible solvent.<sup>[16]</sup>

Interestingly, it is to be stressed that epoxides 7 and 8, bearing an additional *gem*-methyl group on the oxirane ring, were efficiently processed. This is a very noteworthy observation since even the Jacobsen's salen (Co)OAc catalysts, claimed as being the best transition metal based catalysts for achieving the

Substrate	1	2	3	4	5	6	7	8
Reaction time	5 h 15	2 h 05	2 h 05	1 h 10	1 h 45	-	3 h 40	5 h 10
ees	98	98.7	98	98.6	98.5	-	98.3	99.1
een	77.3	13.2	84.3	94.5	85	-	59.0	88.3
Eapp	34	5	52	175	60 <sup>[b]</sup>	nd	17	85
Yieldenox	34	11	37	45	33	-	32	35
Yield <sub>diol</sub>	59.2	84.9	51.3	54.8	39	-	64.1	58

**Table 1.** Results obtained upon A. niger EH-catalysed hydrolytic kinetic resolution of rac-1-8, operated in the presence of iso-octane.<sup>[a]</sup>

<sup>[a]</sup> Standard experimental conditions: Global volume substrate concentration: 50 g/L; 10% iso-octane (v/v); S/E=100; nd= not determined.

<sup>[b]</sup> Experiment achieved using a 10 g/L global volume substrate concentration and S/E = 50.

HKR of epoxides, are known to be inoperative on such trisubstituted substrates.

#### **Scale-Up Experiments**

In order to explore the possibility to scale-up this two liquid-liquid phase BHKR methodology, we decided to further optimise two important parameters, i.e., (a) the agitation rate, which partly governs the intimate nature of the emulsion created and therefore the phase-transfer rate of the substrate and (b) the purity of the enzyme to be used, which is another key factor as far as the overall cost of a large-scale application is concerned. These parameters were studied using epoxides rac-4 and 8, respectively. Epoxide 4 was chosen due to its excellent  $E_{app}$  value ( $E_{app}=175$  at 50 g/L) and to the high activity of the A. niger EH against this substrate. The choice of rac-8 (which showed an  $E_{app} = 85$  at 50 g/L) was motivated by the fact that the best conventional chemical catalysts known to day are inefficient on this type of gem-disubstituted substrate. All experiments were conducted in a standardised stirred-tank reactor (STR) equipped with a rotatory Rushton turbine. This type of reactor is commonly used in ours laboratories as a "scale-up tool" allowing accurate determination of most reactor key parameters, thus facilitating direct extrapolation to large-scale application.

#### **Agitation Rate Optimisation**

Using such a 100-mL reactor, 12.5 g of *rac*-4 were submitted to BHKR at room temperature (27 °C), using a 250 g/L global volume substrate concentration, a 20% (v/v) iso-octane/total liquid volume and an S/E ratio of 200. Four experiments were performed, each of them at a different rotation rate, i.e., at respectively 500, 800, 1000 and 1200 rpm. Aliquots were withdrawn each 15 min and analysed for  $ee_s$  using chiral GC chromatography. In each experiment,

**Table 2.** *A. niger* EH-catalysed BHKR of *rac*-**4** conducted in a "perfectly agitated standardised reactor" at a 250 g/L global volume substrate concentration and at different agitation rates.

Agitation rate (rpm)	500	800	1000	1200
ee <sub>s</sub>	98.6	98.7	99.3	99.0
ee <sub>p</sub>	95.7	96.3	95.4	96.0
E <sub>app</sub>	226	267	250	259

the  $E_{app}$  value was determined on the base of  $ee_s$  and  $ee_p$ , the later being measured after completion of the reaction and extraction and purification of the formed diol. The obtained results are provided on Table 2 and their kinetic profile is illustrated on Figure 3.

It could be observed that, except for the experiment conducted at 500 rpm, the reaction rate as well as the calculated  $E_{app}$  value were very comparable one to each other and stayed nearly unchanged from one experiment to the other. To the contrary, both these parameters were lower for the experiment con-



**Figure 3.** Reaction profile of the BHKR of *rac*-4 at different agitation rates. ●: 500 rpm; ◆: 800 rpm; ■: 1000 rpm; ▲: 1200 rpm.

Enzyme (amount U)	Amount of sub- strate (g)	Reaction volume (mL)	Agitation rate (rpm)	Reaction time (min)	ee <sub>s</sub>	ee <sub>p</sub>	$E_{\text{app}}$	Yield epox- ide [%]	Yield diol [%]
purified (1100)	5	50	800	220	99.2	84.3	63	40	49
crude (1100)	5	50	800	290	99.8	78.8	56	41	49
crude (5600)	25	250	600	150	99.7	78.5	52	42	49

**Table 3.** Influence of the enzyme purity on the *A. niger*-catalysed BHKR of *rac*-4. Experiments conducted at a global volume substrate concentration of 100 g/L and 10% (v/v) iso-octane/water.

ducted at 500 rpm. This result clearly indicated that no substrate phase-transfer limitation did occur provided the agitation rate was maintained at least at 800 rpm. This is a valuable information since minimising energy cost is another important goal for running an industrial process. Hence, it is also interesting to stress that the optimum  $E_{\mbox{\scriptsize app}}$  value (about 250) was noticeably higher in these cases as compared to the value of 175 observed during the semi-preparative scale experiment described above, conducted in a non-optimised reactor. These results clearly indicate that intimate dispersion of the substrate in the medium - achieved by using an optimised reactor set and an adequate agitation rate - is another key factor of this resolution. Using these optimised experimental conditions, a total amount of 50 g of rac-4 was processed in four (identical) experiments of 12.5 g each, which proved to be highly reproducible. These were performed using a 250 g/L global volume substrate concentration, an agitation rate of 800 rpm and an S/ E ratio of 200 (i.e., a total amount of 312.5 mg enzymatic powder). Each of these experiments only necessitated about 210 min to reach an  $ee_s > 99\%$ . Extraction and purification of the remaining epoxide and of the formed diol provided as a pool 27.0 g of (S)-4 (ee 99%, 43% yield) and 29.1 g of the corresponding (*R*)-diol (*ee* 95%, 44% yield).

