Epoxide hydrolase and its application in organic synthesis

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1 Introduction

Organic chemists have become interested in enzymes as catalysts due to their high efficiencies and specificities. Moreover, recent progress in molecular biology and enzyme-related research areas enabled and simplified the production and purification of recombinant enzymes in large quantities and their engineering towards tailor-made biocatalysts using straightforward mutagenesis and screening techniques. This is also true for epoxide hydrolases (EHs), as evidenced by the many published research papers about the synthetic applications of naturally occurring or engineered EHs.
EHs catalyze the opening of oxirane rings, generating a vicinal diol as the final product (**Figure 1**). From a synthetic chemistry point of view, the most valuable EHs are those with either (1) high enantioselectivities or (2) a combination of low enantiopreference with a high level of enantioconvergence. While the former generate enantiopure epoxides with a maximum yield of 50% in a kinetic resolution process, the latter produce ideally an enantiopure diol product with a theoretical yield of 100%. Thus, EHs provide convenient access to enantiopure epoxides or diols from racemic epoxides. Furthermore, the enantiopure diols can then be often chemically transformed back to the corresponding epoxides with no effect on the enantiomeric excess. High values of enantioselectivity or enantioconvergence are a consequence of particular enzyme-substrate interactions, which can be modulated by specific reaction conditions or through the exchange of specific amino acids of the biocatalyst. The final stereochemical outcome of an EH-catalyzed reaction depends solely on the regioselectivity coefficients, which determine the absolute configuration and the enantiomeric excess of the diol product when the reaction reaches 100% conversion. During the reaction, the oxirane ringof each enantiomeris often attacked at either carbon atoms, resulting in a mixture of diol enantiomers (**Figure 2**). Unfortunately, several authors used the product-derived E-value E_P (calculated from c and ee_P using Sih's equation; see Chen et al., 1982) for characterizing the stereochemistry of the diol formation of an EH-mediated hydrolysis reaction (Rui et al., 2005; Chiappe et al., 2007). This is wrong and misleading for epoxide opening reactions, since there are two possible positions of attack for each oxirane enantiomer. The ideal enantioconvergent EH leads to deracemization of a racemic mixture of an epoxide and exhibits reversed regioselectivity for either substrate enantiomer, i.e. α_S = 100% and α_R = 0%, or α_S = 0% and α_R = 100%affording an enantiomerically pure diol in a theoretical 100% yield. However, enantioconvergence levels of above 90% (i.e. ee_p>90% at complete conversion of the substrate) are quite rare with wild-type EHs. EHs can also be used to de-racemize *meso* epoxides and in this case the ee of the formed diol keeps constant during the entire reaction. The resulting ee is here again fixed by the four regioselectivity coefficients. In the ideal case the diol product is obtained in 100% ee and 100% yield.

Enantiopure epoxides and diols are chiral intermediates or building blocks which can be used for the synthesis of biologically active compounds, such as antibiotics (Ueberbacher et al., 2005), antifungal drugs (Monfort et al., 2004), antiandrogens (Fujino et al., 2007), receptor agonists and antagonists (Pedragosa-Moreau et al., 1997; Genzel et al., 2002; Monterde et al., 2004), 11-heterosteroids (Bottalla et al., 2007), HIV-protease inhibitors (Zhang et al., 1995; Pedragosa-Moreau et al., 1996a), nonsteroidal anti-inflammatory drugs (Chen et al., 1993; Cleij et al., 1999) and other natural products (Kroutil et al., 1997b; Orru et al., 1998b; Steinreiber et al., 2001c; Mayer et al., 2002a; Edegger et al., 2004). An EH-triggered cascade reaction enabled the asymmetric synthesis of two antitumor agents which occur naturally in *Panax ginseng* (Mayer et al., 2002b). Further, substituted tetrahydrofurans, whose structural motifs are frequently found in acetogenins and polyether antibiotics, were also accessed through EH-mediated cascade reactions (Ueberbacher et al., 2009).

The scope of this review is to provide a comprehensive overview of the most interesting applications of EHs for the formation of enantiopure epoxides or diols, which are valuable chiral building blocks and key intermediates for the synthesis of various natural products or pharmacologically active compounds.

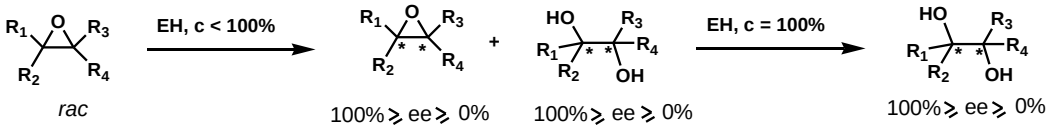


Figure1.EH-catalyzed hydrolysis of a racemic epoxide, resulting in the formation of a vicinal diol with an enantiomeric excess of 100%≥ ee_p ≥0% at complete conversion (c = 100%) of the substrate. Depending on the EH-substrate interactions and consequently its substrate-related enantiomeric ratio or E-value (which depends on c and ee_S and characterizes the ability of the enzyme to discriminate between the two competing substrate enantiomers, Chen et al., 1982), enantiopure epoxide is obtained at a specific degree of conversion within the range of 50%< c<100%. The kinetics of highly enantioselective EHs is characterized by arapid hydrolysis of the preferred epoxide enantiomer, followed by a much slower hydrolysis of the remaining epoxide.

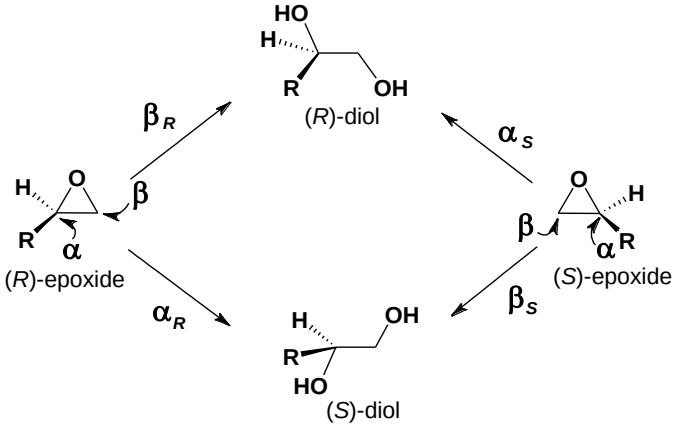


Figure 2. Attack at the oxirane ring can occur at either the terminal carbon atom, which results in a diol product with retained configuration (β-attack), or the carbon atom with the substituent R, resulting in a diol with an inverted configuration (α-attack). The percentages of epoxide molecules following a particular reaction pathway are represented by the corresponding regioselectivity coefficients; the following relationships between the four regioselectivity coefficients are valid: $\alpha_R + \beta_R = 100\%$, and $\alpha_S + \beta_S = 100\%$.

2 Sources and reaction mechanism of EHs

2.1 Sources of EHs

EHs have been found in many prokaryotic and eukaryotic organisms, including bacteria, fungi, yeast, plants, insects, fish and mammals (**Figure 3**). The physiological role of EHs appears to be manifold; they are involved in the detoxification of potentially harmful, naturally occurring or anthropogenic epoxides (Decker et al., 2009), in lipid metabolism in plants and animals (Newman et al., 2005; Morisseau, 2013), and in the metabolism of juvenile hormones in insects (Newman et al., 2005). Recently, a new role of EHs in the biosynthesis of two antibiotics has been established in two *Streptomyces* strains (Lin et al., 2009; Lin et al., 2010). The substrates of EHs are structurally very diverse, representing a broad range of metabolites and xenobiotics. The substrate specificity of individual EHs appears to be diverse as well, being in many cases broad, but occasionally limited to a few available epoxidic compounds (Elfström and Widersten, 2005; van Loo et al., 2006; Kotik et al., 2009). Sources of novel EHs are not limited to known (micro-) organisms. Metagenomic or environmental DNA (eDNA), i.e. the total microbial DNA of a microcosm such as a small soil or groundwater sample, can serve as a source of novel EHs without the need to isolate and cultivate the microorganisms. PCR-based amplification of EH gene fragments in conjunction with genome-walking techniques (Kotik, 2009) have been used to retrieve entire genes encoding α/β-hydrolase fold EHs directly from the metagenomic DNA (Kotik et al., 2009; Kotik et al., 2010). Moreover, activity screening of recombinant clones containing fragments of eDNA and hybridization to EH-specific target sequences led to the discovery of novel eDNA-derived EHs with considerable potential for biotransformations (Zhao et al., 2004).

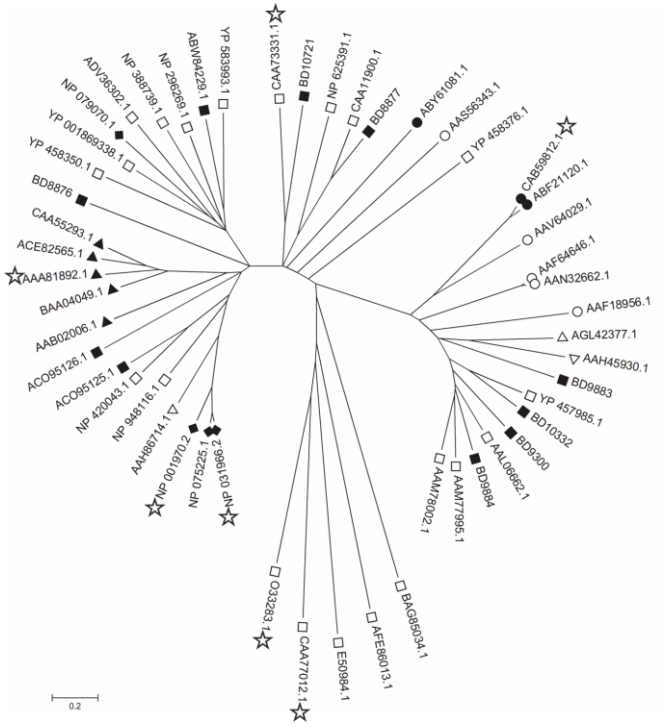


Figure 3. Phylogenetic relationships among protein sequences encoding EHs with confirmed activities. Each sequence is represented by its GenBank accession number; for sequences and further data regarding EHs with codes starting with BD, see Zhao *et al*., 2004. The data set includes sequences of mammalian EHs (◆), plant EHs (▲), fish EHs (▽), insect EHs (△), yeast EHs (○), fungal EHs (●), bacterial EHs (□), and eDNA-derived EHs (■). A star represents an EH with a determined X-ray protein structure: human EH (NP_001970), murine EH (NP_031966), potato EH (AAA81892), a bacterial EH from *Agrobacterium radiobacter* AD1 (CAA73331), a fungal EH from *Aspergillus niger* LCP 521 (CAB59812), and two bacterial EHs which are not members of the α/β-hydrolase fold superfamily (CAA77012 and O33283). The bootstrap consensus tree was inferred from 1000 replicates. The evolutionary distances were computed using the Poisson correction method. The bar represents 0.2 amino acid substitutions per site.

2.2 Heterologous expression of EHs

Most EH expression systems were based on *Escherichia coli* as a host; however, heterologous expression of EHs was also established in mammalian cells (Grant et al., 1993), the baculovirus system with *Spodoptera frugiperda* and *Trichoplusia ni* insect cell lines (Kamita et al., 2013), in the yeast strains *Pichia pastoris*, *Saccharomyces cerevisiae* and *Yarowia lipolytica* (Kim et al., 2006; Labuschagne and Albertyn, 2007; Botes et al., 2008), and in *Aspergillus niger*NW 219 (originally published as *A. niger* NW171; Naundorf et al., 2009). The latter heterologous host offered the possibility to use a low-cost culture medium with inexpensive corn steep liquor as the main component. Further, *E. coli* RE3 as the recombinant host enabled EH production in a minimal growth medium with inexpensive sucrose as the sole carbon source (Grulich et al., 2011). Co-expression of molecular chaperones together with the optimization of culture conditions resulted in lower levels of inclusion bodies in the recombinant strain *E. coli* BL21(DE3) when overexpressing the EH from *Rhodotorula glutinis* (Visser et al., 2003).

2.3 Reaction mechanisms of EHs

A large fraction of EHs belongs to the α/β-hydrolase fold superfamily, which contains – besides EHs – other structurally related hydrolytic enzymes with a characteristic arrangement of α-helices and β-sheets: esterases, haloalkane dehalogenases, lipases, amidases, and some more (Heikinheimo et al., 1999; Lenfant et al., 2013). It appears that all EHs of the α/β-hydrolase fold superfamily share a common three-step reaction mechanism, which involves the action of the active site-located catalytic triad (Asp-His-Glu/Asp), two tyrosine residues and a water molecule (**Figure 4**). In a first step, the carboxylic acid of aspartate attacks an oxirane carbon of the bound epoxide substrate, resulting in a transiently formed ester intermediate. Two conserved tyrosines assist in this step of catalysis, polarizing the epoxide ring by hydrogen bonding with the oxirane oxygen. The second step of the reaction mechanism, which is often rate-limiting, is characterized by the hydrolysis of the ester intermediate, catalyzed by an activated water molecule; the amino acid pair His-Glu/Asp of the catalytic triad is responsible for this activation. In the third and final step the formed diol product is released, leaving behind the restored catalytic triad of the enzyme.

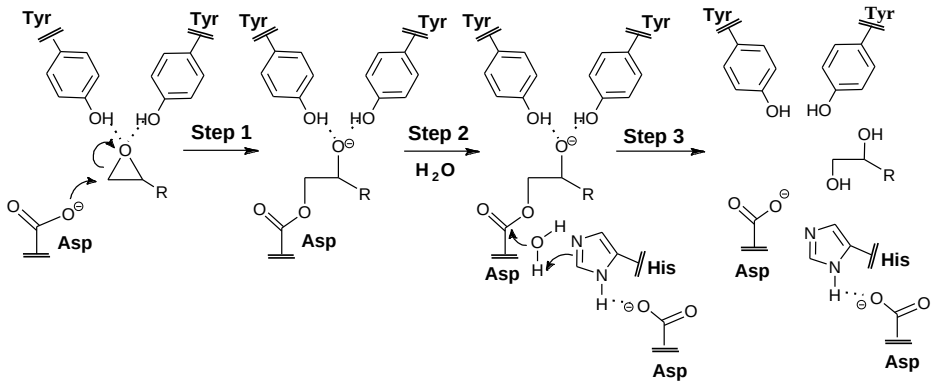


Figure4. Proposed catalytic mechanism of EHs which are members of the α/β-hydrolase fold superfamily.

Some EHs are not members of the α/β-hydrolase fold superfamily, as shown for the limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* (Arand et al., 2003) and the EH from *Mycobacterium tuberculosis* (Johansson et al., 2005). These two enzymes, which are dimers, have similar overall structures, each subunit consisting of a curved six-stranded β-sheet and four helices. The active site is located in a deep pocket with an Asp-Arg-Asp catalytic triad at its bottom. In contrast to the above-mentioned catalytic mechanism of α/β-hydrolase fold EHs, a single-step push-pull mechanism has been proposed, which includes the activation of a water molecule by hydrogen bonding, resulting in a nucleophilic attack at the epoxide ring; at the same time, the epoxide is polarized and thereby activated by making available a proton to the oxirane oxygen (acid catalysis) (**Figure 3**).

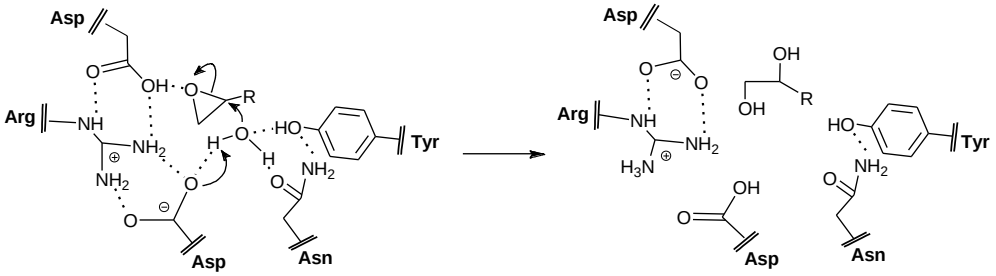


Figure5. Proposed catalytic mechanism of the limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* and the EH from *Mycobacterium tuberculosis*. The catalytic water molecule is activated by the formation of hydrogen bonds involving aspartic acid, asparagine and tyrosine residues. At the same time, polarization and activation of the oxirane ring is achieved by hydrogen bonding with the oxirane oxygen involvinganother aspartic acid residue.

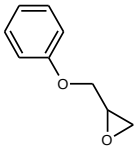
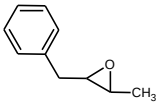
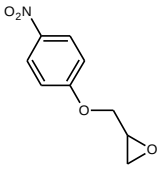
3 Directed Evolution and genetic engineering of EHs

Nature is a very rich source of EHs (**Figure 3**), each enzyme having its own specific substrate range. Nevertheless, the perfect EH, which satisfies all the needs of a chemist for a given application – high chiral selectivity, low product inhibition, acceptable activity and process stability – is rarely found. The generation of improved EH variants by genetic engineering and directed evolution approaches is the solution to the increasing demand for specifically tailored biocatalysts. Mutations in residues which are involved in catalysis or in residues that are flanking the nucleophilic aspartate of the catalytic triad can have a strong influence on the enantioselectivity and EH activity. For instance, replacing the acidic charge relay residue Glu with Asp in the rat microsomal EH led to an increase in V_{max} by more than 20-fold for the substrates styrene oxide and 9,10-epoxystearic acid (Arand et al., 1999). In another example, one of the two oxirane-polarizing tyrosines was exchanged by phenylalanine in the active site of the EH from *Agrobacterium radiobacter* AD1, which resulted in a substantial increase in E-values for racemic styrene oxide and its *para*-nitro and *para*-chloro derivatives. However, the enzyme variants were significantly less active when compared to the wild-type EH (Rink et al., 1999). More recently, the nucleophile-flanking residue Phe-108 was replaced in the same enzyme by one of the remaining 19 amino acids using saturation mutagenesis. Activity measurements of the resulting enzymes revealed an increase in activity and/or enantioselectivity for a number of substrate-enzyme variant pairs (van Loo et al., 2009).

Due to the complexity of biocatalyst engineering and its limitations when using site-directed mutagenesis of a few mutational sites, *in vitro* enzyme evolution has emerged as an additional powerful tool for improving enzymes (Jochens et al., 2011). Using this approach, higher values of enantioselectivity have been achieved for a number of EH-catalyzed reactions (**Table 1**). Three main techniques have been used for the laboratory evolution

of EHs: error-prone PCR, DNA shuffling, and iterative saturation mutagenesis. A critical and thorough comparison of these methods has not been done until recently when Reetz and co-workers compared the efficiencies of various evolution methods for improving a lipase from *Pseudomonas aeruginosa* in the stereoselective hydrolytic kinetic resolution of an ester (Reetz et al., 2010). Their results suggest a significantly higher efficiency of iterative saturation mutagenesis over error-prone PCR, saturation mutagenesis at hot spots and/or DNA shuffling in generating highly enantioselective mutants. In each round of iterative saturation mutagenesis, randomization at two or more sites is performed simultaneously, exploiting the possible occurrence of cooperative effects operating between the randomized sites (Mildvan, 2004). Usually, these randomization sites are selected in the vicinity of the active site, a minimal requirement of this technique being a sound structural model of the enzyme. Improvement in enantioconvergence of EHs was also reported using iterative saturation mutagenesis (**Table 2**; **Figure 6**). For example, higher levels of enantioconvergence towards racemic *para*-chlorostyrene oxide were achieved in only three productive mutagenesis rounds (**Figure 6A**), using a manual two-step screen based on a colorimetric activity assay followed by a chiral GC test for ee_p determination. The wild-type enzyme and the evolved EHs were analyzed for all four regioselectivity coefficients (see **Figure 2**), using the racemic epoxide as a substrate and a chiral GC column for separating both diol enantiomers (**Figure 6B**).

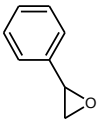
Table 1. Selected EH variants evolved towards higher enantioselectivities.

Source of EH	Racemic substrate	E-values[a](Abs. conf. remaining epoxide)	No. of mutations introduced	Technique of evolution	Screening method	Reference and remarks
Aspergillus niger LCP 521		4.6 → 115 (*R*)	9	Iterative saturation mutagenesis	2-step screening: (1) growth on epoxide-containing agar plates and (2) MS-based system	Reetz et al., 2006; NNK codon randomization
		5 → 160 (*R*)	6 plus insert of 13 residues[b]		Chiral HPLC-based test	Reetz et al., 2011; NDT codon randomization; use of chaperones
		–[c] → >200 (2*R*,3*R*)	3		Cell-based adrenaline test	Reetz et al., 2008; NDT codon randomization
Agrobacterium radiobacter AD1		3.4 → 32 (*S*)	4	Error-prone PCR and DNA shuffling	3-step screening: (1) activity screening using safranin O indicator plates, (2) progress curves with whole cells and (3) cell-free extracts	van Loo et al., 2004; significant reduction in activity for the mutant EHs

[a]The change in E-value from wild-type or initial variant to final mutant enzyme is indicated.
[b]A stretch of 13 residues was accidentally incorporated between sites 318 and 319 during directed evolution. This insertion had a significant enantioselectivity-enhancing effect.
[c]There is virtually no activity of the wild-type enzyme towards this substrate.

Table 2. Selected EH variants evolved towards higher enantioconvergence using iterative saturation mutagenesis (including desymmetrization of *meso*-epoxides).

Source of EH	Substrate	ee_p [%][a] (Abs. conf. of main diol)	No. of mutations introduced	Screening method	Reference and remarks

Aspergillus niger M200		3 → 70 (*R*)	9	2-step screening: (1) activity screening using 4-(4-nitrobenzyl)pyridine and (2) chiral GC	Kotik et al., 2011; NNK codon randomization
Rhodococcus erythropolis DCL 14		14 → 93 (*S*,*S*) 14 → 80 (*R*,*R*)	4 5	2-step screening: (1) cell-based adrenaline test and (2) chiral GC	Zheng and Reetz, 2010; NDT codon randomization

[a] The change in ee_p-value from wild-type to final mutant enzyme is indicated.

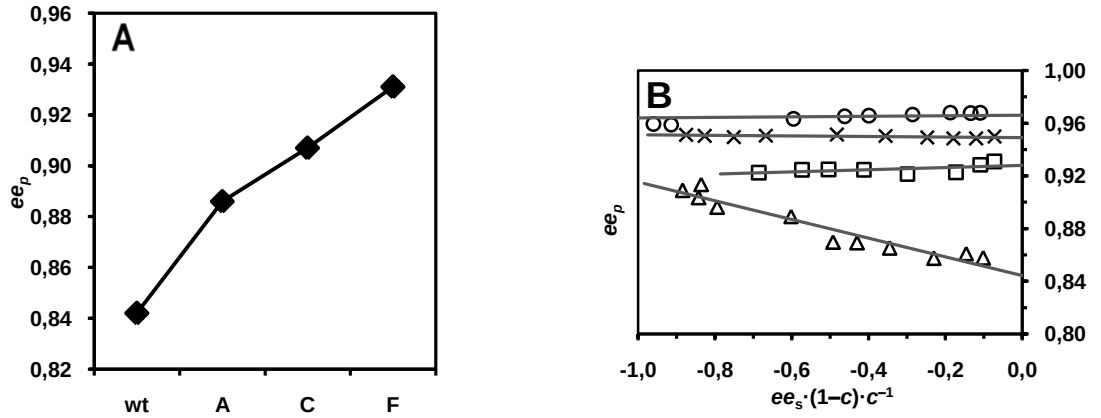


Figure 6. (**A**) Directed evolution of the metagenome-derived EH Kau2 towards an enantioconvergent biocatalyst. Five amino acid substitutions were sufficient to increase the degree of enantioconvergence from 84% ee_p for the wild-type EH (wt) to 93% ee_p for the final evolved variant (F), enabling the production of the *R*-diol with an ee-value of 93% at 28 °C in a complete conversion of racemic *para*-chlorostyrene oxide. A and C represent intermediate variants of the *in vitro*evolution process. Higher ee_p-values of up to 97% were determined in enantioconvergent reactions using lower temperatures (Kotik et al., 2013). (**B**) Determination of the regioselectivity coefficients α_S and α_R of the EH-catalyzed reaction using the wild-type and final evolved Kau2 variantwith racemic *para*-chlorostyrene oxide as the substrate. Plotting ee_p against $ee_s \cdot (1–c) \cdot c^{-1}$ enabled the determination of α_R and subsequently α_S as described in Kotik *et al*. (2010), using the intercept *q* and the slope *m* from the linear regression; for the calculations non-absolute values for ee_p and ee_s were used (see Moussou et al., 1998b). The following relationships are valid: $\alpha_R + \beta_R = 1$, and $\alpha_S + \beta_S = 1$. Wild-type EH at 28°C (△): $\alpha_R = 0.12$, $\alpha_S = 0.95$; final evolved variant at 28°C (□): $\alpha_R = 0.03$, $\alpha_S = 0.96$; at 18°C (×): $\alpha_R = 0.03$, $\alpha_S = 0.98$; at 8°C (○): $\alpha_R = 0.02$, $\alpha_S = 0.98$.

