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Epoxide Hydrolase-Catalysed Kinetic Resolution of a Spiroepoxide, a Key Building Block of Various 11-Heterosteroids

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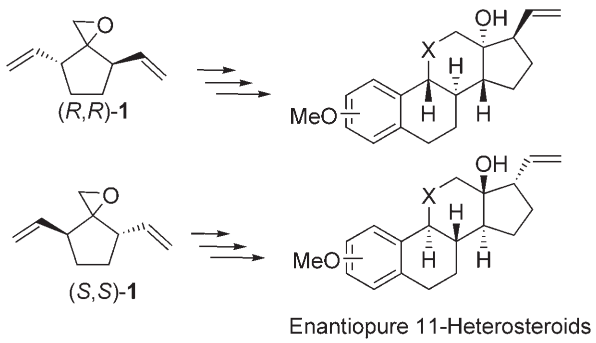
Abstract: Two microbial epoxide hydrolases – i.e., *Aspergillus niger* (*An*EH) and *Rhodococcus erythropolis* (the so-called "Limonene EH": LEH) were used to achieve, for the first time, the biocatalysed hydrolytic kinetic resolution (BHKR) of spiroepoxide *rac*-**1**. This compound is a strategic key building block allowing the synthesis of 11-heterosteroids. Interestingly enough, the two enzymes exhibited opposite and therefore complementary enantioselectivity allowing us to isolate the residual (*R,R*)-**1** (from *An*EH) and the residual (*S,S*)-**1** (from LEH) in nearly enantiopure forms (>98%). Their absolute configurations were determined by X-ray crystallography. An opposite regioselectivity of the oxirane ring opening for both enantiomers of substrate **1**, determined using $H_2{}^{18}O$ labelling and chiral GC-MS analysis, was also observed, corresponding to an attack at the *less* substituted carbon atom using *An*EH, and at the *most* substituted carbon atom using LEH. A chemical process-improving methodology was also developed. This allowed us to obtain both enantiomers of the substrate in high enantiomeric purity (99%) and optimised quantity. In the case of the *An*EH, the use of a biphasic (water/isooctane) reaction medium allowed us to increase the global substrate concentration up to 200 g/L. The preparation of both enantiomers of **1** clearly paves the way to the preparative scale synthesis and biochemical evaluation of the corresponding 11-heterosteroid enantiomers.

Keywords: biotransformations; chiral resolution; enzyme catalysis; epoxide hydrolase; spiroepoxides

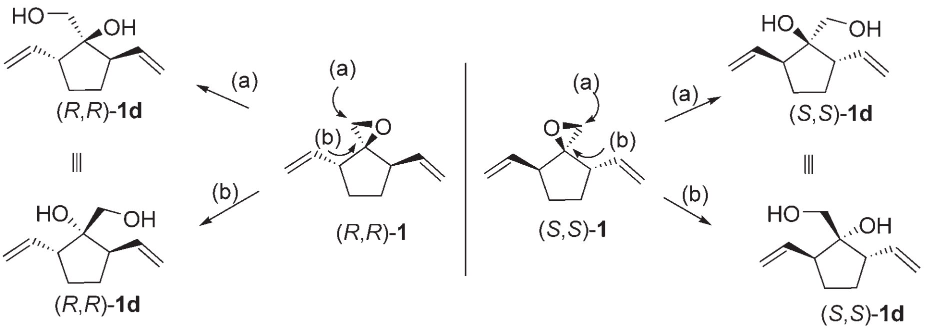
Introduction

The *trans*-spiroepoxide *rac*-**1** is an important building block of 11-heterosteroids,[1] a family of compounds which are of substantial current interest due to their high biological and medicinal potential (Scheme 1).[2] In spite of this, to the best of our knowledge, no chemical or biocatalytic method allowing the preparation of this key spiroepoxide in enantioenriched form has been described up to now.

Over the last years, a high interest has been devoted to the hydrolytic activity of epoxide hydrolases (EHs), a family of enzymes catalysing the hydrolytic kinetic resolution of numerous racemic epoxides.[3] As far as spiroepoxides are concerned, if the kinetic resolution of a range of methyl-substituted 1-oxaspiro-[2,5]octanes by the yeast epoxide hydrolase from *Rhodotorula glutinis* has been recently described,[4] no example of resolution of 1-oxaspiro[2,4]heptanes has been ever carried out. To perform the kinetic resolution of *rac*-**1** we tested two very different EHs, a fungal and a bacterial EH.



Scheme 1. Synthesis of enantiopure 11-heterosteroids from enantiopure spiroepoxides **1**.



Scheme 2. Regioselectivity of the oxirane opening.

