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Microbial Epoxide Hydrolases†

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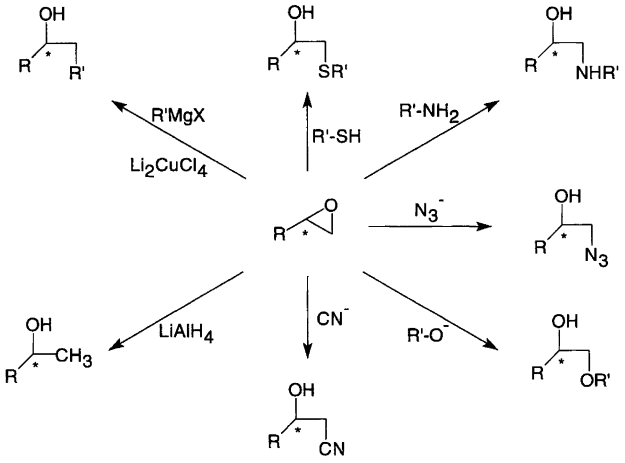
Chiral epoxides and 1,2-diols, which are central building blocks for the asymmetric synthesis of bioactive compounds, can be obtained by using enzymes, which catalyse the enantioselective hydrolysis of epoxides – epoxide hydrolases. These biocatalysts are more widely distributed in fungi and bacteria than previously expected, and sufficient sources from bacteria, such as *Rhodococcus* and *Myobacterium* sp., or fungi, for instance *Aspergillus* and *Beauveria* sp. have recently been identified. The reaction proceeds via an S_N2-specific opening of the epoxide leading to the formation of the corresponding *trans*-configurated 1,2-diols. For the resolution of 2-monosubstituted epoxides and for 2,2-disubstituted substrates fungal cells and several bacteria, respectively, have been shown to possess excellent selectivities. In addition, the use of non-natural nucleophiles such as azide or amine provides access to chiral azido- and amino-alcohols. The synthetic potential of these enzymes is illustrated with recent examples of kinetic resolutions of epoxides from the literature.

1. Introduction

Chiral epoxides and vicinal diols (employed as their corresponding cyclic sulfate or sulfite esters as reactive intermediates) are extensively employed, high-value intermediates in the synthesis of chiral compounds due to their ability to react with a broad variety of nucleophiles (Schemes 1 and 2). In recent years a lot of research has been devoted to the development of catalytic methods for their production.[1,2] The Katsuki–Sharpless method for the asymmetric epoxidation of allylic alcohols[3] and the asymmetric dihydroxylation of alkenes[2] are now widely applied and reliable procedures. Catalysts for the epoxidation of non-functionalized olefins have been developed more recently.[4,5] Although high selectivities have been achieved for the epoxidation of *cis*-alkenes, the selectivities achieved with *trans*- and terminal olefins are less satisfactory using the latter methods.

On the other hand, a number of biocatalytic methods[6–9] have been reported to provide a useful arsenal of methods as valuable alternatives to the above-mentioned techniques. For instance, prochiral or racemic synthetic precursors of epoxides, such as halohydrins, can be asymmetrized or resolved *via* enantioselective ester hydrolysis or esterification by using hydrolytic enzymes,[10,11] in particular esterases and lipases (Scheme 3). This methodology is well developed and high selectivities have been achieved in particular for esters of secondary alcohols, but it is impeded by the requirement of regioisomerically pure halohydrins. α-Haloacid dehalogenases catalyze the S_N2-displacement of a halogen atom at the α-position of carboxylic acids by hydroxy with inversion of configuration, leading to the corresponding α-hydroxy acid.[12] Two drawbacks of α-halo acid dehalogenation are (i) the in-



Scheme 1. Reaction of epoxides with nucleophiles.