4 Immobilized EHs and reactions in non-aqueous media

4.1 Immobilization of EHs

Immobilization can help to increase the operational stability of a biocatalyst, which is a particularly important aspect of preparative and industrial-scale biotransformations. The following immobilization techniques have been reported for EHs: carrier-free approaches, adsorption or covalent attachment of the enzyme to a support, whole-cell encapsulation and entrapment, and immobilized metal ion affinity binding via genetically engineered His tags. The latter technology can be used for both one-step extraction from cellular crude extracts and facile immobilization of the recombinant EH.

Often, enzyme enrichment using recombinant protein production and/or purification precedes immobilization to increase the enzyme load on the support. For instance, a polyethylene glycol-based aqueous two-phase system was established to extract a recombinant metagenome-derived EH from the clarified crude cell lysate. This enriched EH preparation was then used directly for immobilization onto oxirane-activated supports. The highest per-gram activity of the immobilized EH was achieved with Eupergit C 250L, reaching 44 μmol min^{-1} (g of dry support)$^{-1}$ for *trans*-1-phenylpropene oxide at 28 °C. The immobilized EH exhibited an increase in thermal stability compared to the free enzyme (Grulich et al., 2011).

Carrier-free immobilized EHs were prepared using the cross-linked enzyme aggregate technology (CLEA), as exemplified by the CLEA formation of a mixture of two EHs from mung beans. Compared to the free enzymes, the CLEAs exhibited significantly shorter reaction times and higher enantioconvergence for the hydrolysis of styrene oxide in a biphasic *n*-hexane-buffer reaction system, which ensured low levels of nonenzymatic hydrolysis of the substrate (Sheldon et al., 2013).

Immobilization reactions based on biocatalyst adsorption or covalent binding onto supports were described for various EHs, the most interesting results are summarized below. A clarified crude extract of recombinant *E. coli* biomass was used for immobilization of the *Solanum tuberosum* EH onto glyoxyl-agarose in the presence of dextran. This covalently immobilized enzyme preparation exhibited a marked increase in thermostability by a factor of 300 at 60 °C and nearly no change in the regioselectivity coefficients and *E*-value for the hydrolysis of racemic styrene oxide (Mateo et al., 2007).

Two EHs, one from *S. tuberosum* and the other from *Aspergillus niger* LCP 521, were separately immobilized onto DEAE-cellulose by adsorption and used sequentially for the transformation of racemic *para*-chlorostyrene oxide into the corresponding (*R*)-diol with an enantiopurity of 89%. In total, five cycles were performed, each immobilized biocatalyst being recovered after each cycle and re-used in the following biotransformation reaction (Karboune et al., 2005a).

A magnetically separable biocatalyst was created by immobilizing the thermally unstable EH from *Mugil cephalus* within the cavities of magnetite-containing mesoporous silica. First, the purified enzyme was adsorbed onto the carrier material and then cross-linked within the pores of the carrier with glutaraldehyde, creating a so-called nanoscale enzyme reactor. The immobilized biocatalyst exhibited significantly higher stability at 30 °C compared to the free enzyme. Repeated hydrolytic kinetic resolution experiments were performed with racemic styrene oxide as the substrate, the EH-containing nanoparticles being recovered and re-used in a total of seven cycles (Kim et al., 2013).

The results described in the following five reports were all obtained with a partially purified recombinant EH from *A. niger* LCP 521. Yildirim and co-workers covalently immobilized the EH onto modified Florisil and Eupergit C supports, leading to biocatalyst preparations with improved thermostabilities and a twofold higher E-value for the hydrolysis of racemic styrene oxide when compared to the free enzyme (Yildirim et al., 2013).

Petri and co-workers used an epoxide-derivatized silica gel for the covalent immobilization of the EH, resulting in an immobilized enzyme preparation with enhanced stability in a reaction mixture containing dimethylsulfoxide, which was needed as a co-solvent for efficient solubilization of the substrate *para*-nitrostyrene oxide. Nine cycles of repeated batch reactions were performed without significant loss of biocatalyst activity (Petri et al., 2005).

Immobilization onto DEAE-cellulose by ionic adsorption resulted in an EH preparation with significantly reduced half-life of enzyme activity at all investigated reaction temperatures. Nevertheless, the immobilized EH was successfully recycled in a repeated biphasic batch process, kinetically resolving in 5 cycles a total of 3 g of racemic *para*-chlorostyrene oxide at a concentration of 2 M at 4 °C (Karboune et al., 2005b). The same substrate at a concentration of 4 mM and the same EH-containing support were used in a series of hydrolytic kinetic resolutions in heptane which was equilibrated at a water activity of 0.9. A decrease in E-value, but an increase in operational stability during biocatalyst recycling was determined, when compared to the free EH (Karboune et al., 2010).

An enhancement in *E*-value for the hydrolysis of racemic styrene oxide and *para*-chlorostyrene oxide was observed for the above-mentioned EH when immobilized onto an ethylene diamine-modified epoxy-activated support. A repeated batch experiment with 12 cycles of hydrolytic kinetic resolution of racemic styrene oxide was performed using this immobilized biocatalyst preparation without significant loss in enzyme activity (Mateo et al., 2003).

Whole cells of the bacterium *Nocardia tartaricans* with hydrolytic activity towards *cis*-epoxysuccinate were encapsulated in a carrier material consisting of sodium alginate, cellulose sulfate and poly(methylene-*co*-guanidine) for the production of L-(+)-tartrate. The encapsulation led to improved performance of the cells in terms of EH activity and storage stability when compared to the cells that were entrapped in calcium pectate gel beads (Bučko et al., 2005).

Entrapment of *Rhodosporidium toruloides* cells in calcium alginate led to an immobilized EH-containing biocatalyst for the enantioselective kinetic resolution of racemic 1,2-epoxyoctane. The entrapped cells exhibited a much lower loss in enzyme activity during storage at room temperature in the absence of substrate (Maritz et al., 2003).

Recently, several immobilization methods based on the His-tag technology (Hochuli et al., 1988) were reported for EHs. A magnetically separable Fe_3O_4–silica-based composite material with NiO nanoparticles attached to its surface was produced, which enabled the immobilization of a His-tagged EH from *Rhodotorula glutinis*. The immobilized biocatalyst was re-used in repeated batch experiments for the hydrolytic kinetic resolution of racemic styrene oxide using a magnet for separating the biocatalyst from the reaction mixture (Lee et al., 2009). Wang and co-workers synthesized magnetic iron oxide-based nanoparticles with Ni-nitrilotriacetic acid functions on their surface. The His-tagged EH from *Solanum tuberosum* was selectively immobilized on these nanoparticles, and the resulting biocatalyst was re-used in repeated hydrolytic kinetic resolutions with racemic *para*-chlorostyrene oxide as the substrate. The nanoparticle-based biocatalyst retained 80% of its productivity after eight cycles of kinetic resolution (Wang et al., 2011). Co(II)-resorcinol-grafted silica beads were prepared for the one-step extraction-immobilization of His-tagged enzymes. Immobilized *Solanum*

tuberosum EH was prepared in this way and tested in water-saturated cyclohexane for the formation of hydrobenzoin from *trans*-stilbene oxide (Cassimjee et al., 2011).

4.2 EH-catalyzed reactions in organic solvent- or ionic liquid-containing media

The use of non-aqueous reaction media for EH-based biotransformations can be advantegeous: Higher solubilities and stabilities of the relatively hydrophobic epoxidic substrates can be achieved, and in some cases higher enzyme thermostabilities have been reported. Stirred two-phase systems can ensure an optimal reaction environment for the biocatalyst in the aqueous phase and at the same time a high concentration of the epoxidic substrate with a low nonenzymatic hydrolysis rate in the organic phase. For instance, the hydrolytic kinetic resolution of phenyl glycidyl ether was achieved in a stirred water-isooctane two-phase system using whole cells of *Bacillus megaterium* ECU1001 (Gong and Xu, 2005).

An increase in *E*-value in an *n*-hexane-containing two-phase reaction system was determined for the EH-mediated enantioselective hydrolysis of styrene oxide using a freeze-dried cell-free extract from *Sphingomonas* sp. (Liu et al., 2006).

The tendency of epoxides to undergo spontaneous nonenzymatic hydrolysis in water was also reduced in reaction media composed of ionic liquids, as shown by Chiappe and co-workers. Under these reaction conditions with crude or purified EHs from cress or mouse as the biocatalyst, the reaction rates and stereoselectivities for the hydrolysis of *trans*-β-methylstyrene oxide were found to be – with a few exceptions – generally comparable to those of buffer-based reactions (Chiappe et al., 2004). The beneficial effects of ionic liquids on EH-mediated asymmetric hydrolysis reactions, i.e. reducing the nonenzymatic hydrolysis rate and increasing the solubility of the epoxidic substrate, were confirmed in more recent reports, as mentioned below. Chiappe and co-workers tested a number of ionic liquids and established optimal reaction conditions for the kinetic resolution of racemic 2-*tert*-butyloxirane using recombinant soluble and microsomal EHs from mouse, cress and rat. It is worth noting that several of the tested ionic liquids were found to be incompatible with this epoxidic substrate, presumably due to a nonenzymatic reaction between the epoxide and the anion of the ionic liquid compound (Chiappe et al., 2007). Further, Chen and co-workers studied the asymmetric hydrolysis of styrene oxide in two-phase systems using an enriched EH-containing preparation from Mung beans. The biphasic systems were composed of either hydrophobic ionic liquid–buffer or *n*-hexane–buffer in the presence of small amounts of hydrophilic ionic liquids. Using optimized reaction conditions, the two EHs in Mung bean converted the racemic substrate into (*R*)-1-phenyl-1,2-ethanediol with an *ee*-value of up to 97% and a product yield of 49% (Chen et al., 2012a; Chen et al., 2012b).

Compared to an aqueous medium, an increased thermostability in heptane for an enriched and freeze-dried EH preparation from *Aspergillus niger* LCP 521 was reported in heptane. An initial water activity (a_w) of 0.9 for the heptane reaction medium was determined to be optimal in terms of enzyme activity and E-value. On the other hand, the use of heptane greatly increased the apparent K_m-value of the enzyme for *para*-chlorostyrene oxide by a factor of 460, thereby reducing the catalytic efficiency of the enzyme (Karboune et al., 2006).

A high substrate concentration in an aqueous reaction mixture can also lead to biphasic systems with the substrate forming a second phase. Such two-phase systems were established for a number of EH-based reactions (Cleij et al., 1998; Monfort et al., 2004; Doumèche et al., 2006).

5Mono-functional epoxides as chiral building blocks for the synthesis of biologically active compounds

Mono-functional epoxides are the simplest EHs substrates and some of them, such as styrene oxide and derivatives thereof, are routinely used as standard substrates for EH activity measurements. Even within such a simple class of compounds it is possible to distinguish between, for example, mono-, di- and tri-substituted epoxides, aromatic and non-aromatic epoxides, and *meso* epoxides. The following reflects such a subdivision of epoxides and may assist in finding the right enzyme for as yet not studied epoxides just by structure comparison with already described substrates of EHs.

5.1 Mono-substituted aromatic epoxides

5.1.1 Styrene oxide

Both enantiomers of styrene oxide (SO) and phenyl-1,2-ethanediol are common chiral aromatic molecular building blocks for the synthesis of pharmaceuticals and other specialty chemicals. In the last decades, a substantialamount of work has been dedicated to the enantioselective biohydrolysis of SO, the reason probably being the commercial availability of SO and the corresponding diol in both racemic and enantiopure forms. In 1993 Furstoss and co-workers described the first preparative access to both enantiomers of SO by enantioselective hydrolysis of the racemate using cells of two fungal strains that were enantiocomplementary, i.e.

enantioselectively hydrolyzed either of the two SO enantiomers (Pedragosa-Moreau et al., 1993; Pedragosa-Moreau et al., 1995). In addition, it was observed that the hydrolysis of racemic SO using cells of *Aspergillus niger* LCP 521 proceeded with retention of configuration at the chiral center, generating the (*R*)-diol and leaving behind the (*S*)-epoxide as the residual compound. On the other hand, hydrolysis of SO using cells of *Beauveria sulfurescens* ATCC 7159 resulted in the formation of the (*R*)-diol from the (*S*)-epoxide by inversion of configuration, leaving behind the unreacted (*R*)-epoxide. These findings were used for a biohydrolysis reaction with both molds present, resulting in the first enantioconvergent bi-enzymatic process for the production of (*R*)-phenyl-1,2-ethanediol in high yield (92%) and with an ee as high as 89% (**Figure 7**). More recently, this bi-enzymatic enantioconvergent strategy was applied for the preparation of the same (*R*)-diol compound in a higher *ee*, using different combinations of EHs. For example, by mixing two purified EHs, a wild-type EH from *S. tuberosum* and an evolved EH from *A. radiobacter* AD1, SO was rapidly converted to the corresponding (*R*)-diol in 98% ee and 100% yield [Cao et al., 2006). However, this process had to be carried out at a low substrate concentration of 5 mM due to product inhibition of the *S. tuberosum* EH. Moreover, a mixture of recombinant whole cells harboring the EH-encoding genes from *A. niger* LK and *C. crescentus* was also used in a preparative-scale batch reaction for the enantioconvergent hydrolysis of 1.2 g of racemic SO at a concentration of 33 mM. 1.3 g of (*R*)-phenyl-1,2-ethanediol with an enantiopurity of 91% was obtained with an overall yield of 95%. Substrate concentrations exceeding 50 mM could not be used due to product inhibition of the bacterial EH [Hwang et al., 2008b). A similar approach was described later in which the *A. niger* LK EH was replaced by an EH mutant from *Mugil cephalus*, a marine fish (Min et lee, 2012). After optimization of the reaction conditions, (*R*)-phenyl-1,2-ethanediol was obtained in 90% ee and 95% yield from 50 mM racemic styrene oxide. Shen and co-workers isolated two bacterial EHs (SgcF and NcsF2), which are involved in the biosynthesis of enediynes, antitumor antibiotics produced by *Streptomyces globisporus* and *Streptomyces carzinostaticus*. Using SO as a substrate mimic, SgcF and NcsF2 were shown to be enantiocomplementary, leading to the formation of (*R*)-phenyl-1,2-ethanediol in 99% ee and 87% yield from racemic SO (14 mM) when used together in the reaction mixture (Lin et al., 2010). An interesting enantioconvergent process with whole cells of *Aspergillus tubingensis* TF1 was very recently described by Duarah *et al.* (Duarah et al., 2013). (*R*)-phenyl-1,2-ethanediol was isolated from the reaction mixture in 97% ee after 45 min, reaching >99% conversion of racemic SO (8.75 mM). Although a single enzyme was claimed to be responsible for this process, the possibility of two enantiocomplementary EHs with opposite regioselectivity being present in the microorganism cannot be excluded from the described data.

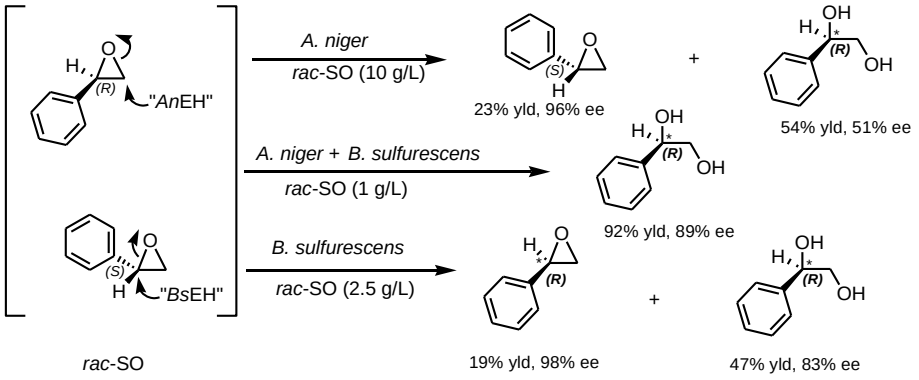


Figure 7. Biohydrolytic kinetic resolution of racemic styrene oxide (*rac*-SO) catalyzed by *Aspergillus niger* LCP 521 (*An*EH) and/or *Beauveria sulfurescens* ATCC 7159 (*Bs*EH). Using both fungi together led to an enantioconvergent production of (*R*)-phenyl-1,2-ethanediol.

Although a great number of wild-type EHs were found to kinetically resolve racemic SO, to our best knowledge no EH showed a very high enantioselectivity level. Nevertheless, several kinetic resolutions at high SO concentrations have been described in the last decade using EHs from yeast, bacteria and plants. For example, (*S*)-SO with 98% ee was obtained in 41% yield from racemic SO at a very high concentration of 1.8 M using a *Pichia pastoris* strainoverexpressingthe EH from *Rhodotorula glutinis*. Such a high substrate concentration in the reaction mixture called for optimized reaction conditions, i.e. the reaction was taking place at 4° C in the presence of 40% (v/v) Tween 20 and 5% (v/v) glycerol (Yoo et al., 2008). Biomass of *Achromobacter* sp. MTCC 5605, which was isolated from a petroleum-contaminated sludge sample, enabled the hydrolytic kinetic resolution of racemic SO at a high concentration of 0.5 M using a biphasic reaction system composed of isooctane and buffer. Under these conditions, an enantiomeric ratio of 64 was determined; the remaining (*S*)-SO (42% yield) and the formed (*R*)-phenyl-1,2-ethanediol were isolated in >99 and 65% ee, respectively (Kamal et al., 2013). It is worth mentioning that an improvement of the enantioselectivity was obtained after covalent immobilization of the multimeric EH from*A. niger* LCP 521 onto Eupergit C which was

partially modified with ethylene diamine (Eupergit C/EDA), resulting in an E-value of 56 instead of 25) (Mateo et al., 2003).

5.1.2 Chlorostyrene oxide

(*R*)-*para*- and (*R*)-*meta*-chlorostyrene oxides are important building blocks for the synthesis of various biologically active molecules. Indeed, these compounds are, for example, essential chiral intermediates for the production of Eliprodil (Pabel et al., 2000), an effective NMDA receptor antagonist, and various β-3-adrenergic receptor agonists such as SR 58611A or AJ-9677 (Harada et al., 2003). Numerous biohydrolytic kinetic resolutions have been described in the literature with the purpose of preparing these chiral synthons in enantiopure form. However, it is well known that one of the general drawbacks of including a resolution step in a chemical synthesis is its intrinsic 50% yield limitation. This is the reason why various enantioconvergent processes with the advantage of approaching the ideal situation of "100% yield and 100% ee" have been elaborated.

As far as *para*-chlorostyrene oxide (*p*-ClSO) is concerned, enzymatic extracts of overexpressed EHs from *A. niger* LCP 521 (*An*EH) and *S. tuberosum* (*St*EH) were shown to efficiently resolve this racemic epoxide (E-values of 100 at 0°C). The enantiocomplementarity of these two enantioselective EHs enabled the preparation of both enantiomers of *p*-ClSO, the (*R*)-*p*ClSO and (*S*)-*p*ClSO being preferentially hydrolyzed by the *An*EH and the *St*EH, respectively. The absolute configuration of the formed diol was determined to be *R* in both cases. Preparative-scale resolutions were performed at the very high substrate concentrations of 306 g/L and 30.6 g/L using respectively *An*EH and *St*EH as the biocatalysts (Manoj et al., 2001). Similar results were also obtained in a repeated batch reaction with both enzymes immobilized onto DEAE-cellulose by ionic adsorption (Karboune et al., 2005).

Based on the complementary enantio- and regioselectivities of these two EHs, an enantioconvergent production of (*R*)-*para*-chlorophenyl-1,2-ethanediol from *rac*-*p*-ClSO was established using a sequential bi-enzymatic strategy. Thus, a preparative-scale experiment was carried out at a substrate concentration of 30.6 g/L using first the *St*EH followed by the *An*EH. The (*R*)-diol was obtained with an overall yield as high as 93% andwith a 96% ee (**Figure 8)** (Manoj et al., 2001). Unfortunately, in this bi-enzymatic process the substrate concentration had to be decreased by a factor of 10, compared to a single-enzyme resolution process with *An*EH, to diminish the inhibitory effect of the formed diol on the *St*EH activity. Later, a repeated batch experiment using these two EHs, separately immobilized onto DEAE-cellulose, was also described (Karboune et al., 2005). More recently, other teams reported a similar strategy using two EHs. A sequential bi-enzymatic hydrolysis of *p*-ClSO was described by Lee *et al.*(Min et al., 2012), using a heterologously expressed EH from *Caulobacter crescentus* and an EH mutant from *Mugil cephalus*. The combined use of whole cells overexpressing these two EHs enabled the production of (*R*)-*para*-chlorophenyl-1,2-ethanediol with 92% enantiopurity and 71% yield, starting from 17 g/L of *rac*-*p*-ClSO. In a previous paper, the same authors described an enantioconvergent process which was based on a mono-enzymatic approach using the EH from *C. crescentus*(Hwang et al., 2008a). This EH was shown to have opposite enantioselectivity and regioselectivity toward either enantiomer of *rac*-*p*-ClSO, which led to the almost exclusive formation of the (*R*)-diol. With the enantioselectivity being not too high (*E*-value = 30), a preparative-scale biohydrolysis at a substrate concentration of 16.8 g/L resulted in the formation of (*R*)-*para*-chlorophenyl-1,2-ethanediol with 98% ee and 78% overall yield. Kotik *et al.* have described the first example of regioselectivity engineering in EHs by directed evolution starting from a non-enantioconvergent enzyme (Kotik et al., 2011). The substrate binding cavity of the EH from *A. niger* M200 was redesigned to generate an enantioconvergent biocatalyst by guiding the point of nucleophilic attack to the benzylic oxirane position of the bound (*S*)-enantiomer. After nine amino acid exchanges, the final mutant enzyme transformed racemic *p*-ClSO to the (*R*)-diol with an *ee* of 70.5%. In the same article, a sequential bi-enzymatic reaction using the wild-type EH from *A. niger* M200 and its evolved variant enabled the preparation of the (*R*)-diol with an ee-value of 88%. More recently, Kotik and co-workers reported that an EH (named Kau2), whose gene was isolated from a biofilter-derived metagenome, exhibited an opposite regioselectivity for the two enantiomers of *p*-ClSO, whichenabled them to obtain (*R*)-*para*-chlorophenyl-1,2-ethanediol in 84% eeat 100% conversion(Kotik et al., 2010). Later,an improvement in the enantioconvergence of Kau2 was achieved using iterative saturation mutagenesis (**Figure 4**), leading to a higher enantiomeric excess of 94% for the diol product at complete conversion of the racemic *p*-ClSO substrate(Kotik et al., 2013).

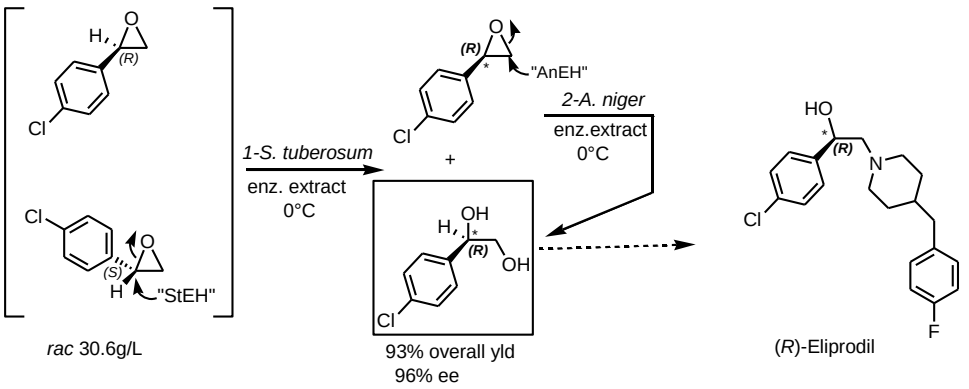


Figure 8. Enantioconvergent biohydrolytic transformation of *para*-chlorostyrene oxide using a bi-enzymatic process. The sequential use of *Solanum tuberosum* and *Aspergillus niger* EHs as biocatalysts led to the formation of enantiopure (*R*)-*para*-chlorophenyl-1,2-diol, a chiral building block for the synthesis of (*R*)-Eliprodil.

An enantioconvergent preparative-scale production of (*R*)-*meta*-chlorophenyl-1,2-ethanediol was described by Monterde *et al*. using the EH from *Solanum tuberosum*. The enzyme exhibited a low enantioselectivity (E-value of 6) in conjunction withan opposite regioselectivity for each enantiomer of *m*-ClSO, which are ideal conditions for an enantioconvergent process. Starting from *rac*-*m*-ClSO, nine cycles of a repeated batch experiment in a stirred reactor, at 10 g/L substrate concentration, furnished the (*R*)-diol with an ee-value of 97% and an 88% overall yield (Monterde et al., 2004. Very recently, Li and co-workers have studied the biohydrolysis of numerous epoxides (including *meso*ones) using the EH from *Sphingomonas* sp. HXN-200 overexpressed in *E. coli* (*Sp*EH) (Wu et al.,2013). This EH was shown to be more enantioselective than any other known EH for the hydrolysis of *rac*-*m*-ClSO with an E-value of 41. Interestingly, *Sp*EH reacted preferentially with the (*R*)-epoxide, forming the (*R*)-diol and leaving behind the (*S*)-*m*-ClSO, which is a useful chiral building block for the preparation of an IGF-1R kinase inhibitor (Witmann et al., 2005). A gram-scale kinetic resolution of *rac*-*m*-ClSO was performed in a two-phase system (buffer/*n*-hexane) at 15 g/L of substrate with resting *Sp*EH cells. Enantiopure (*S*)-*m*-ClSO was obtained in 37.9% yield.

