Production of Chiral Epoxides: Epoxide Hydrolase-catalyzed Enantioselective Hydrolysis

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Abstract   Chiral epoxides are highly valuable intermediates, used for the synthesis of pharmaceutical drugs and agrochemicals. They have broad scope of market demand because of their applications. A major challenge in modern organic chemistry is to generate such compounds in high yields, with high stereo- and regio-selectivities. Epoxide hydrolases (EH) are promising biocatalysts for the preparation of chiral epoxides and vicinal diols. They exhibit high enantioselectivity for their substrates, and can be effectively used in the resolution of racemic epoxides through enantioselective hydrolysis. The selective hydrolysis of a racemic epoxide can produce both the corresponding diols and the unreacted epoxides with high enantiomeric excess (ee) value. The potential of microbial EH to produce chiral epoxides and vicinal diol has prompted researchers to explore their use in the synthesis of epoxides and diols with high ee values.

Keywords: chiral epoxides, resolution, epoxide hydrolase, enantioselective hydrolysis

INTRODUCTION

Chiral Epoxides in Organic Chemistry

Epoxides are versatile intermediates formed during organic synthesis of compounds. Epoxides are easily prepared from various starting materials, and the inherent polarity and strain of their three-membered ring structure make them susceptible to react with a large number of reagents like electrophiles, nucleophiles, acids, bases, reducing agents, and some oxidizing agents [1]. Organic chemists have devoted tremendous efforts for the preparation of enantiomerically pure epoxides and to use them in organic synthesis. There are two practical reasons to concentrate on epoxides. Firstly, epoxides are a common structural element in both simple and complex biologically active molecules. Secondly, they undergo facile, stereoselective ring-opening reactions with a wide range of nucleophiles, thus making them versatile starting materials and intermediates for the synthesis of compounds that have interesting biological activity [2]. The chiral feature of epoxides must be taken into account during the ring-opening reaction. A vast majority of compounds with interesting biological functions are chiral, and the different stereoisomers often have different biological activities. As epoxides are involved in the preparation of biologically useful compounds, it is pertinent that epoxides must be stereochemically pure when used as synthetic substrate. There has been a great interest in the application of chiral epoxides for the synthesis of either key intermediates in the preparation of more complex optically pure bioactive compounds or as end products which also have biological activities.

The synthetic applications of chiral epoxides are shown in Table 1. Amongst many other optically active epoxides, aliphatic epoxides have been used as starting materials in the synthesis of new optically active ferroelectric liquid crystals [3], that have been extensively investigated in recent years. From these optically active epoxides, many kinds of optically active alcohols, carboxylic acids and halohydrins have been produced [4]. Optically active 2-methyl-1,2-epoxyalkanes are useful precursors for the synthesis of optically active tertiary alcohols [5], which can be used in the synthesis of pharmaceutics such as prostaglandins. Other optically active epoxides such as styrene oxide, phenyl glycidyl ethers, epichlorohydrin and 3,3,3-trifluoro-1,2-epoxypropane may also find application as intermediates in the synthesis of pharmaceutical drugs and agrochemicals.

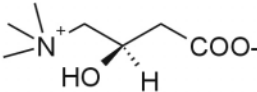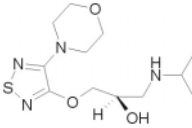### Chemical and Biological Approaches for the Synthesis of Chiral Epoxides
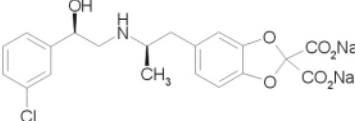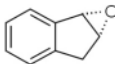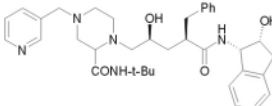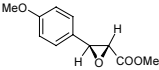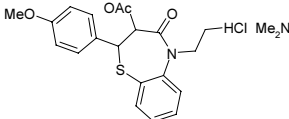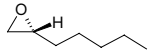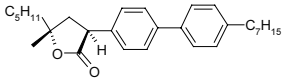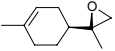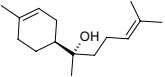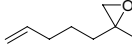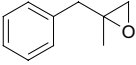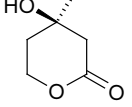
Currently available chemical method for the synthesis of chiral epoxides is asymmetric epoxidation of alkenes, as developed by Sharpless, Jacobsen and Yian Shi. The Sharpless method uses titanium-based catalysts to epoxidize a wide variety of allylic alcohols with optical yields often greater than 90% [6]. This methodology is compatible with a wide range of functionalities and this has led to its extensive use in synthetic chemistry. However, the Sharpless approach suffers a significant drawback, as

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Table 1. Synthetic applications of chiral epoxides [32,33]