## Use of a Crude Enzymatic Preparation

An additional important parameter is the cost of the enzyme production itself. As described previously, the *A. niger* EH is produced by culturing the recombinant fungal strain, which only necessitates a simple and cheap culture medium. In a first step, a straightforward isolation procedure affords the crude enzyme in aqueous solution. In a second step, this can be further purified to obtain the partly purified enzyme in a lyophilised form (which is in fact the material which was used up to now throughout this study). We now checked whether the crude material could be used to perform the BHKR of *rac*-**8** as a model substrate and, for the sake of comparison, the same experiment was conducted using the partly purified EH. For each one of these experiments, 5 g of *rac*-**8** were processed at

room temperature (27°C) using a 100 g/L global volume substrate concentration, 50 mL of a 10% (v/v) iso-octane/water liquid medium, a 800 rpm agitation rate and 1100 U of enzyme.<sup>[18]</sup> The stirred-tank reactor used was the same as the one mentioned above for the BHKR of rac-4. The reactions were followed by controlling the  $ee_s$  using chiral GC and the  $E_{app}$  values were calculated on the base of the  $ee_s$  and ee<sub>p</sub> after extraction and purification of the reaction product. Interestingly enough, no difference between the two experiments did appear, the kinetic profiles of the two reactions being nearly superimposable. The  $E_{app}$  values appeared to be identical as well (Table 3). From these experiments it could be concluded that the purity of the enzyme used influenced neither the reaction rate nor the product formation of the reaction. In particular, no conflicting side reactions, which could have been catalysed by other enzymes present in the crude extract, did occur.

As a validation of this observation, a 25-g preparative-scale experiment using the crude enzyme was operated under the same experimental conditions. Interestingly, due to the improved geometrical characteristics of the 500-mL standardised STR we used, a lower (600 rpm) agitation rate was sufficient and a shorter reaction time was observed.

### Resolution of rac-1-8 using Jacobsen's Catalysts

As far as the resolution of racemic epoxides is concerned, different conventional chemistry-based methodologies have been described over the recent years, the best known implying the use of transition metalbased catalysts. In order to compare the efficiency of our enzymatic methodology versus these chemical approaches, we have explored the possibility to perform the resolution of rac-1-8 using the so-called salen(Co)OAc catalyst, using the best appropriate experimental conditions.<sup>[22]</sup> Thus, 1 mmol of each epoxide was submitted to a HKR using (R,R)-(salen)Co-(OAc) as catalyst. The conversion ratio c and the  $ee_s$ values were determined after extraction of the reaction medium and chiral GC analysis. When appropriate, the *ee* of the formed diol  $(ee_p)$  was similarly determined after chemical derivatisation. The respective

e	( 2)	•		· · /				
Substrate	1	2	3	4	5	6	7	8
c	77.7	61	nd	nd	nd	25.8	2	19.6
ees	23.3	100	97.8	98.6	99.6	0.2	0.1	0.2
eep	nd	nd	79.3	89.0	89.4	nd	nd	nd
E <sub>app</sub> chemical	~1	>30	38	84	110	~1	~1	~1
$E_{app}^{n}$ enzymatic (at 50 g/L)	34	5	52	175	60	nd	17	85

**Table 4.** Analytical scale HKR of *rac*-**1–8** using (*R*,*R*)-(salen)Co(OAc) as catalyst. Experiments conducted on 1 mmol of substrate at 27 °C, using 0.007 mmol (4.76 mg) of catalyst and 0.55 mmol (9.9  $\mu$ L) of water. Reaction time: 48 h.

 $E_{app}$  values were calculated by using either the c and  $ee_{\rm s}$  values or the  $ee_{\rm s}$  and  $ee_{\rm p}$  values. The results obtained are summarised in Table 4. For a comparison purpose, the E<sub>app</sub> values obtained by our A. niger EH enzymatic resolution under biphasic conditions (i.e., 10% v/v iso-octane) at a global volume substrate concentration of 50 g/L are also recalled in Table 4. Our results indicated that the outcome of the reaction was clearly depending upon the substitution pattern of the aromatic ring, as well as of the epoxide moiety. Thus, in the case of the monosubstituted epoxides (a) the ortho-substituted substrate 1 was not resolved by the salen(Co) catalyst, whereas an  $E_{app}$  value of about 34 was measured using the biocatalytic approach, (b) the  $E_{app}$  value of the *meta*-substituted substrate 2 was higher with the salen(Co) catalyst, (c) in the case of the para-substituted epoxides 3, 4 and 5, two out of three substrates showed a better  $E_{app}$  value under enzymatic catalysis, (d) hence, the chemical approach was inoperative for all gem-disubstituted epoxides 6, 7 and 8.

## **Catalytic Efficiency**

In addition to the  $E_{app}$  value, which obviously is of fundamental importance in the context of a preparative-scale resolution process, some other important parameters - which unfortunately are very often not addressed – are (a) the so-called turnover frequency (TOF), (b) the total turnover number (TON), (c) the average turnover frequency (aTOF) (i.e., the TON value divided by the time necessary to reach completion of the reaction), and the space-time yield (STY) of the process.<sup>[4,23]</sup> The first parameter relates to the efficiency of a given catalyst by indicating the number of molecules of substrate transformed - in a given time period - by one molecule of catalyst. This TOF value corresponds in fact to the  $k_{cat}$  value used in enzymology and is generally measured at the beginning of the reaction.<sup>[24]</sup> The second parameter (TON) relates to the overall efficiency and stability of the catalyst in the given experimental conditions (catalyst productivity), by indicating the total number of substrate molecules transformed by one molecule of catalyst until this catalyst is exhausted, independently of the time necessary for this transformation.<sup>[4]</sup> The third value (aTOF) provides a good image of the average efficiency of the catalyst over time. The fourth (STY) parameter describes the overall efficiency of a given reactor by indicating the productivity of the process (generally expressed in grams per litre per hour). Obviously, the higher these TOF, TON, aTOF and STY values, the better the catalyst and the overall process will be. An additional difficulty to the calculation of the correct TON and aTOF values lies in the fact that it is very difficult to accurately tune the minimum amount of (bio)catalyst to be used to just reach this ees and to, simultaneously, exhaust the (bio)catalyst. Therefore, the amount of catalyst used will be higher than strictly necessary, and the experimentally evaluated TON and aTOF will be lower than the "real" values. However, provided the amount of catalyst used is only slightly higher than the minimum required, the calculated value will - although underestimated – provide a good image of the efficiency of the (bio)catalyst.

## Experimental Determination of the Efficiency Parameters (h2)

According to the above described definitions, the different efficiency parameters related to the resolution experiments of each epoxide **1–8** were determined and are summarised in Table 5.