The first one was the recombinant *Aspergillus niger* (*An*EH) overexpressed into another *A. niger* fungus.[5,6] This enzyme, that belongs to the family of α/β-hydrolase fold enzymes (EC 3.3.2.3), as the majority of EHs, was recently made commercially available.[7] We have shown previously that this enzyme is a very powerful and versatile biocatalyst offering high activity and enantioselectivity.[3c] In particular, it allowed us to prepare various enantiopure epoxides using a two-phase reactor methodology which, depending on the substrate, could be performed at a global substrate concentration as high as 500 g/L.[8,9]

The second enzyme, the so-called "Limonene EH", (LEH) has been recently isolated from the bacterium *Rhodococcus erythropolis* (DCL14 strain).[10,11] The natural function of this enzyme is to contribute to a metabolic system that allows the bacterium to grow on limonene as a sole source of carbon and energy, catalysing the limonene 1,2-epoxide hydrolysis. This enzyme belongs to a novel class of EH (EC 3.3.2.8) which strongly differs from the α/β-hydrolase fold enzymes in structural, mechanistic and selectivity aspects.[10] Indeed, in opposition with *An*EH (45 kDa), which hydrolyses epoxides by the action of a catalytic triad *via* a two-step mechanism, this low molecular weight enzyme (17 kDa) has been shown to imply a one-step concerted general acid/general base-catalysed mechanism.[12] Therefore, some differences of selectivity between these two types of EH could be expected.

In this paper, we describe the use of both *An*EH and LEH to achieve the biocatalysed hydrolytic kinetic resolution (BHKR) of the structurally peculiar *trans*-spiroepoxide *rac*-**1**, and to obtain both enantiomers of this substrate in highly enantioenriched form.

Results and Discussion

Theoretical Comments

Determination of the enantiomeric ratio *E* and the regioselectivity coefficients α(*S*) and α(*R*) of these resolutions deserves a special comment. Indeed, it is now well known that, as a general feature, the (formal) nucleophilic attack of a water molecule implied in the enzymatic hydrolysis of an epoxide can occur at either carbon atom of the oxirane ring. Hence, the regioselectivity of this attack can be different from one enantiomer to the other.[13] As a consequence, the absolute configuration and the *ee* of the formed diol (eep) depend upon two combined factors, that is, the enantioselectivity *and* the regioselectivity of the oxirane ring opening. This leads to the conclusion that Sih's[14] [$E=f(c,eep)$] and Straathof's[15] [$E=f(ees,eep)$] equations, both implying the *ee* of the product, can generally not be used without previous determination of the regioselectivity coefficients.[13]

However, in the special case of spiroepoxide **1**, these restrictions are not to be considered, since neither carbon atom of the oxirane ring is a stereogenic centre. Indeed, the chirality of the molecule is *not* born by the epoxide moiety itself, but by the two carbon atoms adjacent to the oxirane (i.e., the ones bearing a vinyl group) which are, in each specific enantiomer, of identical absolute configuration [i.e., (*R,R*) or (*S,S*)].

As a consequence, the absolute configuration of the formed diol is independent of the regioselectivity of the water attack (Scheme 2) and the *E* value can, in this case, be validly calculated using Straathof's equation.

Analytical Scale Experiments

Enantioselectivity Determination

The solubility of **1** in phosphate buffer (10 mM) at 27 °C was established by GC analysis to be about 5.3 mM. Therefore, an analytical scale biohydrolysis was carried out at low substrate concentration (3.3 mM, homogeneous medium) for each enzyme. Under these experimental conditions the chemical aspecific hydrolysis was negligible. The enantiomeric excesses of the residual epoxide **1** and of the formed

Table 1. Results of the biohydrolysis of *rac*-**1** using *An*EH and LEH.

Enzyme	*t* (min)	*c* (%)	ees (%)	eep (%)	*E*
*An*EH	60	65	>99	54	17
LEH	180	64	>99	56	19

diol **1d** being easily determined using chiral GC analysis (Cyclosil B, Agilent) the *E* value could be calculated from these values. Similarly, the conversion ratio could be determined on the basis of the two enantiomeric excesses [$c = \text{ees}/(\text{ees}+\text{eep})$].[14]

When *rac*-**1** was submitted to react with *An*EH, a moderate *E* value of 17 was determined (Table 1). The residual epoxide was shown to be of (*R*,*R*) absolute configuration (see determination of absolute configurations below) allowing us to conclude that the formed diol must be of (*S*,*S*) absolute configuration.

For LEH (Table 1), a similar *E* value of 19 was determined but the residual epoxide was the (*S*,*S*)-**1** enantiomer and the formed diol the (*R*,*R*)-**1d**. It thus appeared that, interestingly, the *An*EH and the LEH were enantiocomplementary.

It should be stressed that, to our best knowledge, these two experiments describe for the first time the kinetic resolution of an epoxide bearing stereogenic centres *remote* from the reaction site.

Regioselectivity Determination using ^{18}O-Labelling

Owing to the very different mechanisms implied in *An*EH and LEH, and to their opposite enantioselectivities in the biohydrolysis of *rac*-**1**, we aimed at accurately determining the regioselectivity coefficients for each epoxide enantiomer and for each one of the two enzymes.