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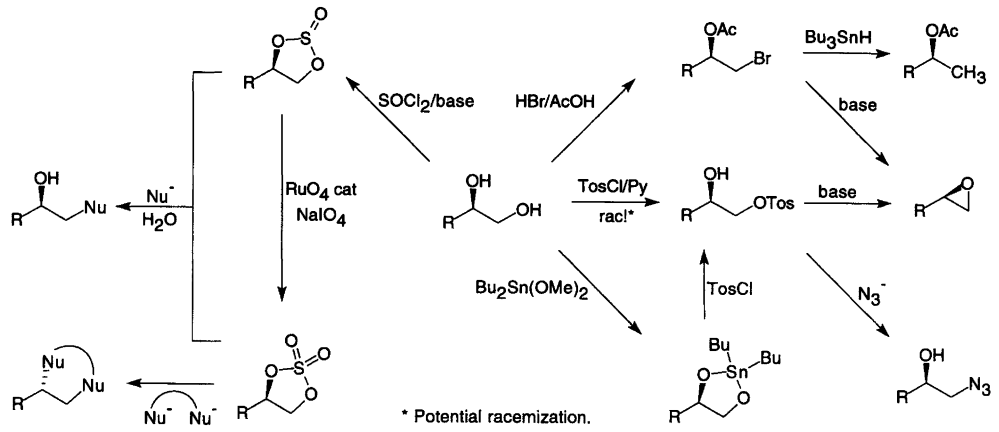
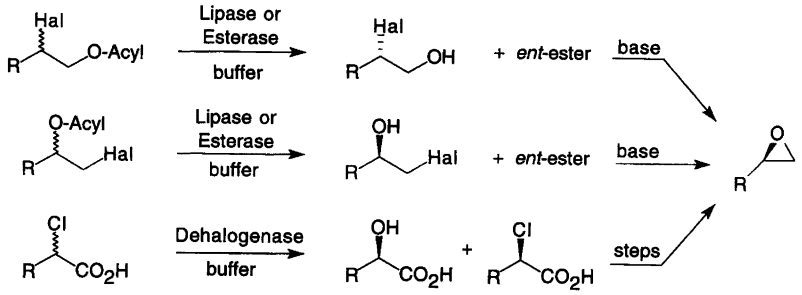


Scheme 2. Syntheses from chiral 1,2-diols.



Scheme 3. Enzymatic syntheses of epoxides using hydrolytic enzymes.

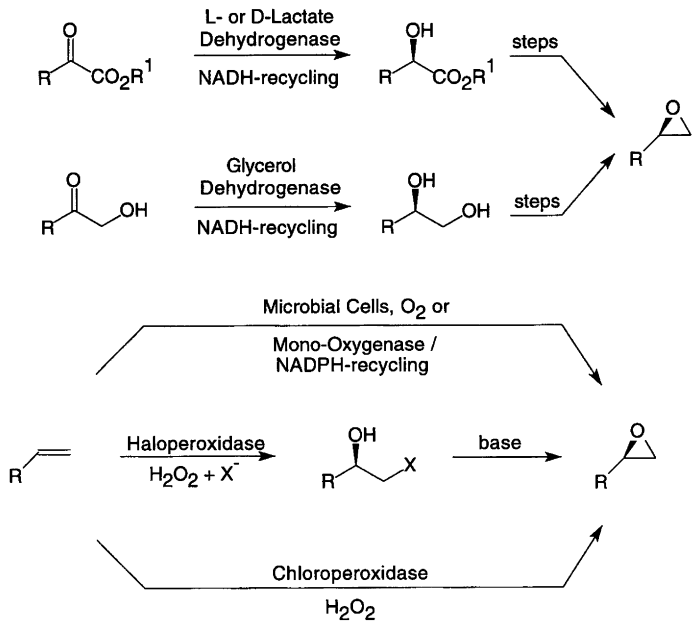
stability of the substrates in aqueous systems – and in particular α-bromoacids – and (ii) the limited substrate tolerance, as only short-chain haloacids are accepted.[13]

Asymmetric biocatalytic reduction of α-keto-acids[14] or -alcohols[15] using D- or L-lactate dehydrogenase or glycerol dehydrogenase, respectively, gives rise to chiral α-hydroxy acids and 1,2-diols, which in turn can be converted into the corresponding epoxides using conventional methodology. Although excellent selectivities are generally achieved, the need for the recycling of redox-cofactors such as NAD(P)H has restricted the number of applications (Scheme 4). Likewise, biocatalytic asymmetric epoxidation of alkenes catalyzed by mono-oxygenases cannot be performed on a preparative scale with isolated enzymes owing to their complex nature and their dependence on a redox cofactor, such as NAD(P)H. Thus whole microbial cells are used instead. Although the toxic effects of the epoxide formed and its further (undesired) metabolism by the cells can be reduced by employing biphasic media, this method is not trivial and requires high bioengineering skills.[16] On the other hand, haloperoxidases are independent of nicotinamide-cofactors, as they produce hypohalous acid from H_2O_2 and halide, which in turn yields a halohydrin from an alkene (Scheme 4). These enzymes are rare in Nature and exhibit usually low selectivities owing to the fact that halohydrin-formation can take place not only in the active site of the enzyme but also without enzyme catalysis.[17] Similar low selectivities have been observed with halohydrin epoxidases, which act like a 'biogenic chiral base' by converting a halohydrin into the corresponding epoxide.[18] Similarly, peroxidases, such as chloroperoxidase, are cofactor-independent and can be used in isolated form for the enzymatic epoxidation of alkenes. Although excellent selectivities were obtained with internal *cis*-olefins, long-chain substrates and terminal alkenes were unreactive.[19]

A valuable alternative to the above existing methods is the use of cofactor-independent epoxide-hydrolases [EC 3.3.2.X]. Although enzymes from mammalian sources – such as liver tissue – have been investigated in great detail during detoxification studies,[20] biotransformations on a preparative scale are hampered by the limited supply of enzyme and they rarely surpass the millimole range.[21] It was only recently that microbial sources for highly selective epoxide hydrolases were identified, which allow an (almost) unlimited supply of these enzymes for preparative-scale applications.