As commented above, the experimental conditions under which the different parameters of Table 5 were recorded were not optimised for each one of the experiments. Therefore, as a general rule, all these values surely could be further improved. Nevertheless, it can be observed that most of them are already very satisfactory. Thus, all the E/S mol% values range at values of about  $10^{-3}$ , which very favourably compare with those generally used for transition metalbased catalysts.<sup>[22]</sup> For example, this value is  $1.1 \cdot 10^{-3}$ mol% for *rac*-4 at S/E = 100 and 50 g/L, i.e., under non-optimised conditions (see Table 1). However, we have demonstrated (see above) that, for this same substrate, an S/E ratio of 200 could be used to achieve the nearly optimised resolution at a 250 g/L global volume substrate concentration. In this case, the cal-

Epoxide	$\mathrm{E}_{\mathrm{app}}$	S/E [w/w]	E/S <sup>[a]</sup> mol %	TON <sup>[a]</sup> [mol sub/mol enz]	<i>a</i> TOF <sup>[a]</sup> [mol sub/mol enz/h]	$STY_{diol}$ [g/L h <sup>-1</sup> ]	$\frac{\text{STY}_{\text{epoxide}}}{[g/Lh^{-1}]}$	
1	34	100 <sup>[b]</sup>	$10^{-3}$	$5.7 \cdot 10^4$	$11.10^{3}$	5.7	3.3	
2	5	100 <sup>[b]</sup>	$10^{-3}$	$8.1 \cdot 10^4$	$39.5 \cdot 10^3$	20.7	2.7	
3	52	100 <sup>b]</sup>	$10^{-3}$	$4.8 \cdot 10^4$	$23.4 \cdot 10^3$	12.5	9	
4	175	$100^{[b]}$	$1.1 \cdot 10^{-3}$	$4.8 \cdot 10^4$	$44.1 \cdot 10^3$	29.9	20.5	
4	267	200 <sup>[c]</sup>	$5.7 \cdot 10^{-4}$	$7.8 \cdot 10^4$	$22 \cdot 10^3$	34	30.7	
5	60	50 <sup>[b]</sup>	$2.4 \cdot 10^{-3}$	$2.10^{4}$	$14.10^{3}$	2.9	2.3	
6	nd	$100^{[b]}$	$1.1 \cdot 10^{-3}$	nc	nc	nc	-	
7	17	$100^{[b]}$	$1.1 \cdot 10^{-3}$	$5.6 \cdot 10^4$	$16.6 \cdot 10^3$	9.4	4.7	
8	85	$100^{[b]}$	$1.1 \cdot 10^{-3}$	$2.6 \cdot 10^4$	$5.1 \cdot 10^3$	5.7	3.4	
8	52	_[d]	$2.3 \cdot 10^{-3}$	$2.2 \cdot 10^4$	$8.7 \cdot 10^3$	21.5	16.8	

Table 5. Process efficiency parameters calculated upon resolution of epoxides 1-8 catalysed by the A. niger EH.

<sup>[a]</sup> Calculated assuming a 25% content of EH in the enzymatic extract and a molecular weight of 45 kDa of the protein. nc: not calculated.

<sup>[b]</sup> Standard experimental conditions described in Table 1.

[c] Experimental conditions corresponding to the preparative scale biohydrolysis of rac-4 at 250 g/L in an STR.

<sup>[d]</sup> Experimental conditions corresponding to the preparative scale biohydrolysis of 25 g of *rac*-8 at 100 g/L in a STR using crude enzymatic extract as biocatalyst.

culated E/S mol% value was as low as  $5.7 \cdot 10^{-4}$ %. Similarly, under the non-optimised experimental conditions, the recorded TON value of this resolution was  $4.8 \cdot 10^4$ , whereas under the optimised conditions, a noticeably higher TON value of about  $7.8 \cdot 10^4$  was calculated.

More generally, the different parameters recorded in Table 5 afford interesting information about the efficiency of the resolution of *rac*-1–8 by the A. *niger* EH. It can be seen that, for all epoxides (but rac-6) the TON values, which lie in the range  $2 \cdot 10^4$  to  $8 \cdot 10^4$ , very favourably compare with those generally accepted for conventional chemical catalysis, which ought to be >1,000 for high-value products and >50,000 for large-scale or less-expensive products.<sup>[23]</sup> Similarly, due to the catalytic efficiency of the enzyme (short reaction time), the aTOF values, which lie in the range  $5 \cdot 10^3$  to  $4.4 \cdot 10^4$  mol sub/mol enz/h, again are excellent as compared to those of chemical processes were *a*TOF values of 500 mol sub/mol cat/h for small and >10,000 mol sub/mol cat/h for large scale products are considered as being good to excellent.<sup>[23]</sup>

Finally, another important parameter – i.e., the STY value – was calculated for the formed diol as well as for the recovered epoxide. The obtained values (Table 5) in particular highlight the fact that, as for instance for *rac-2*, excellent TON, *a*TOF and STY<sub>diol</sub> values do not necessarily reflect an interesting process. This is due to the poor E value (E=5) of this resolution. To the contrary, excellent STY<sub>diol</sub> and STY<sub>epox</sub> values of over 30 g/L h<sup>-1</sup> could be observed for *rac-4*, for which an E value of 267 was estimated at 250 g/L global substrate concentration.

These different results and considerations clearly illustrate the fact that, due to the multiparametric nature of such a resolution process, careful examination and combination of all the different parameters of a specific process have, as a general rule, to be considered for correct evaluation.

## Conclusions

In the course of this work, we have developed different studies aimed at improving the methodology we had previously described to perform the *Aspergillus niger* epoxide hydrolase-catalysed resolution of eight trifluoromethyl-substituted styrene oxide derivatives (*rac*-**1**-**8**). Our present results indicated that, by using a 10 to 20% (v/v) proportion of iso-octane as a cosolvent, we were able to overcome the severe global volume substrate concentration bottleneck observed in our preliminary studies. Thus, very efficient and cost-effective resolution processes could be run. For example, *rac*-**7** could be resolved at a global volume substrate concentration as high as 360 g/L (1.8M) using a 20% iso-octane/demineralised water liquid medium in a two liquid-liquid phase reactor.