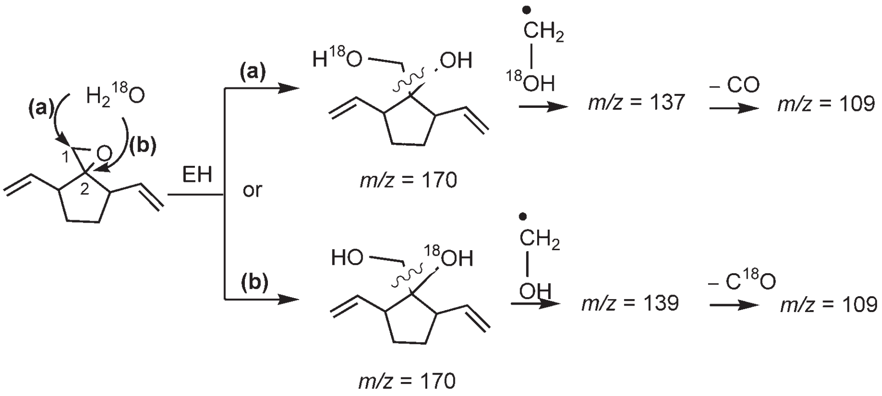
As commented above, neither carbon atom of the oxirane ring is a stereogenic centre. Thus, the regioselectivity of these enzymes against **1** cannot be deduced from the absolute configuration of the formed diol even if the biohydrolysis is carried out using an enantiopure enantiomer (Scheme 2). We therefore decided to determine these parameters by performing the enzymatic hydrolysis of *rac*-**1** in ^{18}O-labelled water and by conducting a GC-MS analysis using a chiral column able to separate the two enantiomers of the formed diol **1d**. Thus an MS analysis of the two enantiomers of **1d** could be achieved in one run.

Depending on the regioselectivity of the $H_2{}^{18}O$ attack – on the less or on the most substituted carbon atom of the oxirane ring (respectively C-1 and C-2) – the formed diol **1d** should be essentially labelled on one of these two carbon atoms (Scheme 3). The location of this label could be determined and quantified by analysis of the MS fragmentation pattern of the diol, due to splitting between the two hydroxy-bearing carbon atoms. The regioselectivity coefficients were calculated from the relative intensities of the $m/z = 137$ and 139 fragments.

Table 2 shows that, for a given EH, similar values were obtained for both epoxide enantiomers but that, as expected from the mechanistic aspect, the *An*EH and LEH did indeed exhibit opposite regioselectivities: *An*EH led to 90–94% attack at C-1 whereas LEH led to about 75–79% attack at C-2. In the *An*EH case, this result is in accordance with the numerous studies carried out on other types of substrates, for which this enzyme showed high specifici-

Table 2. Regioselectivity of each enzyme against (*R*,*R*)-**1** and (*S*,*S*)-**1**, calculated from numerical values of the GC-MS peaks.

Enzyme	Absolute configuration	^{18}O% at C-1	^{18}O% at C-2
*An*EH	(*R*,*R*)-**1**	90	10
	(*S*,*S*)-**1**	94	6
LEH	(*R*,*R*)-**1**	25	75
	(*S*,*S*)-**1**	21	79



Scheme 3. GC-MS regioselectivity determination. Bioconversion in $H_2{}^{18}O$ and expected MS fragmentations.

ties for the sterically less hindered carbon atom.[3c,16] Concerning LEH, our results indicate that the water attack was preferentially oriented at the *most* substituted carbon atom. This stereochemical outcome is interestingly in line with the results previously observed with the most reactive enantiomer of limonene-1,2-epoxide[17] as well as for 1-methylcyclohexene oxide.[18]

Optimisation of the Substrate Concentration

Our objective was to prepare both enantiomers of **1** in enantiopure form in order to allow the further synthesis of optically pure heterosteroids. Thus we aimed at increasing the operational substrate concentration for both resolution processes. As already mentioned, we have described previously various BHKR of *rac*-epoxides using *An*EH as biocatalyst. Some of these could be performed at very high substrate concentration by using two methodologies implying a two-phase approach in which the immiscible organic phase was either the neat epoxide[8] or isooctane (10% v/v) as an additional cosolvent.[19] The choice between these two approaches essentially depends on the possibility to efficiently disperse the specific epoxide in the aqueous phase. In the case of *rac*-**1**, the water/isooctane methodology led to better results than a simple water/epoxide emulsion, for which some mass transfer limitations were observed.[20] Using isooctane we could observe that (a) the reaction could be performed at substrate concentrations much higher than the maximum solubility of the substrate in the aqueous medium, (b) no mass transfer limitation occurred, (c) no product inhibition slowed down the reaction rate. Thus, using this methodology, we were able to increase the global substrate concentration from 20 g/L up to 200 g/L without variation of the *E* value (Figure 1).

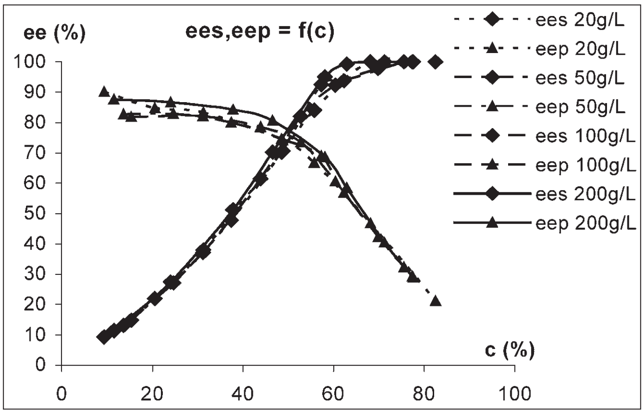


Figure 1. Influence of the *rac*-**1** global substrate concentration on the biohydrolysis rate using *An*EH in a biphasic H_2O/isooctane (90/10) system.