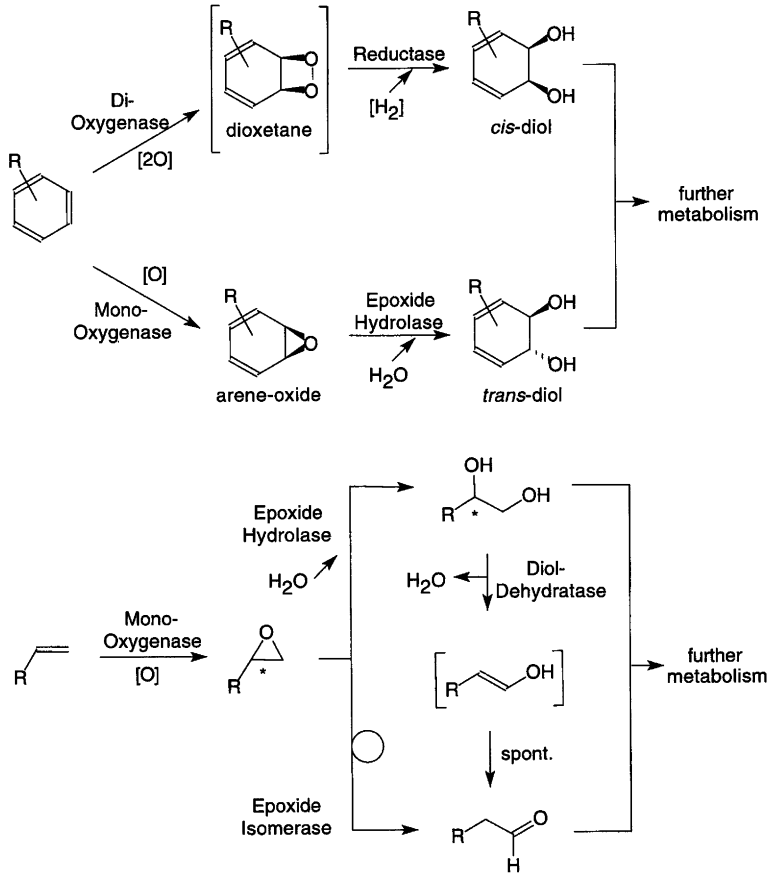
2. Biological role of epoxide hydrolases

In eukaryotes, epoxide hydrolases play a key role in the metabolism of xenobiotics, in particular of aromatic systems.[22,23] On the other hand, these enzymes are essential for the utilization of alkenes as carbon-source in prokaryotes. In living cells, aromatics can be metabolized via two



Scheme 4. Enzymatic syntheses of epoxides using dehydrogenases, mono-oxygenases and peroxidases.

different pathways (Scheme 5). (i) Di-oxygenases catalyze the cycloaddition of molecular oxygen to the C = C double bond by forming a (putative) dioxetane. The latter species is detoxified via reductive cleavage of the O–O



Scheme 5. Epoxide hydrolases in the biodegradation of alkenes and aromatics.

bond yielding a physiologically more innocuous *cis*-1,2-diol. (ii) Alternatively, a mono-oxygenase can introduce a single O-atom into an aromatic system which forms a highly reactive arene oxide. The latter is metabolized via hydrolysis catalyzed by an epoxide-hydrolase to yield a *trans*-1,2-diol. Alkenes can be metabolized in an analogous fashion, i.e. via an epoxide intermediate, which in turn is hydrolyzed to the corresponding 1,2-diol. The latter is degraded either by oxidation or by elimination of water via catalysis of a diol-dehydratase, which leads to an aldehyde.[24] The same species is obtained via direct rearrangement of the epoxide by an epoxide isomerase.[25] Until recently, it was generally accepted that epoxide hydrolases are amply found in eukaryotic cells, such as mammals and fungi, but are rare in (prokaryotic) bacteria. A careful investigation of the literature, however, reveals that this view was certainly too simplistic, considering the fact that bacterial epoxide hydrolases have been isolated from *Corynebacterium*,[26] *Pseudomonas*,[27,28] *Bacillus megaterium*[29] and most recently also from *Rhodococcus* sp.[30]