For the sake of cost effectiveness, other key parameters, i.e., (a) the substrate over biocatalyst S/E ratio, (b) the agitation rate and (c) the purity of the enzyme used, were also optimised. Thus, resolution of epoxide *rac-4* could be performed at 250 g/L global volume substrate concentration (1.22 M), using an S/E ratio of 200. This in fact represents a  $5.7 \cdot 10^{-4}$  mol% ratio of catalyst, a value which very favourably compares with the ones used with the best transition metal-based catalysts. From a practical point of view, this result translates into the fact that, under these experimental conditions, the BHKR of 1 kilogram of *rac-4* could be conducted, within a 4 h period, by using only 5 g of (commercially available) enzymatic powder. As far as

the agitation rate is concerned, its optimum value was determined. Depending on the specific reactor vessel geometry, moderate values of 600 or 800 rpm were shown to be sufficient. Finally, we have demonstrated that the (very easily prepared) crude enzyme could be used in place of the purified one. Indeed, its purity did not influence the reaction rate and no conflicting side reactions, which could have been catalysed by other enzymes present in the crude extract, did occur. As a validation of this observation, a 25 g preparative-scale experiment using this crude enzyme was operated and led to excellent results. Owing to the importance of the efficiency parameters for any industrial process, the total turnover number (TON), the average turnover frequency (aTOF) and the spacetime yield (STY) were determined. Although not optimised, they were shown to be good to excellent and to very favourably compare with those generally accepted for industrial applications of transition metalbased catalysis. According to these results, the biocatalysed hydrolytic kinetic resolution of epoxides proved to be a very mild, cheap, easy-to-use, efficient and cost effective methodology. Therefore, it clearly does contribute to the world-wide effort for development of "environmentally gentle" (bio)chemical processes, since the biocatalyst used can easily be obtained in unlimited amounts, is natural, non-toxic and biodegradable. Work is going on in our laboratory in order to further explore the applicability of this novel two liquid-liquid phase process to the resolution of other interesting epoxides.

## **Experimental Section**

### **General Remarks**

GC analyses were performed with a Shimadzu GC14 A apparatus equipped with an FID detector and helium as carrier gas. Determination of the enantiomeric excesses was performed using 2 different chiral columns (0.25 mm, 25 m), i.e., Chirasil-Dex CB (column I, Varian) and Lipodex E (column II, Macherey–Nagel). <sup>1</sup>H NMR, <sup>13</sup>C NMR and <sup>19</sup>F NMR spectra were recorded on a Bruker AC250 instrument at 250, 62.9 and 235 MHz, respectively. All measurements were carried out at room temperature in CDCl<sub>3</sub>, acetone- $d_6$  or DMSO- $d_6$ .

### **Enzyme Preparation**

**Partly purified enzyme in a lyophilised form:** The recombinant *A. niger* Gbcf 79 containing the EH gene was cultivated in a simple medium with only glucose (10 g/L) and corn steep liquor (20 g/L) as nutrients in 10 L fermentors at 27 °C as previously described.<sup>[5]</sup> After 40 h of culture incubation, the mycelium was recovered by vacuum filtration and resuspended in 1.4 L of 10 mM phosphate buffer, pH 7.1, containing 1 mM EDTA, 1 mM cysteine and 0.3 mM phenylmethanesulfonyl fluoride to prevent enzyme inactivation

(buffer A). The suspension was homogenised using a mechanical grinder and the cells were disrupted by pressure shock (27 kpsi, Cell Disrupter from Constant System Ltd, Daventry Northants, United Kingdom) to liberate the intracellular EH activity. Unbroken cells and cellular debris were precipitated by centrifugation  $(13,300 \times g, 90 \text{ min})$  at 4°C and discarded. The supernatant, containing the EH was treated by microfiltration using a membrane with a cut-off of 0.1 µm (Inceltech, PSFH, France) to obtain a clear extract. The microfiltration process was repeated 3 times after dilution of the retentate with buffer A (2 L). The resulting microfiltrates, were concentrated to 200 mL by tangential flow ultrafiltration through a membrane with a cut-off of 40 kDa (Inceltech, 40UFIB/1/S2, France). The concentrated enzyme solution was finally desalted by gel filtration (Sepharose G-25) using water as eluant. The active fractions were pooled then freeze-dried to furnish the enzymatic powder (1.5 g) stored at 4°C. All procedures were carried out at 4°C.

*Crude enzymatic preparation:* The experimental protocol was the same as described above except that, after centrifugation of the cell debris, the supernatant was just concentrated by microfiltration in order to obtain a final volume of 200 mL. This crude enzymatic solution was frozen in liquid nitrogen then stored at -80 °C.

#### Synthesis of epoxides

Epoxides **1–8** were synthesized from the corresponding aldehyde or ketone precursor by reaction with either  $(CH_3)_3S^+I^-$  (method I) or  $(CH_3)_3SO^+I^-$  (method II) in the presence of NaH.

General procedure:  $(CH_3)_3S^{+I^-}$  [or  $(CH_3)_3SO^{+I^-}$ ] (20 mmol) was dissolved in DMSO (15 mL). To this solution NaH (20 mmol, 60% in oil) was added under nitrogen at room temperature. After stirring for 20 min, the corresponding aldehyde or ketone (17 mmol) dissolved in DMSO (20 mL) was added dropwise within 20 min. After stirring for 1–2 h at room temperature, the reaction mixture was poured into cold water (80 mL) and the epoxide was extracted with ethyl acetate (3×100 mL). The collected organic phase fractions were washed 3 times with water (3× 100 mL), dried over MgSO<sub>4</sub> and concentrated under vacuum. The crude epoxide was purified by bulb-to-bulb distillation under reduced pressure or by flash chromatography.

(2-Trifluoromethylphenyl)-oxirane (1): This epoxide was obtained (method I) as a yellow liquid; yield: 78%; bp 50°C (0.08 mbar); <sup>1</sup>H NMR:  $\delta$ =2.75 (dd, 1H, J=5.5 Hz, J= 2.5 Hz), 3.21 (dd, 1H, J=5.5 Hz, J=4.25 Hz), 4.15 (m, 1H), 7.44 (d, 1H, J=7.75 Hz), 7.54 (t, J=7.5 Hz), 7.63 (t, 1H, J=7.5 Hz), 7.75 (d, 1H, J=7.75 Hz); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>):  $\delta$ = 48.5 (q,  $J_{CF}$ =2.7 Hz, CH), 50.2 (CH<sub>2</sub>), 124.3 (q,  $J_{CF}$ =273.5, C), 125.5 (q,  $J_{CF}$ =5.4 Hz, CH), 125.5 (m, CH), 126.9 (q,  $J_{CF}$ =30.7 Hz, C) 128.2 (CH), 132.9 (m, CH), 136.2 (m, C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-59.62; exact mass calcd. for C<sub>9</sub>H<sub>7</sub>F<sub>3</sub>O: 187.0371; found: 187.0374. Chiral GC analysis [column I, *T*=100°C: (*R*)=3.8 min; (*S*)=4.8 min].