5.1.3 Nitrostyrene Oxide

(*R*)-*para*-nitrostyrene oxide (*p*-NSO) is the key chiral synthon for the synthesis of Nifenalol, a compound showing β-blocking activity and used in the treatment of hypertensive diseases(Murmann et al., 1967). This epoxide, which is mostly insoluble in water, has been resolved with good enantioselectivity by fungal, bacterial and yeast EHs (**Table 3**). The first examples were described with whole cells of the fungi*A. niger* LCP521 and *Beauveria sulfurescens* ATCC7159(Pedragosa-Moreau et al., 1996c).One year later, a cell-free extract from *A. niger* LCP521 was used as a biocatalyst (Morisseau et al., 1997).To improve the solubility of *p*-NSO in the water phase, 20% of a miscible solvent (DMSO or DMF) was added to the reaction mixture. Under these experimental conditions, the hydrolysis was relatively enantioselective (E-value = 48) and could be performed at a high substrate concentration of 54 g/L (330 mM) to produce (*S*)-*p*-NSO (99% ee; 49% yield). Later, high enantioselectivity with an *E*-valueof >100 at 10 °Cwas alsodeterminedwith anotherEH present in the strain *Aspergillus niger* M200, which was isolated from industrial biofilters (Kotik et al., 2005; Kotik and Kyslik, 2006). Starting from the wild-type EH from*Agrobacterium radiobacter* AD1 (E-value = 65) (Spelberg et al., 2002), a substantial improvement of the enantiopreference was obtained byerror-prone PCR and DNA shuffling, reaching an E-value>200(Rink et al., 2000; van Loo et al., 2004).Interestingly, a very high enantioselectivity (E-value>200) together with a reversed (*S*)-enantiopreference was observed for the EH from *Bacillus megaterium* ECU1001,retaining the useful (*R*)-p-NSO for the direct synthesis of (*R*)-Nifenalol.(Zhao et al., 2010). More recently, EHs exhibiting opposite enantiopreference toward the two antipodes of *p*-NSO were also described. Two of them, Kau2 and Kau8, were isolated from biofilter-derived metagenomes (Kotik et al, 2010), and two others, oxy-4 and oxy-10, from yeast(Pienaar et al., 2008). It should be noticed that the remaining epoxides of Kau2- and Oxy-10-mediated kinetic resolutions were determined to be(*R*)-*p*-NSO, which is the correct enantiomer for the synthesis of (*R*)-Nifenalol.

Table 3. Selected kinetic resolutions of *rac*-*p*-nitrostyrene oxide.

Source of EH	Notes	E-value	Substrate conc. (mM)	Abs. Conf. Residual epoxide/ Formed diol	References
Aspergillus niger LCP 521	Enzymatic extract	48	330		Morisseau et al.;

				S/R	1997
Agrobacterium radiobacter AD1	Purified enzyme	65	3	*S/R*	Spelberg et al., 2002
Agrobacterium radiobacter AD1, variant Y215F		>200	3	*S/R*	Rink et al., 2000
Agrobacterium radiobacter AD1, variant S7	Error-prone PCR and DNA shuffling; cell-free extract or purified EH	>200	2	*S/R*	van Loo et al., 2004
Aspergillus niger GBCF 79(recombinant strain)	Enzymatic extract, EH immobilized onto silica gel, co-solvent: 20% DMSO	85	4.5	*S/R*	Petriet al.; 2005
Aspergillus niger M200	Purified enzyme	>100	8	*S/R*	Kotik and Kislik, 2006
Kau2 (metagenome)	Crude extract, EH overexpressed in *E. coli*	80	3.5	*R/R*	Kotik et al.; 2010
Kau8 (metagenome)		65	3.5	*S/R*	
Oxy-4	Whole cells, EH overexpressed in *Y. lipolytica*	>100	500	*S/R*	Pienaar et al., 2008Botes et al. ; 2007
Oxy-10		>100	500	*R/R*	
Bacillus megaterium ECU1001	Crude extract, EH overexpressed in *E. coli*	>200	20	*R/-*	Zhao et al.; 2013

A one-pot chemo-enzymatic enantioconvergent process for the production of (*R*)-*p*-NSO was described by Furstoss *et al.* in 1997 (Pedragosa-Moreau et al., 1997). The strategy was based on the sequential hydrolysis of the (*R*)-epoxide usingan enzymatic extract of*An*EHfollowed by an acid-catalysed hydrolysis of the remaining (*S*)-epoxide (**Figure 9**). A 330 mM (54 g/L) solution of *p*-NSO was hydrolysed within 6 h tofurnishthe (*S*)-epoxide in 49% yield and 99% ee. Then, the controlled acid hydrolysis of the reaction mixtureyielded the (*R*)-diol (80% ee) as a result of steric inversion upon acid hydrolysis of the unreacted (*S*)-epoxide. After recrystallisation, the (*R*)-diol could be easily recycled to the epoxide and transformed into (*R*)-Nifenalol. Interestingly, Botes *et al.*(Pienaar et al., 2008; Botes et al., 2007c)described the enantioconvergent production of the (*R*)-diol in one pot at a high substrate concentration using the combined actionthe Oxy-4 and Oxy-10 biocatalysts (bi-enzymatic-process).

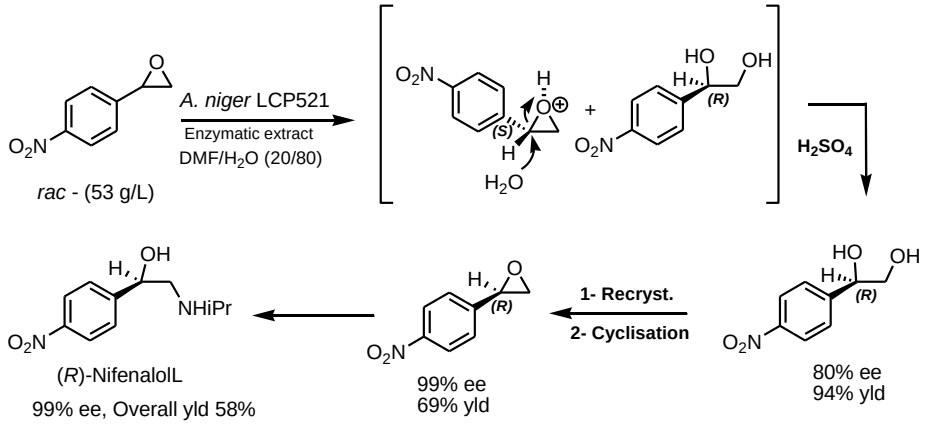


Figure 9. Enantioconvergent synthesis of the β-blocker (*R*)-Nifenalol using a combined chemoenzymatic approach.

5.1.4 Trifluomethylstyrene oxide

Enantiocontrolled synthesis of fluorinated organic compounds has gained tremendous impetus over the recent years because it is well known that the presence of fluorine atoms in a molecule can have dramatic effects on its biological activity (Soloshonok 1999). In this context, the kinetic resolution of a specific trifluoro-methyl-substituted aromatic epoxide family was studied using a recombinant EH from *A. niger* LCP521 EH, and the productivity of the biotransformation process was evaluated (Deregnaucourt et al., 2007). A two liquid-liquid phase methodology with an appropriate co-solvent (isooctane, 10 to 35% (v/v)) and optimized operational conditions, led to a very efficient and cost-effective resolution process. The best results (high *E*-value, high TON and TOFF) were obtained in the case of *para*-substituted CF_3-, OCF_3- and SCF_3-derivatives. For example, resolution of (4-trifluoromethoxyphenyl)-oxirane could be performed at 250 g/L resulting in the residual (*S*)-epoxide and the formed (*R*)-diol in good yields and very high enantiomeric excess (**Figure 10**).

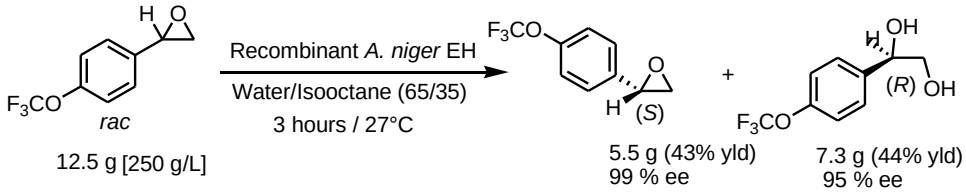


Figure 10. Preparative scale synthesis of enantiopure (4-trifluoromethoxyphenyl)-oxiraneusing apartially purified recombinant EH from *Aspergillus niger* LCP521 as a biocatalyst.

5.1.5 Pyridyl oxirane

Enantiopure 2-,3- and 4-pyridyloxirane are key-building blocks for the synthesis of several biologically active compounds, such as β-adrenergic receptor agonists or antiobesity drugs (Mathvink et al., 1999; Devries et al., 1998; Fisher et al., 1996). Up to now,none of these products could be obtained in a satisfactory enantiopure form using the most effective heavy metal-containingcatalysts (Jacobsen epoxidation, Jacobsen HKR or Sharpless dihydroxylation). Interestingly, the recombinant EH from *A. niger* LCP521 exhibited a rather high enantioselectivity toward all three substrates with *E*-values of 96, 27and 47, respectively, hydrolyzing preferentially the (*R*)-enantiomer and thus enabling the recovery of the slowy reacting (*S*)-epoxide. Unfortunately, it was shown that the *E*-value decreased with increasing substrate concentration. Nevertheless, the preparative scale synthesis of each pyridyloxirane could be performed at about 10 g/L substrate concentration, which enabled obtaining these three target compounds in nearly enantiopure form. (Genzel et al., 2001) In the same year, it was shown that aTyr215Phe mutation in theEHfrom *Agrobacterium radiobacter* AD1 resulted in an enzyme variant thatcould efficiently resolve 2-pyridyloxirane (*E*-value = 55) at a substrate concentration as high as 127 mM (15.5 g/L) (**Figure 11**)(Genzel et al., 2001).

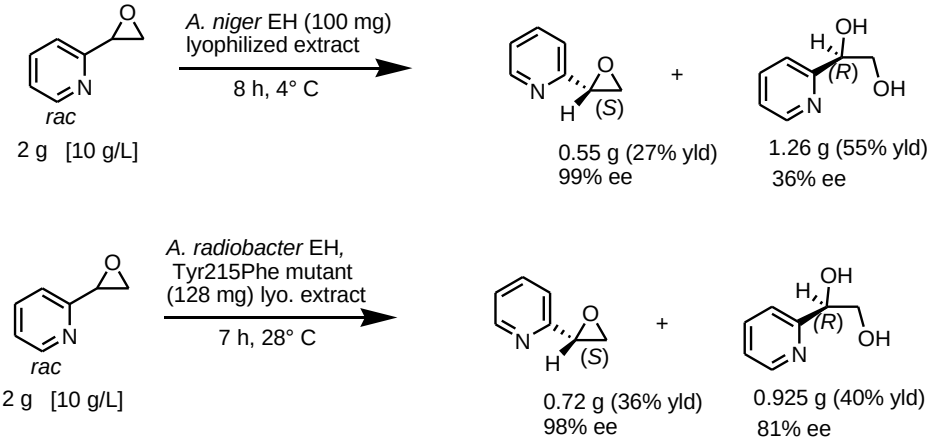


Figure 11. Preparative kinetic resolution of 2-pyridyloxirane with EHs from *A. niger* and *A. radiobacter* AD1 (mutant Tyr215Phe).

5.2 Di-substituted aromatic epoxides

5.2.1 *p*-bromo-,*p*-isobutyl- and *p*-trifluoromethyl-*alpha*-methyl styrene oxide

To overcome the problem of low solubility of aromatic epoxides in the water phase, Furstoss and collaborators studiedin 1998 enzymatic resolutions at high substrate concentration without adding organic solvents(Cleij et al., 1998). They showedthat at a high concentration of 80 g/L of *para*-bromo-α-methyl styrene oxide a biphasic system was formed with the epoxide constituting one phase by itself.Thisenabled a goodkinetic resolution of the aromatic epoxide using an EH-containing extract from *A. niger*LCP521as a biocatalyst. Under these experimental conditions, the residual epoxide was found to be of (*S*) configuration, whereas the formed product was the corresponding (*R*)-diol. Surprisingly, the use of this procedure led to a dramatic enhancement in the enantioselectivity with the E-value increasing from 20 at low substrate concentration (1.7 g/L) to 260 at 80 g/L(**Figure 12**).

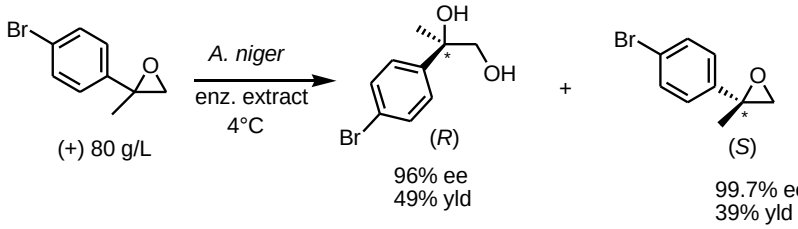


Figure 12. Kinetic hydrolytic resolutionat high substrate concentration of *para*-bromo-α-methyl styrene oxide using an enzymatic extract of *A. niger*.

One year later, the same authors studied the biohydrolysis of seven differently substituted α-methylstyrene oxide derivatives, including the *para*-bromo-α-methyl styrene oxide,using 10 different EHs(Cleij et al., 1999). The best results were obtained withthe EH from *A. niger* LCP521; however, the E-values were relatively moderate. A four-step synthesis of (*S*)-Ibuprofen, a non-steroidal anti-inflammatory drug, was performed to illustrate the synthetic potential of EHs. The strategy was to achieve the enantioselective hydrolysis of *rac*-4-isobutyl-α-methylstyrene oxide using the EH from *A. niger*, which has been shown to specifically hydrolyse the undesired (*R*)-enantiomer, and to further transform the enantiopure residual (*S*)-epoxide into (*S*)-Ibuprofen using classical chemical synthesis. As in the case of the *para*-bromo derivative, the biohydrolysis was performed at a high substrate concentration of 50g/L, leading to a biphasic process, and at a low reaction temperature of 4°C to enhance enzyme stability and decrease the spontaneous hydrolysis of the substrate. Following this strategy, the overall yield of (*S*)-Ibuprofen was only 27%. Recycling of the formed diol via chemical racemisation substantially improved the process yield. Indeed, treatment of the formed diol with HBr/AcOH and subsequent cyclisation of the bromhydrin intermediate under basic conditions afforded racemic 4-isobutyl-α-methylstyrene oxide in 80% yield, which could thus be resubmitted to the enzymatic resolution step. Under these conditions, the overall yield increased from 27 to 47%(**Figure 13**).

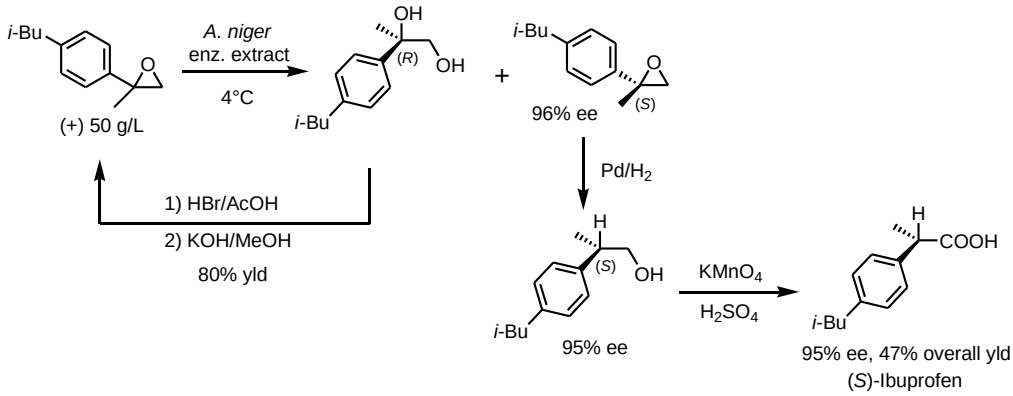


Figure 13. Preparative-scale resolution of *rac*-4-isobutyl-α-methylstyrene oxide using an enzymatic extract of *A. niger*LCP521. A four step enantioconvergent procedure enabled the synthesis of (*S*)-Ibuprofen.

Ten years later, Furstoss's group confirmed the high capacity of the EH from *A. niger* LCP 521 to kinetically resolve *para*-substituted *α*-methyl styrene oxide derivatives by showing that the enzyme could also be used for the efficient resolution of the corresponding *para*-trifluoromethyl epoxide (Deregnaucourt et al., 2007). Indeed, the preparative-scale biohydrolysis at 100 g/L substrate concentration could be performed in a short reaction time in a biphasic reaction medium containing water-organic solvent. Isooctane (25% v/v) wasadded to the reactor to obtain a good substrate emulsion, leading to an optimal transfer of the substrate from the organic phase to the water phase. Under these experimental conditions, a loading of 25 g of racemic epoxide resulted in the formationof 10.5g (42% yield) of (*S*)-epoxide (99.7% ee) and 13.4g (78.5% yield) of (*R*)-diol (78.5% ee) (**Figure 14**).

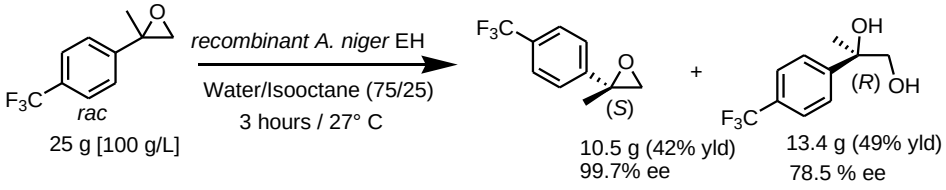


Figure 14. Preparative-scale synthesis of enantiopure *para*-trifluoromethyl-α-methyl styrene oxide using a partially purified recombinant EH from *Aspergillus niger* LCP521 as biocatalyst.

5.2.2 *cis*- &*trans*-β-methylstyrene oxide

trans-β-methylstyrene oxide in enantiopure form has been used as a building block for the synthesis of a potential cocaine abuse therapeutic agent and a drug for the treatment of obesity (Hsin et al., 2003; Lin et al., 2006). Very satisfactory resolutions of *trans*-β-methylstyrene oxide were achieved witha metagenome-derived EH (termed Kau2) (Kotik et al., 2010) and EHs from fungi, e.g.*Beauveria sulfurescens*(Pedragosa et al., 1996b), and yeast(*Rhodotorula glutinis* CIMW147, (Weijers, 1997);*Rhodotorula glutinis* UOFS Y-0123, (Lotter et al., 2004)and *Rotoruloides mucoides* UOFSY-0471 (Botes et al., 2007a)).From a comparison of all the described results,it can be concluded that the hydrolysis of *trans*-β-methylstyrene oxideproceeded with a similar enantioselectivity and stereochemistry. Indeed, in all cases the (1*S*,2*S*)-epoxide was preferentially hydrolysed to the (1*R*,2*S*)-*erythro*-diol, indicating that the enzymatic attack always occurred at the benzylic position. The best result was achieved in a preparative-scale reaction at 80 g/L of substrate concentration, using freeze-dried *E. coli* RE3 cellsharbouring the plasmid pSEKau2 for expression of the EH Kau2. Both (1*R*,2*R*)-epoxide and the corresponding (1*R*,2*S*)-diol were isolated in high enantiomeric excess (>99%) and good yield (>45%), corresponding to a very high enantioselectivity (*E*-value>200) (**Figure 15**).

In contrast to these results, Chiappe *et al.*(Chiappe et al., 2004) reported that the reaction catalyzed by cress soluble EH was partially stereoconvergent,furnishing the corresponding (1*S*,2*R*)-*erythro*-1-phenylpropane-1,2-diol as the main product. Using pure enantiomers as starting epoxides, it was determined that the formal nucleophilic attack of water occurred exclusively at C1 in the case of (1*R*,2*R*)-epoxide, while the same enzyme was practically non-selective for the same carbon of its antipode.

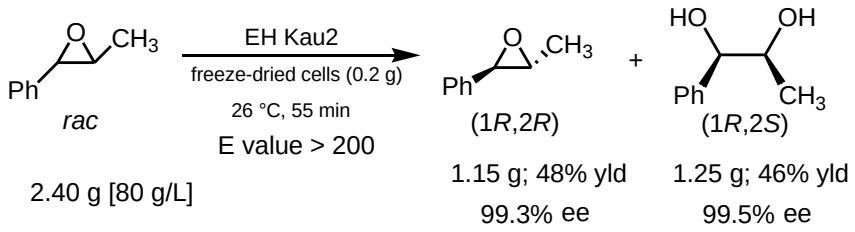


Figure 15. Preparative-scale kinetic resolution of racemic *trans*-β-methylstyrene oxideusing the EH Kau2 at high substrate concentration.

As far as*cis*-β-methylstyrene oxide is concerned, very few EHs with the capacity to hydrolyzethis compound have been described. Three similar and interesting enantioconvergent processes were described using fungal EHs from *Beauveria sulfurescens* ATCC7159(Pedragosa-Moreau et al., 1996b) and*Aspergillus terreus*(Moussou et al., 1998b), and the metagenome-derived EH Kau2 (Kotik et al., 2010). Indeed, it was observed that the two antipodes were hydrolyzed with a low enantioselectivity (*E*-value = 10) and an opposite regioselectivity, leading to the formation of the same (1*R*,2*R*)-diol in almost optically pureand nearly quantitative yield (**Figure 16**). It should be noticed that the modification of the stereochemistry (*trans* to *cis*)of the starting epoxide resulted in a significant decrease of the reaction rate.

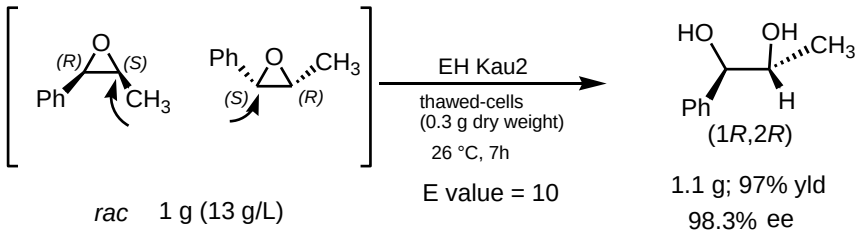


Figure 16. Preparative enantioconvergent biohydrolysis of racemic *cis*-β-methylstyrene oxideusing the EH Kau2.

5.2.3 Indene oxide

Chiral (1*S*,2*R*)-indene oxide is a valuable precursor for the synthesis ofthe side chain of the HIV protease inhibitor MK 639, which wasdeveloped by Merck Research Laboratories. Several teams searched forEHs capable of catalyzing the kinetic resolution of racemic indene oxide.Selected strains of bacteria(Hwang et al., 2006), yeast (Weijers, 1997)and fungi (Zhang et al., 1995; Pedragosa-Moreau et al., 1996a)were shown to be useful forthe preparation of the two antipodes of indene oxide (**Figure 17**). However, low enantioselectivity of the implied EHs and instability of indene oxide in water resulted in low yields when attempting to reach 100% *ee* of the residual epoxide.

Recently,kinetic resolution performed at high substrate concentration was described in a patent by Botes *et al*. (Botes et al., 2007a)using a recombinant yeast EH. Indeed, a preparative-scale biohydrolysis at 264 g/L (2M) substrate concentration was performedusing whole cells of a recombinant *Yarrowia lipolytica* strain expressing the EH from the yeast *Rhodosporidium paludigenum* NCYC 3179. In this process, crystalline indene oxide powder (26.4 g) was directly added to 100 mL of phosphate buffer which contained thawed whole cells (13.5 g wet weight). After 150 minutes at 25°C,the resolution process was complete and 8.5 g (32% yield) of residual enantiopure (1*R*,2*S*)-indene oxide were isolated. Unfortunately, the recovered (1*R*,2*S*)-indene oxide exhibited the wrong stereochemistry forthe synthesis of Indinavir.

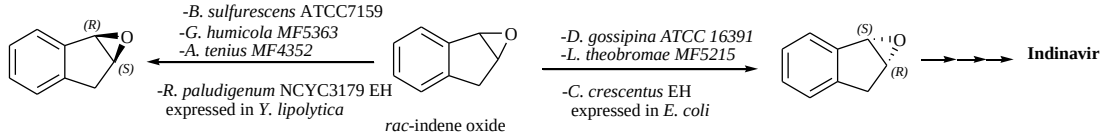


Figure 17.Biohydrolytic kinetic resolution of racemic indene oxide. Access to (1*S*,2*R*)- or (1*R*,2*S*)-indene oxide as a function of the biocatalyst used.