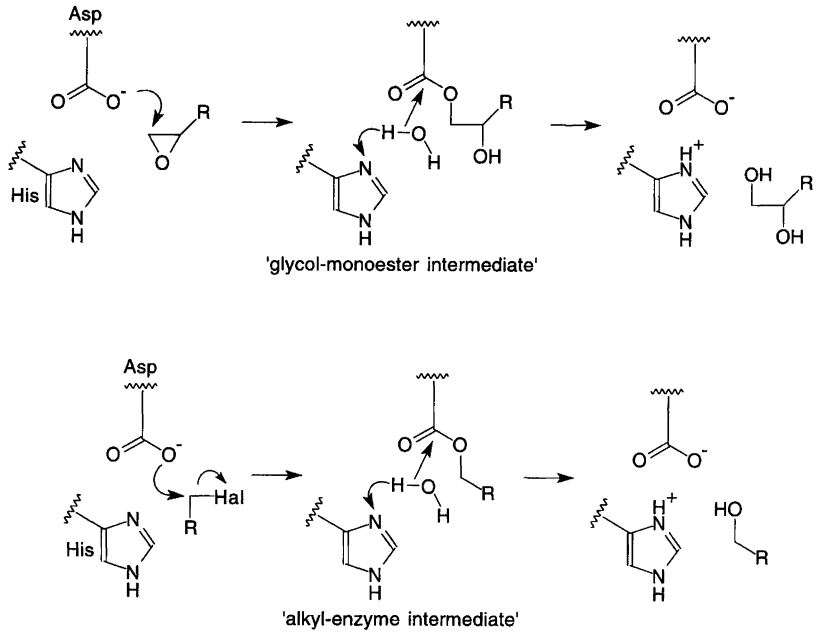
3. Epoxide hydrolase mechanism

The mechanism of epoxide hydrolases, which require neither prostetic groups nor metal ions, has long been debated, and it was formerly assumed, that is comprised a nucleophilic opening of the oxirane ring by a hydroxide ion, which in turn is provided by the aid of a histidine.[31] It was only recently that convincing evidence was provided (at least for microsomal epoxide hydrolase) showing that the reaction occurs via a covalent glycol–monoester–enzyme intermediate[32,33] (Scheme 6). Thus, the epoxide is opened by a nucleophilic attack of an asparate residue by forming a mono-ester of the corresponding 1,2-diol. The latter is subsequently hydrolyzed by hydroxide ion which is provided by water by proton abstraction via a histidine, with concomitant release of the diol. This mechanism shows striking similarities to that of haloalkane dehalogenases, where a halide is displaced by an aspartate in a similar manner. Thus, a closely related alkyl-enzyme intermediate is formed, which is hydrolyzed by OH^- which in turn is provided by a histidine.[34,35] Furthermore, a mechanistic relationship with β-glycosidase, which acts via formation of a covalent glycosyl–enzyme intermediate by retaining the configuration at the anomeric center, may exist.[36]

As a consequence, the epoxide is generally opened in a *trans*-specific manner with one oxygen from water being incorporated into the substrate.[37] For instance, (±)-*trans*-epoxysuccinate was converted to the *meso*-tartrate by an epoxide hydrolase isolated from *Pseudomonas putida*,[37] and in a complementary fashion, *cis*-epoxysuccinate gave D- and L-tartrate, albeit in low optical purity.[38] In addition, it was shown that only one O-atom is derived from water by means of $^{18}OH_2$-experiments using both mammalian epoxide hydrolases[39] and whole fungal cells.[40]

Although two cases for reactions proceeding via a formal *cis*-hydration process have been reported,[41,42] they seem to be rare exceptions and – given the present knowledge of enzyme mechanisms – attempts to explain this phenomenon remain rather speculative.[42]

The above-mentioned facts have important consequences on the stereochemical outcome of the kinetic resolution of asymmetrically substituted epoxides. In contrast with the majority of kinetic resolution of esters (e.g.,



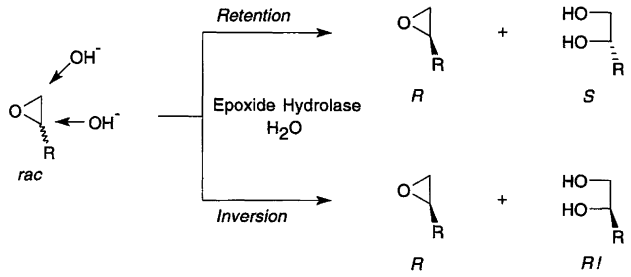
Scheme 6. Mechanism of microsomal epoxide hydrolase and of haloalkane dehalogenase.

by ester-hydrolysis and -synthesis using lipases, esterases and proteases) where the absolute configuration of the stereogenic centre(s) always remains the same throughout the reaction, the enzymatic hydrolysis of epoxides may take place *via* two different pathways (Scheme 7): (i) attack of the (formal) hydroxide ion on the less shielded oxirane carbon atom affecting *retention* of configuration (e.g. by *Aspergillus niger* cells) or (ii) attack on the stereogenic centre, which leads to *inversion* (with *Beauveria* sp.).[43] Although retention of configuration seems to be the more common pathway, inversion of configuration has been reported in some cases.[39,43,44] As a consequence, the absolute configuration of *both* the product and substrate from a kinetic resolution of a racemic epoxide has to be determined.