(3-Trifluoromethylphenyl)-oxirane (2): This epoxide was obtained (method II) as a yellow-orange liquid; yield: 52%; bp 78 °C (1.2 mbar); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 2.79$  (dd, 1H, J=2.5 Hz, J=5.3 Hz), 3.19 (dd, 1H, J=4.0 Hz, J=5.3 Hz),

3.92 (dd, 1 H, J=2.5 Hz, J=4.0 Hz), 7.38–7.65 (m, 4H); <sup>13</sup>C NMR:  $\delta = 51.4$  (CH<sub>2</sub>), 51.7 (CH), 122.3 (q,  $J_{CF}=3.8$  Hz, CH), 124.0 (q,  $J_{CF}=272.0$  Hz, C), 125.0 (q,  $J_{CF}=3.7$  Hz, CH), 128.7 (m, CH), 129.8 (CH), 131.0 (q,  $J_{CF}=32.4$  Hz, C), 138.8 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -62.34$ ; exact mass calcd. for C<sub>9</sub>H<sub>7</sub>F<sub>3</sub>O: 187.0371; found: 187.1324. Chiral GC analysis [column II, T=90°C: (S)=13.2 min; (R)=13.7 min].

(4-Trifluoromethylphenyl)-oxirane (3): This epoxide was obtained (method I) as a yellow liquid; yield: 70%; bp 70°C (0.08 mbar); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$ =2.77 (dd, 1H, *J*= 5.75 Hz, *J*=2.75 Hz), 3.18 (dd, 1H, *J*=5.75 Hz, *J*=4.0 Hz), 3.91 (dd, 1H, *J*=4 Hz, *J*=2.75 Hz), 7.39 (d, 1H, *J*=8 Hz), 7.60 (d, 2H, *J*=8 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$ =51.4 (CH<sub>2</sub>), 51.7 (CH), 124.0 (q, *J*<sub>CF</sub>=271.8 Hz, C), 125.5 (q, *J*<sub>CF</sub>= 4.0 Hz, CH), 125.7 (CH), 130.4 (q, *J*<sub>CF</sub>=32.2 Hz, C), 141 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-62.28; exact mass calcd. for C<sub>9</sub>H<sub>7</sub>F<sub>3</sub>O: 187.0371; found: 187.0374. Chiral GC analysis [column I, *T*=120°C: (*R*)=9.7 min; (*S*)=10.7 min].

(4-Trifluoromethoxyphenyl)-oxirane (4): This epoxide was obtained (method I) as a colourless liquid; yield: 73%; bp 100 °C (0.07 mbar); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$ =2.79 (dd, 1H, J=2.5 Hz, J=5.3 Hz), 3.19 (dd, 1H, J=4.0 Hz, J=5.3 Hz), 3.92 (dd, 1H, J=2.5 Hz, J=4.0 Hz), 7.38–7.65 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$ =51.3 (CH<sub>2</sub>), 51.7 (CH), 120.4 (q,  $J_{CF}$ = 265.5 Hz, C), 121.1 (CH), 126.9 (CH), 136.4 (C), 149.1 (q, J=1.9 Hz, C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-57.35; exact mass calcd. for C<sub>9</sub>H<sub>7</sub>F<sub>3</sub>O<sub>2</sub>: C 52.95, H 3.46, F 27.92; found: C 52.60, H 3.38, F 29.04. Chiral GC analysis [column I, T=120°C: (R)=4.4 min; (S)=4.8 min].

(4-Trifluoromethylthiophenyl)-oxirane (5): This epoxide (method I) was purified by flash chromatography (pentane/ ethyl acetate, 90:10) then distilled (bp 100°C, 0.1 mbar) to provide a colourless liquid; yield: 75%; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$ =2.78 (dd, 1H, *J*=2.5 Hz, *J*=5.5 Hz), 3.18 (dd, 1H, *J*= 4.0 Hz, *J*=55 Hz), 3.89 (dd, 1H, *J*=2.5 Hz, *J*=40 Hz), 7.37 (d, 2H, *J*=8.2 Hz), 7.64 (d, 2H, *J*=8.2 Hz); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$ =66.9 (CH<sub>2</sub>), 73.1 (CH), 124.0 (q, *J*=1.9 Hz, C), 127.9 (CH), 129.6 (q, *J*<sub>CF</sub>=307.6 Hz, C), 135.7 (CH), 147.4 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-43.27; exact mass calcd. for C<sub>9</sub>H<sub>7</sub>F<sub>3</sub>OS: C 49.09, H 3.20, F 25.88, S 14.56; found: C 49.41, H 3.22, F 27.11, S 13.38. Chiral GC analysis [column I, *T*= 130°C: (*R*)=7.2 min; (*S*)=7.6 min].

**2-Methyl-2-(2-trifluoromethylphenyl)-oxirane** (6): This epoxide (method I) was purified by flash chromatography (pentane/ethyl acetate, 95:5) to provide a colourless liquid; yield: 75%; <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  = 24.6 (CH<sub>3</sub>), 55.3 (q, J<sub>CF</sub> = 3.6 Hz, C), 57.6 (CH<sub>2</sub>), 125.1 (q, J<sub>CF</sub> = 271.2, C), 126.8 (q, J<sub>CF</sub> = 5.3 Hz, CH), 127.8 (q, J<sub>CF</sub> = 30.9 Hz, C), 128.6 (CH), 129.9 (CH), 132.8 (CH), 140.6 (m, C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$  = -62.24. Chiral GC analysis [column I, T=90°C: (R) = 10.4 min; (S) = 10.9 min].