5.3Non aromatic epoxides

5.3.1Mono-substituted alkyl epoxides

Highly enantioselective EHs towardmono-aliphatic terminal oxiranes were detected in some species of specific yeast genera (*Rhodotorula* sp., *Rhodosporidium* sp.)(Botes et al., 1998; Botes et al., 1999). Although enantiomeric distinction of these highly flexible molecules is believed to be a difficult task for the enzyme, high *E*-values (>100) were reported in several cases. All enantioselective EHs reacted preferentially with the (*R*)-epoxide, forming (*R*)-diols and leaving behind the (*S*)-epoxide. Using whole cells of *Rhodotorula araucariae* CBS 6031or *Rhodosporidium toruloides* CBS 0349, it was possible to perform a preparative-scale hydrolysis of racemic 1,2-epoxyoctane at a 500 mMsubstrate concentration(**Figure 18**).

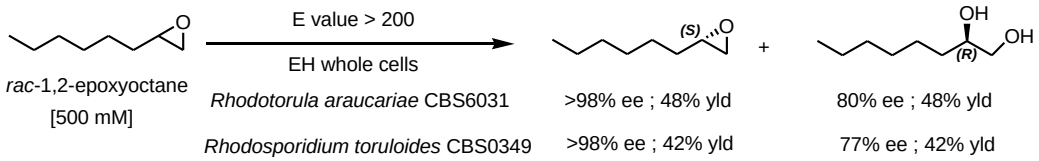


Figure18.Kinetic resolution of 1,2-epoxyoctane at high substrate concentration with two yeast EHs

5.3.2 Glycidyl ether and derivativesthereof

Biohydrolytic kinetic resolution of alkyl and aryl glycidyl ethers, which are important building blocks for the production of various bioactive compounds, have been extensively investigated using EHs from bacteria, yeast and filamentous fungi. Concerning phenyl glycidyl ether (PGE) and various derivatives thereof, interesting results were obtained with the EH from*Bacillus megaterium* ECU1001. In contrast to the majority of other EHs, this enzyme exhibited an unusual (*R*)-enantioselectivity for PGE, retaining the useful (*S*)-PGE for the synthesis of β-blocker compounds;it also exhibited the highest enantioselectivity (*E* =58) among all known wild-type EHs (Zhao et al., 2011). In addition, it was observed that introducing a methyl substituent at the phenyl ring of PGE had a pronounced influence on the enantioselectivity of the hydrolysis reaction. As a general trend, the *E*-value increased as the substituent was shifted from the *para* (*E* = 11) to the *meta* position (*E* = 19),with the*ortho*position exhibiting the highest *E*-value of more than 200. It appears that wild-type EHs are not very efficient catalysts for the preparation of (*R*)-PGE, the best results were obtained with the EHs from*Agrobacterium radiobacter* (*E*-value = 12) (Spelberg, 1998), *Trichosporon loubierii* (*E*-value = 20) (Xu et al., 2001) and a *Rhodobacterales* species (*E*-value = 38) (Woo et al., 2010). However, as described at the beginning of this review, molecular engineering techniques can be used to improve enzymatic properties such as enantioselectivity or enantioconvergence. For example, starting from the wild-type EH from*A. niger* LCP 521 EH, which catalyzes the kinetic resolution of PGE with quite low enantioselectivity (*E*-value = 4.6), Reetz and collaborators generated by directed evolution an highly evolved enantioselective mutant (LW202 exhibiting an *E*-valueof 115)(Reetz et al., 2009). Interestingly, in all cases involving other monosubstituted epoxides (substituted glycidyl ethers, alkyl and aromatic epoxides), a substantial increase in*E*-value of the evolved EH compared to the wild-type EH was observed, leading to the Reetz's conclusion:"The traditional credo in directed evolution, "You get what you screen for", can be extended by the corollary, "You may get more than what you originally screened for". Two years later, Reetz and collaborators managed to improve the expression efficiency and enantioselectivity of the LW202 EH variant by laboratory evolution again (Reetz et al., 2011). The strategy was to focus first on expression and then improvethe enantioselectivity. The expression of the generated mutant

EH222 was 50 times higher than that of the wild-type*An*EH and a very high enantiomeric ratio (*E*-value = 160) in favor of the (*S*)-diol was detected (**Figure 19**).

Kotik *et al.* screened 270 microbial isolates from biofilters and petroleum-contaminated bioremediation sites for enantioselective EHs using *tert*-butyl glycidyl ether, benzyl glycidyl ether and allyl glycidyl ether as substrates. The best results were obtained withthe most substituted ether, *tert*-glycidyl ether, for which a moderate enantioselective EH activity (*E*-value = 30) was found in a fungal isolate identified as an *Aspergillus niger* species(Kotik et al., 2005). Under optimized biotransformation conditions which included a low reaction temperature of 5 °C, a low substrate concentration of 5 mM and a high biocatalyst concentration, the enantiomeric ratio could be increased to 100, and enantiopure (*R*)-*tert*-butyl glycidyl ether and (*S*)-3-butoxy-1,2-propanediol were isolated as residual epoxide and formed diol.

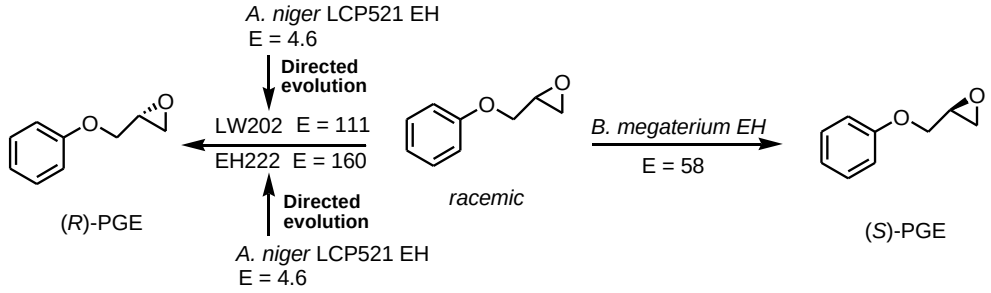


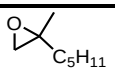
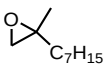
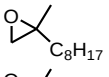
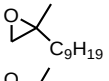
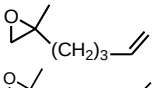
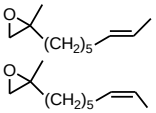
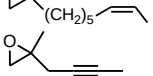
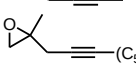
Figure 19. Biohydrolytic kinetic resolutions of racemic-PGE. Use of enantiocomplementary wild-type and evolved mutant EHs for the preparation of two antipodes of PGE.

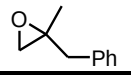
5.3.3 Di-substituted alkyl epoxides

5.3.3.1 *gem*-disubstituted oxiranes

gem-disubstituted oxiranesbearing linear alkyl, alkenyl, alkynyl or benzylsubstituents were enantioselectively hydrolyzed by EHs of the following bacterial genera*: Rhodococcus*, *Mycobacterium*, *Nocardia*, *Methylobacterium*, and *Arthrobacter*. Only EHs reacting with the (*S*)-enantiomer have been found so far, forming the (*S*)-diol (**Table 4**). Using these results, Faber and co-workers developed chemo-enzymatic approaches for the deracemisation of several *gem*-disubstituted oxiranes (**Table 4**, entries 1, 5, 9, 10). Thus, enantioselective biohydrolysis of the (*S*)-epoxide proceeded with retention of configuration,resulting in the corresponding (*S*)-1,2-diol. In a subsequent step, acid-catalyzed hydrolysis of the residual (*R*)-epoxide took place exclusively at the substituted oxirane atom with complete inversion of configuration, yielding the same (*S*)-1,2-diol. The combination of both reaction steps resulted in the formation of the (*S*)-1,2-diol in enantiopure form and in almost quantitative yield (**Figure 20**).Valuable illustrations of these enantioconvergent processes are the synthesis of natural compoundssuch as Frontalin (**Table 4**, entry 5),Fridamycin (**Table 4**, entry 9) and Mevanolactone (**Table4**, entry 10), whichwill be described in detail inchapter 7.

Table4. Selected kinetic resolutions of some *gem*-disubstituted-alkyl epoxides.

Entry	Substrate	Source of EH	*E*-value	Subs. conc.	Abs. Conf.[a]	References
1		*Rhodococcus ruber* DSM 43338 *Nocardia* H8, TB1 and EH1	 >200	 20 g/L	 *R/S*	 Osprian et al., 1997; Orru et al., 1998a
2		*Rhodococcus* sp. NCIMB 11216	126	2.5 g/L	*R/S*	Wandel et al., 1995
3		*Rhodococcus* sp. NCIMB 11216 *Arthrobacter* sp. DSM 312	>200 172	 5 g/L	 *R/S*	Osprian et al., 2000
4		*Rhodococcus* sp. NCIMB 11216	>200	2.5 g/L	*R/S*	Wandel et al., 1995
5		*Nocardia* EH1	>200	20 g/L	*R/S*	Orru et al., 1998a
6		*Rhodococcus* sp. NCIMB 11216	125	5 g/L	*R/S*	Osprian et al., 2000
7		*Rhodococcus* sp. NCIMB 11216	142	5 g/L	*R/S*	Osprian et al., 2000
8		*Rhodococcus* sp. NCIMB 11216	>200	5 g/L	*R/S*	Osprian et al., 2000
9		*Methylobacterium* sp. FCC 031	>200	10 g/L	*R/S*	Ueberbacher et al., 2005

10		*Nocardia* EH1	123	40 g/L	*R/S*	Orru et al., 1998b

[a]Absolute configuration of the remaining epoxide and the formed diol, respectively.

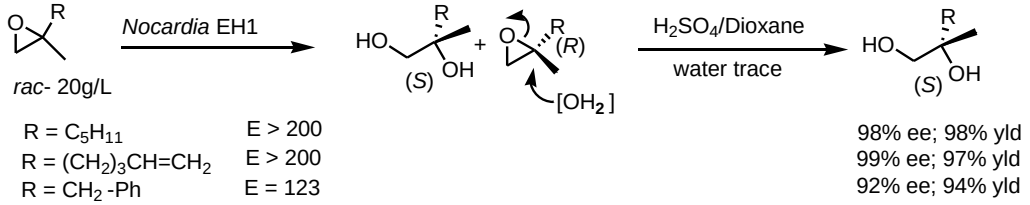


Figure20.Chemoenzymatic deracemization of *rac-gem*-disubtituted oxiranes.

The kinetic resolution of a range of methyl-substituted 1-oxaspiro[2,5]octanes was investigated by Carel *et al.* in 2005 usinga yeast EH from *Rhodoturola glutinis*(Carel et al.,2005). It was observed that the placement of substituents close to the spiroepoxide carbon atom resulted in a decreased reaction rate but increasedenantioselectivity. The best enantioselectivity with an *E*-value>100 was obtained for O-axial or O-equatorial 4-methyl-1-oxaspiro[2,5]octane (**Figure 21**).

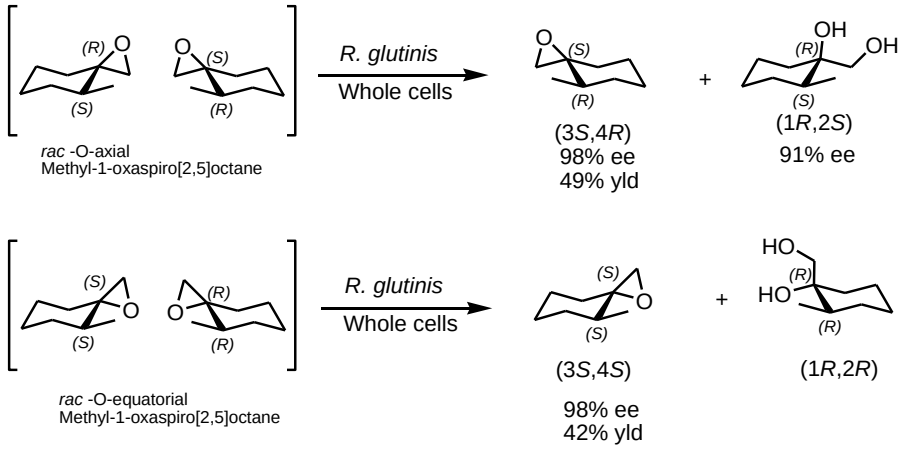


Figure 21. Kinetic resolution of O-axial or O-equatorial 4-methyl-1-oxaspiro[2,5]octane by *Rhodotorula glutinis* EH.

The first deracemisation of a *trans*-divinyl spiroepoxide, a strategic key building block of 11-heterosteroids, was described in 2007 using two enantiocomplementary microbial EHsas biocatalysts (Bottalla et al., 2007). Oneenzyme was the partially purified recombinant EH from *A niger* LCP 521 (*An*EH), the other enzyme, the so-called"Limonene EH" (LEH), was isolated from the bacterium *Rhodococcus erythropolis*. The residual (*R*,*R*)-spiroepoxide (from the *An*EH-mediated reaction) and the residual (*S*,*S*)-spiroepoxide (using LEH) were isolated in nearly enantiopure forms (99% *ee*). However, because of the moderate *E*-values ofaround 20in these two kinetic resolutions, the respective reaction yields did not exceed 26%. A process-improving strategy enabled transformationof the formed enantiomerically enriched diols of opposite absolute configuration, i.e. (*S*,*S*)-spirodiol and (*R*,*R*)-spirodiol, back to the corresponding epoxides, which werethen submitted to a second enzymatic resolution cycle using the enantiocomplementary enzyme (i.e. the LEH for (*S*,*S*)-spiroepoxide or the *An*EH for (*R*,*R*)-spiroepoxide). In conclusion, both enantiomers of the substrate could be obtained in high enantiomeric purity (99%) and reasonable yield (50%) (**Figure 22**).

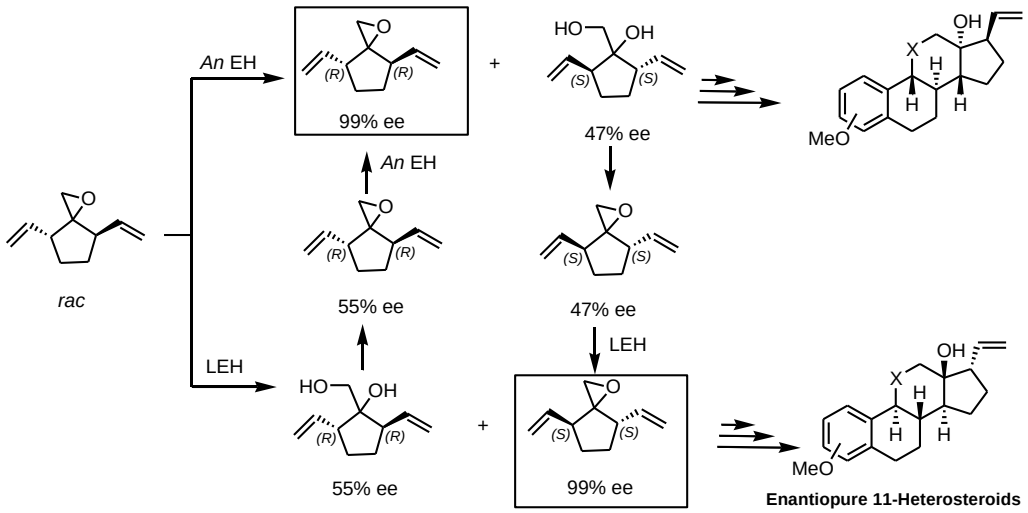


Figure22. Preparative hydrolytic resolution of*trans*-divinylspiroepoxide using *An*EH and LEH as biocatalysts allowing the synthesis of enantiopure 11-heterosteroids.

5.3.3.2*cis*- and *trans*-disubstituted epoxides

2,3-disubstituted aliphatic oxiranes have been reported to be hydrolyzed by EHs from fungi(Moussou et al., 1998a), yeast and bacteria. The most interesting results were observed withyeast and bacterial EHs.As far as kinetic resolution is concerned, it was shown by Weijers (Weijers, 1997) that*Rhodotorulaglutinis*catalyzed the enantioselective hydrolysis of *cis*-2,3- and *trans*-2,3-epoxypentane resulting in residual (2*R*)-epoxides with yields that approached the theoretical maximum of 50%. More interestingly, biocatalytic transformations of racemic 2,3-disubstituted oxiranes to vicinal diols with high *ee* at total conversion were obtained using bacterial EHs. Satisfactory results were described for the first time by Faber's group after a screening of 18 strains. Lyophilized biomass of *Nocardia* EH1 proved to be the best biocatalyst, leading to almost deracemisation of racemic *cis*-2,3-epoxy-heptane at a concentration of 16 g/L, thus producing the corresponding (*R*,*R*)-diol in 79% overall yield and 91% *ee* in gram-scale amounts (**Figure23**)(Kroutil et al., 1997a). It was shown by ^{18}O-labelling that the hydrolysis of both enantiomers occured with opposite regioselectivity via attack at the (*S*)-configured oxirane carbon atom with concomitant inversion of the configuration for both enantiomers. It is worth mentioning that, although the *trans*-isomers were easily hydrolyzed by all the tested strains, no enantioconvergent hydrolysis occurred. Based on these results, the same group developed several preparative syntheses of natural products such as antitumor agents (two stereoisomers of Panaxytriol), (+)-Pestalotin, and a constituent of Jamaican rum (see chapter 7).

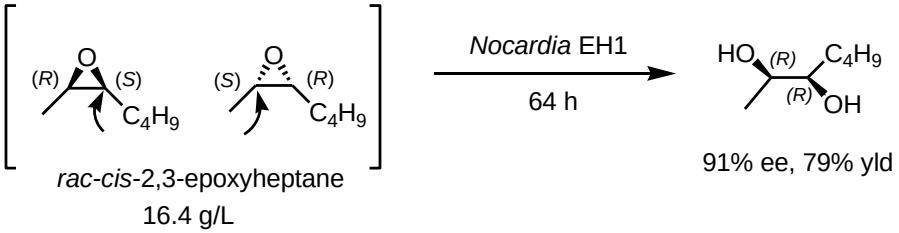


Figure 23. Deracemization of *rac-cis*-2,3-epoxyheptane via enantioconvergent biohydrolysis using *Nocardia* EH1 leading to the formation of an (*R*,*R*)-diol.

Recently, Reetz and co-workers reported on a laboratory evolution experiment with the *A. niger* LCP 521 EH. Several EH variants were isolated that accepted *trans*-2-benzyl-3-methyloxirane as a substrate, in contrasttothe wild-type EH, which was found to be inactive. Two variants exhibited high enantioselectivity (*E*>200) towards this epoxide (Reetz et al., 2008). Later, using the same substrate, enantioconvergence was detected with one of these selected mutants, *i.e.* enantiopure (2*R*,3*S*)-diol at a high conversion ratio of 92% was formed. Directed evolution with this mutant identified second-generation mutants showing higher reaction rates while maintaining the enantioconvergence(Reetz et al., 2010).

5.3.4 Tri-substituted epoxides

One of the first examples of biohydrolytic kinetic resolution of tri-substituted epoxides was the synthesis of both enantiomers of the Bower's compound, which is a potent analogue of the insect juvenile hormone (Archelas et al., 1993). The enantioselective biohydrolysis of the racemic Bower's compound using whole cell of the fungus *Aspergillus niger* LCP521 as a biocatalyst led to the corresponding (6*S*)-diol in 48% yield and 70% ee (**Figure 24**). The remaining (6*S*)-epoxide (96% ee) was isolated in 36% yield and starting from this pure enantiomer, its antipode was easily prepared by chemical means in two steps. Interestingly, it was shown that the (6*R*)-enantiomer was 10 times more active against the yellow meal worm *Tenebrio molitor* than its antipode.

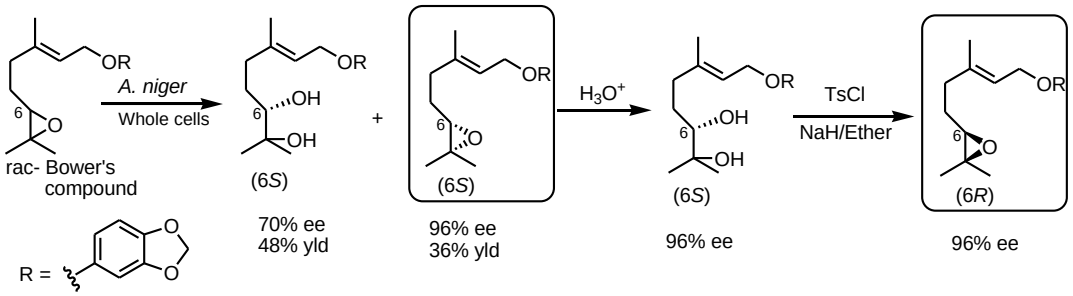


Figure 24. Synthesis of both enantiomers of Bower's compound using whole cells of the fungus *A. niger LCP521* as biocatalyst.

In 1996 Archer *et al.* reported ahighly enantioselective chemoenzymic resolution of *rac*-1-methyl-1,2-epoxycyclohexane using whole cells of *Corynebacterium* C12, giving rise to the (1*R*,2*S*)-epoxide in >99% ee (30% yield) and the (1*S*,2*S*)-diol in 92% ee (42% yield)(Archer et al., 1996). Further, an additional efficient chemoenzymatic deracemization process was run in tandem using the EH from*Corynebacterium* C12 and perchloric acid for the acid-catalyzed ring opening of the residual epoxide.(**Figure25**).

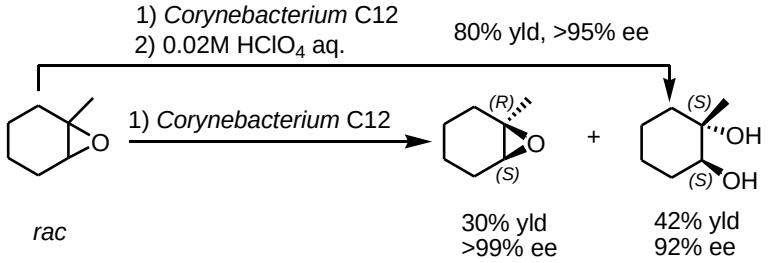
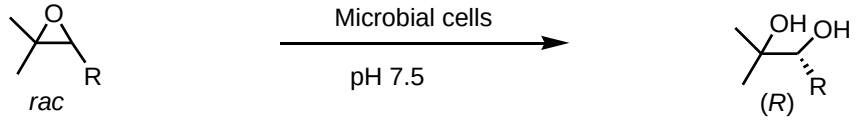


Figure25.Chemoenzymic resolution and deracemisation of *rac*-1-methyl-1,2-epoxycyclohexane using whole cells of *Corynebacterium* C12.

Although enantioconvergent biohydrolysis of trisubstituted epoxides seemed rather unlikely due to their steric bulkyness, Faber's group succeeded in finding several bacterial strains that could perform such a reaction. The biohydrolysis of three racemictrialkyl epoxides (5−6 g/L), using lyophilized cells of *Rhodococcus* and *Mycobacterium*sp., resulted in the formation of the corresponding (*R*)-diols with enantiopurities exceeding 80% *ee* (**Figure26**)(Steinreiber et al., 2001d). The same group used the EH from *Rhodococcus ruber* for the preparative biohydrolysis of two trisubstituted epoxides bearing an olefinic side chain with one or two double bonds. The formed corresponding diolsenabled them to prepare natural compounds such as (*R*)-Myrcenediol and a beer-aroma constituent (see chapter 7).



R = n-C_4H_9	*M. paraffinicum* NCIMB 10420	89% ee; 76% yld
R = n-C_5H_{11}	*R. ruber* SM 1789	85% ee; 85% yld
R = n-C_6H_{13}	*M. paraffinicum* NCIMB 10420	83% ee; 90% yld

Figure26. Deracemization of three *rac*-trialkyl oxiranes via enantioconvergent biohydrolysis using whole cells of *Mycobacterium paraffinicum* and *Rhodococcus ruber*.

5.4*meso*-epoxides

Interestingly, desymmetrization of *meso*-epoxides can produce optically enriched vicinal (*R*,*R*)-diols or (*S*,*S*)-diols in 100% theoretical yield through a stereoselective attack at only one carbon atom of the oxirane. It is worth mentioning that the enantiopurity of the formed diol does not change with reaction time or conversion ratio.Desymmetrization of *meso*-epoxides obtained by classical microbial cultivation techniques are scarce. For example, EHs from the yeast *R. glutinis*ATCC 201718 (Weijers, 1997)and from the bacterium*Sphingomonas* sp. HXN-200 (Chang et al., 2003b)have been used to stereoselectively hydrolyse cyclohexene oxide to the corresponding (*R*,*R*)-diols with enantiomeric excesses of 90% and 87% respectively.It should be also mentioned that Botes and co-workers described in a patent that they found various yeast strains that were able to produce optically active vicinal diols from *meso*-epoxides (Botes et al., 2007b). Recombinant *Yarrowia lipolytica* cells expressing exogenous yeast EHs enabled them to obtain (*R*,*R*)-vicinal diols in high enantiomeric excess starting from *meso*-epoxides such as *cis*-epoxybutane and cyclopentene oxide.