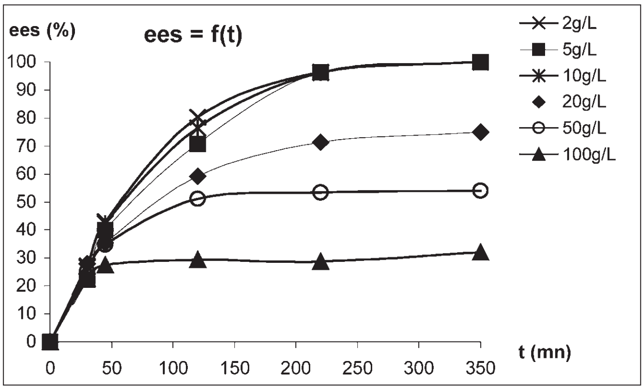


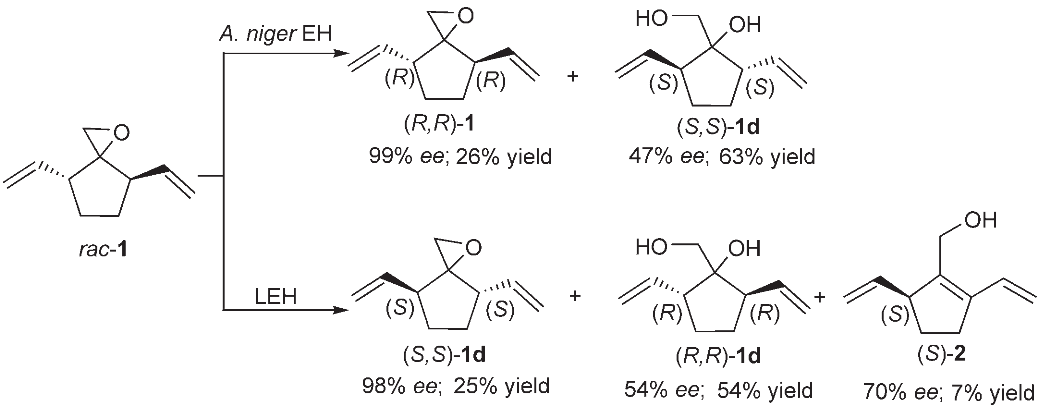
Figure 2. Influence of the *rac*-**1** concentration on the biohydrolysis rate using LEH in a 100 mM Tris-HCl buffer solution.

As far as the LEH is concerned, the substrate concentration was increased from 2 to 100 g/L in a 100 mM Tris-HCl buffer solution. The results indicated (Figure 2) that the resolution of *rac*-**1** could be efficiently performed up to 10 g/L. However, in contrast with the *An*EH-catalysed reaction, substrate concentrations above 10 g/L were deleterious to the enzyme activity. Indeed, above this value, the *ee*s culminated at 70, 50 and 30% at concentrations of 20, 50 and 100 g/L, respectively. This pattern suggested that the formed diol **1d** could be an inhibitor of this enzyme. This was confirmed by an additional experiment carried out at a substrate concentration of 2 g/L in which 10 g/L of diol (*R*,*R*)-**1** was added at the beginning of the reaction (results not shown). In these conditions, the *ee*s stayed below 30% and the kinetic profile was practically identical to the one obtained at a substrate concentration of 100 g/L (Figure 2). The use of isooctane (10% v/v) did not allow us to increase the substrate concentration as in the case of *An*EH. Therefore the preparative scale experiment was conducted at an 8 g/L substrate concentration only.

Gram-Scale Reactors

Gram-scale hydolysis of *rac*-**1** (1 g) catalysed by the *An*EH was carried out at 27 °C at a 100 g/L global substrate concentration, using a biphasic H_2O/isooctane (90/10 v/v) medium. This afforded the (*R*,*R*)-**1** epoxide in nearly enantiopure form (99% *ee*) in a 26% purified yield and the (*S*,*S*)-**1d** diol (47% *ee*) in a 63% purified yield (Scheme 4).

Similarly, a gram-scale biohydrolysis with LEH was carried out at 27 °C at an 8 g/L substrate concentration in a 100 mM Tris-HCl buffer. Thus, 1 g of *rac*-**1** afforded the (*S*,*S*)-**1** epoxide, showing a 98% *ee*, in a 25% purified yield, and the (*R*,*R*)-**1d** diol (54% *ee*) in a 54% purified yield. Surprisingly, a secondary



Scheme 4. Preparative hydrolytic kinetic resolution of *rac*-**1** using *An*EH and LEH as biocatalyst.

product of which the structure was determined as being alcohol (*S*)-**2**, (7% purified yield, 70% *ee*) was also formed during this bioconversion (Scheme 4).

Its (*S*) absolute configuration was deduced from chiral GC analysis (see determination of absolute configurations below). Chemical acid-catalysed hydrolysis of *rac*-**1** interestingly afforded the same alcohol **2** as the major product (53% yield), suggesting that (*S*)-**2** could have been obtained by spontaneous hydrolysis of (*S*)-**1** during biohydrolysis.[1a] However, three arguments strongly suggest that (*S*)-**2** was formed *via* enzymatic hydrolysis. First, the fact that the atypical one-step concerted general acid/general base-catalysed mechanism of LEH is very similar to acidic chemical hydrolysis.[18] Second, the high enantiomeric excess of the product obtained (70%), which indicates that (*S*)-**2** was essentially formed from the less reactive (*S*)-**1** enantiomer and third, the observation that no formation of **2** did occur during *An*EH biocatalysed hydrolysis.