Chiral epoxides	Final products	Applications
		(R)-Carnitine, β-hydroxy-γ-N-trimethylaminobutyrate (Vitamin B$_T$) Energy generating compound from fat degradation
		(S)-Timolol β-adrenergic blocking agent antiarrhythmic and antihypertensive drugs
		Antidiabetic and antiobesity agent (CL 316,243)
		HIV Protease inhibitor L-735,524 Indinavir®
		Diltiazem Calcium channel blocker
		γ-lactone compounds for ferroelectric liquid crystal (FLC)
		(4S,8S)-α-Bisabolol used for the preparation of skin-care creams, lotions and ointments
		(S)-(-)-frontalin central aggregation pheromone of pine beetles of the *Dendroctonus* family
		(R)-(-)-mevalonolactone key intermediate from a broad spectrum of cellular biological process and their regulation

the alkenes must have hydroxyl functionality in the allylic position. In contrast to the Sharpless reaction, the asymmetric epoxidation methodologyy developed by Jacobsen and Katsuki, employs optically active (salen)manganese (III) complexes and does not require allylic alcohols [7]. However, the scope of the reaction is limited due to the steric and electronic nature of the catalysts, and hence the appropriate substrates for the synthesis are *cis*-alkenes conjugated with aryl, acetylenic and alkenyl groups. The requirement of a substrate greatly limits the applicability of aforementioned method. Shi Yan's asymmetric epoxidation method, which uses oxiranes derived from potassium peroxomonosulphate (Oxone) and chiral ketones, is effective for the synthesis of *trans*- and disubstituted ole-

fins [8]. However, the use of Oxone and the catalytic efficiency are two barriers that hamper its industrial application. A different strategy of preparing chiral epoxides and diols is *via* hydrolytic kinetic resolution of racemic epoxides. The method currently used in industry is based on the (salen)cobalt catalysts developed by Jacobsen, and is quite efficient on terminal epoxides [9]. However, it is ineffective for the internal epoxides. In addition, it is not applicable for many heteroatom-containing substrates (*e.g.*, pyridyl-type epoxides) due to interference of these atoms with the metal catalysts. All of the chemical methods discussed above are limited in their application for the practical synthesis of chiral compounds because of the factors such as use of expensive metal catalysts, low substrate/

catalyst ratios, and limited efficiency and productivity with varying degrees of enantioselectivities. To overcome these obstacles, attention has been turned towards biocatalysts [10]. Direct stereospecific epoxidation of alkenes by monooxygenases (*e.g.*, cytochromes P450s or other monooxygenases) has previously been reported [11]. This enzyme-catalyzed reaction often gives high enantiomeric excess, but with low yields and requires cofactor. Epoxides may be produced indirectly from alkenes by haloperoxidases, *via* initial halohydrin formation and subsequent ring closure by halohydrin epoxidase [10]. Although these enzymes possess great potential for their use in the synthesis of enantiopure epoxides, there are also severe limitations for their industrial applications as they have low enantioselectivity, complex multi-component structures and generally are not very stable.

Kinetic Resolution of Epoxides *via* Epoxide Hydrolase-catalyzed Enantioselective Hydrolysis

Epoxide hydrolases (EH) are known to be promising biocatalysts for the preparation of chiral epoxides and vicinal diols. They exhibit high enantioselectivity for their substrates, and can be effectively used in the resolution of racemic epoxides prepared by chemical means. As shown in Fig. 1, the selective hydrolysis of a racemic epoxide can generate both the corresponding diols and the unreacted epoxides with high enantiomeric excess (ee) values.

In fact, EHs from mammalian origin have been known and studied for several decades because of their involvement in xenobiotic detoxification processes. However, more recent studies conducted by different groups indicate that EHs are in fact ubiquitous in nature, and found in various living cells such as plants, insects, bacteria, yeast and filamentous fungi. In spite of very interesting results, the use of mammalian EHs is still severely hampered in large-scale industrial production of epoxides. Even overexpressed enzymes are not currently available in large quantities at a reasonable price. Therefore, the use of enzymes from other origins, and in particular from microbial sources which can be cultivated in almost unlimited amounts, was another very promising track for such applications. In fact, the real breakthrough in using EH produced by bacteria or fungi for fine organic chemistry was recently published. In both the cases, it was found that these enzymes could be excellent biocatalysts for achieving the resolution of several racemic epoxides as summarized in Table 2. It can be concluded from the various results that numerous continuous efforts have been devoted for the synthesis of enantiopure epoxides over the last twenty to thirty years which has led to important fundamental knowledge on this topic. The use of EH in enantioselective hydrolysis is commercially attractive because (a) recently they have been shown to be ubiquitous in nature, (b) they are cofactor independent enzymes, (c) they can be produced easily from various microorganisms, (d) they can be partially purified and used as an enzymatic powder without noticeable loss of enzymatic activity upon storage, (e) they can act in the presence of organic solvents, allowing the use of water-
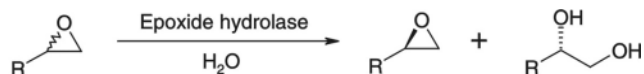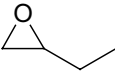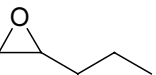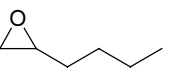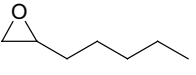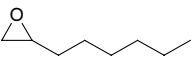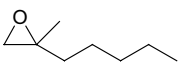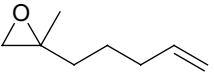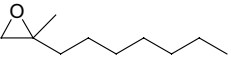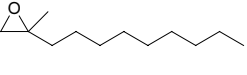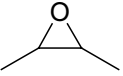