**2-Methyl-2-(3-trifluoromethylphenyl)-oxirane** (7): This epoxide was obtained (method II) as a colourless liquid; yield: 99%; bp 117°C (39 mbar); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$ =1.55 (s, 3H), 2.75 (d, 1H,  $J_{\rm HH}$ =5.4 Hz), 2.98 (d, 1H, J=5.4 Hz), 7.40–7.59 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$ =21.5 (CH<sub>3</sub>), 56.2 (CH<sub>2</sub>), 57.0 (C), 122.2 (q,  $J_{\rm CF}$ =3.8 Hz, CH), 124.1 (q,  $J_{\rm CF}$ =272.5 Hz, C), 124.3 (q,  $J_{\rm CF}$ =3.8 Hz, CH), 128.7 (CH), 128.9 (CH), 130.8 (q,  $J_{\rm CF}$ =32.1 Hz, C), 142.4 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-62.27; exact mass calcd. for C<sub>10</sub>H<sub>9</sub>F<sub>3</sub>O:

201.0527; found: 201.0533. Chiral GC analysis [column I, T=100 °C: (R) = 8.5 min; (S) = 9.1 min].

**2-Methyl-2-(4-trifluoromethylphenyl)-oxirane** (8): This epoxide was obtained (method II) as a colourless liquid; yield: 99%; bp 40°C (0.07 mbar); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$ = 1.73 (s, 3H), 2.76 (d, 1H, *J*=5.4 Hz), 3.00 (d, 1H, *J*= 5.4 Hz), 7.48 (d, 2H, *J*=8.1 Hz), 7.59 (d, 2H, *J*=8.1 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$ =22.2 (CH<sub>3</sub>), 57.1 (CH<sub>2</sub>), 57.8 (C), 124.9 (q, *J*<sub>CF</sub>=270.3 Hz, C), 126.1 (q, *J*<sub>CF</sub>=3.8 Hz, CH), 126.5 (CH), 130.5 (q, *J*<sub>CF</sub>=31.9 Hz, C), 146.1 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta$ =-62.13; exact mass calcd. for C<sub>10</sub>H<sub>9</sub>F<sub>3</sub>O: 201.0527; found: 201.0536. Chiral GC analysis [column I, *T*=120°C: (*R*)=6.4 min; (*S*)=7.1 min].

## Analytical Scale Biohydrolysis of *rac-2* using DMSO as Co-solvent

20  $\mu$ L of an *A. niger* solution (0.37–7.5 mgmL<sup>-1</sup> in demineralised water, S/E ratio of 67) were added to *rac*-**2** (0.5– 10 mg, final epoxide concentration of 0.5–10 gL<sup>-1</sup>) previously mixed into a mixture of DMSO (250  $\mu$ L) containing 3-(trifluoromethyl)-acetophenone (0.3–0.6  $\mu$ L) as an internal standard and demineralised water (780  $\mu$ L). After mixing for 20 min at 27 °C the enzymatic resolution was stopped by adding acetonitrile (500  $\mu$ L) into the medium. The remaining epoxide was extracted with iso-octane (1 mL) and its enantiomeric excess and the conversion ratio were determined by chiral GC analysis.

### Analytical Scale Biohydrolysis of *rac-7* using Isooctane as Co-solvent

50  $\mu$ L of an *A. niger* solution (3.44–124 mgmL<sup>-1</sup> in demineralised water to obtain a constant S/E ratio of 58) were added to *rac*-**7** (final epoxide concentration of 10–360 gL<sup>-1</sup>) previously mixed into a mixture of iso-octane (100  $\mu$ L or 200  $\mu$ L) containing 3-(trifluoromethyl)-acetophenone (20  $\mu$ L) as an internal standard and demineralised water (390– 840 $\mu$ L, final total volume 1 mL). The kinetic resolution (27 °C, under vigorous magnetic stirring) was followed by taking aliquots from the reaction medium (1–10  $\mu$ L) immediately mixed with ethyl acetate (500  $\mu$ L) to stop the enzymatic reaction and to extract the remaining epoxide and the formed diol. The enantiomeric excess of the remaining epoxide and the conversion ratio were determined by chiral GC analysis.

### General Procedure for Semi-preparative Biohydrolysis of 1–8 at 50 g/L

Typically, 1 mL of an *A. niger* EH solution (12.5 mgmL<sup>-1</sup> in demineralised water) was added to *rac*-**1–8** (1.25 g) mixed into an emulsion of iso-octane (2.5 mL) and water (21.5 mL). The reaction was performed at 27 °C. Aliquots (1  $\mu$ L) were withdrawn at regular time intervals and immediately added to ethyl acetate (40  $\mu$ L). After extraction the enantiomeric excess of residual epoxide was determined by chiral GC chromatography until *ee* >97%. Then the enzymatic reaction was stopped and the remaining epoxide was extracted by addition of ethyl acetate (30 mL). After decantation the water phase was extracted two times using ethyl acetate (2×50 mL) then the collected organic fractions were washed with brine (30 mL) before drying over MgSO<sub>4</sub>.

The ethyl acetate was removed under atmospheric pressure and the residue was subjected to flash chromatography (silica gel: hexane/ethyl acetate, gradient). The isolated remaining epoxide and formed diol were further purified by bulb-to-bulb distillation. The <sup>1</sup>H NMR spectra of the enantiopure epoxides 1-8 were identical to those of the racemates.

All diols were isolated as white solids at room temperature.

(S)-1: (ee 97.9%);  $[\alpha]_{D}^{22}$ : +62.4 (c 0.89, CHCl<sub>3</sub>).

(*R*)-1d: (*ee* 77.3%);  $[\alpha]_{D}^{22}$ : -47.9 (*c* 0.98, CHCl<sub>3</sub>); mp 51°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 2.45$  (s, 1 H), 3.00 (s, 1 H), 3.67 (m, 2H), 5.23 (m, 1H) 7.44–7.65 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta = 67.8$  (CH<sub>2</sub>), 74.4 (CH), 125.6 (q,  $J_{CF} = 5.8$  Hz, CH), 124.2 (q, J<sub>CF</sub>=273.7 Hz, C), 127.3 (q, J<sub>CF</sub>=30.3 Hz, C), 128.0 (CH), 128.2 (m, CH), 132.2 (m, CH), 139.2 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -57.93$ ; exact mass calcd. for C<sub>9</sub>H<sub>9</sub>F<sub>3</sub>O<sub>2</sub>: 205.0476; found: 205.0468. Chiral GC analysis after derivatisation to the corresponding dimethoxy ether [column I, T = 80 °C: (S) = 22.0 min; (R) = 22.6 min]

(S)-2: (*ee* 98.7%);  $[\alpha]_{D}^{22}$ : +9.1 (*c* 0.92, CHCl<sub>3</sub>), Lit.<sup>[25]</sup>  $[\alpha]_{D}^{22}$ : + 2.7 (c 0.92, CHCl<sub>3</sub>).