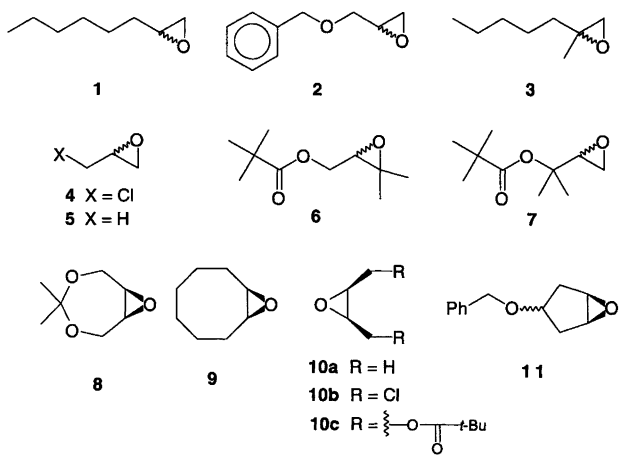
4. Screening for bacterial epoxide hydrolases

It was only recently that a detailed search for epoxide hydrolases from microbial sources has been undertaken by the group of Furstoss[43,45] and in our laboratory,[38,46,47] bearing in mind that the use of microbial enzymes allows an (almost) unlimited supply of biocatalyst. The use of bacteria seemed to be particularly attractive to us due to the fact that (i) they do not tend to form dense mycelia, which may impede agitation of large-scale reactions when whole-cell systems are employed, and (ii) cloning of bacterial enzymes is generally less problematic. Although several micro-organisms are known to possess epoxide hydrolases, the number of applications for preparative organic transformations reported to date is remarkably small.[38,43,45–52] In the majority of cases, the hydrolysis of an epoxide was observed during the microbial epoxidation an alkene, and it was not further investigated since it constitutes an undesired side reaction through degradation of the product.[16]

Our search for microbial epoxide hydrolases was triggered by an observation during a study on the chemoselective hydrolysis of nitriles employing a crude enzyme preparation derived from *Rhodococcus* sp.,[53,54] where we observed an undesired enzymatic hydrolysis of an epoxide moiety. As a consequence, we started our search for microbial epoxide hydrolases with a screening by employing a variety of lyophilized bacterial cells.[55] The selection of strains was done in accord with two arguments. Firstly, *Rhodococcus* spp. were particularly attractive owing to the (purely coincidental) epoxide-hydrolase activity of the Novo nitrilase preparation. Secondly, we anticipated that those strains, which are capable of epoxidizing alkenes with high enantioselectivities, would also possess a corresponding epoxide hydrolase with 'matching' selectivity for the further degradation of the toxic intermediate. Since enzyme induction is still a largely empirical task, cells were grown on non-optimized standard media. Thus, a set of representative substrates with various structural steric requirements (**1–11**, Scheme 8) were subjected to microbial hydrolysis employing lyophilized whole bacterial cells in Tris-buffer at pH 7–8. The results of the screening are summarized in Table 1. In contrast with mammalian systems, where, in general, epoxide hydrolases have to be induced (e.g. by feeding rats with phenobarbital), four *Rhodococci* and one *Myobacterium* sp. exhibited sufficient activity even when the cells were grown on a non-optimized standard medium. Both of the 2-mono- and the 2,2-di-substituted epoxides (**1–3**) were



Scheme 7. Microbial hydrolysis of epoxides proceeding with retention or inversion of configuration.



Scheme 8. Substrates and non-substrates for bacterial epoxide hydrolases.

Table 1. Screening of substrates.[a]

Micro-organism	(±)-1	(±)-2	(±)-3
Rhodococcus sp. NCIMB 11215	+	+	+
Rhodococcus sp. NCIMB 11216	+	+	+
Rhodococcus sp. NCIMB 11540	+	+	+
Rhodococcus equi IFO 3730	+	−	+
Mycobacterium paraffinicum NCIMB 10420	+	−	+
Pseudomonas aeruginosa NCIMB 9571	−	n.d.	−
Pseudomonas aeruginosa mucosus ATCC 17933	−	n.d.	−
Pseudomonas oleovorans ATCC 29347	−	n.d.	−
Corynebacterium glutamicum ATCC 13032	−	n.d.	−

[a] + Denotes activity, − no activity, n.d. = not determined.