Fig. 1. EH-catalyzed enantioselective hydrolysis for the synthesis of chiral epoxides and diols.

insoluble substrates, and (f) they often lead to excellent ee for the remaining epoxide, but also in certain cases for the diol formed, which can itself be used as such or can be either cyclized back to the enantiopure epoxide or derivatized into reactive epoxide-like chiral synthons (cyclic sulfite or sulfates). The recent practical improvements described by various groups, *i.e.* the possibility of using lyophilized powders of either whole cells or crude enzymatic extracts – which make these biocatalysts "easy-to-use" tools for the organic chemist – as well as the possibility of getting these enzymes to work either in the presence of water-miscible solvents or in a two-liquid-phase system, are obviously highly interesting arguments in favor of the use of these enzymes.
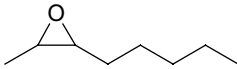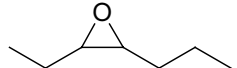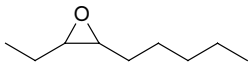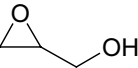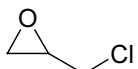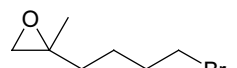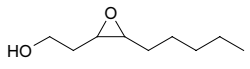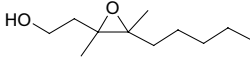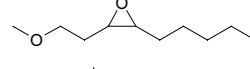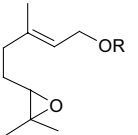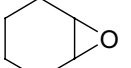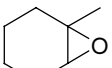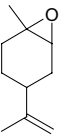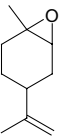
Epoxide Hydrolase

EHs (EC 3.3.2.3) convert epoxides to the more water-soluble and usually less toxic diols, and are therefore key enzymes in the defense mechanism of human against the hazardous properties of xenobiotic compounds [12]. EHs comprise a group of functionally related enzymes that catalyze the addition of water to oxirane compounds, thereby usually generating vicinal *trans*-diols. EHs have been found in all types of living organisms, including mammals, invertebrates, plants, fungi and bacteria. Interest in microbial EH has recently arisen because of the potential of this class of enzymes as enantioselective biocatalysts. Over the last decade, EHs have been characterized and cloned from animals, plants, fungi, yeast and bacteria, as shown in Table 3. Most of these turned out to be members of a large superfamily of enzymes with a common three-dimensional structure, the α/β hydrolase fold enzymes [13]. Amino-acid sequence similarity between the different members of this family is usually very low and mostly restricted to the α/β hydrolase fold domain. For instance, direct comparison between the two mammalian xenobiotic EHs, namely the microsomal EH (mEH) an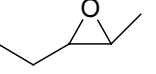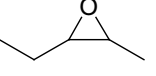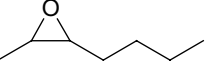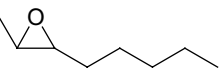d the soluble EH (sEH), does not indicate any phylogenetic relationship between these two enzymes [14]. Both EHs have sequence similarity with bacterial enzyme, haloalkane dehalogenase at different regions. Only with this indirect sequence comparison, they are classified as common phylogeny [15-17]. About fifteen EH genes have been isolated from different organisms, like mammals, insects, plants, fungi, yeast, and bacteria. All enzymes appear to be structurally and mechanistically similar, including two recently cloned bacterial EHs. Many detailed studies have been performed on microsomal and soluble EHs from mammalian origin. They include substrate and inhibitor studies, site-directed mutagenesis studies on catalytic residues, and a recently performed pre-steady-state kinetic analysis with rat mEH. EHs are classified as α/β
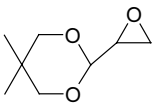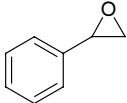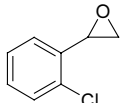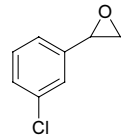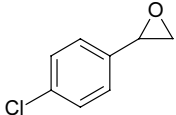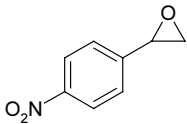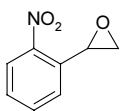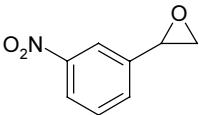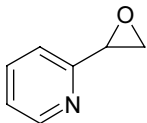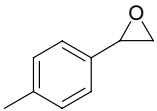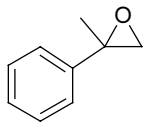
Table 2. Kinetic resolution of epoxides *via* epoxide hydrolase-catalyzed enantioselective hydrolysis