(*R*)-2d: (*ee* 13.2 %);  $[\alpha]_{D}^{22}$ : -5.7 (*c* 0.98, CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 2.26$  (m, 1H), 2.87 (m, 1H), 3.57 (dd, 1H, J =11 Hz, J=8.25 Hz), 3.73 (dd, 1H, J=11 Hz, J=3.25 Hz), 4.81 (dd, 1H, J=3.25 Hz, J=8.25 Hz) 7.44–7.65 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta = 67.9$  (CH<sub>2</sub>), 74.0 (CH), 122.8 (q,  $J_{CF} =$ 3.8 Hz, CH), 124 (q,  $J_{\rm CF}$ =3.7 Hz, CH), 124.8 (q,  $J_{\rm CF}$ =272.4 Hz, C), 129.0 (CH), 129.4 (CH), 130.9 (q,  $J_{\rm CF}$ = 32.3 Hz, C), 139.4 (C). Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T=140 °C:  $(S) = 5.3 \min; (R) = 5.8 \min).$ 

(*S*)-**3**: (*ee* 97.9%);  $[\alpha]_{D}^{22}$ : +18 (*c* 1.13, CHCl<sub>3</sub>).

(*R*)-**3d:** (*ee* 84.3%);  $[\alpha]_{D}^{22}$ : -39.3 (*c* 1.03, CHCl<sub>3</sub>), Lit.<sup>[26]</sup>  $[\alpha]_{D}^{22}$ : -57 (c 1, CHCl<sub>3</sub>); mp 101 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta =$ 2.07 (m, 1H), 2.70 (m, 1H), 3.88 (m, 2H), 4.89 (m, 1H), 7.50 (d, 2H, J=8.5 Hz), 7.63 (d, 2H, J=8.5 Hz); <sup>13</sup>C NMR (acetone- $d_6$ ):  $\delta = 68.6$  (CH<sub>2</sub>), 74.7 (CH), 125.5 (q,  $J_{CF} =$ 269.4 Hz, C), 125.6 (q, J<sub>CF</sub>=3.8 Hz, CH), 127.8 (CH), 129.5 (q,  $J_{CF}$ =31.9 Hz, C), 148.5 (C); <sup>19</sup>F NMR (acetone- $d_6$ ):  $\delta$  = -62.21; exact mass calcd. for C<sub>9</sub>H<sub>9</sub>F<sub>3</sub>O<sub>2</sub>: 205.0476; found: 205.0457. Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T=140 °C: (S)=5.5 min; (R) = 6.1 min].

(*S*)-4: (*ee* 98.6%);  $[\alpha]_{D}^{22}$ : + 13.7 (*c* 1.58, CHCl<sub>3</sub>).

(*R*)-4d: (*ee* 94.5%);  $[\alpha]_{D}^{22}$ : -41.5 (*c* 1.04, CHCl<sub>3</sub>); mp 64°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 2.13$  (m, 1H), 2.69 (m, 1H), 3.70 (m, 2H), 4.84 (m, 1H), 7.21 (d, 2H, J=8.75 Hz), 7.41 (d, 2H, J = 8.5 Hz); <sup>13</sup>C NMR (acetone- $d_6$ ):  $\delta = 68.7$  (CH<sub>2</sub>), 74.5 (CH), 121.4 (CH), 121.5 (q,  $J_{CF}$ =253.3 Hz, C), 128.8 (CH), 143.1 (C), 148.9 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -57.61$ ; exact mass calcd. for C<sub>9</sub>H<sub>9</sub>F<sub>3</sub>O<sub>3</sub>: 221.0426; found: 205.0457; anal. calcd. for C<sub>9</sub>H<sub>9</sub>F<sub>3</sub>O<sub>3</sub>: C 48.66, H 4.08, F 25.65; found: C 48.79, H 4.08, F 26.41. Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T=140 °C:  $(S) = 5.5 \min; (R) = 6.1 \min].$ 

(S)-5: (ee 98.5%);  $[\alpha]_D^{22}$ : + 21.1 (c 1.2, CHCl<sub>3</sub>). (R)-5d: (ee 85%):  $[\alpha]_D^{22}$ : -5 (c 1.18, CHCl<sub>3</sub>); mp 72°C; <sup>1</sup>H NMR (DMSO- $d_6$ ):  $\delta = 3.46$  (m, 2H), 4.61 (dd, 1H, J =4.25 Hz, J = 5.75 Hz), 4.83 (dd, 1H, J = 5.75 Hz, J = 5.75 Hz), 5.47 (d, 1H, J = 4.25 Hz), 7.51 (d, 2H, J = 8 Hz), 7.67 (d, 2H, J = 8 Hz; <sup>13</sup>C NMR (DMSO- $d_6$ ):  $\delta = 67.9 \text{ (CH}_2$ ), 74.7 (CH),

120.8 (C), 130.0 (q,  $J_{CF}$ =318.4 Hz, C), 127.9 (CH), 135.8 (CH), 147.4 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -42.22$ ; exact mass calcd. for C<sub>0</sub>H<sub>0</sub>F<sub>3</sub>O<sub>2</sub>S: 237.0197; found: 237.0190; anal. calcd. for C<sub>9</sub>H<sub>9</sub>F<sub>3</sub>O<sub>2</sub>S: C 48.35, H 3.81, F 23.92, S 13.46; found: C 46.04, H 3.68, F 24.62, S 13.69. Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T= $140 \,^{\circ}\text{C}: (R) = 10.9 \,\text{min}; (S) = 12.7 \,\text{min}$ ].

(S)-7: (*ee* 98.3%);  $[\alpha]_{D}^{22}$ : +8.3 (*c* 1, CHCl<sub>3</sub>).

(*R*)-7d: (*ee* 59%);  $[\alpha]_{D}^{22}$ : -5.9 (*c* 1.25, CHCl<sub>3</sub>); mp 48°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 1.52$  (s, 3H), 2.37 (m, 1H), 2.99 (m, 1H), 3.69 (m, 2H), 7.43–7.73 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta = 26.0$  (CH<sub>3</sub>), 70.7 (CH<sub>2</sub>), 74.7 (C), 122.1 (q,  $J_{CF} = 3.8$  Hz, CH), 124.0 (q,  $J_{CF}$ =3.8 Hz, CH), 124.2 (q,  $J_{CF}$ =272.4 Hz, C), 128.6 (CH), 128.8 (CH), 130.7 (q,  $J_{CF} = 32.1$  Hz, C), 142.4 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -61.98$ ; exact mass calcd. for  $C_{10}H_{11}F_3O_2$ : 219.0633; found: 219.0630; anal. calcd. for C<sub>10</sub>H<sub>11</sub>F<sub>3</sub>O<sub>2</sub>: C 54.55, H 5.04, F 25.88; found: C 53.57, H 5.03, F 25.68. Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T=120 °C: (S)= 9.0 min; (R) = 9.6 min].