An interesting desymmetrisation of a *meso*-bis-epoxide was recently described by Faber and co-workers(Ueberbacher et al., 2009). EHs from various sources (bacteria, fungi and plants) were found to catalyze the transformation of 6,7:9,10-bis(epoxy)pentadecane by hydrolysis/cyclisation cascades leading to different tetrahydrofuran derivatives with excellent *de* and *ee* values (**Figure 27**). The reaction pathway was initiated by epoxide-hydrolase-catalyzed hydrolysis of an oxirane moiety followed by spontaneous ring-closure of the epoxy-diol intermediate. Based on these results, the authors suggested that the formation of tetrahydrofuran moieties found in numerous natural products such as acetogenins proceeds through a nucleophilic cascade mechanism starting from bis-epoxide without involvement of a "cyclase".

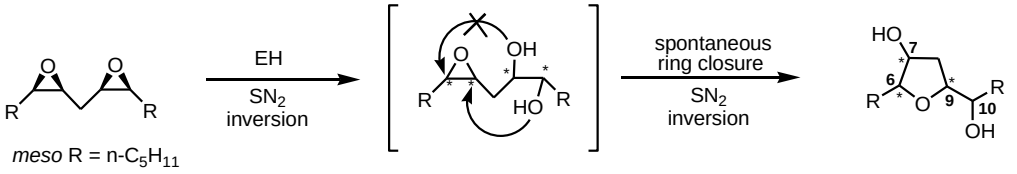


Figure27. Stereochemical courses of epoxide-hydrolase-catalyzed hydrolysis/cyclisation cascades of 6,7:9,10-bis(epoxy)pentadecane to yield tetrahydrofurane derivatives.

Three other strategies were used for the discoveryof EHs capable of desymmetrization of*meso*-epoxides. The first was based on high-throughput screening of DNA libraries which were generated from environmental samples. Using this approach, 50 novel microbial EHshave been discovered and among these11were able to desymmetrize not only cyclic *meso*-epoxides (cyclopentyl and cyclohexyl epoxides) but also bulky internal epoxides such as *cis*-stilbene oxide with various substituents including dipyridyl analogues (**Figure 28**) (Zhao et al. 2004). The second strategy comprised a screening of genomic databases for the presence of EH-encoding genes(van Loo et al., 2006). Five recombinant EHswere found to be active toward *meso*-epoxides such as cyclohexene oxide and *cis*-2,3-epoxybutane.Finally, Reetz and co-workers applied an iterative saturation mutagenesis strategy to the limonene EH (LEH) from *Rhodococcus erythropolis* DCL 14 and obtained three LEH variants (H150, H173, H178) that catalyzed the desymmetrization of cyclic *meso*-epoxides and *cis*-1,2-homo-disubstituted *meso*-epoxides with stereoselective formation of either the (*R*,*R*)- or the (*S*,*S*)-diolon an optional basis (Zheng and Reetz, 2010).

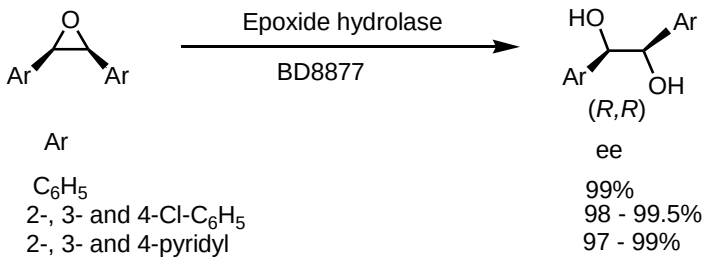


Figure 28. Desymmetrization of aryl *meso*-epoxides with BD887 obtained from DNA libraries of environmental samples.

Very recently, Li and coworkers described the gram-scale preparative desymmetrization of cyclohexene oxide,cyclopentene oxide and N-benzyloxycarbonyl-3,4-epoxypyrrolidine using the EH from *Sphingomonas* sp.HXN-200 expressed in *E. coli*(*E. coli* (SpEH))(Wu et al., 2013). Desymmetrization of 10 g of cyclohexene oxide(500 mM substrate concentration) with resting cells of *E. coli* (SpEH)(10g cdw/L) afforded 10.3 g (89% isolated yield) of (1*R*,2*R*)-1,2-cyclohexanediol in 86% ee. Desymmetrization of the two other *meso*-epoxides (200 mM substrate concentration) afforded (1*R*,2*R*)-1,2-cyclopentanediol in 87% ee and 70.4% isolated yield and (3*R*,4*R*)-N-benzyloxycarbonyl-3,4-dihydroxypyrrolidine in 93% ee and 94.1% isolated yield, respectively.

6 Application to the preparation of valuable chiral building blocks for the synthesis of biologically active compounds starting from bi-functional epoxides

6.1 Halogenated-epoxide

6.1.1Alkyl Chloro-epoxide

6.1.1.1 Epichlorohydrine

Bi-functional small molecules are particularly useful synthons giving access to various valuable biologically active products. A typical example is epichlorohydrin, which has been used (Kasai et al., 1998) for the synthesis of -among others- the nutritional supplement L-Carnitine, the β-adrenergic blocking agent (*S*)-atenolol, (+)-Trehazolin, which is a potent inhibitor of glycosidases and (*S*)-Ipsenol, a beetle pheromone. Both (*S*)- and (*R*)-epichlorohydrin have been obtained optically or nearly optically pure through the kinetic resolution of the racemate at high to very high substrate concentrations using the purified EH from *Agrobacterium radiobacter* overexpressed in *E. coli*(Jin et al., 2013b) (**Figure 29**) or the purified EH from *Agromyces mediolanus*(Xue et al., 2014) (**Figure 30**). In the former case the highest concentrations of racemic epichlorohydrin call for a biphasic system with 40 % cyclohexane.

Figure 29. Kinetic resolution of racemic epichlorohydrin using purified EH from *Agrobacterium radiobacter* leading to the formation of enantiomerically pure (*R*)-epichlorohydrin.

Figure 30. Kinetic resolution of racemic epichlorohydrin using the purified EH from *Agromyces mediolanus* leading to the formation of enantiomerically pure *(S)*-epichlorohydrin.

6.1.1.2 Aromatic chloro-epoxide

Furstoss and collaboratorsshowed that the partially purified recombinant EH from *A niger* LCP 521 can efficiently catalyzed the kinetic resolution of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane at a very high substrate concentration of 500 g/L in a biphasic process) (Montfort et al., 2004a). The unreacted (*S*)-chloro-epoxide and the formed (*R*)-chloro-diol could be obtained in nearly enantiopure form and nearly quantitative

yield. Due to the fact that the formed (*R*)-chloro-diol can be easily chemically transformed into the (*S*)-chloro-epoxide, an enantioconvergent process could thus be set up. Using the difference in chemical reactivity between the oxirane ring and the chlorine substituent, it was possible to use this enantiopure chloro-epoxide as a building block for the synthesis of D0870, a triazole drug derivative known to display efficient activity against human fungal infections (**Figure 31**). It was also shown (Montfort et al., 2004b) that this enantioselective hydrolysis can be performed with the corresponding epoxy-triazole compound at 50 g/L.

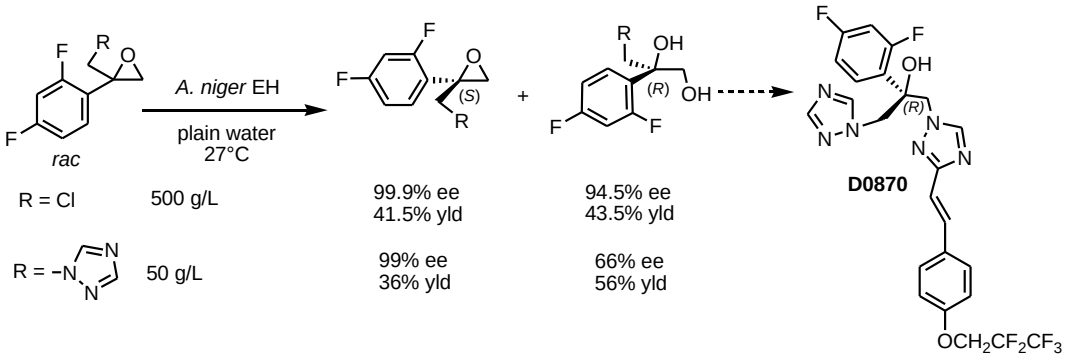


Figure 31. Preparative hydrolytic resolution of *rac*-1-chloro- and 1- triazole-2(2,4-difluorophenyl)-2,3-epoxypropane using *An*EH as biocatalyst. The latter compound is a useful building block for the synthesis of enantiopure D0870,

6.1.2 Bromo-epoxide

Acompany (Oxyrane Ltd.) has established *Yarrowia lipolytica* as a heterologous host for EH overexpression (Pienaar et al., 2008). The origin of some EHs used various biotransformation reactions was released in different patents (Botes et al., 2005; Botes et al., 2007a; Botes et al., 2008). The following biotransformation was performed with the *Y. lipolytica* Oxy-9 strain overexpressing an EH whose origin was not released. Thus, racemic 4-bromo-1,2-epoxybutane (60 g) was kinetically resolved with five grams of wet cells in 40 mL of 0.1 M phosphate buffer pH 7.5 (Pienaar et al., 2008). The reaction was stopped after 90 minutes and after extraction and purification, (*S*)-4-bromo-1,2-epoxybutane in 26% yield and 98.6% *ee* was obtained (**Figure 32**). The residual epoxide was then easily transformed to a hydroxy pyrrolidine derivative which can be used as a chiral synthon for the synthesis of the calcium antagonist Barnidipine (Li et al., 2001).

Figure 32. Kinetic resolution of racemic 4-bromo-1,2-epoxybutane using purified Oxy-9 EH and its use in the synthesis of (*S*)-N-benzyl-3-hydroxypyrrolidine.

6.2Epoxyamide

Using another EH overexpressed in *Y. lipolytica* (Oxy-10), the kinetic resolution of an epoxyamide was performed at 50% conversion, resulting in the optically pure residual (*R*,*R*)-epoxide and the formed *threo*-diol with yields of 32 and 22%, respectively (Pienaar et al., 2008) (**Figure 33**). The remaining (*R*,*R*)-epoxide is of synthetic interest, because it is used for the synthesis of the α-mannosidase inhibitor (–)-Swainsonine.

Figure 33. Kinetic resolution of racemic epoxyamide using purified Oxy-10 EH and application to the synthesis of (*S*)-N-benzyl-3-hydroxypyrrolidine en route to (–)-Swainsonine. (i) See Calvez et al., 1998; (ii) See Haddad et al., 2001 and Ferreira et al., 1997.

6.3Protected Epoxy-alcohols

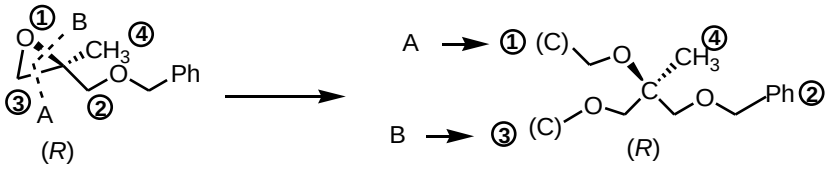
With yet another overexpressed EH in *Y. lipolytica* (Oxy-3), (*S*)-4-acetoxy-buten-1,2-oxide was obtained in 20% yield and 97.8% ee starting from the racemate. The (*S*)-epoxide is easily converted to (*S*)-3-hydroxytetrahydrofuran (**Figure 34**), a compound used for the synthesis of the HIV protease inhibitors Amprenavir and Fosamprenavir (Honda et al., 2004).

Figure 34. Chemo-enzymatic access to (*S*)-3-hydroxytetrahydrofuran. (i) See Yuasa et al., 1997; 79%

Another protected epoxy-alcohol, *rac*-2-methylglycidyl benzyl ether, has been the subject of numerous investigations. Two types of EH proved to be useful: those from various *Rhodococcus* strains (Steinreiber et al., 2001; Fuchs et al., 2009; Hellstrom et al.,2001; Simeó at al., 2006) and the one from *Bacillus subtilis*(Fujimo et al., 2007; Shimizu et al., 2010). Both types of EHs exhibited the same enantioselectivity, generating the (*R*)-diol and leaving behind the unreacted (*R*)-epoxide (see note below), and have been used for the enantioconvergent access to the highly enantio-enriched (*R*)-diol (**Figure 35**). Enantiopure (*R*)-3-benzyloxy-2-methylpropane-1,2-diol was the starting material for the synthesis of (*R*)-Bicalutamide, a synthetic antiandrogen (Fujino et al., 2007). The same (*R*)-diol was used as the starting material for the bio-assisted synthesis of the intermediate (*R*)-3-hydroxy-3-methyl-5-hexanoic acid *p*-methoxybenzyl ester, which was then used in the synthesis of Taurospongin A (Fujino et al., 2008), a natural product inhibiting DNA polymerase β and HIV reverse transcriptase.

Figure 35. Enantioconvergent chemo-enzymatic access to (*R*)-3-benzyloxy-2-methylpropane-1,2-diol. See note and Figure 36 concerning the correct determination of epoxide absolute configuration.

Note: In several papers it was mentioned that the biohydrolysis led to a diol with an inverted absolute configuration when compared to the configuration of the reacting epoxide, the reason being a switch in the Cahn-Ingold-Prelog (CIP) priority and not an inversion at the stereogenic center. This conclusion is wrong according to the CIP rule applied to chiral centers in a ring (Eliel, 1985; Prelog and Helmchen, 1982). Application of this rule(»*Each cyclic ligand is detached at the distal end, where it doubles back upon the chiral center; the chiral center is placed as a phantom atom (with no further ligands) at the end of the chain so generated*«; seeEliel, 1985) leads to the conclusion that the absolute configurationat the stereogenic centers of the epoxides should be reversed in these publications (Steinreiber et al., 2001; Hellstrom et al.,2001; Simeó at al., 2006; Fujino et al., 2007; Fuchs et al., 2009; Shimizu et al., 2010) (**Figure 36**). In these cases the (*R*)-diol is formed by retention of configuration of the (*R*)-epoxide, the opening of the oxirane ring occurring thus at the terminal carbon atom.



A&B: ligand detachment at the distal end; (C): phantom carbon atom

Figure 36. Application of the CIP-rule for the determination of the absolute configuration of an epoxide.

6.4 Epoxy-ester

As a result of a screening for microorganisms with high EH activities using enrichment cultures with alkenes as the sole carbon source, Choi et al. isolated a bacterial strain, identified as *Acinetobacter baumannii*, that showed high selectivity in the kinetic resolution of ethyl-3,4-epoxybutyrate (Choi et al., 2008). Using wet cells of *Acinetobacter baumannii*, ethyl-3,4-epoxybutyrate was kinetically resolved at a concentration of 60 mM, affording after 2 hours of reaction pure (*R*)-ethyl-3,4-epoxybutyrate (*ee*>99%) in 46% yield and (*S*)-ethyl-3,4-dihydroxybutyrate (*ee* = 80%) in 49% yield (**Figure 37**). The unreacted (*R*)-epoxide is a valuable intermediate that can be used in the synthesis of (*R*)-GABOB, (*R*)-Carnitine, the anti-cancer agent Lobatamide C and the statin Lipitor®.

Figure 37. Kinetic resolution of *rac*-ethyl-3,4-epoxybutyrate by *Acinetobacter baumannii*.

Other bi-functional epoxy-esters such as some *trans*-(+/-)-3-phenyl glycidates have been studied as substrates for EH-mediated reactions (Devi et al., 2008) in order to obtain the useful chiral synthons (2*S*,3*R*)- or (2*S*,3*R*)-3-phenyl glycidate, which can used to get access to the Taxol side chain or Diltiazem, respectively (**Figure 38**).

Figure 38. Kinetic resolution of two *trans*-(+/-)-3-phenyl glycidates by immobilized Mung bean EH.

To perform such kinetic resolution the EH from Mung bean has been immobilized in a gelatin gel and di-isopropyl ether used as an immiscible organic co-solvent to minimize spontaneous hydrolysis of the substrates. In both cases the (2*S*,3*R*)-glycidate ester was obtained in 45% yield (90% of the theoretical value) and very high enantiomeric purity (>99%).

6.5 Epoxy-aldehyde

Glycidyl acetal derivatives, i.e. C3 chiral building blocks bearing one stereogenic centre and two different and chemically differentiable functions (such as protected aldehyde and epoxide) located on a short carbon skeleton, are of particular interest due their high chemical versatility. A methodology allowing for the preparation of five enantiopure glycidyl acetal derivatives was performed by using partially purified recombinant EH from *Aspergillus niger* LCP 521 as a biocatalyst (Doumèche et al., 2006). All the epoxides of (*R*) absolute configuration could be prepared in high enantiomeric excess (ee>99%), whereas the formed diols were of (*S*) absolute configuration and showed moderate to excellent ees (45-97%) (**Figure 39**). The E-values were shown to be modest to excellent, depending on the structure of the acetal moiety. The best results were obtained when the protecting group was a cyclic acetal (E-value = 126) and a diisopropyl acetal (E-value > 200). As a proof-of-principle, a 50-g scale resolution of glycidaldehyde 2,2-dimethyltrimethylene acetal was performed at 200 g/L substrate concentration, leading to 22.8 g of residual (*R*)-epoxide (ee > 98%) and 25.4 g of the formed (*S*)-diol (ee = 92%). It is interesting to notice that this enzymatic transformation was performed as a biphasic process using solely demineralised water as a solvent.

Figure 39. Gram scale and high substrate concentration preparation of enantiopure glycidaldehyde 2,2-dimethyltrimethylene acetal using partially purified recombinant EH from *Aspergillus niger* LCP 521 as biocatalyst.

With the aim to explore the metabolism of fructose, Boltes and co-worker synthesized 4-deoxy-D-fructose 6-phosphate in four steps,which included two enzymatic reactions (Guérard et al., 1999), starting from racemic 1,1-diethoxy-3,4-epoxybutane. In the first step, optically pure (3*S*)-1,1-diethoxy-3,4-epoxybutane was obtained using anEH-catalysed kinetic resolution. After a screening of several fungal strains, the best results were obtained with the *Aspergillus niger* LCP521 EH. Although the enantioselectivity was only moderate (E-value = 15), a large scale resolution with 15 g of substrate at 50 g/L enabled to isolate 4.5 g of the (*S*)-residual epoxide in high enantiomeric excess (98% ee). Opening of the epoxide with inorganic phosphate followed by deprotection of the acetal moiety in acidic conditions led to the formation of enantiopure (*S*)-2-hydroxy 4-oxobutyl 1-phosphate. In the last step, the transketolase-catalysed reaction of this enantiomer with L-erythrulose enabled the

stereochemical control of the second asymmetric center of the formed enantiopure 4-deoxy-D-fructose 6-phosphate. Recycling of the NADH cofactor, necessary to shift the equilibrium of the transketolase-mediated reaction towards the formation of 4-deoxy-D-fructose 6-phosphate by removing the formed glycoaldehyde, was achieved with formate dehydrogenase (**Figure 40**).

Figure 40. Synthesis of enantiopure 4-deoxy-D-fructose 6-phosphate implying two enzymatic steps.

7 Application to natural Product Synthesis

7.1 Disparlure

Severalsyntheses of natural productshave been achieved with EHs as the key steps to introduce chirality. One of the first was the synthesis of the biologically active enantiomer of Disparlure, the sex pheromone of the moth *Lymantria dispar*, which causes severe damage to trees in some parts of the world (Otto et al., 1998). The synthesiswas based on the kinetic resolution, mediated by a *Pseudomonas* strain, of *rac*-9,10-epoxy-15-methyl hexadecanoic acid, a precursor of the pheromone. The residual epoxide was then chemically converted (Kolbe reaction) into (+)-Disparlure in 95% ee (**Figure 41**).

Figure 41. Chemo-enzymatic synthesis of Disparlure.

7.2 Linalool

Enantiopure *trans*- and *cis*-Linalool oxides, which are constituents of several plants and fruits, are among the main aroma components of oolong and black teas. These oxides were prepared from (3*RS*,6*R*)-2,3-epoxylinalyl acetate *via* a chemo-enzymatic route (Mischitz and Faber, 1996). The key step was the separation of the diastereomeric mixture of the starting compound using *Rhodococcus* sp. NCIMB 11216, yielding diol and remaining epoxide in excellent diastereomeric excess (de >98%). It followed chemical transformations, which gave both linalool oxide isomers on a preparative scale in excellent diastereomeric and enantiomeric purities. From a mechanistic point of view, it was shown by means of ^{18}O-labelling that the enzyme-catalyzed reaction proceeded in an enantioconvergent fashion leading to (3*S*,6*R*)-diol at 100% conversion (**Figure 42**).

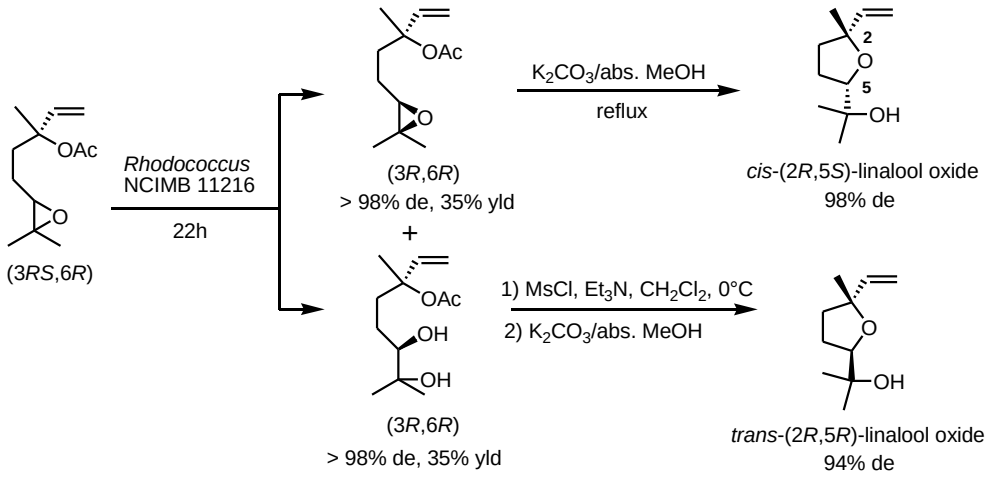


Figure 42. Chemo-enzymatic synthesis of *cis*- and *trans*-Linalool oxide using bacterial EH.

7.3 Bisabolol

Using the fungal strain*A. niger* LCP521, Furstoss and collaborators described the synthesis of the four stereoisomers of bisabolol, one of them, (-)-(4*S*,8*S*)-α-Bisabolol, being of industrial value for the cosmetic industry(Chen et al., 1993). The approach was based on the diastereoselective biohydrolysis of a mixture of 8,9-epoxylimonene diastereomers, obtained from (*S*)- or (*R*)-limonene, implying whole cells of *A. niger* LCP521. The biohydrolysis of the racemic mixture of (4*S*,8*RS*)-epoxides resulted in the unreacted (4*S*,8*S*)-epoxide and the (4*S*,8*R*)-diol (**Figure 43**). The former was chemically converted into (4*S*,8*R*)-épi-α-Bisabolol, and the diol was cyclised to give the corresponding (4*S*,8*R*)-epoxide, which enabled the synthesis of (4*S*,8*S*)-Bisabolol. These two bisabolol stereoisomers were isolated with high enantiomeric excess (99% ee) and diastereomeric excess (94% de).

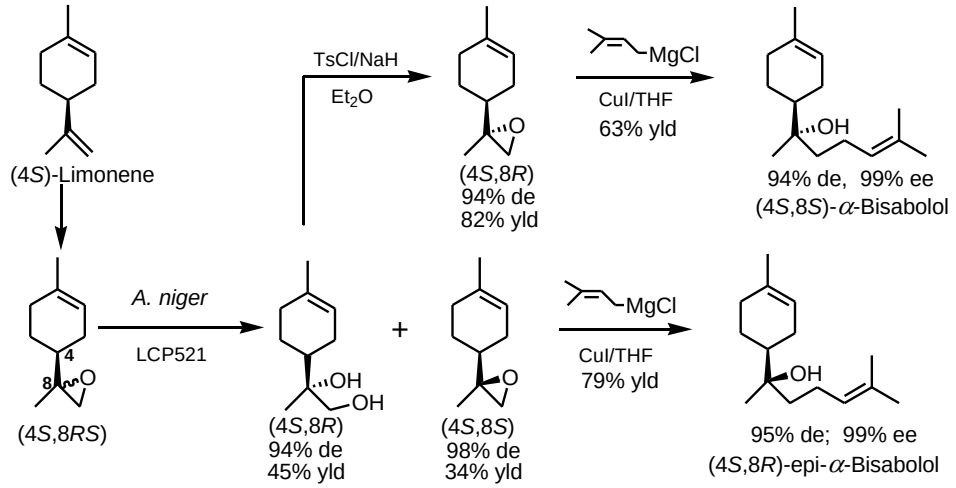


Figure 43. Diastereoselective biohydrolysis of (4*S*,8*RS*)-limonene oxideusing whole cells of the strain*A. niger* LCP521 as biocatalyst, leading to the synthesis of α-Bisabolols.