Substrates	Biocatalyst	Epoxide		Diol		Ref
		ee (%)/abs. conf.	yield (%)	ee (%)/abs. conf.	yield (%)	
	Rhodotorula glutinis ATCC 201718	>98/(S)	15	30/(R)	ND	[34]
	Rhodotorula glutinis ATCC 201718	>98/(S)	21	29/(R)	78	[35]
	Rhodotorula glutinis ATCC 201718	>98/(S)	40	66/(R)	54	[35]
	Rhodotorula glutinis ATCC 201718	>98/(S)	48	83/(R)	47	[35]
	Rhodosporidium toruloides UOFS Y-0471	>98/(S)	100 [a]	ND/(R)	ND	[36]
	Rhodotorula glutinis ATCC 201718	>98/(S)	44	73/(R)	52	[35]
	Rhodosporidium toruloides UOFS Y-0471	>98/(S)	100 [b]	ND/(R)	ND	[36]
	Rhodotorula araucariae CBS 6031	>98/(S)	49	87/(R)	48	[37]
	Rhodotorula glutinis ATCC 201718	>98/(S)	38	55/(R)	60	[35]
	Mortierella isabellina ATCC 42613	97/(S)	18	35/(R)	54	[38]
	Rhodosporidium toruloides UOFS Y-0471	>98/(S)	100 [c]	ND/(R)	ND	[36]
	Chryseomonas luteola	98/(S)	38	86/(R)	44	[39]
	Aspergillus niger LCP 521	99/(S)	22	32/(R)	62	[38]
	Nocardia TB1	>99/(R)	>200 [d]	>99/(S)	ND	[40]
	Nocardia EH1	96/(R)	2	98/(S)	98	[41]
	Nocardia EH1	99/(R)	3	99/(S)	97	[41]
	Rhodococcus sp. NCIMB 11216	25/(R)	ND	98/(S)	ND	[42]
	Rhodococcus sp. NCIMB 11216	55/(R)	ND	>99/(S)	ND	[42]
	Rhodotorula glutinis ATCC 201718	>98/(2R,3R)	47	meso	ND	[34]
	Xanthobacter Py2	>98/(2R,3R)	>45	ND	ND	[43]
	Rhodotorula glutinis ATCC 201718	ND/meso	<2	90/(2R,3R)	ND	[34]
	Rhodotorula glutinis ATCC 201718	>98/(2R,3R)	48	54/(2S,3R)	ND	[34]
	Rhodotorula glutinis ATCC 201718	>98/(2R,3S)	48	>98/(2R,3R)	ND	[34]
	Rabbit liver microsomal epoxide hydrolase	>98/(2R,3S)	ND	>98/(2R,3R)	79	[44]
	Nocardia EH1	ND	ND	90/(2R,3R)		[44]
	Mortierella isabellina ATCC 42613	98/(1R,2R)	11	59/(1R,2S)	62	[38]
	Chaetomium globosum LCP 679	97/(1S,2S)	12	78/(1R,2S)	60	[38]
	Syncephalastrum racemosum	>98/(1R,2R)	ND	ND	ND	[45]
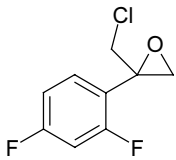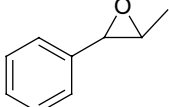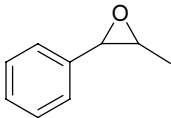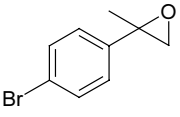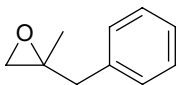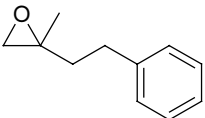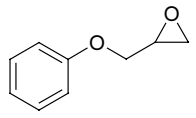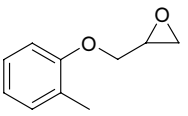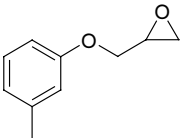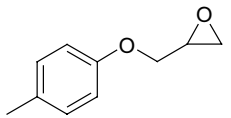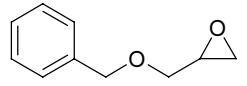
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Substrates	Biocatalyst	Epoxide		Diol		Ref
		ee (%)/abs. conf.	yield (%)	ee (%)/abs. conf.	yield (%)	
	Chaetomium globosum LCP 679	97/(1*R*,2*S*)	8	58/(1*R*,2*R*)	59	[38]
	Rabbit liver microsomal epoxide hydrolase	14/ND	ND	>98/(3*R*,4*R*)	ND	[46]
	Rabbit liver microsomal epoxide hydrolase	56/(3*R*,4*S*)	ND	>98/(3*R*,4*R*)	ND	[46]
	Aspergillus niger *Acetobacter pasteurianus*	5.4/(*R*) >99/(*R*)	41 16 [e]	ND ND	ND ND	[47] [48]