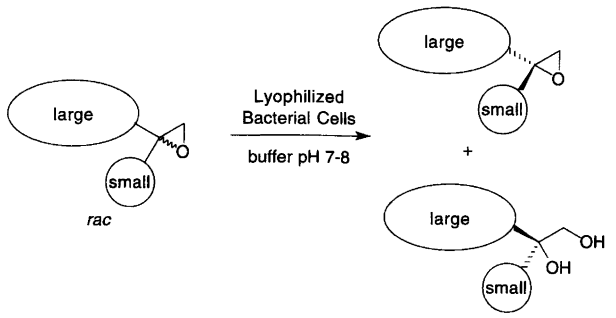
generally hydrolyzed with good activity, whereas small epoxides (**4**, **5**), or those having sterically hindered epoxide moieties (**6**, **7**) or *meso*-substrates (**8–11**), were not hydrolyzed by all *Rhodococcus* sp. Interestingly, *Corynebacterium glutamicum* and three *Pseudomonas* strains did not show any epoxide hydrolase activity. All attempts to induce epoxide hydrolase activity in *Pseudomonas aeruginosa* NCIMB 9571 and *Pseudomonas oleovorans* ATCC 29347 by growing the cells on an alkane (decane) or alkene (1-octene) as the sole carbon source failed.[56]

5. Asymmetrization of *meso*-epoxides

The asymmetrization of a *meso*-epoxide would be the most elegant application of microbial epoxide hydrolases, by leading to a single *trans*-diol in 100% theoretical yield. Unfortunately, only a few applications have been reported in this context, and the results are rather discouraging. For instance, cyclohexene oxide was hydrolyzed using *Corynesporium cassiicola* cells yielding *trans*-cyclohexane-1,2-diol in low ee.[57] It was only due to further metabolism, involving an oxidation–reduction sequence by dehydrogenases present in the cells, that the latter was transformed into optically pure (*S*,*S*)-cyclohexane-1,2-diol. In a related experiment, asymmetric hydrolysis of *cis*-epoxysuccinate using a crude enzyme preparation derived from *Rhodococcus* sp. led to D- and L-tartaric acid in almost racemic form.[38] Similar discouraging results were obtained using baker's yeast.[58]

6. Kinetic resolution of racemic epoxides

In contrast with the asymmetrization of *meso*-epoxides, the kinetic resolution of racemic epoxides by whole fungal and bacterial cells has proved to be highly selective. For instance, styrene oxide was resolved by whole cells of *Aspergillus niger* and *Beauveria bassiana* via two different



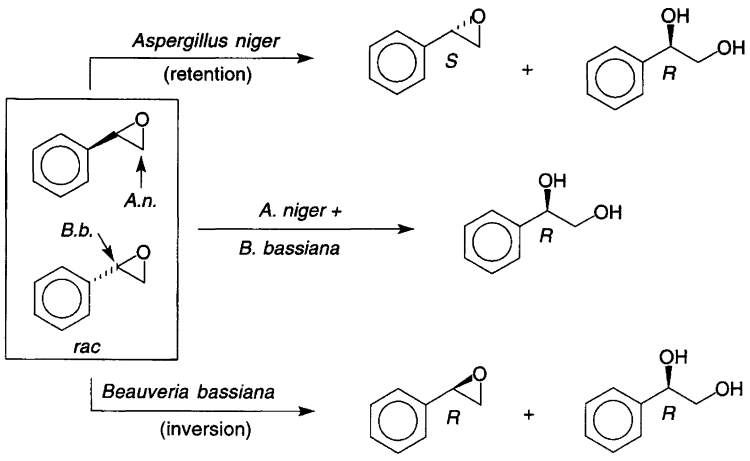
Scheme 10. Resolution of 2,2-disubstituted oxiranes by bacterial cells.

pathways with excellent selectivities (Scheme 8). Combination of the two biocatalysts employing a 'deracemization process' in a single reactor led to (*R*)-phenylethane-1,2-diol in 89% ee, as the sole product.[43]

Whereas bacterial cells have exhibited only low to moderate selectivities with 2-monosubstituted epoxides such as 1-epoxyoctane (**1**, $E<5$) or benzyl glycidyl ether (**2**, $E<2$), with 2,2-disubstituted oxiranes the specificities (expressed as the enantiomeric ratio '*E*'[59]) were excellent (Scheme 10). As may be deduced from Table 2, the selectivities increased with increasing difference in the size of the two alkyl substituents. Also, substrates bearing synthetically useful functional groups were accepted with high selectivities.[60]

An example of the resolution of a 2,3-disubstituted oxirane having a *cis*-configuration is shown in Scheme 11. Thus, by using an enzyme preparation from *Pseudomonas* sp., the (9*R*,10*S*)-enantiomer was hydrolyzed in a *trans*-specific fashion (i.e. via inversion of configuration at C-10) yielding the (9*R*,10*R*)-*threo*-diol. The remaining (9*S*,10*R*)-epoxide was converted into (+)-disparlure, the sex pheromone of the gypsy moth in >95% ee.[51]

An example of the resolution of a 2,2,3-trisubstituted terpene epoxide[52] ('Bowers' compound') is shown in