As an overall result of this gram-scale study, each enantiomer of *rac*-**1** was obtained with an *ee* higher than 98% by using the two different EHs. However, because of the moderate *E*-values, the respective reaction yields did not exceed 30%. A methodology allowing us to increase the overall preparative yield was therefore highly desirable.

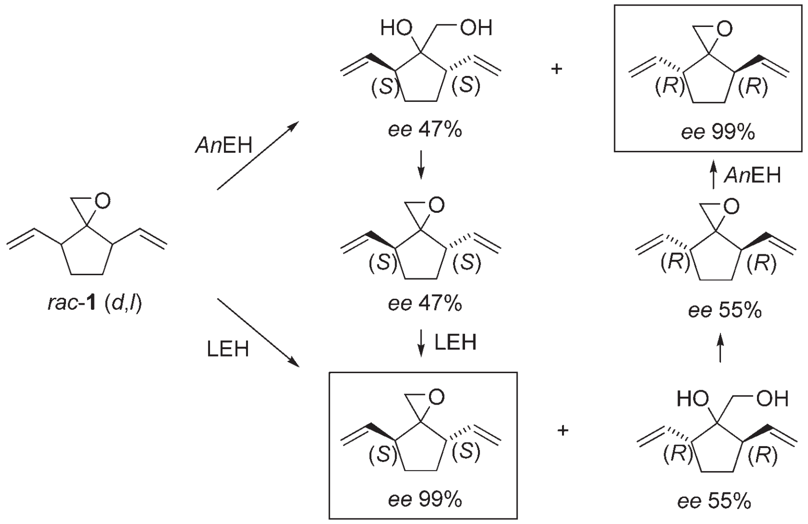
Process-Improving Strategy

One of the general drawbacks of any kinetic resolution is obviously the fact that – in principle – half of the starting substrate (i.e., one of its two enantiomers) will, due to its inappropriate absolute configuration, be unexploitable for further synthesis of a given enantiomerically enriched target product. In order to overcome this cost ineffective aspect, several strategies have been developed, including the use of an enantioconvergent enzyme,[21] of two enantiocomplementary enzymes[22] or elaboration of a combined chemoenzymatic strategy.[23] Such an approach was developed in the present work. Its principle is illustrated in Scheme 5 and is based on the exploitation of the stereo-complementarity of our two epoxide hydrolases. It implies the combined use of enzymatic hydrolysis and of chemical recyclisation of the obtained diols. Thus, during a first cycle experiment, we obtained the recovered (less reactive) epoxide as well as the enantiomerically enriched formed diol of opposite absolute configuration. In order to recycle this diol [i.e., either the (*S,S*)-**1d** or the (*R,R*)-**1d** diol, respectively, obtained from biohydrolysis with *An*EH or LEH], it was cyclised back into the corresponding spiroepoxide **1** (without noticeable loss of enantiomeric purity). Then, the thus obtained spiroepoxide was submitted to a second enzymatic resolution cycle, using the enantiocomplementary enzyme [i.e., the LEH for (*S,S*)-**1** or the *An*EH for (*R,R*)-**1**, respectively].

Thus, recyclisation of (*S,S*)-**1d** (47% *ee*) – initially obtained by an *A. niger*-catalysed BHKR – afforded (*S,S*)-**1** (47% *ee*) in 98% yield. The second BHKR cycle of this epoxide – using this time LEH – then afforded nearly enantiopure epoxide (*S,S*)-**1** (99% *ee*, 50% yield) as well as the (*R,R*)-**1d** diol (13% *ee*, 39% yield) and the secondary product (*S*)-**2** (90% *ee*, 10% yield).

Similarly, recyclisation of (*R,R*)-**1d** (55% *ee*) – initially obtained *via* LEH-catalysed biohydrolysis – afforded (*R,R*)-**1** (55% *ee*) in 87% yield. The second BHKR cycle of this product – using this time *An*EH – led to nearly enantiopure epoxide (*R,R*)-**1** (99% *ee*, 47% yield) and (*S,S*)-**1d** diol (3% *ee*, 35% yield).

As an overall result, the use of these combined strategies (conducted starting from two times 1 g of *rac*-**1**) allowed us to recycle the diol formed in each BHKR (and obtained with an only moderate *ee*), thus affording a second lot of the other epoxide enantiomer in nearly enantiopure form (*ee* >99%). Starting from two times 1 g of *rac*-**1**, we thus obtained, respectively, 556 mg of (*S,S*)-**1** and 479 mg of (*R,R*)-**1**. Obtaining the same amount of these two enantiomers

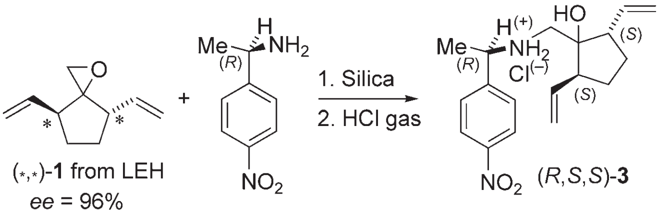


Scheme 5. Combined process-improving strategy.