	Aspergillus niger *Rhodotorula glutinis* ATCC 201718 Recombinant *Pichia pastoris* from *Rhodotorula glutinis*	100/(*S*) >98/(*R*) 100/ (R)	20 10 26	ND 22/(*S*) ND	ND ND ND	[49] [34] [81]
	Nocardia H8	>99/(*R*)	>200[f]	>99/(*S*)	ND	[50]
	Rabbit liver microsomal epoxide hydrolase	40/(3*R*,4*S*)	ND	90/(3*R*,4*R*)	ND	[46]
	Rabbit liver cytosolic prep. (sEH)	>98/(3*R*,4*S*)	ND	>98/(3*R*,4*R*)	ND	[51]
	Rabbit liver microsomal epoxide hydrolase	>98/(3*R*,4*S*)	ND	80/(3*R*,4*R*)	ND	[46]
	Aspergillus niger	97/(*S*)	ND	40/(*S*)	47	[52]
	Rhodotorula glutinis ATCC 201718	Meso	<2	>98/(1*R*,2*R*)	ND	[34]
	Rhodotorula glutinis ATCC 201718	Meso	<2	90/(1*R*,2*R*)	ND	[34]
	Corynebacterium sp. C12	95/(1*S*,2*R*)	42	ND	ND	[53]
g 	*Rhodotorula glutinis* ATCC 201718 *Rhodococcus erythropolis* DCL14	>98/(1*S*,2*R*,4*S*) ND	48 ND	>98/(1*R*,2*R*,4*S*) 100/(1*R*,2*R*,4*S*)	ND ND	[34] [54]
h 	*Rhodotorula glutinis* ATCC 201718 *Rhodococcus erythropolis* DCL14	>98/(1*S*,2*R*,4*R*) ND	28 ND	30/(1*R*,2*R*,4*R*) 100/(1*S*,2*S*,4*R*)	ND ND	[34] [54]
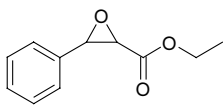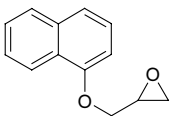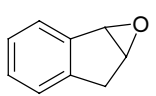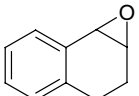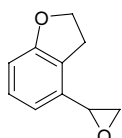
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Substrates	Biocatalyst	Epoxide		Diol		Ref
		ee (%)/abs. conf.	yield (%)	ee (%)/abs. conf.	yield (%)	
	Aspergillus niger	99/(*R*)	40	92/(*R*)	42	[55]
	Aspergillus niger LCP 521	100/(*S*)	32	ND	ND	[47]
	Rhodotorula glutinis ATCC 201718	>98/(*S*)	18	48/(*R*)	ND	[34]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	99/(*S*)	33	ND	ND	[56]
	Aspergillus niger LCP 521	99/(*S*)	28	65/(*R*)	50	[57]
	Beauveria sulfurescens ATCC 7159	98/(*R*)	34	83/(*R*)	45	[57]
	Aspergillus niger LCP 521 and *Beauveria sulfurescens* ATCC 7159	ND	ND	89/(*R*)	92	[58]
	Rhodosporidium kratochvilovae SYU-08	>99/(*S*)	38	ND	ND	[82]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(*S*)	35	ND	ND	[56]
	Human soluble epoxide hydrolase (sEH)	>98/(*R*)	ND	ND	ND	[45]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(*S*)	27	ND	ND	[56]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(*S*)	34	ND	ND	[56]
	Aspergillus niger	>99/(*S*)	44	66/(*R*)	49	[59] [60]
	Aspergillus niger CGMCC0496	98/(*S*)	34	>99/(R)	38	[61]
	Trichosporon loubierii ECU 1040	97/(R)	41	74/(S)	42	[62]
	Trichosporon loubierii ECU 1040	>98/ND	29	ND	ND	[62]
	Agrobacterium radiobacter AD1	>99/(S)	35	71/(R)	57	[28]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(*S*)	36	ND	ND	[56]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(*S*)	27	ND	ND	[56]