7.4 Frontalin

In 1997 Faber and co-workers described the synthesis of (*S*)-(–)-Frontalin in 94% ee, an important aggregation pheromone of pine beetles of the *Dentroctonus* family, in five steps (but rather low overall yield) via a chemoenzymatic route which impliedan enzymatic kinetic resolution of an epoxide (Kroutil et al., 1996). In the key step, racemic 2-methyl-2-(pent-4-en-1-yl)oxirane was enzymatically resolved (E-value = 39) using freeze-dried whole cells of *Rhodococcusequi* IFO 3730. The reaction was stopped at 18% conversion enabling the isolation ofthe formed (*S*)-diol in 94% ee. Via Wacker-oxidation and subsequent ketalization, the latter compound was chemically transformed in a one-pot reaction into the pheromone (*S*)-(–)-Frontalin, (**Figure44**). An improvement of the synthesis was reported in a review three years later (Orru et al., 1999). The improvement was based on the fact that racemic 2-methyl-2-(pent-4-en-1-yl)oxirane could be more efficiently resolved with

an E-value >200 using the *Nocardia* EH1 biocatalyst instead of the *Rhodococcus* EH(Orru et al., 1997). A subsequent chemical hydrolysis of the remaining (*R*)-epoxide using sulfuric acid in dioxane with a trace amount of water afforded (*S*)-2-methyl-hept-6-ene-1,2-diol in 97% yield and 99% ee. Therefore, (*S*)-Frontalin was obtained in higher yield and ee-value using the same route.

Figure 44. Key steps for the chemoenzymatic synthesis of (*S*)-Frontalin using whole cells of *Rhodococcus* IFO 3730.

7.5Mevalonolactone

Natural (*R*)-Mevalonolactone, a key intermediate in a broad spectrum of cellular processes and their regulation, was synthesized in eight steps in 55% overall yield and >99% optical purity employing an enantioconvergent chemoenzymatic process (**Figure 45**). In the key step, 10 g of 2-benzyl-2-methyl oxirane were deracemized using lyophilized cells of *Nocardia* EH1 followed by aqueous sulfuric acid treatment in dioxane. In this way the substrate could be resolved with high enantioselectivity (E-value = 123), the subsequent chemical hydrolysis of the remaining (*R*)-epoxide resulted inthe (*S*)-diol in 94% chemical yield and 94% ee, thusestablishing an efficient enantioconvergent process. The limiting step in this total synthesis was the ruthenium tetroxide-based oxidation of the aryl moiety to form the carboxylic acid. Saponification followed by acidic lactonization gave the (*R*)-mevalonolactone in 47% overall yield and 94% ee.

Figure 45. Asymmetric chemo-enzymatic totalsynthesis of (*R*)-Mevanolactone.

7.6 Myrcenediol and Beer Aroma

Enantioconvergent preparative-scale production of (*R*)-Myrcenediol, a plant constituent isolated from the roots of *Bidens graveolens* and from flowers of *Tanacetum annuum*, was accomplished in one step using lyophilized cells of *Rhodococcus ruber* DSM 44540 (Steinreiber et al;, 2001c) and *rac*-6,7-epoxy-7-methyl-3-methylene-1-octene to afford (*R*)-Myrcenediol in 92% yield and 83% ee. (**Figure46**).

Figure46. Synthesis of (*R*)-myrcenediol *via* an enantioconvergent biohydrolysis using *Rhodococcus ruber* DSM 44540 EH as biocatalyst.

Chemo-enzymatic asymmetric total synthesis of (*S*)-7,7-dimethyl -6,8-dioxabicyclo[3.2.1]octane, which is a volatile contributor to the aroma of beer, was also accomplished using lyophilized cells of *Rhodococcus ruber* DSM 44540 with an enantioconvergent-enzymatic step. Inversion of the stereogenic centre of the (*R*)-2-methyl-7-octene-2,3-diol obtained from the biohydrolysis of the corresponding racemic epoxide was achieved via an epoxide-closing-reopening sequence, which proceeded with inversion and retention of absolute configuration. Oxidation of the (*S*)-diol by ozonolysis, followed by acid-catalyzed ring-closure in a one pot reaction gave finally (*S*)-7,7-dimethyl -6,8-dioxabicyclo[3.2.1]octane in 77% yield and 94% ee (**Figure47**).

Figure47. Enantioconvergent biohydrolytic access to a volatile contributor of beer aroma.

7.7 Pityol

Using lyophilized cells of *Rhodococcus ruber* DSM 44540, Faber and co-workers described the synthesis of two enantiopure diastereoisomers of the bark beetle pheromone Pityol (Steinreiber et al., 2001a). Their approach was based on a diastereoconvergent biohydrolysis of a mixture of (3*RS*,6*S*)- or (3*RS*,6*R*)-6-bromo-2-methyl-2-heptene oxide diastereomers, obtained respectively from (*R*)- and (*S*)-Sulcatol after bromination and epoxidation of the double bond. As exemplified below the (3*RS*,6*R*)-6-bromo-2-methyl-2-heptene oxide mixture of diastereomers afforded upon EH catalysis the corresponding (3*R*,6*S*)-bromo-diol formed as ~~a~~ the sole intermediate. Due to the presence of a bromine atom in the molecule this intermediate underwent spontaneous ring-closure affording (2*R*,5*R*)-Pityol in 54% yield and 98% ee after separation of the formed minor (2*S*,5*R*) diastereomer (12% yield) (**Figure 48**). Both enantiomers of Sulcatol were themselves prepared in enantiopure form via *Candida antarctica* lipase B-catalyzed kinetic resolution *via* an acyl-transfer or ester hydrolysis.

Figure48. Synthesis of the pheromone (2*R*,5*R*)-Pityol via a diastereoconvergent biohydrolysis using *Rhodococcus ruber* DSM 44540 EH as biocatalyst.

7.8 Pestalotine- Jamaican rum constituent

In 2002, Faber and co-workers described a chemoenzymatic route to two natural products(a constituent of Jamaican rum and the second the (+)-antipode of the Gibberellin synergist (-)-Pestalotin) starting from*rac-cis*-1-chloro-2,3-epoxyheptane (Mayer et al., 2002a).The strategy was based on an enzyme-triggered cascade-reaction. First, an enantioconvergent biocatalytic hydrolysis of *rac-cis*-1-chloro-2,3-epoxyheptane, using lyophilized cells of *Mycobacterium paraffinicum* NCIMB 10420, afforded the corresponding *threo*-(2*S*,3*R*)-diol as an intermediate. Due to the presence of a chlorine atom in the molecule, this intermediate underwent spontaneous ring-closure to yield (2*R*,3*R*)-1,2-epoxy-3-heptanol in 81% yield, 99% de and 93% ee as the sole product. That almost enantiopure epoxy-alcohol was used as a building block for the first total synthesis of (+)-exo-7-butyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane, a constituent of Jamaican rum as well as for the synthesis of (+)-Pestalotin (**Figure49**).

Figure49.Enantioconvergent enzyme-triggered cascade reaction of *rac-cis*-1-chloro-2,3-epoxyheptane as a key step for the total synthesis of a (+)-Jamaican rum constituent and (+)-Pestalotin.

7.9 Panaxtriol

A total asymmetric synthesis of two enantiopure diastereoismers components of *Panax ginseng* showing antitumor activity was accomplished by connecting two chiral building blocks obtained via two distinct enantioconvergent enzymatic processes implying epoxide hydrolase and lipase as biocatalysts (Mayer et al., 2002b). The first building block was obtained *via* an enantioconvergent biocatalytic hydrolysis of *rac-cis*-1-chloro-2,3-epoxydecane, using lyophilized cells of *Streptomyces sp.* FCC008 as biocatalyst. The corresponding *threo*-(2*S*,3*R*)-diol was formed as an intermediate that underwent acidic spontaneous ring-closure to yield (2*R*,3*R*)-1,2-epoxy-3-decanol in 92% yield and 95% ee as the sole product. For the second building block *Candida antartica lipase* B-catalyzed kinetic resolution *via* acyl-transfer or ester hydrolysis was used to obtain separately both enantiomers of hept-1-ene-4,6-diyn-3-ol. In addition, both kinetic resolutions were linked to an *in situ* inversion to avoid the occurrence of the undesired enantiomer. Thus (*R*)- and (*S*)-hept-1-ene-4,6-diyn-3-ol were obtained in >98% ee from the racemate in 82% and 80% yield respectively. The last steps of the synthesis of (3*R*,9*R*,10*R*)- and (3*S*,9*R*,10*R*)-Panaxytriol was the protection of the free hydroxyl of the first building blockleading to (2*R*,3*R*)-*tert*-butyldimethylsilyloxy-1,2-epoxy-3-decane and its chemical coupling with (*S*)- or (*R*)-hept-1-ene-4,6-diyn-3-ol (**Figure50**).

Figure50. Synthesis of (3*R*,9*R*,10*R*)- and (3*S*,9*R*,10*R*)-Panaxytriol *via* two distinct enantioconvergent enzymatic processes implying EH from *Streptomyces* sp. FCC008 and *Candida antartica* lipase B as biocatalysts.

7.10 Fridamycin E

A total synthesis of the antibiotic (*R*)-Fridamycin E was accomplished using as a key chiral building block the (*S*)-diol product obtained by EH-catalyzed kinetic resolution of 2-methyl-2-(oct-2-yn-1-yl)oxiranefollowed by stereo-inversion of the remaining (*R*)-epoxide by subsequent chemical hydrolysis (**Figure51**). The biocatalytic kinetic resolution(E-value = 66) carried out using lyophilized cells of *Methylobacterium* sp. FCC

031 followed by chemical hydrolysis using sulfuric acid in dioxane containing a trace amount of water provided the desired building block in 84% ee and 82% overall yield from the racemate (Ueberbacher et al., 2005).

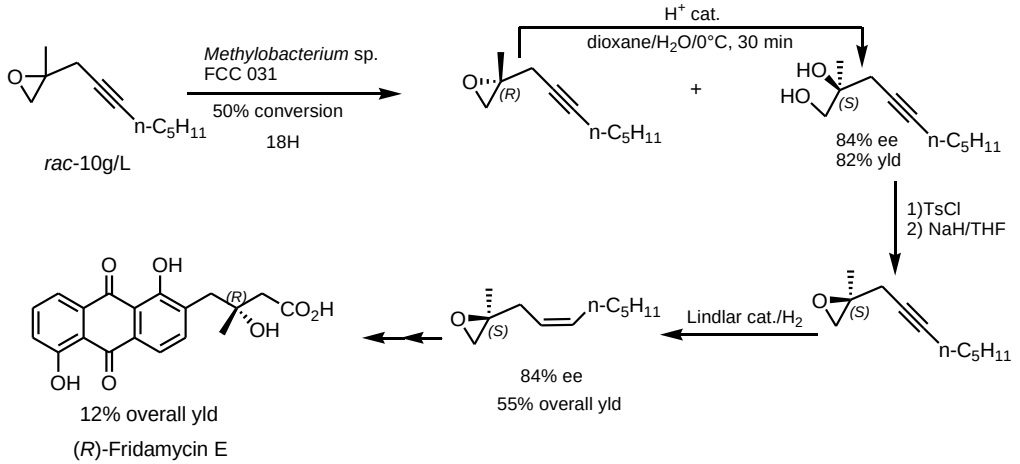


Figure51. Asymmetric total synthesis of (*R*)-Fridamycin E.

8 Bienzymatic process implying one epoxide hydrolase

Enzymes work within biosynthetic or biodegradation pathways, the product of one enzyme being the substrate of the following. It is thus tempting to use two or more enzymes in a biomimetic fashion for multistep transformations of specific compounds; this is especially true for one-pot transformations, thereby simplifying the experimental procedures. Only a few examples of multi-enzyme processes involving EHs have been described to date. In one such an example, *Sphingomonas* sp. HXN-200 was used to conduct the enantioselective *trans*-specific dihydroxylation of N-substituted 1,2,5,6-tetrahydropyridines (**Figure 52**) owing to both monooxygenase and EH activities found in this strain (Chang et al., 2003a). The formed *trans*-diol was obtained with excellent *ee* and in good yield, suggesting that either the EH was enantioconvergent for the intermediate epoxide or that the monooxygenase exhibited a high degree of selectivity with the EH being highly regioselective.

Figure 52. *Trans*-specific dihydroxylation of N-substituted 1,2,5,6-tetrahydropyridines using *Sphingomonas* sp. HXN-200 exhibiting monooxygenase and EH activities.

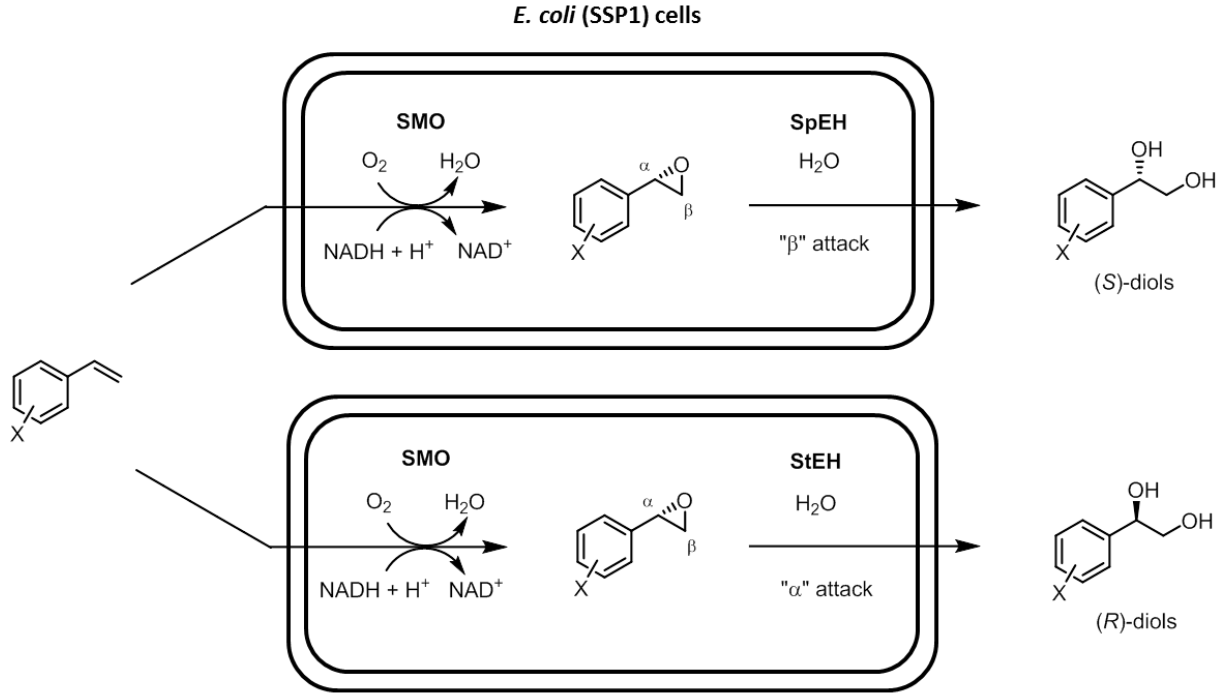
A few years later (Xu et al., 2009), a biphasic reaction was established using a recombinant *E. coli* strain exhibiting styrene monooxygenase activity in conjunction with an EH preparation from *Sphingomonas* sp. HXN-200 to generate highly enantiomerically enriched diols (*ee*>99%) from various styrene derivatives (**Figure 53**)

Figure 53. *Trans*-specific dihydroxylation of styrene using *E. coli* JM101 (pSPZ10) with styrene monooxygenase activity and the EH from *Sphingomonas* sp. HXN-200 in a biphasic system.

In this case, the monooxygenase was particularly stereoselective, leading to the formation of (*S*)-styrene oxide in very high *ee* (>99%). The EH from *Sphingomonas* sp. attacked as a highly regioselective biocatalyst the less hindered carbon atom of the epoxide functionality, resulting in the formation of the corresponding (*S*)-diol.

In a continuous effort to develop a widely applicable biocatalytic system that would permit both *S*- and *R*-specific dihydroxylation of mono-substituted aryl olefins as well as *cis*- and *trans*-specific dihydroxylation of α,β-disubstituted aryl olefins through cascade reactions, Li's group (Wu et al., 2014) recently reported the engineering of a *E. coli* strain expressing both styrene monooxygenase and either one of the two regio-complementary EHs from *Sphingomonas* sp. HXN-200 or *Solanum tuberosum*. The two *E. coli* strains, which were named SSP1 and SST1, were first tested as resting cells for the biotransformation of 15 variously substituted styrene derivatives as well as styrene itself (**Table 5**). The reaction was conducted at 20 mM substrate concentration in a biphasic (1:1) phosphate buffer/*n*-hexadecane reaction medium with 10 g cdw/L of either SSP1 or SST1 biomass for 8 hours.

Table 5. Enantioselective *S*- and *R*-specific dihydroxylation of styrene oxide and some of its mono-substituted derivatives (adapted from Wu *et al.*, 2014).



Cells	Anal. yield %	Diol abs. conf./ ee %	Anal. yield %	Diol abs. conf./ ee %	Anal. yield %	Diol abs. conf./ ee %	Anal. yield %	Diol abs. conf./ ee %
	X=H		**X = *p*-F**		**X = *p*-Cl**		**X = *p*-Br**	
SSP1	92	(*S*) /98.1	88	(*S*) /97.9	67	(*S*) /97.8	34	(*S*) /97.7
SST1	93	(*R*) /95.5	90	(*R*) /95.2	89	(*R*) /95.6	86	(*R*) /94.4
	X = *p*-CH_3		**X = *p*-OCH_3**		**X = *p*-CF_3**		**X = *m*-F**	
SSP1	86	(*S*) / 93.9	67	(*S*) /83.2	25	(*S*) /97.5	>99	(*S*) /98.4
SST1	85	(*R*) /87.7	65	(*R*) /85.4	19	(*R*) /87.7	>99	(*R*) /94.2
	X = *m*-Cl		**X = *m*-Br**		**X = *m*-CH_3**		**X = *m*-OCH_3**	
SSP1	95	(*S*) /97.5	67	(*S*) /97.5	91	(*S*) /93.1	96	(*S*) /97.6
SST1	95	(*R*) /95.8	86	(*R*) /84.2	92	(*R*) /98.2	>99	(*R*) /87.3
	X = *m*-CF_3		**X = *o*-F**		**X = *o*-Cl**		**X = *o*-CH_3**	
SSP1	46	(*S*) /97.6	94	(*S*) /98.6	34	(*S*) /92.2	34	(*S*) /65.7
SST1	13	(*S*) /74.0	89	(*R*) /68.1	10	(*R*) /36.9	15	(*R*) /89.9

The analytical yields were mostly excellent with *ee*s of the formed diols reaching values of up to 98.6%. The lowest yields and/or the lowest *ee*s were obtained for styrene derivatives bearing electron-withdrawing substituents or substituents in *ortho*-positions. The use of either SSP1 or SST1 cells enabled the preferential formation of the (*S*)- and (*R*)-diol enantiomer, respectively. Using the same experimental conditions as described

above, the substrate range was then extended to some di- and tri-substituted or cyclic aryl olefins. Once again, high yields and high selectivities were generally determined, however with some exceptions (**Figure 54**).

Figure 54. Enantioselective *cis*-, *trans*-, *R*- or *S*-specific dihydroxylation of some di-, tri- or cyclic-aryl olefins.

In order to demonstrate the usefulness of the developed process as a tool in synthetic chemistry, the substrate concentration and the biomass concentration were increased to 50 mM and 20 g cdw/L, respectively, and the performance of the biotransformation set up was tested with some of the best substrates using either the SSP1 or SST1 cells. Very good isolated yields as well as high selectivities were obtained enabling the isolation of about 300 mg of each diol. Finally, the dihydroxylation of styrene was conducted with growing SST1 cells in a bioreactor. In this case, 120 mM (16.6 g/L) of (*R*)-1-phenyl-1,2-ethanediol were obtained in 96.2% *ee* with a volumetric productivity of 3.3 g/L/h, styrene being added continuously over a period of 5 h.
The same group also developed a one-pot bi-enzymatic sequential process to access *trans*-diols from cyclic olefins using a lipase-mediated epoxidation of olefins in the presence of H_2O_2 and lauric acid in conjunction with the EH-catalyzed hydrolysis of the formed epoxide. The method was developed with cyclohexene and *N*-benzyloxycarbonyl 3-pyrroline as substrates. The first chemo-enzymatic reaction, *i.e.* the lipase-mediated epoxidation, was conducted in acetonitrile, which was compatible with the next biocatalyst suspended in phosphate buffer, *i.e.* whole cells from *Sphingomonas* sp. HXN-200 bearing EH activity. The initial substrate concentration was 0.5 M, that of H_2O_2 was 1.25 M and that of lauric acid either 0.25 M with cyclohexene as substrate or 1.25 M with the pyrroline derivative as substrate. 25 or 35 g/L of Novozyme 435 lipase and 24 or 15 g/L of *Sphingomonas* sp. HXN-200 cells were used to obtain after 72 h 9.5 mM of 1*R*,2*R*-*trans*-cyclohexane diol (84% *ee*) or after 168 h 9.4 mM of 3*R*,4*R*-*trans*-N-benzyloxycarbonyl 3-pyrroline diol (93% *ee*), respectively (**Figure 55**).

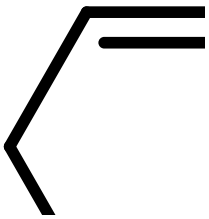


Figure 55. Asymmetric *trans*-dihydroxylation of cyclohexene and *N*-benzyloxycarbonyl 3-pyrroline.

Another example of an EH-based bi-enzymatic transformation, which involved an a haloalcohol dehalogenase was recently described with 1,3-dichloro-2-propanol as the substrate (Jin et al., 2013a). The two enzymes were expressed in two different *E. coli* strains. The cells were then immobilized onto perlite and placed in two distinct bubble reactor columns, the final reaction medium containing 40% cyclohexane. The first column exhibiting haloalcohol dehalogenase activity was fed with 10 mM 1,3-dichloro-2-propanol and after 150 min the reaction reached an equilibrium generating racemic epichlorohydrin (7.3 mM, 73% yield). The second column with EH activity was then filled with the reaction medium of the first column (without the cells), and after 30 minutes, 2.51 mM of essentially enantiopure (*R*)-epichlorohydrin (*ee* 99%, 25% yield) were obtained (**Figure 56**).

Figure 56. Access to (*R*)-epichlorohydrin using two successive biotransformation reactions in a bi-enzymatic system involving haloalcohol dehalogenase and EH.

The EH enabled the kinetic resolution of the racemic epichlorohydrin which was formed via the enzymatic dehalogenation of the starting compound 1,3-dichloro-2-propanol.
From those examples it becomes apparent that enzymes which are involved in the synthesis of epoxides are particularly well suited to be used in conjunction with EHs for the generation of optically active diols or epoxides. As these compounds are highly attractive chiral synthons, one can expect that in the future more and more bi- (or tri-)-enzymatic processes will be developed with EHs as the key components.

9 Conclusions

For more than twenty years now EHs have proved to be outstanding biocatalysts in a large number of applications in the field of fine chemistry. This success is largely due to the fact that EHs are robust enzymes which do not need any cofactors for catalysis. Furthermore, although these enzymes catalyse a hydrolytic reaction, they are tolerant to both water-miscible and water-immiscible organic solvents. From a practical point of view, this allows to increase the used substrate concentration and consequently, in a lot of cases, it enables the

alleviation of one of the main drawbacks in using EHs, *i.e.* substrate and product inhibition. Indeed, the presence of a water-immiscible solvent limits the substrate and product concentration in the aqueous phase, which consequently limits enzyme inhibition to a large extent. Another interesting property of EHs is their relatively large substrate spectrum as exemplified in this review. Even trisubstituted epoxides could act as substrates for some EHs. Numerous types of biocatalytic reactions have been performed with EHs; this should arouse in the near future the interest of many organic chemists in these catalysts. Beside the classical kinetic resolution of a racemate with its intrinsic limitation of a 50% maximum theoretical yield in both residual epoxide and formed diol, EHs can also be used in stereoselective desymmetrization of *meso*-epoxides or in the enantioconvergent hydrolysis of racemic epoxides, both processes offering the possibility to generate enantiopure diol products in theoretically100% yield. The latter case is a characteristic of EHs and is the result of the intrinsic capacity of some of these enzymes to react with either of the two oxirane carbon atoms in an enantiomer-dependent fashion, resulting in the same diol enantiomer at complete conversion of the racemic substrate. Such a situation is also encountered in classical kinetic resolutions when the remaining epoxide is chemically transformed (under acidic conditions for example) into the same enantiomer of the enzymatically formed diol product, or when two enantiocomplementary enzymes are used together.