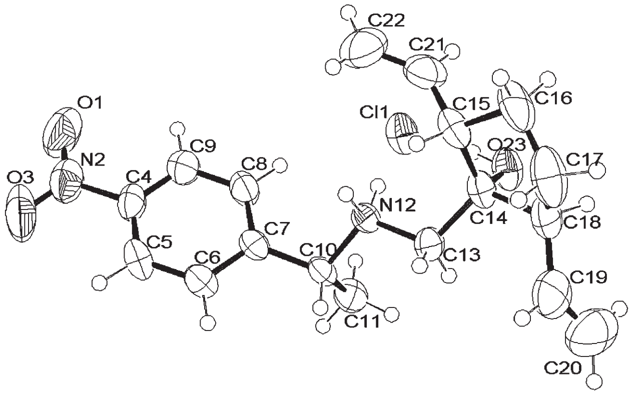
using a single BHKR cycle would have necessitated starting from a two-fold amount (two times 2 g – that is, 4 g) of *rac*-**1**, due to loss of the corresponding diol.

Determination of the Absolute Configurations

As mentioned in the introduction part, these results describe the first preparation of spiroepoxide **1** in optically active form. Therefore, the absolute configuration of the thus obtained enantiomers still had to be unambiguously determined. Hence, the absolute configuration of the enantiomer of **1** (96% *ee*) obtained *via* BHKR catalysed by LEH, was determined using X-ray crystallography of the hydrochloride **3** obtained by condensation of this epoxide with (commercial) optically pure (*R*)(+)-1-(4-nitrophenyl)ethylamine (Scheme 6). The crystal structure of **3** (Scheme 7) indicated an (*R*,*S*,*S*) absolute configuration of this derivative, showing that the starting epoxide **1** was of (*S*,*S*)-**1** configuration. The residual spiroepoxide from *An*EH biohydrolysis was therefore the (*R*,*R*)-**1** antipode. As a consequence, the absolute configuration of the formed diols could be deduced as being respectively (*S*,*S*)-**1d** (for the *An*EH produced diol) and (*R*,*R*)-**1d** (for the LEH produced diol).



Scheme 6. Preparation of chlorhydrate **3** by condensation of enantiopure spiroepoxide **1** obtained from LEH bioconversion with *R*(+)-1-(4-nitrophenyl)ethylamine.



Scheme 7. ORTEP drawing of **3**.

The (*S*) absolute configuration of alcohol **2** formed during LEH biohydrolysis (Scheme 4) was determined by comparison of the chiral GC retention times with the product obtained by acidic chemical hydrolysis of enantiopure (*R*,*R*)-**1**.

Conclusions

In the course of this work, we have explored the behaviour of two microbial epoxide hydrolases – that is, the *Aspergillus niger* and the *Rhodococcus erythropolis* EHs – against the stereochemically peculiar spiroepoxide *rac*-**1**. The two enzymes interestingly exhibited both an opposite enantioselectivity and an opposite regioselectivity for the oxirane ring opening. This translated into an attack at the *less* substituted carbon atom with the *A. niger* EH, and at the *most* substituted carbon atom for the *R. erythropolis* (i.e., limonene epoxide hydrolase LEH). Using these two enzymes allowed us to prepare, for the first time, both enantiomers of **1** in high enantiomeric purity. With the *A. niger* EH, the substrate concentration could be increased up to 200 g/L thanks to the use of a biphasic water/isooctane medium. To the contrary, the LEH-catalysed process was limited to a substrate concentration of 10 g/L due to toxicity of the formed diol against the enzyme. A process-improving methodology, based on the combined use of enzymatic hydrolysis catalysed by the two enzymes, chemical cyclisation and exploitation of the stereocomplementarity of the two epoxide hydrolases was developed. This allowed us to recycle the obtained diols and to prepare an additional lot of each enantiomer of spiroepoxide **1** of very high *ee* (>98%).

It should be stressed that, to our best knowledge, this approach is presently the only way to obtain these two enantiomers in nearly enantiopure form. This achievement thus clearly paves the way to the further synthesis and biochemical evaluation of the different related 11-heterosteroid enantiomers.

Experimental Section

General Remarks

GC analyses were performed with a Shimadzu GC14 A apparatus equipped with FID detector and helium as carrier gas. Determination of the enantiomeric excesses was performed using two different chiral columns, i.e., Cyclosil B (30 m, 0.25 mm, 0.25 μm film, Agilent) for compounds **1** and **1d** and Lipodex A (25 m, 0.25 mm, 0.25 μm film, Macherey–Nagel) for compound **2**. NMR spectra were recorded in $CDCl_3$ using a Bruker AC-250 instrument.