(Continued)

Substrates	Biocatalyst	Epoxide		Diol		Ref
		ee (%)/abs. conf.	yield (%)	ee (%)/abs. conf.	yield (%)	
(structure: 2-(chloromethyl)-2-(2,4-difluorophenyl)oxirane)	*Aspergillus niger* (commercial EH, Fluka)	>99/(S)	42	95/(R)	44	[63]
(structure: 2-methyl-3-phenyloxirane, trans)	*Rhodotorula glutinis* ATCC 201718	>98/(1R,2R)	45	>98/(1R,2S)	ND	[34]
	Aspergillus terreus	>98/(1R,2R)	ND	ND	ND	[45]
(structure: 2-methyl-3-phenyloxirane, cis)	*Aspergillus terreus*	>98/(1S,2R)	ND	ND	ND	[45]
	Beauveria sulfurescens ATCC 7159	20/(1R,2S)	42	99/(1R,2R)	42	[64]
(structure: 2-(4-bromophenyl)-2-methyloxirane)	*Aspergillus niger*	>99/(S)	39	96/(R)	49	[65]
(structure: 2-benzyl-2-methyloxirane)	*Nocardia* EH1	75/(R)	42 [i]	90/(S)	ND	[40]
(structure: 2-methyl-2-phenethyloxirane)	*Nocardia* TB1	45/(R)	13 [j]	80/(S)	ND	[40]
(structure: 2-(phenoxymethyl)oxirane)	*Aspergillus niger*	100/(R)	26	ND	ND	[47]
	Recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1	>99/(R)	28	ND	ND	[56]
(structure: 2-((o-tolyloxy)methyl)oxirane)	*Aspergillus niger*	100/(R)	29	ND	ND	[47]
(structure: 2-((m-tolyloxy)methyl)oxirane)	*Aspergillus niger*	100/(R)	17	ND	ND	[47]
(structure: 2-((p-tolyloxy)methyl)oxirane)	*Aspergillus niger*	100/(R)	23	ND	ND	[47]
(structure: 2-((benzyloxy)methyl)oxirane)	*Aspergillus niger*	100/(R)	28	ND	ND	[47]
	Rhodotorula glutinis ATCC 201718	>98/(R)	14	33/(S)	ND	[34]

(Continued)

Substrates	Biocatalyst	Epoxide		Diol		Ref
		ee (%)/abs. conf.	yield (%)	ee (%)/abs. conf.	yield (%)	
(structure)	*Pseudomonas* sp. BZS21	95/(2R,3S)	26	ND	ND	[66]
(structure)	*Aspergillus niger*	100(R)	1	ND	ND	[47]
(structure)	*Rhodotorula glutinis* ATCC 201718	>98/(1R,2S)	22	54/ND	ND	[34]
	Beauveria sulfurescens ATCC 7159	>98/(1R,2S)	20	69/(1R,2R)	48	[67]
	Diplodia gossipina ATCC 16391	100/(1S,2R)	14	ND	ND	[67]
(structure)	*Beauveria sulfurescens* ATCC 7159	>98/(1R,2S)	38	77/(1R,2R)	49	[64]
(structure)	*Rhodotorula glutinis* SC 16293	>99/(S)	45	ND	ND	[68]
	Aspergillus niger SC 16311	97/(S)	45	ND	ND	[68]

a, b, c, d, e, f, i, j: Enantiomeric ratio (E), g: (-)-limonene-1,2-epoxide, h: (+)-limonene-1,2-epoxide, ND: not determined.

hydrolase fold enzymes. The topology of this class of enzymes shows two domains. A main domain consisting of a central β-sheet surrounded by α-helices involves in substrate binding [18]. The mammalian EH contain additional *N*-terminal domain which has sequence similarity to either another class of hydrolytic enzymes or a membrane anchor protein. The cloned bacterial EHs are simple enzymes, as they do not contain the extra *N*-terminal domains [19]. The α/β hydrolase fold enzymes hydrolyse their substrates by the action of a catalytic triad in a two-step mechanism. In all the EHs, an aspartic acid residue acts as a catalytic nucleophile that attacks the substrate (epoxide ring), and leads to the intermediate formation of a substrate-enzyme ester [20,21]. This intermediate is subsequently hydrolyzed by a water molecule in the catalytic centre of the enzyme, which is activated by a so-called charge-relay system composed of a proton-abstracting histidine residue that is supported by hydrogen bonding to an acidic residue, as depicted in Fig. 2 [22-25].