The development of molecular biology tools has had a great impact on many aspects of biocatalysis. Numerous chemistry labs are now familiar with gene cloning and protein overexpression techniques, which offer the possibility to obtain as-yet uncharacterized enzymes. Especially, screening complex samples of high biodiversity for a specific activity can uncover enzymes with novel properties in terms of activity, substrate range and stereo-, regio- and chemoselectivity. As a consequence, recombinant hosts such as *Escherichia coli* or yeast overexpressing EHs are now routinely used in biotransformation reactions as a powerful alternative to naturally occurring EH-containing microorganisms. The high attainable level of overexpression enables extremely high substrate concentrations to be used, in conjunction with significantly shorter reaction times. Consequently, the time-consuming preparation of purified or partially purified enzymes is no longer necessary to obtain high enzymatic activities. Site-directed mutagenesis and laboratory evolution (error-prone PCR, DNA shuffling and iterative saturation mutagenesis) further offer the possibility to modify existing enzymes in order to improve one or more enzyme characteristics. Such tailor-made catalysts can now be developed to fulfil specific needs of the chemical industry. Another emerging area is the use of multienzyme-based transformations to perform several consecutive reactions in one pot. Combining epoxide-generating enzymes with EHs in a single process or host can considerably reduce the complexity of biotransformation processes. In conclusion, EHs are extremely useful enzymes in organic synthesis due to their ease of use, robustness, ubiquity and applicability and will probably continue to be a field of intense research in the near future, both from an applied and fundamental point of view.

References:

Arand, M., Müller, F., Mecky, A., Hinz, W., Urban, P., Pompon, D., Kellner, R. and Oesch, F. (1999) Catalytic triad of microsomal epoxide hydrolase: replacement of Glu^{404} with Asp leads to a strongly increased turnover rate. *Biochem. J.*, **337**, 37–43.

Arand, M., Hallberg, B.M., Zou, J., Bergfors, T., Oesch, F., van der Werf, M.J., de Bont, J.A.M., Jones, T.A. and Mowbray, S.L. (2003) Structure of *Rhodococcus erythropolis* limonene-1,2-epoxide hydrolase reveals a novel active site. *EMBO J.*, **22**, 2583–2592.

Archelas, A., Delbecque, J.P. and Furstoss, R. (1993) Microbiological transformations.30. Enantioselective hydrolysis of racemic epoxides : the synthesis of enantiopure insect juvenile hormone analogs (Bower's compound). *Tetrahedron: Asymmetry*,**4**, 2445-2446.

Archer, I.V.J., Leak, D.J. and Widdowson D.A. (1996) Chemoenzymatic resolution and deracemisation of (±)-1-methyl-1,2-epoxycyclohexane: the synthesis of (1-*S*,2-*S*)-1-methylcyclohexane-1,2-diol. *Tetrahedron Lett*., **37**, 8819–8822.

Botes, A.L., Steenkamp, J.A., Weijers, C.A.G.M. and van Dyk, M.S. (1998) Biocatalytic resolution of 1,2-epoxyoctane using resting cells of different yeast strains with novel epoxide hydrolase activities. *Biotechnol. Lett.*, **20**, 421–426.

Botes, A.L., Weijers, C.A.G.M., Botes, P.J. and van Dyk, M.S. (1999) Enantioselectivities of yeast epoxide hydrolases for 1,2-epoxides. *Tetrahedron-Asymmetry*, **10**, 3327–3336.

Botes, A.L., Lotter, J., Labuschagne, M. and Mitra, R.K. (2005) Methods for the preparation of optically active epoxides and vicinal diols from styrene epoxides using enantioselective epoxide hydrolases derived from yeasts. WO Pat. 100569 A2 (CSIR, Oct. 27, 2005).

Botes, A.L., Labuschagne, M., Roth, R., Mitra, R.K., Lotter, J., Abrahams, N., Simpson, C. and Van der Westhuizen, C. (2007a) Methods for obtaining optically active epoxides and diols from 2,3-disubstituted and 2,3-trisubstituted epoxides. WO Pat. 069079 A2(CSIR, June 21, 2007).

Botes, A.L., Lotter, J. and Labuschagne, M. (2007b) Methods of obtaining optically active epoxides and vicinal diols from meso-epoxides. U.S. Pat. 0275448 A1 (CSIR, Nov.29, 2007)

Botes, A.L., Mitra, R. and Plenaar, D. (2007c) Epoxide Hydrolases: Process Applications *Innov. Pharma Technol.*, 90-92.

Botes, A.L., Labuschagne, M., Roth, R., Mitra, R.K., Lotter, J., Lalloo, R., Ramduth, D., Rohitlall, N., Simpson, C. and Van Zyl, P. (2008) Recombinant yeasts for synthesizing epoxide hydrolases. U.S. Pat. 0171359 A1 (Oxyrane (UK) Ltd, Jul. 17, 2008).

Bottalla, A.L., Ibrahim-Ouali, M., Santelli, M., Furstoss, R. and Archelas, A. (2007) Epoxide hydrolase-catalyzed kinetic resolution of a spiroepoxide, a key building block of various 11-heterosteroids. *Adv. Synth. Catal*.,**349**, 1102–1110.

Bučko, M., Vikartovská, A., Lacík, I., Kolláriková, G., Gemeiner, P., Pätoprstý, V. and Brygin, M. (2005) Immobilization of a whole-cell epoxide-hydrolyzing biocatalyst in sodium alginate–cellulose sulfate–poly(methylene-co-guanidine) capsules using a controlled encapsulation process. *Enzyme Microb. Technol.*,**36**, 118–126.

Calvez, O., Chiaroni, A. and Langlois, N. (1998) Enantioselective synthesis of 2,3-disubstituted piperidines from (*S*)-methylpyroglutamate. *Tet. Lett*. **39**, 9447-9450.

Cao, L, Lee, J., Chen, W. and Wood, T.K. (2006) Enantioconvergent production of (*R*)-1-phenyl-1,2-ethanediol from styrene oxide by combining the *Solanum tuberosum* and an evolved *Agrobacterium radiobacter* AD1 epoxide hydrolases. *Biotech. Bioeng*., **94**, 522–529.

Cassimjee, K.E., Kourist, R., Lindberg, D., Larsen, M.W., Hong Thanh, N., Widersten, M., Bornscheuer, U.T. and Berglund P. (2011) One-step enzyme extraction and immobilization for biocatalysis applications. *Biotechnol.J.*, **6**, 463–469.

Chang, D., Heringa, M.F., Witholt, B. and Li, Z. (2003a) Enantioselective trans dihydroxylation of nonactivated C-C double bounds of aliphatic heterocycles with *Sphingomonas* sp. HXN-200. *J. Org. Chem.*,**68**, 8599-8606.

Chang, D., Wang, Z., Heringa M.F., Wirthner, R., Witholt, B. and Li, Z. (2003b) Highly enantioselective hydrolysis of alicyclic *meso*-epoxides with a bacterial epoxide hydrolase from *Sphingomonas* sp. HXN-200: simple synthesis of alicyclic vicinal *trans*-diols. *Chem. Commun*., 960–961.

Chen, C.-S., Fujimoto, Y., Girdaukas, G. and Sih, C.J. (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc*., **104**, 7294–7299.

Chen, W.-J., Lou, W.-Y. and Zong, M.-H. (2012a) Efficient asymmetric hydrolysis of styrene oxide catalyzed by Mung bean epoxide hydrolases in ionic liquid-based biphasic systems. *Bioresource Technol*., **115**, 58–62.

Chen, W.-J., Lou, W.-Y., Yu, C.-Y., Wu, H., Zong, M.-H. and Smith, T.J. (2012b) Use of hydrophilic ionic liquids in a two-phase system to improve Mung bean epoxide hydrolases-mediated asymmetric hydrolysis of styrene oxide. *J. Biotechnol*., **162**, 183–190.

Chen, X-J., Archelas, A. and Furstoss, R. (1993) Microbiological transformations. 27. The first examples for preparative-scale enantioselective or diastereoselective epoxide hydrolases using microorganisms. An unequivocal access to all four bisabolol stereoisomers. *J. Org. Chem.*, **58**, 5528–5532.

Chiappe, C., Leandri, E., Lucchesi, S., Pieraccini, D., Hammock, B.D. and Morisseau, C. (2004) Biocatalysis in ionic liquids: the stereoconvergent hydrolysis of *trans*-β-methylstyrene oxide catalyzed by soluble epoxide hydrolase. *J. Mol. Catal. B: Enzym*., **27**, 243–248.

Chiappe, C., Leandri, E., Hammock, B. and Morisseau, C. (2007) Effect of ionic liquids on epoxide hydrolase-catalyzed synthesis of chiral 1,2-diols. *Green Chem.*, **9**, 162–168.

Choi, W.J., Puah, S.M., Tan, L.L. and Ng, S.S. (2008) Production of (R)-ethyl-3,4-epoxybutyrate by newly isolated *Acinetobacter baumannii* containing epoxide hydrolase. *Appl. Microbiol. Biotechnol*. **79**, 61-67.

Choi, S.H., Kim, H.S., Lee, I.S. and Lee, E.Y. (2010) Functional expression and magnetic nanoparticle-based immobilization of a protein-engineered marine fish epoxide hydrolase of *Mugil cephalus* for enantioselective hydrolysis of racemic styrene oxide. *Biotechnol. Lett.*, **32**, 1685–1691.

Cleij, M., Archelas, A. and Furstoss, R. (1998) Microbiological transformations. Part 42: a two-liquid-phase preparative scale process for an epoxide hydrolase catalysed resolution of *para*-bromo-α-methyl styrene oxide. Occurrence of a surprising enantioselectivity enhancement. *Tetrahedron:Asymmetry*, **9**, 1839–1842.

Cleij, M., Archelas, A. and Furstoss, R. (1999) Microbial transformations 43. Epoxide hydrolase as tools for the synthesis of enantiopure alpha-methylstyrene oxides: a new and efficient synthesis of (*S*)-Ibuprofen. *J. Org. Chem.*, **64**, 5029–5035.

Decker, M., Arand, M. And Cronin, A. (2009)Mammalian epoxide hydrolases in xenobiotic metabolism and signalling *Arch. Toxicol.*,**83**, 297–318.

Deregnaucourt, J., Archelas, A., Barbirato, F., Paris, J-M. and Furstoss, R. (2007) Enzymatic Transformations 63. High-Concentration Two Liquid-Liquid Phase *Aspergillus niger* Epoxide Hydrolase-Catalysed Resolution: Application to Trifluoromethyl-Substituted Aromatic Epoxides. *Adv. Synth. Catal*., **349**,1405-1417.

Devi, A.V, Lahari, C., Swarnalatha, L. and Fadnavis, N.W. (2008) Gelozymes in organic synthesis. Part IV: Resolution of glycidate esters with crude Mung bean Phaseolus radiatus) epoxide hydrolase immobilized in gelatin matrix. *Tetrahedron: Asymmetry***19**, 1139-1144.

Devries, K. M., Dow, R. L. and Wright, S. W. (1998)Procédé de preparation de pyridines substituées WO Pat. 021184A1 (May 22, 1998).

Doumèche, B., Archelas, A. and Furstoss, R. (2006) Enzymatic transformations 62. Preparative scale synthesis of enantiopure glycidylacetals using an *A. niger* epoxide hydrolase catalysed kinetic resolution. *Adv. Synth. Catal.,348*, 1948-1957.

Duarah, A., Goswami, A., Bora, T.C., Talukdar, M. and Gogoi, B.K. (2013) Enantioconvergent biohydrolysis of racemic styrene oxide to *R*-phenyl-1,2-ethanediol by a newly isolated filamentous fungus *Aspergillus tubingensis* TF1 *Appl. Biochem. Biotechnol*., **170**, 1965-1973.

Edegger, K., Mayer, S.F., Steinreiber, A. and Faber, K. (2004) Chemo-enzymatic enantio-convergent asymmetric synthesis of (R)-(+)-Marmin. *Tetrahedron*, **60**, 583-588.

Elfström, L.T. and Widersten, M. (2005) The S*accharomyces cerevisiae* ORF YNR064c protein has characteristics of an 'orphaned' epoxidehydrolase.*BBA-Proteins Proteomics*, **1748,** 213-221.

Eliel, E.L. (1985) The R/S system. A method for assignment and some recent modifications. *J. Chem. Educ*.**62**, 223-224.

Ferreira, F., Greck, C. and Genêt, J.P. (1997) Stereocontrolled syntheses of *trans*-3-hydroxypipecolic acids and application to (-)-Swainsonine. *Bull. Soc. Chim. Fr.***134**, 615-621.

Fisher, M. H., Naylor, E. M., Ok, D., Weber, A. E., Shih, T. and Ok, H. (1996)Substituted sulfonamides as selective β3 agonists for the treatment of diabetes and obesityUS Pat. 5561142 A (Merck&Co, Inc., Oct.1, 1996).

Fuchs, M., Simeo, Y., Ueberbacher, B.T., Mautner, B., Netscher, T. and Faber, K. (2009) Enantiocomplementary Chemoenzymatic Asymmetric Synthesis of (*R*)- and (*S*)-Chromanemethanol. *Eur. J. Org. Chem.***6**, 833-840.

Fujino, A. and Sugai, T. (2008) Chemoenzymatic approach to enantiomerically pure (*R*)-3-hydroxy-3-methyl-4-pentenoic acid ester and its application to a formal total synthesis of taurospongin A. *Adv. Synth. Catal*. **350**, 1712–1716.

Fujino, A., Asano, M., Yamaguchi, H., Shirasaka, N., Sakoda A., Ikunaka, M., Obata, R., Nishiyama S. and Sugai, T.(2007) *Bacillus subtilis* epoxide hydrolase-catalyzed preparation of enantiopure 2-methylpropane-1,2,3-triol monobenzyl ether and its application to expeditious synthesis of (*R*)-bicalutamide. *Tetrahedron Lett.*,**48**, 979–983.

Genzel,Y., Archelas, A., Broxterman, Q.B., Schulze, B. and Furstoss, R. Microbiological transformations. 47. A step toward a green chemistry preparation of enantiopure (*S*)-2-, -3-, and -4-pyridyloxirane via an epoxide hydrolase catalysed kinetic resolution. (2001a) *J. Org. Chem.*,**66**, 538-543.

Genzel, Y., Archelas, A., Lutje, J.F.H., Janssen, D.B. and Furstoss, R. (2001b) Microbiological transformations. Part 48: Enantioselective biohydrolysis of 2-,3- and 4-pyridyloxirane at high substrate concentration using the *Agrobacterium radiobacter* AD1 epoxide hydrolase and its Tyr215Phe mutant. *Tetrahedron*,**57**, 2775-2779.

Genzel, Y., Archelas, A., Broxterman, Q.B., Schulze, B. and Furstoss, R. (2002) Microbiological transformations 50: selection of epoxide hydrolases for enzymatic resolution of 2-, 3-, or 4-pyridyloxirane. *J. Mol. Catal. B: Enzym.*, **16**, 217–222.

Gong, P.-F. and Xu, J.-H. (2005) Bio-resolution of a chiral epoxide using whole cells of *Bacillus megaterium* ECU1001 in a biphasic system. *Enzyme Microb Technol*., **36**, 252–257.

Grant, D.F., Storms, D.H. and Hammock, B.D. (1993) Molecular cloning and expression of murine liver epoxide hydrolase. *J.Biol. Chem.*, **268**, 17628–17633.

Grulich, M., Maršálek, J., Kyslík, P., Štěpánek, V. and Kotik, M. (2011) Production, enrichment and immobilization of a metagenome-derived epoxide hydrolase. *Process Biochem.*,**46**, 526–532.

Guérard, C., Alphand, V., Archelas, A., Demuynck, C., Hecquet, L., Furstoss, R. and Bolte, J. (1999) Transketolase-Mediated synthesis of 4-deoxy-D-fructose 6-phosphate by epoxide hydrolase-catalysed resolution of 1,1-diethoxy-3,4-epoxybutane. *Eur. J. Org. Chem.*, 3399-3402.

Haddad, M. and Larchevêque, M. (2001) An alternative stereoselective synthesis of *trans*-(2*R*,3*R*)-3-hydroxypipecolic acid. *Tet. Lett*. **42**, 5223-5225.

Harada, H., Hirokawa, Y., Suzuki, K., Hiyama, Y., Oue, M., Kawashima, H., Yoshida, N., Furutani, Y. and Kato, S. (2003) *Bioorg. Med. Chem. Lett.*,**13**, 1301–1305.

Heikinheimo P., Goldman A., Jeffries C. and Ollis D.L. (1999) Of barn owls and bankers: a lush variety of α/β hydrolases. *Structure*,**7**, R141-R146.

Hellstrom, H., Steinreiber, A., Mayer, S.F. and Faber, K. (2001) Bacterial epoxide hydrolase-catalyzed resolution of a 2,2-disubstituted oxirane: optimization and upscaling. *Biotech. Lett*. **23**, 169-173.

Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. and Stüber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Nat. Biotechnol*.,**6**, 1321–1325.

Hsin, L-W., Prisinzano, T., Wilkerson, C.R., Dersch, C.M., Horel, R., Jacobson, A.E., Rothman, R.B. and Rice, K.C. (2003) Synthesis and dopamine transporter affinity of chiral 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(2-hydroxypropyl)piperazines as potential cocaine abuse therapeutic agents. *Bioorg. Med. Chem. Lett.*,**13**, 553–556.

Hwang, S., Hyun, H., Lee, B., Park, Y., Lee, E.Y. and Choi, C. (2006) Purification and Characterization of a Recombinant *Caulobacter crescentus* Epoxide Hydrolase *Biotech. Bioprocess Bioeng*., **11**, 282-287.

Hwang, S., Choi, C.Y. and Lee, E.Y. (2008a) Enantioconvergent bioconversion of *p*-chlorostyrene oxide to (*R*)-*p*-chlorophenyl-1,2-ethandiol by the bacterial epoxide hydrolase of *Caulobacter crescentus. Biotechnol. Lett.*,**30**, 1219-1225.

Hwang, S., Choi, C.Y. and Lee, E.Y. (2008b) One-pot biotransformation of racemic styrene oxide into (*R*)-1,2-phenylethanediol by two recombinant microbial epoxide hydrolases. *Biotech. Bioprocess Eng*., **13**, 453–457.

Jin, H.-X., Liu, Z.Q., Hu, Z.-C. and Zheng, Y.-G. (2013a) Production of (*R*)-epichlorohydrin from 1,3-dichloro-2-propanol by two-step biocatalysis using haloalcohol dehalogenase and epoxide hydrolase in two-phase system. *Biochem. Eng. J.*,**74**, 1-7.

Jin, H-X., Liu, Z-Q., Hu, Z-C. and Zheng, Y-G. (2013b) Biosynthesis of (*R*)-epichlorohydrin at high substrate concentration by kinetic resolution of racemic epichlorohydrin with a recombinant epoxide hydrolase *Eng. Life Sci*.**13**, 385-392.

Johansson, P., Unge, T., Cronin, A., Arand, M., Bergfors, T., Jones, T.A. and Mowbray, S.L. (2005) Structure of an atypical epoxide hydrolase from *Mycobacterium tuberculosis* gives insights into its function. *J. Mol. Biol.*, **351**, 1048–1056.

Kamal, A., Khanna, R., Kumar, C.G., Shaik, A.B. and Kumar, M.S. (2013) A novel bacterial strain of *Achromobacter* sp. MTCC 5605 and a highly enantioselective epoxide hydrolase isolated therefrom. WO Pat. 030851 A1 (March 7, Council of Scientific & Industrial Research).

Kamita, S.G., Oshita, G.H., Wang, P., Morisseau, C., Hammock, B.D., Nandety, R.S. and Falk, B.W. (2013) Characterization of Hovi-mEH1, a microsomal epoxide hydrolase from the glassy-winged sharpshooter *Homalodisca vitripennis*. *Arch.Insect Biochem. Physiol.*, **83**, 171–179.

Karboune, S., Archelas, A., Furstoss, R. and Baratti, J. (2005a) Immobilization of the *Solanum tuberosum* epoxide hydrolase and its application in an enantioconvergent process. *Biocatal. Biotransform.*,**23**, 397–405.

Karboune, S., Archelas, A., Furstoss and R., Baratti, J. (2005b) Immobilization of epoxide hydrolase from *Aspergillus niger* onto DEAE-cellulose: enzymatic properties and application for the enantioselective resolution of a racemic epoxide. *J. Mol. Catal. B: Enzym.*,**32**, 175–183.

Karboune, S., Archelas, A. and Baratti, J. (2006) Properties of epoxide hydrolase from *Aspergillus niger* for the hydrolytic kinetic resolution of epoxides in pure organic media. *Enz. Microbiol. Technol.*, **39**, 318–324.

Karboune, S., Archelas, A. and Baratti, J.C. (2010) Free and immobilized *Aspergillus niger* epoxide hydrolase-catalyzed hydrolytic kinetic resolution of racemic *p*-chlorostyrene oxide in a neat organic solvent medium. *Process Biochem.*,**45**, 210–216.

Kasai, N., Suzuki, T. and Furukawa, Y. (1998) Chiral C3 epoxides and halohydrins: Their preparation and synthetic application *J. Mol. Catal. B: Enzymatic***4**, 237–252.

Kim, H.S., Lee, S.J. and Lee, E.Y. (2006) Development and characterization of recombinant whole-cell biocatalysts expressing epoxide hydrolase from *Rhodotorula glutinis* for enantioselective resolution of racemic epoxides. *J. Mol. Catal. B: Enzym.*,**43**, 2–8.

Kim, Y.H., Lee, I., Choi, S.H., Lee, O.K., Shim, J., Lee, J., Kim, J. and Lee, E.Y. (2013) Enhanced stability and reusability of marine epoxide hydrolase using ship-in-a-bottle approach with magnetically-separable mesoporous silica. *J. Mol. Catal. B: Enzym.*,**89**, 48–51.

Kotik, M., Brichac, J. and Kyslik, P. (2005) Novel microbial epoxide hydrolases for biohydrolysis of glycidyl derivatives. *J. Biotechnol.*,**120**, 364-375.

Kotik, M. and Kyslik, P. (2006) Purification and characterisation of a novel enantioselective epoxide hydrolase from *Aspergillus niger* M200. *Biochem. Biophys. Acta*,**1760**, 245-252.

Kotik, M. (2009)Novel genes retrieved from environmental DNA by polymerase chain reaction: Current genome-walking techniques for future metagenome applications. *J. Biotechnol.*, **144**, 75–82.

Kotik, M., Štěpánek, V., Marešová, H., Kyslík, P. and Archelas, A. (2009) Environmental DNA as a source of a novel epoxide hydrolase reacting with aliphatic terminal epoxides. *J. Mol. Catal. B: Enzym.*, **56**, 288–293.

Kotik, M., Štěpánek, V., Grulich, M., Kyslík, P. and Archelas, A. (2010) Access to enantiopure aromatic epoxides and diols using epoxide hydrolases derived from total biofilter DNA. *J. Mol. Catal. B: Enzym.*, **65**, 41–48.

Kotik, M., Archelas, A., Faměrova, V., Oubrechtova, P. and Křen, V. (2011) Laboratory evolution of an epoxide hydrolase – Towards an enantioconvergent biocatalyst. *J. Biotechnol.*, **156**, 1–10.

Kotik, M., Zhao, W., Iacazio, G. and Archelas, A. (2013) Directed evolution of metagenome-derived epoxide hydrolase for improved enantioselectivity and enantioconvergence *J. Mol. Catal. B: Enzym.*, **91**, 44–51.

Kroutil, W., Mischitz, M. and Faber, K. (1997a) Deracemisation of (±)-2,3-disubstituted oxiranes via biocatalytic hydrolysis using bacterial epoxide hydrolases: kinetics of an enantioconvergent process. *J. Chem. Soc. Perkin Trans 1*, 3629–3636.

Kroutil, W., Osprian, I., Mischitz, M. and Faber, K. (1997b) Chemoenzymatic synthesis of (*S*)-(-)-Frontalin using bacterial epoxide hydrolases. *Synthesis*, 156–158.

Labuschagne, M. and Albertyn, J. (2007) Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*. *Yeast*, **24**, 69–78.

Lee, K.S., Woo, M.H., Kim, H.S., Lee, E.Y. and Lee, I.S. (2009) Synthesis of hybrid Fe_3O_4–silica–NiO superstructures and their application as magnetically separable high-performance biocatalysts. *Chem. Commun*., 3780–3782.

Lenfant, N., Hotelier, T., Velluet, E., Bourne, Y., Marchot, P. and Chatonnet, A. (2013) ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions Nucleic Acids Research., **41**, D423-D429.

Li Z., Feiten, H.-J., Chang, D., Duetz, W.A., van Beilen, J.B. and Witholt, B. (2001) Preparation of (*R*)- and (*S*)-*N*-protected 3-hydroxypyrrolidines by hydroxylation with *Sphingomonas* sp. HXN-200, a highly active, region- and stereoselective, and easy to handle biocatalyst. *J. Org. Chem.* **66**, 8424-8430.

Lin, L.S., Lanza, T.J. Jr., Jewell, J.P., Liu, P., Shah, S.K., Qi, H., Tong, X., Wang, J., Xu, S.S., Fong, T.M., Shen, C-P., Lao, J., Xiao, J.C., Shearman, L.P., Stribling, D.S., Rosko, K., Strack, A., Marsh, D.J., Feng, Y., Kumar, S., Sumuel, K., Yin, W., Van der Ploeg, L.H.T., Goulet, M.T. and Hagmann, W.K. (2006) Discovery of N-[(1*S*,2*S*)-3-(4-chlorophenyl)-2-(3-cyanophenyl)-1-methylpropyl]-2-methyl-2-{[5-(trifluoro-methyl)pyridin-2-yl]oxy}propanamide (MK-0364), a novel, acyclic cannabinoid-1 receptor inverse agonist for the treatment of obesity. *J. Med. Chem.*,**49**, 7584–7587.