Preparation of the Enzymatic Extracts

The crude powder of recombinant *A. niger* EH was obtained by overexpression in *A. niger* cells as previously described.[24] *E. coli* BL21(DE3) was used as the host for cloning and functional expression of the EH gene from *R. erythropolis*. The recombinant plasmid (pGEF-LEH) was used to transform the *E. coli* competent cells. To produce native EH, cells grown to an initial OD_{600} of 0.8 in a 1-L fermentor were induced for 20 h with 400 μM IPTG (20 °C, LB broth, 100 $\mu g\,mL^{-1}$ ampicillin). Cells were harvested by centrifugation, resuspended in ammonium carbonate buffer (40 mL, 20 mM, pH 7.6) then broken by a single pass through a French press. After centrifugation at 20 000 × *g* for 30 min at 4 °C, the supernatant was freeze-dried to produce about 800 mg of crude enzymatic extract. This powder was used as such in all experiments.

Synthesis of (±)-1-Oxa-4,7-divinylspiro[4,2]heptane (*rac*-1)

The starting compound *rac*-**1** was synthesised following the procedure described by Cachoux et al.[1e] The secondary product (*meso* chlorohydrin, 10.5%) formed during the first step of this synthesis was removed by flash chromatography (pentane/AcOEt, 95/5), then *rac*-**1** was purified by bulb-to-bulb distillation (2 mbar, 65 °C) to furnish a colourless oil; yield: 41%. ^1H NMR: $\delta = 1.80$ (m, 4H), 2.50 (d, $J = 4.8$ Hz, 1H), 2.73 (d, $J = 4.8$ Hz, 1H), 2.65 (m, 2H), 4.95 (m, 4H), 5.56 (m, 2H).

General Procedure for Biohydrolysis of *rac*-1

***Analytical scale*:** In a 50-mL reactor, EH powder (10 mg in the case of *An*EH, 25 mg for LEH) was dissolved in a buffer (25 mL, phosphate buffer, 10 mM, pH 7.4 for *An*EH; 25 mL, Tris-HCl buffer, 100 mM, pH 7 for LEH). *rac*-**1** (12.5 mg) was added, then the mixture was stirred at 27 °C. The kinetic resolution was followed by withdrawing aliquots (80 μL) from the reaction medium at different time intervals in which acetonitrile (50 μL) was added to stop the enzymatic hydrolysis. Epoxide was extracted with isooctane (100 μL), then diol was extracted with AcOEt (100 μL). Chiral GC analysis (Cyclosil B): epoxide **1** 80 °C: (*R,R*) 26 min, (*S,S*) 28 min; diol **1d** 130 °C: (*S,S*) 29 min, (*R,R*) 31 min.

Gram scale, AnEH-catalysed biohydrolysis: In a 50-mL reactor, to *rac*-**1** (1 g) dissolved in isooctane (1 mL) was added a solution of *An*EH powder (0.5 g) in demineralised water (9 mL) then the mixture was stirred at 500 rpm with a mechanical engine, and maintained at 27 °C. The reaction was stopped when the *ee* of the residual epoxide reached 99% (1 h) by adding AcOEt (15 mL). The aqueous layer was saturated with NaCl then epoxide and diol were extracted twice with AcOEt (25 mL). After drying over magnesium sulfate, solvents were removed under vacuum. Separation by flash chromatography (pentane/AcOEt, 50/50) afforded the (*S,S*)-**1d** diol; yield: 0.7 g (62.5% purified yield), *ee* 47%, $[\alpha]_D^{25}$: −13.4 (*c* 1.0, ethanol), and the (*R,R*)-**1** epoxide. This epoxide was then purified by bulb-to-bulb distillation (2 mbar, 65 °C) that afforded (*R,R*)-**1**; yield: 0.26 g (26% purified yield), *ee* 99%, $[\alpha]_D^{25}$: −5.0 (*c* 0.875, ethanol).

Gram scale, LEH-catalysed biohydrolysis: In a 250-mL reactor, *rac*-**1** (1 g) was added to a solution of LEH powder (3 g) in Tris-HCl buffer (125 mL, 100 mM, pH 7). The stirred mixture was maintained at 27 °C. The reaction was stopped when the *ee* of the residual epoxide reached 98% (2 h). Extraction and purification of the products were carried out as described above to furnish the epoxide (*S,S*)-**1**; yield: 0.25 g (25% purified yield), *ee* 98%, $[\alpha]_D^{25}$: +6.4 (*c* 0.85, ethanol), the diol (*R,R*)-**1d**; yield: 0.60 g (54% purified yield, *ee* 54%, $[\alpha]_D^{20}$: +17.6 (*c* 0.95, ethanol), and the alcohol (*S*)-**2**; yield: 0.07 g (7% purified yield), *ee* 70%.

Regioselectivity Determination

Bioconversions of *rac*-**1** (2 $g\,L^{-1}$) were done with LEH (20 $g\,L^{-1}$) and *An*EH (5 $g\,L^{-1}$) in 500 μL $H_2{}^{18}O$ (95% isotopic enrichment, Sigma–Aldrich) as described previously (analytical scales). After 2 h, products were extracted with AcOEt (200 μL) then analysed using a GC-MS apparatus (HP6890-HP5973) equipped with a chiral column (Cyclosil B).