Preparative Scale EH-catalyzed Resolution of Epoxides

EH-mediated chiral resolutions were scaled up to gram scale in order to demonstrate the usefulness of EH for organic synthesis. EH from whole cells of *Rodotorula glutinis* was employed for the preparative resolution of 1,2-epoxyhexane using a cascade hollow-fiber membrane bioreactor [26]. Dodecane was used as an organic phase in order to manage the chemical instability and low solubility of the substrate (epoxide) in the aqueous phase. Moreover, the choice of the membrane reactor was made in order to minimize the toxicity of the organic solvent towards the EH of the yeast and to continuously remove the inhibitory amounts of the formed diol. In addition, continuous mode of operation was also carried out using the membrane bioreactor with reasonable ee and yield [27]. Another interesting case is the preparation of (*S*)-2-pyridyloxirane using *Aspergillus niger*. It is to be emphasized that this product could not be obtained in a satisfactorily enantiopure form using conventional chemistry approaches, and the biocatalyzed resolution could be achieved even by using plain water instead of a buffer solution [28].

Directed Evolution and High-throughput Screening

Several EHs are enantioselective in nature and have been tested in kinetic resolutions to obtain an enantiopure epoxide and an optically active diol with the opposite stereochemistry at the chiral center. The yield of the remaining substrate and the enantiopurity of the diol are often not very high due to the low enantioselectivity of the enzyme. Therefore, it is desirable to improve the enantioselectivity of EHs. With *Agrobacterium radiobacter* EH, rational design using site-directed mutagenesis of the ring-opening tyrosines resulted in 2- to 5-fold improved enantioselectivity towards a number of aromatic

Table 3. Characterization of epoxide hydrolases from microbial sources

Origin [ref.]	structure	size	characteristics
Corynebacterium sp. C12 [69]	multimeric (probably tetrameric) α/β-hydrolase-fold family	32,140 Da (subunit size)	similar to mammalian, plant sEH and EH from *A. radiobacter* AD1 constitutively expressed at low level induced by cyclohexene oxide no enantioselectivity
Pseudomonas sp. AD1 [70,71]	monomeric α/β-hydrolase-fold family	34 kDa (294 aa)	later identified as *A. radiobacter* AD1 constitutively expressed induced by epichlorohydrin
Rhodosporidium toruloides CBS 0349 [72,73]	monomeric	about 54 kDa	highly glycosylated act analogous to lipases enantioselectivity
Rhodotorula glutinis ATCC 201718 [74,75]	homodimer	46.3 kDa (subunit size, 409 aa) ORF 1,230 bp interrupted by 9 introns	membrane-associated similar to mEH enantioselectivity α/β-hydrolase-fold family homology with mEH
Rhodococcus sp. NCIMB 11216 [76]	monomeric	about 35 kDa	soluble constitutively expressed enantioselectivity
Rhodococcus erythropolis DCL 14 [77,78]	monomeric	16.5 kDa (149 aa)	cytoplasmic enzyme, new class of EH absence of any significant homology with other known EHs induced by monoterpenes
Xanthophyllomyces dendrorhous [79]	ND	46,185 Da (411 aa) ORF 1236bp interrupted by 8 introns	membrane-associated α/β-hydrolase-fold family homology with mEH enantioselectivity
Aspergillus niger LCP 521 [80]	dimer	two 44 kDa subunits 9 exons and 8 introns	sequence similarity with mEH lacks membrane anchor, soluble enantioselectivity
Nocardia sp. EH1 [44]	monomer	about 34 kDa	ND

epoxides [29]. Recently, the enantioselectivity of the EH from *Aspergillus niger* towards phenyl glycidyl ether was improved 2-fold by random mutagenesis, but the enantioselectivity of the best mutant (E=10.8) was not higher than that of the *Agrobacterium radiobacter* wild-type enzyme (E=11) [30]. The enantioselectivity of EH from *Agrobacterium radiobacter* was improved using error-prone PCR and DNA shuffling. An agar plate assay was used to screen the mutant libraries for EH activity. Screening for improved enantioselectivity was subsequently done by spectrophotometric progress curve analysis of the conversion of para-nitrophenyl glycidyl ether (pNPGE). Kinetic resolutions showed that eight mutants were obtained with up to 13-fold improved enantioselctivity towards pNPGE and with at least three other epoxides. The large enhancement in enantioselectivity towards epichlorohydrin and 1,2-epoxyhexane indicated that pNPGE acts as an epoxyalkane mimic. Active site mutations were found in all the shuffled mutants, which can be explained by an interaction of the affected amino acid with the epoxide oxygen or the hydrophobic moiety of the substrate. Several mutations in the shuffled mutants had additive effects [31].