Lin, S., Horsman, G.P. and Shen, B. (2010) Characterization of the epoxide hydrolase NcsF2 from the neocarzinostatin biosynthetic gene cluster. *Org. Lett.*, **12**, 3816–3819.

Liu, Z., Michel, J., Wang, Z., Witholt, B. and Li, Z. (2006)Enantioselective hydrolysis of styrene oxide with the epoxide hydrolase of *Sphingomonas* sp. HXN-200. *Tetrahedron:Asymmetry*, **17**, 47–52.

Lotter, J., Botes, A.L., van Dyk, M.S. and Breytenbach, J.C. (2004) Hydrolytic kinetic resolution of the enantiomers of the structural isomers trans-1-phenylpropene oxide and (2,3-epoxypropyl)benzene by yeast epoxide hydrolase. *Biotech. Lett.*, **15**, 1197-1200.

Manoj, K.M., Archelas, A., Baratti, J. and Furstoss, R. Microbiological transformations. Part 45. A green chemistry preparative scale synthesis of enantiopure building blocks of Eliprodil: elaboration of a high substrate concentration epoxide hydrolase-catalyzed hydrolytic kinetic resolution process. (2001) *Tetrahedron*,**57**, 695-701.

Maritz, J., Krieg, H.M., Yeates, C.A., Botes, A.L. and Breytenbach, J.C. (2003) Calcium alginate entrapment of the yeast *Rhodosporidium toruloides* for the kinetic resolution of 1,2-epoxyoctane. *Biotechnol. Lett*., **25**, 1775–1781.

Mateo, C., Archelas, A., Fernandez-Lafuente, R., Guisan, J.M. and Furstoss, R. (2003) Enzymatic transformations. Immobilized *A. niger* epoxide hydrolase as a novel biocatalytic tool for repeated-batch hydrolytic kinetic resolution of epoxides. *Org. Biomol. Chem.*,**1**, 2739–2743.

Mateo, C., Fernandez-Lafuente, R., Archelas, A., Guisan, J.M. and Furstoss, R. (2007) Preparation of a very stable immobilized *Solanum tuberosum* epoxide hydrolase. *Tetrahedron:Asymmetry*,**18**, 1233–1238.

Mathvink, R. J., Barritta, A. M., Candelore, M. R., Cascieri,M. A., Deng, L., Tota, L., Strader, C. D., Wyvratt, M. J., Fisher, M. H. and Weber, A. E. (1999) *Bioorg. Med. Chem. Lett*., **9**, 1869-1874.

Mayer, S.F., Steinreiber, A., Goriup, M., Saf, R. and Faber, K. (2002a) Chemoenzymatic asymmetric total syntheses of a constituent of Jamaican rum and of (+)-Pestalotin using an enantioconvergent enzyme-triggered cascade reaction. *Tetrahedron:Asymmetry*,**13**, 523-528.

Mayer, S.F., Steinreiber, A., Orru, R.V.A. and Faber, K. (2002b) Chemoenzymatic asymmetric total synthesis of antitumor agents (3*R*,9*R*,10*R*)- and (3*S*,9*R*,10*R*)-Panaxytriol and (*R*)- and (*S*)-Falcarinol from *Panax ginseng* using an enantioconvergent enzyme-triggered cascade reaction. *J. Org. Chem*., **67**, 9115–9121.

Mildvan, A.S. (2004) Inverse thinking about double mutants of enzymes. *Biochemistry*, **43**, 14517–14520.

Min, J.Y. and Lee, E.Y. (2012) Biosynthesis of (*R*)-1,2-phenylethanediol and (*R*)-4-chloro-1,2-phenylethanediol by using two recombinant cells expressing enantiocomplementary epoxide hydrolases *J. Ind. Eng*., **18**, 160-164.

Minami, A., Migita, A., Inada, D., Hotta, K., Watanabe, K., Oguri, H. and Oikawa, H. (2011)Enzymatic epoxide-opening cascades catalyzed by a pair of epoxide hydrolases in the ionophore polyether biosynthesis. *Org. Lett*., **13**, 1638-1641.

Mischitz, M. and Faber, K. (1996) Chemo-enzymatic synthesis of (2*R*,5*S*)- and (2*R*,5*R*)-5-(1-hydroxy-1-methylethyl)-2-vinyl-tetrahydrofuran ('linalool oxide'): preparative application of a highly selective bacterial epoxide hydrolase. *Synlett*, 978-980.

Monfort, N., Archelas, A. and Furstoss, R. (2004a) Enzymatic transformations. Part 55: Highly productive epoxide hydrolase catalysed resolution of an azole antifungal key synthon.*Tetrahedron*, **60**, 601–605.

Monfort, N., Archelas, A. and Furstoss, R. (2004b) Unpublished results.

Monterde, M.I., Lombard, M., Archelas, A., Cronin, A., Arand, M. and Furstoss, R. (2004) Enzymatic transformations. Part 58: Enantioconvergent biohydrolysis of styrene oxide derivatives catalysed by the *Solanum tuberosum* epoxide hydrolase. *Tetrahedron:Asymmetry*, **15**, 2801–2805.

Morisseau C. (2013) Role of epoxide hydrolases in lipid metabolism. *Biochimie*,**95**, 91–95.

Morisseau, C., Nellaiah, H., Archelas, A., Furstoss, R. and Baratti, J.C. (1997) Asymmetric hydrolysis of racemic para-nitrostyrene oxide using an epoxide hydrolase preparation from aspergillus niger. *Enzyme Microb. Technol.*,**20**, 446-452.

Moussou P., Archelas A. and Furstoss R. (1998a) Microbiological transformations 40. Use of fungal epoxide hydrolases for the synthesis of enantiopure alkyl epoxides. *Tetrahedron*, **54**, 1563-1572.

Moussou, P., Archelas, A., Baratti, J. and Furstoss, R. (1998b) Microbiological transformations. Part 39: determination of the regioselectivity occuring during oxirane ring opening by epoxide hydrolases: a theoritical analysis and a new method for its determination. *Tetrahedron: Asymmetry*,**9**, 1539-1547.

Murmann, W., Rumore, G. and Gamba, A. (1967) *Boll. Chim. Farm.*, **106**, 251-268.

Naundorf, A., Melzer, G., Archelas, A., Furstoss, R. and Wohlgemuth, R. (2009)Influence of pH on the expression of a recombinant epoxide hydrolase in *Aspergillus niger*. *Biotechnol.J.*, **4**, 756–765.

Newman, J.W., Morisseau, C. and Hammock, B.D. (2005) Epoxide hydrolases: their roles and interactions with lipid metabolism. *Progress in Lipid Research*,**44**, 1–51

Orru, R.V.A., Mayer, S.F., Kroutil, W. and Faber, K. (1998a) Chemoenzymatic deracemizationof (+-)-2,2-disubstituted oxiranes. *Tetrahedron*, **54**, 859-874.

Orru, R.V.A., Osprian, I., Kroutil, W. and Faber, K. (1998b) An Efficient Large-Scale Synthesis of (*R*)-(-)-Mevalonolactone Using Simple Biological and Chemical Catalysts. *Synthesis*,1259-1263.

Orru, R.V.A., Archelas, A., Furstoss, R. and Faber, K. (1999) Epoxide hydrolases and their synthetic applications. *Adv. Biochem. Engineering*, **63**, 145-167.

Osprian, I., Kroutil, W., Mischitz, M. and Faber, K. (1997) Biocatalytic resolution of 2-methyl-2-(aryl)alkyloxiranes using novel bacterial epoxide hydrolases. *Tetrahedron-Asymmetry*, **18**, 65–71.

Osprian, I., Stampfer, W. and Faber, K. (2000) Selectivity enhancement of epoxide hydrolase catalyzed resolution of 2,2-disubstituted oxiranes by substrate modification. *J. Chem. Soc. Perkin Trans. 1*, 3779–3785.

Otto, P.PJ.H.L., Stein, F. and Van Der Willigen, C.A. (1988) A bio-organic synthesis of (+)-disparlure, sex pheromone of *Lymantria dispar* (gypsy moth). *Agric. Ecosys. Environ*., **21**, 121-123.

Pabel, J., Hofner, G. and Wanner, K.T. (2000) *Bioorg. Med. Chem. Lett.*, **10**, 1377–1380.

Pedragosa-Moreau, S., Archelas, A. and Furstoss, R. (1993) Microbiological transformations. 28. Enantiocomplementary epoxide hydrolyses as a preparative access to both enantiomers of styrene oxide. *J. Org. Chem.*, **58**, 5533-5536.

Pedragosa-Moreau, S., Archelas, A. and Furstoss R. (1995) Preparative access to both enantiomers of styrene oxide by hydrolysis of the racemate using *Aspergillus niger* or *Beauveria sulfurescens*. In "*Preparative Biotransformation*", Wiley & Sons, Ltd, **1**, 18.1-18.7.

Pedragosa-Moreau, S., Archelas, A. and Furstoss, R. (1996a) Microbiological transformations. 31: Synthesis of enantiopure epoxides and vicinal diols using fungal epoxide hydrolase mediated hydrolysis. *Tetrahedron Lett.*, **37**, 3319–3322.

Pedragosa-Moreau, S., Archelas, A. and Furstoss, R. (1996b) Microbial transformations 32: use of epoxide hydrolase mediated biohydrolysis as a way to enantiopure epoxides and vicinal diols: application to substituted styrene oxide derivatives. *Tetrahedron*,**52**, 4593-4606.

Pedragosa-Moreau, S., Morisseau, C., Zylber, J., Archelas, A., Baratti, J. and Furstoss, R. (1996c) Microbiological transformations. 33. Fungal epoxide hydrolases applied to the synthesis of enantiopure para-substituted styrene oxides. A mechanistic approach. *J. Org. Chem*., **61**, 7402-7407.

Pedragosa-Moreau, S., Morisseau, C. , Baratti, J. , Zylber, J. , Archelas, A. and Furstoss, R. (1997) Microbiological transformations 37. An enantioconvergent synthesis of the *beta*-blocker ®-Nifénalol using a combined chemoenzymatic approach. *Tetrahedron*, **53**, 9707-9714.

Petri, A., Marconcini, P. and Salvadori, P. (2005) Efficient immobilization of epoxide hydrolase onto silica gel and use in the enantioselective hydrolysis of racemic *para*-nitrostyrene oxide. *J. Mol. Catal. B: Enzym.*,**32**, 219–224.

Pienaar, D.P., Mitra, R.K., van Deventer, T.I. and Botes, A.L. (2008) Synthesis of a variety of optically active hydroxylated heterocyclic compounds using epoxide hydrolase technology. *Tetrahedron Lett*., **49**, 6752-6755.

Prelog, V. and Helmchem, G. (1982) Basic Principles of the CIP-System and Proposals for a Revision. *Angew. Chem. Int. Ed. Engl.***21**, 567-583.

Reetz, M.T. and Zheng, H. (2011) Manipulating the expression rate and enantioselectivity of an epoxide hydrolase by using directed evolution. *ChemBioChem*, **12**, 1529–1535.

Reetz, M.T., Wang, L.-W. and Bocola, M. (2006) Directed evolution of enantioselective enzymes: iterative cycles of CASTing for probing protein-sequence space. *Angew. Chem. Int. Ed.*, **45**, 1236–1241.

Reetz, M.T., Kahakeaw, D. and Lohmer, R. (2008) Addressing the numbers problem in directed evolution. *ChemBioChem*, **9**, 1797–1804.

Reetz, MT., Bocola, M., Wang, L-W., Sanchis, J., Cronin, A., Arand, M, Zou, J., Archelas, A., Bottalla, A-L., Naworyta, A. and Mowbray, S.L. (2009) Directed Evolution of an Enantioselective Epoxide Hydrolase: Uncovering the Source of Enantioselectivity at Each Evolutionary Stage. *J. Am. Chem. Soc*., **131**, 7334-7343.

Reetz, M.T., Prasad, S., Carballeira, J.D., Gumulya, Y. and Bocola, M. (2010) Iterative saturation mutagenesis accelerates laboratory evolution of enzyme stereoselectivity: rigurous comparison with traditional methods. *J. Am. Chem. Soc.*, **132**, 9144–9152.

Rink, R., Lutje Spelberg, J.H., Pieters, R.J., Kingma, J., Nardini, M., Kellogg, R.M., Dijkstra, B.W. and Janssen, D.B. (1999) Mutation of tyrosine residues involved in the alkylation half reaction of epoxide hydrolase from *Agrobacterium radiobacter* AD1 results in improved enantioselectivity. *J. Am. Chem. Soc.*, **121**, 7417–7418.

Rink, R., Kingma, J., Spelberg, H. L. and Janssen, D. (2000) Tyrosine residues serve as proton donor in the catalytic mechanism of epoxide hydrolase from *Agrobacterium radiobacter*. *Biochem*., **39**, 5600-5613.

Rui, L., Cao, L., Chen, W., Reardon, K.F. and Wood, T.K. (2005) Protein engineering of epoxide hydrolase from *Agrobacterium radiobacter* AD1 for enhanced activity and enantioselective production of (*R*)-1-phenylethane-1,2-diol. *Appl. Environ. Microbiol.*, **71**, 3995–4003.

Sheldon, R.A., van Pelt, S., Kanbak-Aksu, S., Rasmussen, J. and Janssen, M.H.A. (2013) Cross-linked enzyme aggregates (CLEAs) in organic synthesis. *Aldrichimica Acta*, **46**, 81–93.

Shimizu, K.I, Sakamoto, M., Hamada, M., Higashi, T., Sugai, T. and Shoji, M. (2010) The scope and limitation of the regio- and enantioselective hydrolysis of aliphatic epoxides using *Bacillus subtilis* epoxide hydrolase, and exploration toward chirally differentiated tris(hydroxymethyl)methanol.*Tetrahedron: Asymmetry***21**, 2043-2049.

Simeó, Y. and Faber, K. (2006) Selectivity enhancement of enantio- and stereo-complementary epoxide hydrolases and chemo-enzymatic deracemization of (±)-2-methylglycidyl benzyl ether. *Tetrahedron: Asymmetry***17**, 402-409.

Soloshonok, V.A. (1999) EnantioControlled Synthesis of Fluoro-organic Compounds: Stereochemical Challenges and Biomedicinal Targets, Wiley, Chichester.

Spelberg, J.H.L., Rink, R., Kellogg, R.M. and Janssen, D.B. (1998) Enantioselectivity of a recombinant epoxide hydrolase from *Agrobacterium radiobacter*. *Tetrahedron: Asymmetry*, **9**, 459-466.

Spelberg, J.H.L., Rink, R., Archelas, A., Furstoss, R. and Janssen, D. (2002) Biocatalytic potential of the epoxide hydrolase from *Agrobacterium radiobacter* AD1 and a mutant with enhanced enantioselectivity. *Adv. Synth. Catal.*,**344**, 980-985.

Steinreiber, A., Edegger K., Mayer, S.F. and Faber K. (2001a) Enantio- and diastereo-convergent synthesis of (2*R*,5*R*)- and (2*R*,5*S*)-Pityol through enzyme-triggered ring closure. *Tetrahedron: Asymmetry*, **12**, 2067-2071.

Steinreiber, A., Hellstrom, H., Mayer S.F., Orru, R.V.A. and Faber, K. (2001b) Chemo-enzymatic enantioconvergent synthesis of C4-building blocks containing a fully substituted chiral carbon center using bacterial epoxide hydrolases *Synlett* 111-113.

Steinreiber, A., Mayer, S.F. and Faber, K. (2001c) Asymmetric Total Synthesis of a Beer-Aroma Constituent Based on Enantioconvergent Biocatalytic Hydrolysis of Trisubstituted Epoxides *Synthesis*,**13**, 2035-2039.

Steinreiber, A., Mayer, S.F., Saf, R. and Faber, K. (2001d) Biocatalytic asymmetric enantioconvergent hydrolysis of trisubstituted oxiranes. *Tetrahedron: Asymmetry*, **12**, 1519–1528.

Ueberbacher, B.J., Osprian, I., Mayer, S.F. and Faber, K. (2005) A chemoenzymatic, enantioconvergent, asymmetric total synthesis of (*R*)-Fridamycin E. *Eur. J. Org. Chem.*, 1266–1270.

Ueberbacher, B.T., Oberdorfer, G., Gruber, K. and Faber, K. (2009) Epoxide-hydrolase-initiated hydrolysis/rearrangement cascade of a methylene-interrupted bis-epoxide yields chiral THF moieties without involvement of a "cyclase". *ChemBioChem*, **10**, 1697–1704.

van Loo, B., Spelberg, J.H.L., Kingma, J., Sonke, T., Wubbolts, M.G. and Janssen, D.B. (2004) Directed Evolution of Epoxide Hydrolase from *A. radiobacter* toward Higher Enantioselectivity by Error-Prone PCR and DNA Shuffling. *Chemistry & Biology*,**11**, 981-990.

van Loo, B., Kingma, J., Arand, M., Wubbolts, M.G. and Janssen, D.B. (2006) Diversity and biocatalytic potential of epoxide hydrolases identified by genome analysis. *Appl. Environ. Microbiol.*, **72**, 2905–2917.

van Loo, B., Kingma, J., Heyman, G., Wittenaar, A., Lutje Spelberg, J.H., Sonke, T. and Janssen, D.B. (2009) Improved enantioselective conversion of styrene epoxides and *meso*-epoxides through epoxide hydrolases with a mutated nucleophile-flanking residue. *Enzyme Microb. Technol.*, **44**, 145–153.

van Loo, B., Lutje Spelberg, J.H., Kingma, J., Sonke, T., Wubbolts, M.G. and Janssen, D.B. (2004) Directed evolution of epoxide hydrolase from *A. radiobacter* toward higher enantioselectivity by error-prone PCR and DNA shuffling. *Chem. Biol.*, **11**, 981–990.

Visser, H., de Oliveira Villela Filho, M., Liese, A., Weijers, C.A.G.M. and Verdoes, J.C. (2003) Construction and characterisation of a genetically engineered *Escherichia coli* strain for the epoxide hydrolase-catalysed kinetic resolution of epoxides. *Biocatal. Biotransform.*, **21**, 33–40.

Wandel, U., Mischitz, M., Kroutil, W. and Faber, K. (1995) Highly selective asymmetric hydrolysis of 2,2-disubstituted epoxides using lyophilized cells of *Rhodococcus* sp. NCIMB 11216. *J. Chem. Soc. Perkin Trans. 1*, 735–736.

Wang, W., Wang, D.I.C. and Li, Z. (2011) Facile fabrication of recyclable and active nanobiocatalysts: purification and immobilization of enzyme in one pot with Ni-NTA functionalized magnetic nanoparticles. *Chem. Commun*., **47**, 8115–8117.

Weijers, C.A.G.M. (1997) Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by *Rhodotorula glutinis*. *Tetrahedron:Asymmetry*,**8**, 639-647.

Weijers, C.A.G.M., Meeuwse, P., Herpers, R.L.J.M., Franssen, M.C.R. and Sudhoelter, E.J.R. (2005) Stereoselectivity and Substrate Specificity in the Kinetic Resolution of Methyl-Substituted 1-Oxaspiro[2.5]octanes by *Rhodotorula glutinis* Epoxide Hydrolase. *J. Org. Chem.,***70**, 6639-6646.

Widersten, M., Gurell, A. and Lindberg, D. (2010) Structure-function relationships of epoxide hydrolases and their potential use in biocatalysis. *Biochim. Biophys. Acta*. **1800**, 316-326.

Wittman, M., Carboni, J., Attar, R., Balasubramanian, B., Balimane, P., Brassil, P., Beaulieu, F., Chang, C., Clarke, W., Dell, J., Eummer, J., Frennesson, D., Gottardis, M., Greer, A., Hansel, S., Hurlburt, W., Jacobson, B., Krishnananthan, S., Lee, F.Y., Li, A., Lin, T.-A., Liu, P., Ouellet, C., Sang, X., Saulnier, M.G., Stoffan, K., Sun, Y., Velaparthi, U., Wong, H., Zang, Z., Zimmermann, K., Zoeckler, M. and Vyas, D. (2005) Discovery of a 1H-benzoimidazol-2-yl)-1H-pyridin-2-one (BMS-536924) inhibitor of insulin-like growth factor I receptor kinase with in vivo antitumor activity. *J. Med. Chem*., **48**, 5639–5643.

Woo, J-H., Kang, J-H., Hwang, H-O., Cho, J-C., Kim, S-J. and Kang, S.G. (2009) Biocatalytic resolution of glycidyl phenyl ether using a novel epoxide hydrolase from a marine bacterium, *Rhodobacterales bacterium* HTCC2654. *J. Biosci. Bioeng.*, **109**, 539-544.

Wu, S., Li, A., Chin, Y.S. and Li, Z. (2013) Enantioselective Hydrolysis of Racemic and Meso-Epoxides with Recombinant *Escherichia coli* expressing Epoxide Hydrolase from *Sphingomonas* sp. HXN-200: Preparation of Epoxides and vicinal Diols in High ee and High Concentration *ACS Catalysis*,**3**, 752-759.

Wu, S., Chen, Y., Xu, Y., Li, A., Xu, Q., Glieder, A. and Li, Z. (2014) Enantioselective trans-dihydroxylation of aryl olefins by cascade biocatalysis with recombinant *Escherichia coli* coexpressing monooxygenase and epoxide hydrolase. *ACS Catal.***4**, 409-420.

Xu, Y, Xu, J-H., Pan, J. and Tang, Y-F. (2004) Biocatalytic resolution of glycidyl aryl ethers by *Trichosporonloubierii* : cell/substrate ratio influences the optical purity of (*R*)-epoxides. *Biotechnol. Lett.*, **26**, 1217-1221.

Xu, Y., Jia, X., Panke, S. and Li, Z. (2009) Asymmetric dihydroxylation of aryl olefins by sequential enantioselective epoxidation and regioselective hydrolysis with tandem biocatalysts. *Chem. Commun.*, 1481-1483.

Xu, Y., Li, A., Jia, X. and Li, Z. (2011) Asymetric *trans*-dihydroxylation of cyclic olefins by enzymatic or chemo-enzymatic sequential epoxidation and hydrolysis in one pot" *Green Chem.*,**13**, 2452-2458.

Xue, Feng, Liu, Z-Q., Zou, S-P. , Wan, N-W., Zhu, W-Y. , Zhu, Q. and Zheng, Y-G. (2014) A novel enantioselective epoxide hydrolase from *Agromyces mediolanus* ZJB120203: Cloning, characterization and application *Process Biochem*. **49**, 409-417.

Yildirim, D., Tükel, S.S., Alptekin, Ö. and Alagöz, D. (2013) Immobilized *Aspergillus niger* epoxide hydrolases: cost-effective biocatalysts for the preparation of enantiopure styrene oxide, propylene oxide and epichlorohydrin. *J. Mol. Catal. B: Enzym.*, **88**, 84–90.

Yoo, S.S., Park, S. and Lee, E.Y. (2008) Enantioselective resolution of racemic styrene oxide at high concentration using recombinant Pichia pastoris expressing epoxide hydrolase of *Rhodotorula glutinis* in the presence of surfactant and glycerol. *Biotech. Lett.*, **30,** 1807-1810.

Yuasa, Y. and Tsuruta, H. (1997) Practical Syntheses of (*S*)-4-Hydroxytetrahydrofuran-2-one, (*S*)-3-Hydroxytetrahydrofuran and Their (*R*)-Enantiomers *Liebigs Ann. Recl.*, **9**, 1877-1879.

Zhang, J., Reddy, J., Roberge, C., Senanayake, C., Greasham, R. and Chartrain, M. (1995) Chiral bio-resolution of racemic indene oxide by fungal epoxide hydrolases. *J. Ferment. Bioeng.*,**80**, 244–246.

Zhao, J., Chu, Y-Y., Li, A-T., Ju, X., Kong, X-D., Pan, J., Tang, Y. and Xu, J-H. (2013) An Unusual (*R*)-Selective Epoxide Hydrolase with High Activity for Facile Preparation of enantiopure Glycidyl Ethers. *Adv. Synth. Catal.*, **353**, 1510-1518.

Zhao, L., Han, B., Huang, Z., Miller, M., Huang, H., Malashock, D.S., Zhu, Z., Milan, A., Robertson, D.E., Weiner, D.P. and Burk, M.J. (2004) Epoxide hydrolase-catalyzed enantioselective synthesis of chiral 1,2-diols via desymmetrization of *meso*-epoxides. *J. Am. Chem. Soc.*, **126**, 11156–11157.

Zheng, H. and Reetz, M.T. (2010) Manipulating the stereoselectivity of limonene epoxide hydrolase by directed evolution based on iterative saturation mutagenesis. *J. Am. Chem. Soc*., **132**, 15744–15751.

Zheng, H., Kahakeaw, D., Acevedo, J.P. and Reetz, M. (2010) Directed evolution of enantioconvergency: the case of an epoxide hydrolase-catalyzed reaction of a racemic epoxide. *ChemCatChem*, **2**, 958–961.