Substrate-Concentration Optimisation

AnEH: H_2O-isooctane emulsion. To keep constant the enzyme/substrate ratio, solutions of *rac*-**1** (for a final concentration of 20–200 $g L^{-1}$) in isooctane (50 µL, 10% of the total volume) were added in an *An*EH solution (10–100 $g L^{-1}$ in 450 µL of water). The mixture was stirred vigorously at 27 °C. Kinetic resolution was followed by taking off aliquots (5 µL) as described above.

LEH: Same procedure as analytical scales, with increased concentrations of *rac*-**1** (2–100 $g L^{-1}$), LEH concentration was increased to keep constant the enzyme/substrate ratio.

Product Toxicity against LEH

In a minivial (1 mL), 10 mg of the diol (*R,R*)-**1d** (from gram-scale LEH bioconversion, 53% *ee*) was added to LEH solution (10 $g L^{-1}$, 1 mL). The biohydrolysis was started by adding *rac*-**1** (2 $g L^{-1}$) then the mixture was stirred at 27 °C. The kinetic resolution was followed by taking off aliquots (80 µL) as described above.

Acidic Chemical Hydrolysis of *rac*-1

In a 25-mL flask on ice, to *rac*-**1** (150 mg) dissolved in THF (10 mL), a H_2SO_4 solution (2 mL, 1 M) was added dropwise. After complete reaction, a saturated $NaHCO_3$ solution (5 mL) was added. Products were extracted with AcOEt, and then concentrated under vacuum. After a flash chromatography column (pentane/AcOEt, 7/3), *rac*-**1d** (yield: 70 mg, 41%) and *rac*-**2** (yield: 81 mg, 53%) were obtained.

Cyclisation of (*R,R*)-1d and (*S,S*)-1d to the Corresponding Epoxides

These reactions were carried out following the procedure of tosylation described by Kolb and Sharpless.[25] After purification by flash-chromatography (pentane/AcOEt, 9/1), 1 g of (*S,S*)-**1d** afforded 876 mg of the (*S,S*)-**1** spiroepoxide (98% purified yield), and 318 mg of (*R,R*)-**1d** afforded 246 mg of the (*R,R*)-**1** spiroepoxide (87% purified yield).

Assignment of the Absolute Configurations

***Synthesis of hydrochloride* 3:** Basic hydrolysis of the commercially available (*R*)-1-(4-nitrophenyl)ethylamine hydrochloride (68 mg, 0.34 mmol), was carried out using a KOH solution (200 µL, 3 N). After extraction with diethyl ether (100 µL), organic layers were dried under sodium sulfate. (*R*)-(+)-1-(4-nitrophenyl)ethylamine (yield: 55 mg, 97%) was obtained after removal of the solvents under vacuum.

To a stirred mixture of (*R*)-(+)-1-(4-nitrophenyl)ethylamine (55 mg, 0.33 mmol, 5 equivs.) and (*,*)-**1** obtained from LEH bioconversion (*ee* = 96%, 10 mg, 0.067 mmol, 1 equiv.) in anhydrous ethanol (200 µL), 1 mg of silica was added.[26] After 6 days at room temperature, a 96% yield was observed (GC). After concentration under vacuum and flash chromatography (pentane/diethyl ether, 7/3), the purified amino alcohol was transformed (HCl gas) into its corresponding hydrochloride **3**. This solid was recrystallised in cyclohexane/ethanol, then in water before X-ray analysis.

***X-ray analysis of* 3:** Crystal size 0.4×0.01×0.01 mm, colourless. Crystal and intensity data were obtained on a Bruker-Nonius KappaCCD diffractometer using graphite-monochromated Mo-Kα radiation. 13468 reflections were measured using 2° ϕ rotations. Reflection integration and lattice parameter calculation were performed with Denzo-Scalepack.[27] Crystal data: $C_{18}H_{24}ClN_2O_3$, $M_r = 351.84$, orthorhombic, space group $P2_12_12_1$, $a = 5.8662(1)$, $b = 15.6473(4)$, $c = 20.6010(7)$ Å, $V = 1890.87(9)$ Å^3, $Z = 4$, $D_x = 1.236$ g·cm^{-3}, F(000) = 748, μ(Mo-Kα) = 0.219 cm^{-1}. 2045 unique reflections were measured. The structure was solved by direct methods using SIR92[28] and 217 parameters were refined using Shelxl-97[29] to final indices $R[F^2 > 4\sigma F^2$: 1624 reflections] = 0.049, wR [2045 reflections] = 0.1227 [$w = 1/[\sigma^2(Fo^2) + (0.0513P)^2 + 0.7384P]$ where $P = (Fo^2 + 2Fc^2)/3$]. All H atoms were introduced at geometrical positions, included in the calculation but not refined except the H atoms from the hydroxy and ammonium groups which were located in a last Fourier map. The final difference Fourier map positive and negative peaks were 0.157 and −0.192 e Å^{-3} respectively.[30]

***Absolute configuration of* 2:** A chemical hydrolysis of the enantiopure (*R,R*)-**1** compound (99% *ee*) (*cf.* procedure of acidic chemical hydrolysis of *rac*-**1**) has been done to identify the GC retention time of (*R*)-**2**. Compound **2** obtained from LEH bioconversion was compared to this one, and the (*S*) configuration was so deduced (Lipodex A GC-column, 90 °C, (*R*)-**2**: 29 min, (*S*)-**2**: 30 min).

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