CONCLUSION

Chemical compounds that are utilized in both established and emerging chemical, pharmaceutical, agrochemical, and fine chemical markets must meet stringent economical and environmental standards. Expensive processes, which produce harmful byproducts and which suffer from poor or inefficient catalysis, often hamper the synthesis of target products. Enzymes have a number of remarkable advantages, which can overcome these problems in catalysis, like they can act on single functional groups, and can distinguish between similar functional groups on a single molecule, and also between enantiomers. In addition, they are biodegradable in nature and function at very low mole fractions in reaction mixtures. Moreover, because of their chemo-, regio- and stereo-specificity, they present a unique opportunity to optimally achieve desired selective
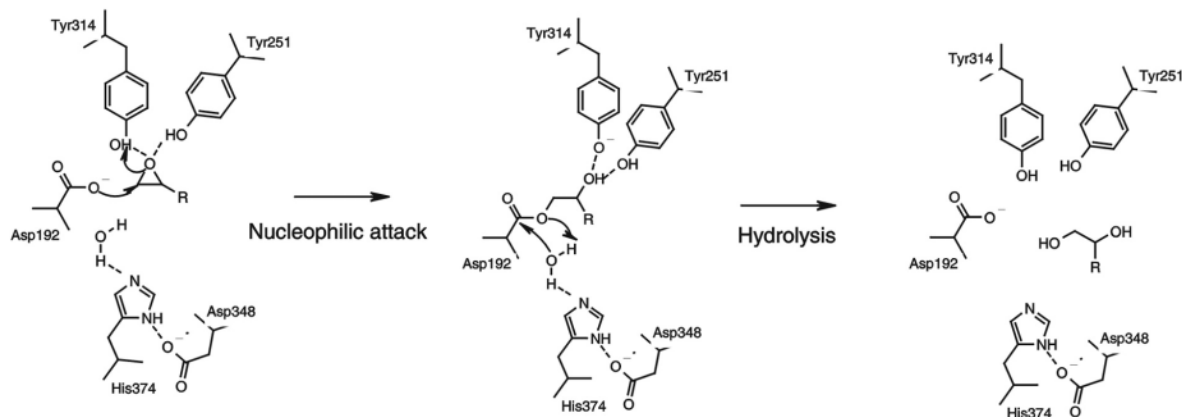
Fig. 2. Mechanism of epoxide hydrolysis by EH from *Aspergillus niger*; residues are shown as close as possible to their actual relative orientation and position. Initial binding and positioning of the substrate in the active center of the enzyme is thought to be directed by hydrogen bonding between the epoxide ring oxygen and the hydroxyl groups of Tyr251 and Tyr314. In the first step of the enzymatic reaction, the carboxylate side chain of the catalytic nucleophile Asp192 attacks the unsubstituted carbon atom of the oxirane ring, forming an ester bond and opening the ring. The process is facilitated through simultaneous proton donation by one of the tyrosine residues. Both the parts together comprise a classic push-pull mechanism. The second step of the reaction consists of the hydrolysis of the ester intermediate *via* the attack of a water molecule. The water molecule is activated by proton abstraction by the His374/Asp348 charge-relay system, possibly further assisted by Glu123 (not shown).

transformations. The elimination of the need for selectivity, the ability to carry out multi-step transformations in a single reaction vessel, along with the concomitant reduction in environmental burden, has led to the increased demand for enzymes in chemical and pharmacetical industries. Enzyme-based processes have been gradually replacing many conventional chemical-based methods. A current limitation to more widespread industrial use is primarily due to the relatively small number of commercially available enzymes. Amongst more than 3,000 catalytically active enzymes, at present, only about 300 enzymes are commercially available. The potential demonstrated by the microbial EHs has prompted researchers to explore their use in the synthesis of epoxides and diols with high ee values. However, several obstacles must be overcome before a broad industrial platform for EH-catalyzed synthesis of epoxides and diols can be realized. Firstly, the number of enzymes available is still small and those that are promising in synthetic applications are even more rare. In particular, only two kinds of EHs, originated from microbial sources, like *Aspergillus niger* and *Rhodococcus rhodochrous* are currently commercially available from Fluka. Current discovery of new EHs through screening available strains is hampered by limited culture collections and the lack of powerful screening assays. Secondly, the available enzymes have limited substrate scope. Lastly, in most of the preparations, high concentrations of enzymes (either whole cells or crude extract) and rather low substrate concentrations had to be used because of the enzymes' low catalytic efficiency. Regarding current market of industrial biocatalysts, novel EH is urged to be developed for a practical application of EH-based resolution for enantiopure epoxides production. Based on the rapid growing demand for chiral epoxides, development of biocatalytic process using novel EH-catalyzed enantiose-

lective hydrolysis will lead to a breakthrough in the production process of chemical and pharmaceutical industry.

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