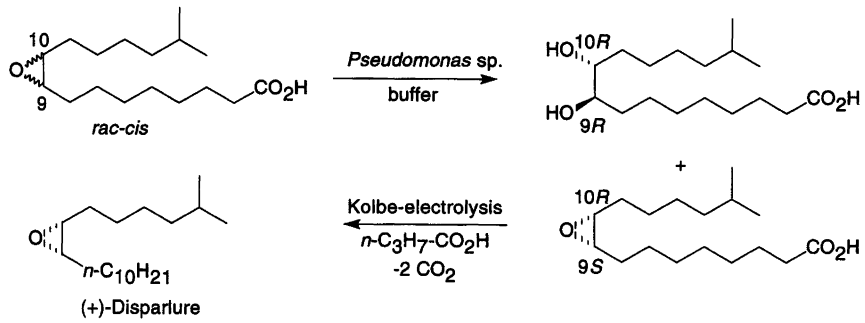


Scheme 9. Resolution and deracemization of styrene oxide by fungal cells.

Table 2. Selectivities in the resolution of 2,2-disubstituted oxiranes by bacterial cells (see Scheme 10).

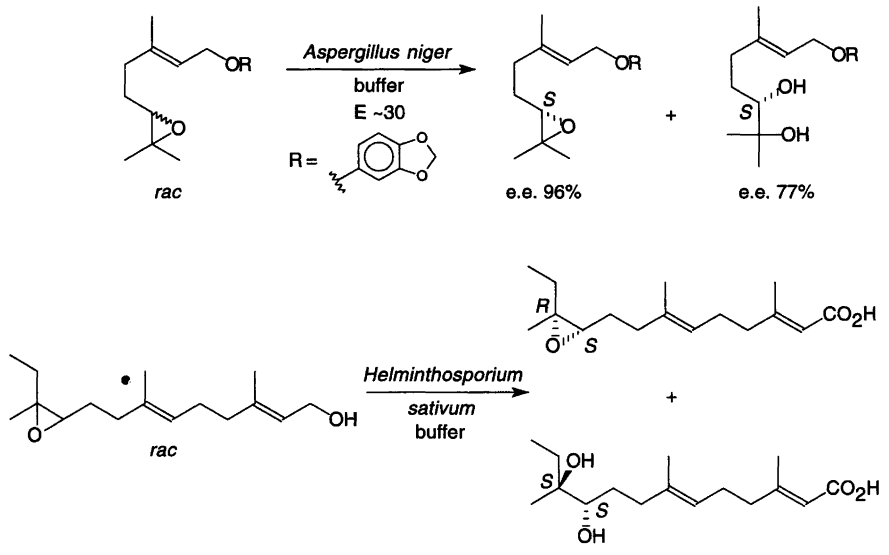
Substituent			
Small	Large	Biocatalyst	Selectivity (*E*)
H	C_6H_{13}	*Rhodococcus* sp. NCIMB 11216	2.8[a]
CH_3	C_5H_{11}	*Rhodococcus* sp. NCIMB 11216	105
C_2H_5	C_5H_{11}	*Rhodococcus* sp. NCIMB 11216	7
CH_3	C_7H_{15}	*Rhodococcus* sp. NCIMB 11216	125
CH_3	C_9H_{19}	*Rhodococcus* sp. NCIMB 11216	>200
CH_3	C_5H_{11}	*Rhodococcus equi* IFO 3730	194
CH_3	C_5H_{11}	*Mycobacterium paraffinicum* NCIMB 10420	49
CH_3	C_7H_{15}	*Rhodococcus equi* IFO 3730	>200
CH_3	C_7H_{15}	*Mycobacterium paraffinicum* NCIMB 10420	>200
CH_3	C_9H_{19}	*Rhodococcus equi* IFO 3730	>200
CH_3	C_9H_{19}	*Mycobacterium paraffinicum* NCIMB 10420	>200
CH_3	$(CH_2)_3$–CH=CH_2	*Rhodococcus equi* IFO 3730	39
CH_3	CH_2–O–CH_2–Ph	*Mycobacterium paraffinicum* NCIMB 10420	>200

[a] Opposite configuration as shown.



Scheme 11. Resolution of a *cis*-2,3-disubstituted epoxide.

Scheme 12. Using *Aspergillus* sp. cells, the resolution proceeded with a selectivity of $E \approx 30$ and the remaining epoxide was isolated in 96% ee. The latter is a potent analogue of a juvenile hormone of the yellow meal worm.[61] In a related transformation, a racemic allylic terpene alcohol containing a *cis*-trisubstituted epoxide moiety was



Scheme 12. Resolution of 2,2,3-trisubstituted oxiranes by fungal cells.

hydrolyzed by *Helminthosporium sativum* to yield the (*S*,*S*)-diol with concomitant oxidation of the alcoholic group; other minor metabolic products were observed. The mirror image (*R*,*S*)-epoxide was not transformed. Both optically pure enantiomers were then chemically converted into a juvenile hormone.[49]