(S)-8: (ee 99.1%);  $[\alpha]_{D}^{22}$ : +16.7 (c 0.89, CHCl<sub>3</sub>).

(*R*)-8d (*ee* 88.3 %);  $[\alpha]_{D}^{22}$ : -9.4 (*c* 1.03, CHCl<sub>3</sub>); mp 59°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta = 1.55$  (s, 3H), 1.84 (dd, 1H, J = 5 Hz, J = 7.25 Hz), 2.68 (s, 1 H), 3.74 (ddd, 2 H, J = 11 Hz, J = 5 Hz, J=7.25 Hz), 7.58 (d, 2H, J=8.75 Hz), 7.63 (d, 2H, J=8.75 Hz); <sup>13</sup>C NMR (acetone- $d_6$ ):  $\delta = 26.4$  (CH<sub>3</sub>), 71.5 (CH<sub>2</sub>), 750 (C), 125.4 (q,  $J_{CF}$  = 3.8 Hz, CH), 125.6 (q,  $J_{CF}$  = 269.6 Hz, C), 127.1 (CH), 128.3 (q,  $J_{CF}$ =32.1 Hz, C), 152.6 (C); <sup>19</sup>F NMR (CDCl<sub>3</sub>):  $\delta = -61.98$ ; exact mass calcd. for C<sub>10</sub>H<sub>11</sub>F<sub>3</sub>O<sub>2</sub>: 219.0633; found: 219.0614. Chiral GC analysis after derivatisation to the corresponding acetonide [column I, T = 130 °C: (S) = 8.1 min; (R) = 8.6 min].

### Preparative Scale Biohydrolysis of *rac*-4 at 250 g L<sup>-1</sup> in a Standardised STR

In a standardised STR (100 mL total volume) equipped with a rotatory Rushton turbine and thermostated at 27°C, rac-4 (12.5 g, 61.3 mmol) was added in iso-octane (10 mL) and demineralised water (20 mL). An emulsion was formed by agitation at 800 rpm before careful addition of the enzyme (62.5 mg, containing 688 U,<sup>[19]</sup> dissolved in 7.5 mL of demineralised water). The kinetic resolution was followed by taking aliquots from the reaction medium (100 µL) which were immediately mixed with a mixture of acetonitrile (300  $\mu$ L) and iso-octane (1 mL) to stop the enzymatic reaction and to extract the remaining epoxide. After vigorous vortexing of samples (30 s) then centrifugation (13.000 rpm, 2 min) the organic phase was analysed using a chiral GC column to determine the enantiomeric excess of the remaining epoxide. The reaction was stopped when the ee of the residual epoxide reached 99% (210 min) by adding AcOEt (30 mL) directly into the reactor. After decantation the aqueous phase was extracted twice with ethyl acetate (2×30 mL). The collected organic fractions were washed with brine then dried over MgSO<sub>4</sub> and concentrated in vacuum. Using these experimental conditions a total amount of 50 g of rac-4 was processed in four experiments of 12.5 g each leading to a total amount of 64.9 g of crude extract. Addition of hexane to this crude extract allowed us to obtain the crystallization of a part of the formed diol-4d. After filtration 24.4 g of diol-4d were isolated as white crystals. The filtrate was concentrated in vacuum then a part of the remaining epoxide 4 (22.1 g) was isolated by distillation (75 °C, 10 mbar). Addition of hexane to the residue of distillation led to the crystallization of a second part of diol-4d (4.7 g). After filtration the filtrate was concentrated and the residue was finally subjected to flash chromatography (silica gel: pentane/ethyl acetate, 90/10) which gave 4.9 g of 4. Altogether, extraction and purification of 4 and 4d provided as a pool 27.0 g of (S)-4 (ee 99%, 43% yield) and 29.1 g of (R)-4d (ee 95%, 44% yield).

## Preparative Scale Biohydrolysis of *rac*-8 at 100 g $L^{-1}$ in a Standardised STR

In a standardised STR (500 mL total volume) equipped with a rotatory Rushton turbine and thermostated at 27 °C, *rac*-8 (25 g, 123.8 mmol) was added in iso-octane (25 mL) and demineralised water (112.5 mL). An emulsion was formed by agitation at 600 rpm before addition of a crude enzymatic solution (112.5 mL containing 5600 U<sup>[19]</sup>). The reaction was stopped when the *ee* of the residual epoxide reached 99 % (150 min) by adding AcOEt (60 mL) directly into the reactor. After decantation the aqueous phase was extracted with ethyl acetate (2×60 mL). The collected organic fractions were washed with brine then dried over MgSO<sub>4</sub> and concentrated in vacuum. Separation by flash-chromatography (pentane/Et<sub>2</sub>O, 80/20 then 0/100) afforded the (*S*)-8 epoxide (10.5 g, 42 % yield, *ee* 99.7 %) and the (*R*)-8d diol (13.41 g, 49 % yield, *ee* 78.5 %).

# General Procedure for Resolution of *rac*-1–8 using Jacobsen's Catalyst

The (R,R)-(salen)Co(III)(OAc) catalyst was prepared using the (R,R)-(salen)Co(II) precatalyst commercially available from Aldrich. The best results were obtained when a mixture of (R,R)-(salen)Co(II) (202 mg, 0.332 mmol), CH<sub>2</sub>Cl<sub>2</sub> (1.66 mL), and acetic acid (66 µL, 1.33 mmol) was stirred while open to the air for 10 min at room temperature.<sup>[22]</sup> The solvent was removed by rotary evaporation, and the brown residue was dried under vacuum. In a mini-vial (1 mL) rac-1-8 (1 mmol) were added to Co(III) complex (4.76 mg, 0.007 mmol) previously prepared. Water (9.9 µL, 0.55 mmol) was added and the reaction mixture was stirred at room temperature for 48 h. The remaining epoxide was extracted with iso-octane (10 mL) containing 3-(trifluoromethyl)-acetophenone  $(0.5 \text{ gL}^{-1})$  as a standard and its enantiomeric excess and the conversion ratio were determined by chiral GC analysis.

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