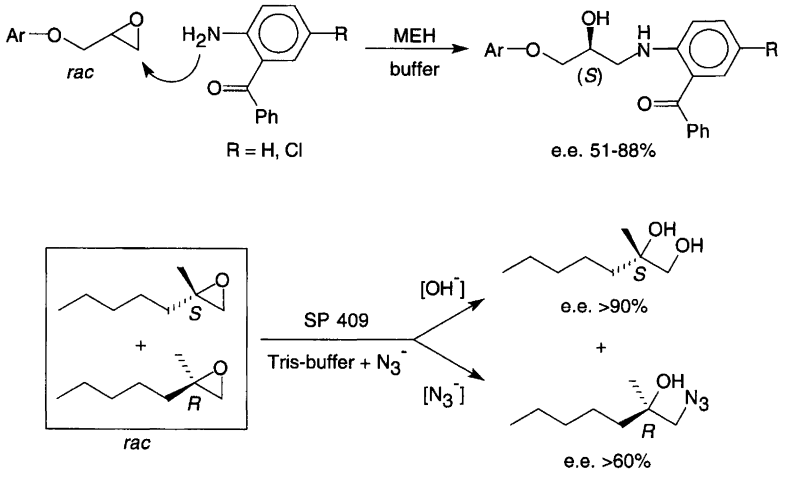
7. Use of non-natural nucleophiles

In reactions catalyzed by hydrolytic enzymes of the serine-hydrolase type, which form covalent acyl–enzyme intermediates during the course of the reaction, it has been shown that the 'natural' nucleophile (water) can be replaced with 'foreign' nucleophiles[62] such as an alcohol, amine, hydroxylamine, hydrazine and even hydrogen peroxide. As a consequence, a wealth of synthetically useful reactions, which are usually performed in organic solvents of low water content, can be performed in a stereoselective manner. Although one requirement is fulfilled with epoxide hydrolases (i.e. a covalent enzyme–substrate intermediate is formed) the high sensitivity of epoxide hydrolase to most of the water-miscible or -immiscible organic solvents[30,63] poses a problem towards the use of non-natural nucleophiles in enzymatic epoxide hydrolysis. However, two types of transformation, i.e. the aminolysis and azidolysis of an epoxide have been reported as having been carried out in an aqueous system (Scheme 12).

When racemic aryl glycidyl ethers were subjected to aminolysis in aqueous buffer catalyzed by hepatic microsomal epoxide hydrolase from rat, the corresponding (*S*)-configurated amino-alcohols were obtained in 51–88% ee.[64] On the other hand, when azide was employed as the nucleophile for the asymmetric opening of 2-methyl-1,2-epoxyheptane (±)-(**3**) in the presence of an immobilized crude enzyme preparation derived from *Rhodococcus* sp., the reaction revealed a complex picture.[65] The (*S*)-epoxide from the racemate was hydrolyzed (as in the absence of azide), and the less readily accepted (*R*)-enantiomer was transformed into the corresponding azido-alcohol (ee >60%). Although at present only speculations can be made about the actual mechanism of both the aminolysis and azidolysis reaction, in both cases is was proved that the reaction was catalyzed by an enzyme, and that no reaction was observed in the absence of biocatalyst or by using a heat-denatured preparation. However, a recent related report on the aminolysis of epoxides employing crude porcine pancreatic lipase[66] may likewise be explained by catalysis by a chiral protein surface rather than true lipase-catalysis since the latter enzyme, being a serine hydrolase, is irreversibly deactivated by epoxides.

8. Summary and outlook

Epoxide hydrolases from microbial sources are only just being recognized as highly versatile biocatalysts for the preparation of enantiopure epoxides and 1,2-diols and the near future will bring a number of useful applications of these systems to the asymmetric synthesis of chiral bioactive compounds. These biocatalysts are easy to use owing to their independence of cofactors and a number of microbial strains permitting a sufficient supply of enzyme is available from culture collections. An additional benefit of these enzymes is in the fact that, in contrast with epoxide hydrolases from mammalian systems, enzyme induction does not seem to be necessary. Furthermore, whole lyophilized cells, which can be stored for several months without significant loss of activity, can be used instead of isolated enzymes. The enantioselectivity of microbial epoxide hydrolysis is often low, but can be excellent depending in the substrate structure. As this field is currently still in a developmental stage, more information is needed to enable predictions of suitable microbial



Scheme 13. Enzyme-catalyzed aminolysis and azidolysis of epoxides.

strains possessing epoxide hydrolase activity for a given substrate. This will certainly be helped once the first three-dimensional X-ray structure of an epoxide hydrolase has been be solved. In addition to the 'natural' hydrolysis reaction, aminolysis and azidolysis has been shown to be possible. The use of other nucleophiles and the application of bacterial epoxide hydrolases for the synthesis of pheromones and vitamins is currently being investigated in our laboratory.

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